Review

GnRH and GnRH receptors in metazoa: A historical, comparative, and evolutive perspective

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Abstract

About 50 years after Harris’s first demonstration of its existence, GnRH has strongly stimulated the interest and imagination of scientists, resulting in a high number of studies in an increasing number of species. For the endocrinologist, GnRH, via its actions on the synthesis and release of pituitary gonadotrophins, is first an essential hormone for the initiation and maintenance of the reproductive axis, but recent data suggest that GnRH emerged in animals lacking a pituitary. In this context, this review intends to explore the current status of knowledge on GnRH and GnRH receptors in metazoa in order to see if it is possible to draw an evolutive scenario according to which GnRH actions progressively evolved from the control of simple basic functions in early metazoa to an indirect mean of controlling gonadal activity in vertebrates through a sophisticated network of finely tuned neurons developing in a rather fascinating way. This review also intends to provide an evolutive scenario based on the recent advances of whole genome sequencing possibly explaining the number of GnRH and GnRH receptor variants according to the 2R and 3R theories accompanied by gene losses.

Keywords: GnRH; GnRH receptor; Evolution; Reproduction; Genome duplication

1. Introduction: the early steps

Exactly 150 years ago, in 1856, a Spanish medical doctor, Aureliano Maestre de San Juan (1828–1890), published an article entitled “Complete absence of olfactory nerves with anosmia in an individual with a congenital atrophy of the testis and the penis” (Maestre de San Juan, 1856). Without knowing it, Maestre reported the first case of what is now mostly referred to as the Kallman-de Morsier syndrome, caused by a deficiency in the proper development of neurons synthesizing a factor called GnRH. In this pioneer article, Maestre reported observations made on a male patient on the occasion of an autopsy in 1849: he noted the lack of development of external genitalia (hypogonadism), but also expressed his surprise when discovering the total absence of olfactory nerves. In addition, by questioning the patient’s family Maestre learnt from a sister that her brother was able to remain in stinking places without being bothered (anosmia).

Virtually 100 years later, GnRH was discovered following pioneer studies conducted by a series of early neuroendocrinologists, notably Geoffrey Harris who for example showed that if one cut the pituitary stalk in a female ferret, the cyclicity of the ovary disappeared (Donovan and Harris, 1954). But, the surprise came from the fact that, after some time, regeneration of the portal vessels connecting the median eminence to the anterior lobe was accompanied by a return of ovarian cyclicity. This and many other experiments paved the way to the discovery of GnRH. In the early 70s, two competing groups published the primary structure of a decapeptide, named LHRH, isolated on the basis of its luteinizing hormone (LH) releasing activity (Burgus et al., 1971; Matsuo et al., 1971). As LHRH was then also shown to stimulate (follicle-stimulating hormone...
FSH) release, it is now commonly named GnRH for gonadotrophin-releasing hormone and mGnRH in the case of the mammalian peptide. In 1976, Roger Guillemin and Andrew Schally shared the Nobel Prize “for their discoveries concerning the peptide hormone production of the brain” including GnRH.

The basic pattern of organization of the GnRH system was rapidly established by Julien Barry’s group in Lille (France) thanks to the first GnRH antibody (developed against synthetic GnRH by the late Dr. Maurice P. Dubois). This group was working on guinea pig and they first reported the presence of LHRH-immunoreactive terminals in the median eminence, before establishing the existence of a preoptico-median eminence pathway (Barry and Dubois, 1974; Barry et al., 1973; Leonardelli et al., 1973), a basic pattern that was then found in many mammalian species with minor variations. It must be stressed out that, ironically, the guinea pig is precisely the only mammalian species known for expressing a variant called guinea pig GnRH (Jiménez-Liñán et al., 1997) different from mGnRH (see Table 1), already pointing out the problem of the specificity of antibodies to the GnRH decapetides.

Since then, a considerable amount of studies was devoted to all aspects of GnRH which is mostly known in vertebrates as a key player in the control of the reproductive axis. As an illustration, searching PubMed for “GnRH review” retrieves 3415 review articles (14th of November 2006) devoted to all aspects of GnRH biology. The most documented aspect of GnRH of course concerns its function as a hypophysiotropic peptide occupying a central position in the regulatory loop controlling reproduction. Extensive studies in mammals have indeed shown that GnRH is liberated in a pulsatile manner in the portal blood at the level of the median eminence. The amplitude and frequency of these GnRH pulses finely controls the synthesis and liberation of FSH and LH (Counis et al., 2005), which stimulate ovarian activity and the production of steroids. Very importantly, steroids in turn feedback on GnRH neurons (Wintermantel et al., 2006) to participate in controlling their activity in concert with a plethora of neuropeptides and neurotransmitters (Ojeda et al., 2006).

For more than 50 years, given its clinical and agroindustrial applications, GnRH has been and still is the topic of very active research, mainly in mammals, but also in other vertebrate groups and now in invertebrates. Over the years, biochemical, neuroanatomical, pharmacological, physiological, and molecular studies on a large diversity of experimental models have constantly fuelled our reflection and challenged our vision of these GnRH peptides and their receptors. As a result, the GnRH field is a good example of the kind of inputs that comparative endocrinology studies can have on our understanding not only of the mechanisms underlying the regulation of endocrine functions, but also on the evolution of these mechanisms in vertebrates and now in invertebrates.

This review certainly does not pretend to address all aspects of GnRH and GnRH receptors. Its ambition is more limited, but still quite ambitious, as it intends to provide a comparative and evolutive perspective aiming at proposing scenarios for the evolution of the GnRH peptides, GnRH receptors, GnRH systems, and GnRH functions over 650 millions years of evolution.

Table 1
Primary structure of the 24 known GnRH variants taking mGnRH as the reference

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2. The GnRH family: so far 24 members

Since the characterization of mGnRH, the number of GnRH variants increased gradually to now reach a total of 24 known forms. With the only exception of mGnRH, all these peptides were originally named after the first species in which they have been discovered and are presented in Fig. 1. The first two forms discovered in non-mammalian vertebrates were chicken GnRH I, cGnRH-I (King and Millar, 1982; Miyamoto et al., 1982), and salmon GnRH, sGnRH (Sherwood et al., 1983), characterized in the early 80’s. Very rapidly, a second form was characterized in chicken and named chicken GnRH-II, cGnRH-II (Miyamoto et al., 1984), and certainly the discoverers of this peptide did not imagine at that time that this peptide would become so intriguing. At the present time, 14 variants have been found in vertebrates (Guilgur et al., 2006; Morgan and Millar, 2004; Vickers et al., 2004), nine in tunicates (Adams et al., 2003; Powell et al., 1996) and one in Octopus, a cephalopod mollusc (Iwakoshi et al., 2002). In vertebrates, original variants were mostly found (a large majority by Nancy Sherwood’s laboratory) in teleost fishes which altogether present eight forms including six original variants (references in Table 1): herring GnRH; whitefish GnRH; sea-bream GnRH; pejerrey GnRH; catfish GnRH, mGnRH, and cGnRH-II. Other variants were found in guinea pig (gpGnRH), frog (frGnRH), dogfish (dfGnRH), and lamprey (lamprey I and III: lGnRH-I and lGnRH-III). A major outcome of these comparative biochemical studies was to show that in most gnathostomes examined, there are at least two GnRH forms, one of these forms always corresponding to cGnRH-II which appears almost ubiquitous (Fig. 1). The other form varies from one class to another and in teleost fish from one order to another (Guilgur et al., 2006; Lethimonier et al., 2004; Morgan and Millar, 2004).

In basal representatives such as, in actinopterygians, the sturgeons, and the alligator gar (Lepretre et al., 1993; Sherwood et al., 1991) or, in sarcopterygians, the lungfish (King et al., 1995), mGnRH, and cGnRH-II were characterized, strongly suggesting that common ancestors of these two lineages already possessed two genes encoding peptides related to mGnRH and cGnRH-II (Fig. 1). In sharks, the closest lineage preceding the separation of actinopterygians and sarcopterygians, two GnRH forms have also been found, dogfish GnRH (dfGnRH), and cGnRH-II, again suggesting the presence of two genes in these early vertebrates (Forlano et al., 2000). It is interesting to mention that dfGnRH only shows one substitution compared to cGnRH-II, possibly indicating a common origin, but this is only a hypothesis so far as the corresponding cDNAs have not been cloned. Finally, Stacia Sower’s group characterized two forms of GnRH in lamprey, lamprey GnRH-I, and lamprey GnRH-III (Sherwood et al., 1986; Sower et al., 1993). Recent data on the cDNA encoding the precursors of these early variants suggests that these two lamprey forms are closely related to each other (Silver et al., 2004; Suzuki et al., 2000), but the relationships between these early forms and the variants found in other vertebrates are unclear at the moment. Certainly, the current sequencing of the genomes of lamprey and dogfish will rapidly allow resolving these issues.

3. GnRHs in vertebrates

Of course, it is in vertebrates that most data on GnRHs and GnRH receptors are available. During the 80s and 90s, a growing number of GnRH forms were identified in different vertebrate groups sometimes giving the impression that multiple GnRH systems occurred in the brain. However, when one considers the recent advances in the knowledge of genome duplication and evolution (Fig. 2; Meyer and Van de Peer, 2005; Steinke et al., 2006a,b,c), and the most recent data from neuroanatomical and developmental studies, a clearer picture is now emerging.

3.1. Vertebrate GnRH genes cluster in three branches

Because GnRHs are short peptides, it is difficult to establish their phylogeny on the basis of the decapptide sequence. However, like most other proteins and peptides, GnRHs are enzymatically processed from larger precursors. The complementary DNA consists of the decapptide, extended at the N-terminus by a signal peptide, and at the
The tremendous expansion and diversity of this group emergence of teleosts, and could be largely responsible for teleost lost in certain phyla making the relationships between the fact that, during evolution some GnRH genes have been gift of these three branches and this is most likely due to the recent data on W...in teleost speci W...the teleost situation to all vertebrates. Original but it seems that this idea was more based on a premature claimed to be present in various tetrapods as a third form, sGnRH and lGnRH-III were presence of three genes in tetrapods as stated in some stud- W...1998). Branch 3 only contains teleost sequences all generating the same peptide, salmon GnRH, the first teleost specific peptide characterized in 1983 by Nancy Sherwood (Sherwood et al., 1983). What is not always clear is the origin of these three branches and this is most likely due to the fact that, during evolution some GnRH genes have been lost in certain phyla making the relationships between the remaining genes difficult to establish.

For the moment, there is no evidence supporting the presence of three genes in tetrapods as stated in some stud-ies. At a certain stage, sGnRH and lGnRH-III were claimed to be present in various tetrapods as a third form, but it seems that this idea was more based on a premature extension of the teleost situation to all vertebrates. Original variants like gpGnRH, cGnRH-I, or frog GnRH clearly cluster with the tetrapod type 1 genes and thus appear to be orthologs of mGnRH. Given that a third gene only occurs W...in teleost fish, it is very likely that the type 3 gene arose from the teleost specific gene duplication, now referred to as the 3R (third round) duplication, posterior to the divergence of fish and land vertebrates (Fig. 2). According to recent data on fish genomes, such a duplication occurred in teleost fish about 320–350 millions years ago, after the emergence of teleosts, and could be largely responsible for the tremendous expansion and diversity of this group (Meyer and Van de Peer, 2005; Vollff, 2005). In addition, the distribution of type 1 and type 3 GnRH neurons and their embryonic origin in teleost fish strongly suggest that they have a common ancestor.

3.1.1. The type 1 GnRH system

According to Russ Fernald’s nomenclature, type 1 GnRH neurons correspond to the classical hypophysiotropic systems first reported by Barry’s group. Apart from reptiles in which there is little information, a GnRH system similar to what was first reported for the mammalian type 1 GnRH system is present in birds, amphibians, and fish. These neurons send projections to the median eminence in tetrapods or pituitary in fish and this system is clearly implicated in the regulation of gonadotrophin release, thus a very clear role in reproduction. We also know that type I GnRH neurons originate from the olfactory region and migrate into the forebrain (Schwanzel-Fukuda and Pfaff, 1990; Wray et al., 1989). If migration does not occur, GnRH neurons do not establish connections with the pituitary and animals remain at the prepubertal stage and are infertile (Hardelin et al., 2000). It is now known that the rare X-linked form of the Maestre-Kallman-de Morssier syndrome is caused by mutation of the Kal 1 gene encoding anosmin 1 a cell adhesion molecule with branch-promoting and guidance activity having a crucial role in the development of the olfactory cortex (Soussi-Yanicostas et al., 2002), but also in the migration of GnRH neurons (Cariboni et al., 2004).

3.1.2. The type 2 GnRH system

In 1984, while looking at the distribution of salmon GnRH in the brain of the goldfish, we observed large GnRH neurons in an unusual location, the tegument of the midbrain (Kah et al., 1986). Such cells had already been mentioned in the platyfish in 1981 (Munz et al., 1981). Several years later, Kei Li Yu identified the presence of both sGnRH and cGnRH-II, the later being more abundant in the caudal brain of the goldfish (Yu et al., 1988) and a similar finding was made in the Masu salmon (Okuzawa et al., 1990). We thus started to suspect that these large tegmental GnRH neurons could be the ones expressing cGnRH-II. But finally, it is in the Masu salmon that it was established for the first time, using cGnRH-II antibodies, that the mid-brain expressed cGnRH-II (Amano et al., 1991). Since then, such neurons were found in all fish species and in many other vertebrates such as the stingray (Forlano et al., 2000), a frog (Conlon et al., 1993), the quail (van Gils et al., 1993) or the musk shrew, a primitive placental mammal (Dello-vade et al., 1993), and fetal rhesus monkey (Lescheid et al., 1997). All these studies stimulated the search of a second GnRH gene in humans which was finally found and characterized by Russ Fernald’s group (White et al., 1998). This gene had the same intron–exon organization that all GnRH genes and the prepro human cGnRH-II mRNAs appeared to have a larger distribution than prepro human cGnRH-II.

Fig. 2. Diagram showing a phylogenetic tree of gnathostomes and the potential position of whole genome duplication events. The timing of 1R is still uncertain (adapted from Steinke et al., 2006a).
mRNAs, in particular in the bone marrow, the prostate, and the kidney (White et al., 1998). Available information in frog, fish, and primate suggest that cGnRH-II neurons do not develop from the olfactory regions but appear first in a mesencephalic primordium originating from the neural crest (Gonzalez-Martinez et al., 2004b; Muske et al., 1994; White and Fernald, 1998; Whitlock, 2005).

3.1.3. The type 3 GnRH system: only in teleost fish

By the mid 90s, the situation in fish was similar to that reported in other vertebrates with an anterior forebrain system producing different variants according to the species, and a midbrain system consistently expressing chicken GnRH-II. Everything seemed clear when in 1994, appeared an astonishing paper showing for the first time the presence of three forms of GnRH in the brain of a vertebrate species, the gilthead sea bream a highly evolved perciform fish (Powell et al., 1994). This third form was named sea bream GnRH and is similar to mammalian GnRH except for a serine in position 8. Very rapidly some other papers appeared showing the same situation in other species of evolved teleosts, an African cichlid (perciform) or the medaka (beloniform), and establishing the distribution of the cells expressing the corresponding messengers. The data showed that apparently cells expressing these different variants had a clear differential distribution. (Gothilf et al., 1996; Okubo et al., 2000; White et al., 1995).

Based on these data some people started to claim that, given their different distribution, these cells also had three different origins. sGnRH neurons developing from the olfactory region, cGnRH-II appearing straight in the midbrain, and seabream GnRH cells developing in the preoptic area. However, more detailed studies showed that the distribution of type 1 and type 3 GnRH neurons clearly overlap in the forebrain and that both populations migrate from the olfactory placode region. At the present day, the European sea bass is the only teleost species in which the organization of the three GnRH systems was studied at both the messenger and protein level (Fig. 3).

3.1.3.1. Type 1 and type 3 GnRHs share a common distribution: the sea bass example.

Three different GnRH cDNAs were characterized from the European sea bass, another perciform, of high economical interest in Europe. These cDNAs generate three active biological GnRH decapeptides, sbGnRH, sGnRH and cGnRH-II, and riboprobes corresponding to the GAP regions were used to perform in situ hybridization. The results showed that both sGAP and sbGAP mRNAs had a broad expression in the olfactory bulbs, ventral telencephalon, and preoptic region, whereas cIIGAP mRNA expression was confined to large cells of the nucleus of the medial longitudinal fascicile. In the olfactory bulbs, both the signal intensity and the number of positive cells were higher with the sGAP probe, whereas sbGAP mRNA-expressing cells were more numerous and intensely stained in the preoptic region. Additional isolated sbGAP-positive cells were detected in the ventrolateral hypothalamus. Similar data showing a clear overlapping of sGAP- and sbGAP-expressing cells in the forebrain of the European sea bass (Gonzalez-Martinez et al., 2001, 2002a) were obtained in the Atlantic croaker (Mohamed and Khan, 2006).

In order to have detailed data on the projections of these neurons and to avoid the problem of specificity of GnRH antibodies, antisera were raised against recombinant GAP peptides which are longer and highly divergent in the same species (Gonzalez-Martinez et al., 2002a). The salmon GAP immunostaining was mostly detected in terminal nerve neurons but also in ventral telencephalic and preoptic perikarya. Salmon GAP-immunoreactive (ir) fibers were observed mainly in the forebrain, although sGAP-ir projections were also evident in the optic tectum, mesencephalic tegmentum, and ventral rhombencephalon. The pituitary only receives a few sGAP-ir fibers (Fig. 3). The seabream GAP-ir cells were mainly detected in the preoptic area. Nevertheless, sbGAP-ir neurons were also found in olfactory bulbs, ventral telencephalon, and ventrolateral hypothalamus. The sbGAP-ir fibers were only observed in the ventral forebrain, innervating strongly, and only the pituitary gland. Finally, chicken-II GAP immunoreactivity was only detected in large synencephalic cells, which are the origin of a profuse innervation reaching the telencephalon, preoptic area, hypothalamus, thalamus, pretectum, posterior tuberculum, mesencephalic tectum, and tegmentum, cerebellum, and rhombencephalon. However, no cIIGAP-ir fibers were detected in the hypophysis (Gonzalez-Martinez et al., 2002a).

3.1.3.2. Type 1 and type 3 GnRH neurons share a common origin.

Detailed immunohistochemistry and in situ hybridization studies on the ontogeny of the three GnRH populations showed that cGnRH-II neurons appeared first at day 4 post-hatching, then at day 7, the first sGnRH
neurons were detectable in the olfactory placode region and then they seem to move caudally (Gonzalez-Martinez et al., 2004b, 2002b). These data suggested that type 1 and type 3 GnRH neurons have a common origin in the olfactory placode region. This was recently confirmed using a transgenic approach in medaka (Okubo et al., 2006). Fish expressing GFP in the type 1 or type 3 GnRH neurons were produced, clearly showing in vivo that both populations appear first in the olfactory placode area and then migrate caudally. In this article Okubo and colleagues also show that the kal1.1 gene, one of the two kal1 genes in the medaka, is absolutely necessary for proper migration of both type 1 and type 3 GnRH neurons (Okubo et al., 2006).

All these data showing a similar overall distribution of type 1 and type 3 GnRH neurons, a similar origin and common mechanisms controlling their migration strongly suggest that GnRH 3 and GnRH 1 share a common ancestor.

3.2. A scenario for the evolution of the GnRH family in vertebrates

Recent information based on whole genome studies has provided highly interesting inputs on the evolution of the GnRH genes in actinopterygians and sarcopterygians (Fig. 2). According to these studies, two rounds of genome duplication (1R and 2R) occurred early in the vertebrate evolution (Panopoulou et al., 2003). More recently, other studies have strongly suggested the occurrence of a third duplication (3R) shortly after emergence of teleost fish (Meyer and Van de Peer, 2005; Steinke et al., 2006a,c; Volff, 2005).

3.2.1. The actinopterygian lineage, a high plasticity in the use of three GnRH genes

Originally, it was believed that the presence of three GnRH genes was a characteristic of evolved teleosts such as perciforms or pleuronectiforms, whereas only two forms have been found in well-studied species such as salmon, goldfish or carps. However, Nancy Sherwood’s group showed the presence of three GnRH forms in the herring (Carolsfeld et al., 2000), a rather primitive teleost and, very interestingly, her group also characterized three forms in the whitefish, a basal salmonid (Vickers et al., 2004). Therefore, the presence of three GnRH genes in teleosts seems to become more the rule than the exception. Thus, the evolution of the GnRH genes in teleosts supports the occurrence of the teleost-specific genome duplication (3R). If, as we saw before the number of GnRH genes in primitive bony fish (sturgeons, amia, bichir, paddlefish) was 2, then teleosts should potentially have four GnRH genes, but none appears to have a second copy of the cGnRH-II gene that was probably lost soon after the duplication which is what happens to most gene duplicates (Fig. 4). According to recent genome analyzes, 50% to 80% of duplicate genes are lost shortly after a genome duplication event (Lynch and Force, 2000). This probably explains why teleosts have an odd number of GnRH genes. With respect to the type 1 gene, it is then likely the 3R teleost specific gene duplication gave two paralog genes and, for some reason, one of these paralogs was lost in some families (Fig. 5). What is interesting is that it is not always the same gene that was lost. For example, goldfish, zebrafish, and salmon do not seem to have a type 1 GnRH gene, whereas in catfish or eel, it is GnRH type 3 that is missing. In contrast, all fish so far appear to have a single cGnRH-II gene except for some recent duplication that occurred in cyprinids or salmonids (Fig. 5). Similarly recent duplication caused the presence of two copies of the type 1 GnRH gene (catfish) or type 3 GnRH gene (salmon, trout).

Nevertheless, we already stressed out (Lethimonier et al., 2004), that if one looks at the distribution of the GnRH neurons in the ventral forebrain of fish, one always finds a similar overall pattern whatever the genes expressed. Basically, we have three groups: those fish like the eel and the catfish having only a type 1 gene, fish like salmon or trout who have only type 3, fish like the perciforms, and the whitefish-expressing both type 1 and type 3. A special mention should go to the goldfish in which strikingly chicken GnRH-II is expressed in the ventral forebrain (Kim et al., 2006). It thus seems that, after the 3R duplication, different cases of gene loss occurred in the teleost lineage. However, in the case of the perciforms, we are probably facing a situation of partition of functions, subfunctionalization, a mechanism whereby both members of a duplicate pair experience degenerative mutations that reduce their joint levels and patterns of activity to that of the single ancestral gene (Lynch and Force, 2000). It seems indeed quite clear, at least in the case of the sea bass, that type 1 and type 3 GnRH neurons have a common origin and an overall similar pattern of distribution. However, while the type 3 neurons project widely to the forebrain and very little to the pituitary, type 1 GnRH neurons only project to the pituitary.
3.2.2. The sarcopterygian lineage: towards the loss of cGnRH-II in mammals

In the second lineage of vertebrates, the sarcopterygians, we do not have enough data in a large sample of species, but it is likely there are only two genes belonging to the type 1 and the type 2 branches (Fig. 5). This latter cGnRH-II system seems to be well developed in frogs where cGnRH-II neurons are abundant in the mesencephalon but also in the diencephalon. In frogs, the type 1 GnRH corresponds to mGnRH (Conlon et al., 1993) or frGnRH (Yoo et al., 2000). In ancestral reptiles, both cGnRH-I and cGnRH-II are thought to be present prior to the diversification in three living reptilian subclasses. However, it was shown that both cGnRH-I and cGnRH-II occur in turtle and alligator, cGnRH-I in snake, and only cGnRH-II in lizard (Sherwood and Whittier, 1988). Certainly, more detailed studies are needed in reptiles. Both cGnRH-I and cGnRH-II are well expressed in birds, notably chicken, and quail, in which a large group of cGnRH-II neurons is present in the midbrain (van Gils et al., 1993).

Until recently, it was believed that cGnRH-II generated from a type 2 GnRH gene was present in all tetrapods, including humans (White et al., 1998). However, if, as mentioned above, type 2 GnRH neurons are present in primitive mammals (Dellovade et al., 1993), cGnRH-II seems to be either not functional or even lost in some species (J.J. Lareyre, unpublished; Morgan et al., 2006). Indeed, recent surveys of the mammalian sequence genomes indicate that the cGnRH-II gene is apparently not present in certain mammalian species like rodents (rat, mouse) or chimpanzee. An interesting situation has been recently found in bovine in which the prepro-GnRH-II gene encodes a unique decapeptide with an arginine in position 3 making this peptide non-functional. Indeed, bovine GnRH-II has no binding at mammalian type II or type I GnRH receptors and does not stimulate IP production (Morgan et al., 2006). Furthermore, in a number of species, the type II GnRH receptor gene is disrupted by frame shifts and premature stop codon has already documented in human (Cheng and Leung, 2005; Millar, 2005; Morgan et al., 2006).

Thus, although cGnRH-II appears to be highly conserved in non-mammalian vertebrates suggesting that this peptide serves crucial functions, it seems that many mammalian species are doing very well without a functional type 2 GnRH system and of course the question is: why?
4. How many GnRH receptors in vertebrates?

In parallel to the increase in the number of GnRH potential ligands, the number of GnRH receptors has also increased leading to some confusion in the nomenclature and the interpretation of the data.

4.1. The early steps: the mammalian type I GnRH receptor lacking an intracellular C-terminus domain

For many years, there was a single GnRH receptor first cloned from mouse pituitary cells (Reinhart et al., 1992; Tsutsumi et al., 1992) and then in a number of mammalian species, including humans (Chi et al., 1993; Kakar et al., 1993). All these mammalian receptors exhibited a number of features of the seven transmembrane domains GPCR receptors, but remarkably lack an intracellular tail. As a result these receptor do not desensitize sensu stricto and internalizes very poorly (Counis et al., 2005; Maudsley et al., 2004). In the pituitary gonadotrophs, activation of the GnRH receptor stimulates a variety of intracellular signalling systems. A major transduction system is through phospholipase C stimulation which generates inositol 1,4,5-trisphosphate and diacylglycerol, leading to calcium release, and activation of protein kinase C, respectively (Anderson, 1996; Maudsley et al., 2004). Because a second GnRH receptor was later found in some mammals, this first type is now referred to as human GnRH receptor type I. In 1997, Tensen and colleagues reported in African catfish the cloning of the first non-mammalian receptor (Tensen et al., 1997). In contrast to its mammalian counterpart, this receptor had a long intracellular tail that, added to the rat receptor, allowed rapid internalization of the mammalian receptor (Blomenrohr et al., 1999). This paper is the first to document the distinct efficacies of two endogenous ligands (in that case, cfGnRH, and cGnRH-II) on a single cognate receptor. Interestingly, on both receptors, cGnRH-II was more potent than cfGnRH, the putative hypophysiotropic ligand, in stimulating inositol phosphate (IP) production.

4.2. Two or more GnRH receptor types in non-mammalian vertebrates, all having an intracellular tail and a preference for cGnRH-II

Because it was becoming obvious that most vertebrates had two GnRH genes, some groups were looking for a second GnRH receptor and, indeed, in 1999 two GnRH receptor cDNAs were found in goldfish brain and pituitary, GfA and GfB (Illing et al., 1999). These two receptors shared 71% of identity and both had a long intracellular tail. Although there was some difference in the ligand selectivity of these receptors expressed in COS-1 cells, both of them had a clear preference for cGnRH-II in producing IP. This important paper stimulated a series of studies showing the presence of two receptors in the same fish species, such as the medaka (Okubo et al., 2001), the catfish (Bogerd et al., 2002) or the sea bass (Lethimonier et al., 2004). At the same time the cloning of a second mammalian GnRH receptor was reported in mammalian species, namely the marmoset (Millar et al., 2001), the rhesus monkey and the green monkey (Neill et al., 2001). This second receptor named type II was presumed to be a receptor for cGnRH-II as this peptide, in contrast to the type I receptor, was much more efficient than mGnRH in stimulating IP production from this new receptor (Millar et al., 2001; Neill et al., 2001).

However, the situation soon became more complicated when several studies showed the presence of three or more receptors in certain species. This was notably shown in the bullfrog, a diploid species, in which three GnRH receptors were cloned and characterized, bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 (Wang et al., 2001). These sequences showed less than 53% amino acid identity between them indicating a long evolutionary history (compared for example with the 71% of identity between GfA and GfB from goldfish, as mentioned above). The bfGnRHR-1 mRNA was expressed predominantly in the pituitary, whereas bfGnRHR-2 and -3 mRNAs were expressed in brain. These three receptors are functional as their transient expression in COS-7 cells caused a ligand-dependent increase in inositol phosphate production. Importantly, although mGnRH is the type 1 GnRH endogenous ligand in the fullfrog, for all three receptors, cGnRH-II has a much higher potency than mGnRH (Wang et al., 2001). It is however worth mentioning that highest activity of mGnRH, the putative hypophysiotropic ligand, is observed on bfGnRHR-1, the putative putitary receptor (see below).

However, again, it is in fish that the highest number of GnRH receptors has been reported, first in the medaka with three cDNAs (Okubo et al., 2003), then in Masu salmon in which five partial potential sequences were reported (Jodo et al., 2003). Unfortunately, there is no information on the pharmacological properties of these five sequences, some of which are highly related to each other (above 95%), suggesting that they emerged by recent duplication event in the salmonid branch. The recent availability of whole genomes from fish allowed us to perform in silico analysis of two fish genome, the fugu Takifugu rubripes (Aparicio et al., 2002) and the zebrafish (http://www.ensembl.org/Danio_rerio/index.html) allowed us to retrieve five different loci showing open reading frames (termed GnRH-R1 to GnRH-R5) in the fugu and four open reading frames, GnRH-R1 to GnRH-R4 in the zebrafish (Lethimonier et al., 2004). The five sequences are also found in the tetraodon, Tetraodon nigroviridis, genome (http://www.ensembl.org/Tetraodon_nigroviridis/index.html). Phylogenetical analysis of these sequences and other known teleost GnRH-R indicated the presence of two main types, originally termed 1 and 2 and now referred to as, type II-1 and type II-2. Accordingly, three of the putative fugu receptors (GnRH-R1, GnRH-R2, and GnRH-R3) belong to type II-1 whereas GnRH-R4 and GnRH-R5 cluster with type II-2 receptors. Two of the zebrafish sequences belong to type II-1 receptors, while the other two are found among
type II-2. It is interesting to mention that, in agreement with their high percentage of identity, the two GnRH-R cloned in goldfish (Illing et al., 1999), and catfish (Bogerd et al., 2002) cluster in the same branch (type 2), and thus are subtypes of type II-2. In contrast, the two receptors identified in tilapia (Parhar et al., 2002) or the three found in the medaka (Okubo et al., 2003), two Acanthopterygii, are found in the two main branches (Lethimonier et al., 2004).

In agreement with the presence of 5 GnRH receptors in the genome of the fugu, five GnRH receptors have been cloned and characterized in the European sea bass, another highly evolved teleost (Gonzalez-Martinez et al., 2004a; Lethimonier et al., 2004; Moncaut et al., 2005). These five GnRH receptors are functional as shown by transient transfection in COS-7 cells and all of them have much more affinity for cGnRH-II (which is not a major hypophysiotropic endogenous ligand in this species; Gonzalez-Martinez et al., 2002a). Only one of these receptors (dGnRHR-II.1A) shows some affinity for sbGnRH and for sGnRH (C. Lethimonier, JJ. Lareyre, N. Moncaut and A. Canario, unpublished data), two potential hypophysiotrophic ligands in the sea bass (Gonzalez-Martinez et al., 2002a; Lethimonier et al., 2004). Interestingly, this receptor is strongly expressed in the pituitary gonadotrophs, FSH, and LH (Gonzalez-Martinez et al., 2002a). So, it appears that in the sea bass, one of the five GnRH receptors, which all have a clear preference for cGnRH-II, is evolving in order to better mediate type 1 and type 3 ligands effects at the pituitary level.

4.3. Except the mammalian type I GnRH receptor, all other GnRH receptors belong to the type II

Until now, all GnRH receptors from vertebrates are “type II” receptors in the sense that they have a higher affinity for the type 2 GnRH ligand, cGnRH-II. Table 2 summarizes the currently available information and clearly shows that the only exception is the mammalian type I GnRH receptor which obviously shows higher efficacy when activated by type 1 GnRH ligand, mGnRH (Millar, 2005; Neill et al., 2001). Thus, the situation is rather paradoxical as, except in mammals, the GnRH receptor type present in the pituitary has less affinity for the hypophysiotropic type 1 GnRH than for cGnRH-II. How, can we explain this intriguing situation? Again, we have to look at this question in the context of evolution.

Attempts to draw phylogenetical trees of vertebrate GnRH receptor including mammalian sequences failed. Indeed, whatever the method employed, the GnRH-R type I sequences from mammals stick together, as expected, but the branching of that sub-group is not satisfactory because it diverges before the diversification of vertebrates, which is of course impossible. This probably indicates that sequences allowing proper branching of the type 1 mammalian receptors onto some ancestral mammalian or tetrapod branches are missing and, if they are missing, it is either because these sequences have not been found yet or because this ancestral gene has been lost. Thus, these sequences are now so divergent from other vertebrate GnRH-R genes that they form an out-group, resulting in trees that are difficult to interpret and sometimes have no sense in terms of evolution.

4.4. A hypothesis for the evolution of GnRH receptors in vertebrates

We have already seen that chicken GnRH-II is already present in chondrichthyes (sharks), then is found in all vertebrates and probably tends to disappear in evolved mammals. This strongly suggests that cGnRH-II is an ancestral form with crucial functions and thus under strong conservation pressure. Thus, one may expect that functions of this ancestral type 2 peptide were mediated by an ancestral type II GnRH receptor.

The 2R theory strongly suggests that two rounds of whole genome duplication occurred before the emergence of vertebrates (Panopoulou et al., 2003; Steinke et al., 2006a,c). According to the 2R hypothesis, each invertebrate gene is expected to have at least four vertebrate orthologs (1:4 rule). As we shall see below, at least one GnRH-R exists in invertebrates (Kanda et al., 2006; Rodet et al., 2005) and some evidence suggests that there is only one GnRH/GnRH receptor pair in Octopus (Kanda et al., 2006). Thus, according to the 2R theory, one would expect to have four potential GnRH receptor sequences in basal representatives of vertebrates and, according to the 3R theory (Panopoulou et al., 2003; Steinke et al., 2006a,b), the number of potential sequences in teleost fish would then be 8 (1:8 rule). Fig. 6 provides a tentative evolutive scenario for GnRH receptors in Vertebrates which supports the 3R theory accompanied by GnRH-R gene loss. Indeed, it is known that extensive gene loss follows whole genome duplication, and only very rarely can the 1:4 or 1:8 rules be applied sensu stricto.

The majority of the duplicates in the human, mouse, and Fugu genomes are organized in two member families (Panopoulou et al., 2003), which is also verified for the GnRH and GnRH receptors (two GnRH branches and two main GnRH-R branches; this paper). However, it is clear that if GnRH receptors can be grouped in two main types, not all potential genes can be found in these two main types. In actinopterygians, out of the eight potential ancestral sequences, a maximum of five (three in one branch, two in the other) have been retrieved so far in perciforms and tetraodontiforms apart form the results of more recent (tetraploidization events in salmonids for instance), indicating loss of three sequences (see above). In sarcopterygians, from the four potential sequences, a maximum of three was found in the diploid bullfrog (Fig. 6), again indicating that some sequences were lost along the way. In addition, although it seems clear that in both lineages, most receptors have a clear preference for cGnRH-II and have a wide tissue distribution, there seems to be a tendency towards the emergence of a “pituitary specific receptor” shifting from GnRH type 2 preferences to GnRH type 1.
Indeed, in sarcopterygians, as mentioned earlier, three GnRH receptor sequences were found in the bullfrog (Wang et al., 2001) and, although all three bullfrog receptors show higher affinity for cGnRH-II, one of them, bfGnRHR-1 is strongly expressed in the pituitary and shows a much better affinity than bfGnRHR-2 and bfGnRHR-3 for mGnRH, the putative type 1 GnRH ligand in the pituitary of the bullfrog. Thus, frogs probably lost one of the four putative potential GnRH type II receptors (redundancy) while at the same time, one of the remaining sequences is evolving to better accommodate a pituitary type 1 GnRH ligand (Wang et al., 2001). Unfortunately, there is a lack of information in

Table 2
Summary of the available information regarding GnRH receptors, their potency and their binding affinity in vertebrates

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Proposed nomenclature</th>
<th>Original nomenclature/species</th>
<th>Intracellular tail</th>
<th>Biopotency (IP production)</th>
<th>Binding affinity</th>
<th>References</th>
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(1) Maiti et al. (2003).
(2) Lethimonier et al. (unpublished data).
(3) Millar et al. (2001).
(4) Okubo et al. (2001).
(5) Okubo et al. (2003).
(6) Tensen et al. (1997).
(7) Bogerd et al. (2002).
(9) Gault et al. (2004).
(10) Sun YM et al. (2001).
(11) Morgan et al. (2006).
(14) Knox et al. (1994).
reptiles. The only known reptilian receptor cloned in gecko is considered as a cGnRH-II receptor and has low expression in the pituitary of males and females gecko, suggesting that another unidentified receptor mediates the pituitary effects of either cGnRH-II or a so-far unidentified ligand, possibly cGnRH-I (Ikemoto and Park, 2005). It is very likely that at least one other GnRH receptor occur in reptiles. Recent studies in chicken have established the presence of at least two different GnRH receptors: a first sequence, cloned several years ago showed higher affinity for cGnRH-II ($K_i = 0.6 \pm 0.01\ \text{nM}$) than for cGnRH-I ($K_i = 5.3 \pm 0.5\ \text{nM}$) (Sun et al., 2001), had a wide tissue distribution and lower expression level in the pituitary that a new sequence recently characterized also in chicken (Shimizu and Bedecarrats, 2006). This second receptor appeared to be well expressed in the pituitary where its expression correlates with the gonadotropic activity. Nevertheless, as the first receptor GNRHR1, chicken GNRHR2 also showed higher affinity to cGnRH-II (log ED50: 8.80) than to cGnRH-I (log ED50: 8.20). The minor difference in the efficacy of the two ligands, suggests that possibly this second sequence is evolving to better accommodate hypophysiotropic effects of cGnRH-I (Shimizu and Bedecarrats, 2006). In chicken, there is for the moment no evidence for the presence of additional sequences in the genome (Ensemble Chicken Genome 2 http://www.ensembl.org/Gallus_gallus/index.html) and thus it is likely that chicken has lost two of the ancestral GnRH receptors genes. In the actinopterygian lineage, we have discussed above the case of the sea bass in which the only pituitary receptor is the one showing the highest affinity for type 1 or type 3 ligands. Although there is limited data for the moment, we can predict that similar situations will be found in other fish orders. Given the high diversification and the above-mentioned variability in the use of the three GnRH genes in teleosts, it will be very interesting to see how receptors have evolved at the same time in the different main orders. Indeed, as already mentioned, receptors obviously remain to be found in salmonids, cyprinids and silurids. Given their basal situation, elopomorphs also deserve special attention.

4.5. Towards a highly efficient GnRH type I/GnRH receptor type I pair in mammals

Mammals present a trend towards a situation in which a unique and highly efficient type 1 GnRH receptor has emerged showing clear preference to mGnRH, the hypophysiotropic type 1 GnRH form in mammals (Barran et al., 2005; Millar, 2005). This results in a very efficient GnRH type I/GnRH receptor type I system specialized in the

Fig. 6. Schematic representation of a hypothesis on the evolution of GnRH receptors in vertebrates. Starting with one putative ancestral GnRH-R sequence in invertebrates, the 1R and 2R genome duplications, whose exact positions still remain to be documented, potentially gave birth to four potential sequences in basal representatives of the sarcopterygian and actinopterygian lineages. This hypothesis is supported by the fact that three GnRH receptors have been found in the bullfrog, a diploid species. In the teleost fish, the fact that more than four GnRH sequences have been found in some species also supports the 3R teleost specific genome duplication which gave birth to eight putative GnRH receptor sequences. However, the current data suggest that at least three (sea bass, fugu) or four (zebrafish) genes of the initial set of eight potential genes have been lost along the way. In the sarcopterygian lineage, one (in Xenopus), two (in gallus or marmoset) or three (in rat, mouse or human) genes of this initial receptor set have been lost or are currently being inactivated. The sequencing of the genome in lamprey and dogfish will be of great interest to further document the precise timing of the 1R and 2R duplications. Data on a larger sample of species, notably in mammals and fish, will also be essential to refine this hypothesis.
control of pituitary functions (Cheng and Leung, 2005; Millar, 2005). Indeed, as mentioned above, the GnRH type 2/GnRH receptor type II, which most likely represents the ancestral pair, seems, and this is unique in vertebrates, to be disappearing in evolved mammals. We already mentioned that the chicken GnRH-II gene, although existing in some species, notably in humans (White et al., 1998), has not been found in a number of well studied models such as the mouse or the rat, or encodes a non-functional peptide like in the bovine (Morgan et al., 2006). In addition, recent studies have documented that the mammalian type II GnRH receptor is functional in only a limited number of species (marmoset, green monkey, and rhesus monkey) due to frame shift and premature stop codon (Cheng and Leung, 2005; Morgan et al., 2006). This leaves us with two hypotheses: either there is no longer need for cGnRH-II and a cGnRH-II receptor because the functions of cGnRH-II were lost, or the functions of this system are sustained by the GnRH type I/GnRH receptor type I couple. The fact that the cGnRH-II gene seems to disappear more or less concomitantly with its receptor would favour this second hypothesis. Clearly further studies are needed in primitive mammals in order to solve this important issue and to determine the evolutive trends that have conducted to the emergence of the unique mammalian tail-less type I GnRH receptor.

5. Why is cGnRH-II so conserved in non-mammalian species?

So far, the only established function of GnRH-II is the inhibition of M currents (K+ channels) in amphibian sympathetic ganglia, an effect mediated by a type II GnRH receptor (Jones, 1987). However, in the literature, the list of potential functions of cGnRH-II is rather impressive, and includes roles in gonadotrophin secretion, sexual behaviour, stimulation of cell adhesion or migration, and role in peripheral endocrine regulation (Millar, 2003, 2005; Millar et al., 2004). Given the emerging data based on genome sequencing programs indicating that in many mammalian species the GnRH type 2/GnRH receptor type II is not functional (Morgan et al., 2006), all these data need to be carefully revisited, at least in mammals, since most of them are likely to be pharmacological. This is well exemplified with respect to the well-documented anti-proliferative effects of cGnRH-II on cell proliferation which are now considered to be mediated by GnRH type I receptors (Kim et al., 2005; Maudsley et al., 2004; Millar et al., 2004).

Such effects of GnRH peptides on cell proliferation and apoptosis of cancer cells imply transduction mechanisms that are distinct from the classical pituitary mechanism and are possibly reminiscent of the reported effects of GnRHs on the gonads, notably on apoptosis in both the ovary and the testis (Andreu-Vieyra et al., 2005; Andreu-Vieyra and Habibi, 2000). Because most of the attention was focused on the role of GnRH as a neuroendocrine peptide, such effects have largely been overlooked and one just starts to envision the pleiotropic effects of GnRH-like peptides, notably cGnRH-II, on cell functions. Recent studies on different prostate, breast, and endometrial cell lines have documented the variety of intracellular pathways that are used in response to GnRH stimulation according to the cell types (Kraus et al., 2006). It is very likely that cGnRH-II in non-mammalian vertebrates serves essential functions, others than the classical hypophysiotropic functions, that cannot be mediated by a GnRH ligand other than cGnRH-II because all their receptors are type II receptors. It will be essential in the future to perform careful analyzes of the precise distribution of the different GnRH peptides, notably in the gonads.

6. GnRH: a peptide in search of its ancestral function

There is accumulating evidence that GnRH and GnRH receptors exist in protochordates and invertebrates. Given that these animals do not have a pituitary sensu stricto (Christiaen et al., 2002; Deyts et al., 2006), such functions deserve special attention as potential ancestral functions of GnRH peptides.

6.1. GnRHs and GnRH receptors in protochordates

Most evidence on the presence and functions of GnRH in protochordates comes from the tunicates which, on the basis of recent phylogenetic data have been relocated as the closest living relatives of vertebrates (Delsuc et al., 2006). First evidence that GnRH was present in protochordates comes from an article by Danielle Georges and Maurice P. Dubois showing, with an antibody against mGnRH (the same that was used in guinea pig), that immunoreactive cells were localized between the nervous ganglion and the neural gland and also along the small nervous fibres near the dorsal vessel (Georges and Dubois, 1980). These pioneer observations were confirmed and detailed in several other studies (Dufour et al., 1988; Powell et al., 1996; Tsutsui et al., 1998) and it is now known that, in different species of Ciona, there are two GnRH genes, each encoding three GnRH as triplets separated by basic amino acids representing potential peptide cleavage sites (Adams et al., 2003; Powell et al., 1996). Because protochordates have a unique Hox cluster, it is believed that two rounds of whole genome duplications occurred between protochordates and basal gnathostomes (four Hox clusters). This suggests that, in the case of the Ciona GnRH genes, exon duplication occurred before a tunicate-specific genome duplication (Adams et al., 2003). A total of nine GnRH variants, all of which with substitutions in position 5 to 8, have now been identified on the basis of biochemical characterization and genome analyzes in different species of Ciona (Adams et al., 2003; Powell et al., 1996; Vickers et al., 2004). Another tenth form was also found in the Ciona genome (Tello et al., 2005). Immunohistochemical studies have reported the presence of immunoreactive structures in cells and fibers of the cerebral ganglion, along the inner wall of the dorsal blood sinus, as
well as on the anterior surface of the ovary (Georges and Dubois, 1980; Powell et al., 1996; Tsutsui et al., 1998). Interestingly, various tunicate GnRHs have been shown to stimulate gamete release, when injected in the body cavity, suggesting direct and rapid actions on gamete liberation (Adams et al., 2003; Terakado, 2001).

Two GnRH-R were cloned from the neural gland in Ciona intestinalis (Kusakabe et al., 2003), but sequencing of Ciona genome also allowed to retrieve two other potential sequences (Tello et al., 2005). When expressed in COS-7 cells, three of these receptors (GnRH receptors 1, 2, and 3) were activated by all six C. intestinalis peptides resulting in increased cAMP accumulation. Only one Ciona GnRH variant (tGnRH-6) caused inositol phosphate production in COS-7 cells expressing GnRH receptors 1 (Tello et al., 2005). The Ciona receptors have wide expression in reproductive and non-reproductive tissues, further suggesting implication of GnRHs in a wide range of functions (Sherwood et al., 2005).

Importantly, phylogenetic analyzes showed that the four Ciona GnRH receptors are more similar between them than they are from vertebrate receptors. This suggests that, similar to the GnRH genes, duplications occurred within the tunicate lineage (Sherwood et al., 2005, 2006).

6.2. GnRH in invertebrates

There is now no doubt that GnRH-like peptides are present in invertebrates. The strongest evidence comes from molluscs, but more recently also from cnidarians, although some weak indications for the presence of GnRHs in echinoderms and annelids have also been presented (Kah et al., 2004; Tsai, 2006). Interestingly, the presence of GnRH peptides is not documented in arthropods, insects or crustaceans (Kah et al., 2004; Rastogi et al., 2002). A sequence annotated for a long time as a Drosophila GnRH receptor as now been reannotated as the fruit fly AKH (adipokinetic hormone) receptor, which would share a common ancestor with GnRH receptors (Cazzamali et al., 2002; Staubli et al., 2002). Nevertheless, AKHs are involved in sugar, and fat mobilization and thus there is a strong need for further information regarding the link between AKH and GnRH peptides.

6.2.1. GnRH in molluscs

Bivalves represent a basal group of molluscs in which there is now strong evidence for the occurrence of GnRH peptides, GnRH receptors, and GnRH actions on the gonads. It was first shown that GnRH-immunoreactive material is present in neurons of the cerebral ganglia, close to the cerebral commissure, a putative neurohemal organ, and in the posterior part of the pedal ganglia (Pazos and Mathieu, 1999). Although immunoreactive GnRH was not detected in the gonads, it is likely that GnRH has gonadal actions as various GnRHs were able to stimulate gonial proliferation in the testis of mussels and oysters (Pazos and Mathieu, 1999). Furthermore, a putative GnRH receptor having a high degree of identity with both vertebrate GnRH receptors and insect AKH receptors has recently been cloned in oyster. In addition, this receptor is specifically expressed in the male and female gonads and strongly regulated during the reproductive cycle, suggesting a role of the putative ligand in the regulation of spawning (Rodet et al., 2005).

In the more evolved gastropods, there is also solid evidence for the presence of GnRH-like peptides. Early studies on snails showed that extracts of central nervous systems stimulated goldfish gonadotrophs and that GnRH elicited electrophysiological responses on various neurons. Other studies in Aplysia have documented the presence of many immunoreactive structures including in the central nervous system and in the osphradium, a chemosensory organ (Tsai, 2006; Tsai et al., 2003; Zhang et al., 2000).

However, the most conclusive evidence for the presence of GnRH in invertebrates comes from Octopus (cephalopod, highly evolved mollusc) in which a GnRH dodecapeptide was isolated (Iwakoshi et al., 2002). Despite two extra residues at the N-terminus (see Fig. 1) this peptide resembles other GnRHs by a number of structural features, including a pyroglutamate at the N-terminus and the sequence Pro-Gly-NH₂ at the C-terminus (Iwakoshi et al., 2002). The corresponding cDNA has been cloned and possesses the usual organization of preproGnRHs with a signal peptide, the GnRH dodecapeptide, a GKR cleavage site and a GnRH-associated peptide. Interestingly, immunoreactive cell bodies were detected in the subpedunculate lobe that controls the optic-gland activity (Iwakoshi et al., 2002). The subpedunculate lobe-optic gland complex of Octopus is presumed to be the functional equivalent of the hypothalamo-pituitary complex of vertebrates (Iwakoshi-Ukena et al., 2004). Additional studies have shown the presence of GnRH positive neurons in the fusiform ganglion, a peripheral ganglion that projects to the oviduct and the oviducal gland in the female, and the seminal vesicle in the male (Di Cosmo and Di Cristo, 1998; Di Cristo et al., 2002). Octopus GnRH was shown to stimulate cardiac rhythm in addition of modulating oviduct contractions (Iwakoshi-Ukena et al., 2004). Very recently, an authentic Octopus GnRH receptor (oct-GnRHR) was cloned that shows high structural similarities with its vertebrate counterparts and sensitivity to Octopus GnRH. Of high significance is the fact that Oct-GnRH induces synthesis of testosterone, progesterone, and 17β-oestradiol in Octopus ovary and testis, where oct-GnRHR was abundantly expressed (Kanda et al., 2006).

6.2.2. GnRH in cnidarians

Several years ago, a first study documented the presence of GnRH in the sea pansy, a soft coral, in which two peaks of GnRH-immunoreactivity had been found by HPLC (Anctil, 2000). In the sea pansy or the starlet anemone, positive bi- or multipolar neurons with neurites extending in the endoderm over parietal muscles or over the mesenteric filaments associated with gonad tissues (Anctil, 2000). In this study, the only activity of GnRH-like peptides was an
inhibition of the peristaltic activity of the sea pansy colonies (Anctil, 2000). A recent study in a scleractinian coral, *Euphyllia ancora*, also showed the presence of GnRH-immunoreactivity detected by HPLC and radioimmunoassay (Twan et al., 2006a). Extracts were able to stimulate LH release from fish pituitary cells in vitro, an effect blocked by GnRH-antagonists. These corals synchronously release their gametes during a brief annual spawning period, determined by the lunar cycle (Twan et al., 2006b), and it was shown that GnRH-immunoreactivity increases by three times during this mass spawning event in correlation with aromatase activity and sex steroid production (Twan et al., 2006a). These date strongly suggest a role for GnRH in the regulation of mass spawning under the control of external factors. Of particular interest is the possibility of an already-existing functional link between sexual steroids, GnRH, and environmental factors in this coral.

### 7. An evolutive scenario over 650 millions years

Based on the existing information reviewed above, it is possible to start building an evolutionary scenario. It is probably quite safe now to claim that GnRH is a very ancient peptide, at least 650 millions years old, with a clear role in reproductive functions in a wide range of organisms, which does not exclude potential other functions. It has been suggested that the tridecapeptide mating pheromone of yeast (Saccharomyces cerevisiae), has structural homology with GnRH. This peptide blocks haploid cells of opposite mating types in the G1 stage, prior to fusion with another cell to produce diploid zygotes. Interestingly, although high doses were required, α-mating factor was found to bind to rat pituitary GnRH receptors and to stimulate the release of luteinizing hormone from gonadotrophs in vitro (Loumaye et al., 1982). Whether or not, the yeast α-mating factor is a true GnRH, on a theoretical point of view, it is interesting to mention this link between a GnRH-like molecule, some sort of a pheromonal activity and a primitive form of reproduction.

What seems more and more certain is that GnRHs are found in both cnidaria and bilateria indicating that they already existed in their common ancestor close to the most primitive forms of metazoa around 650 millions years ago. It is then interesting to mention that in bilateria, the presence of GnRH peptides is strongly documented in deuterostomia (echinoderms, tunicates, vertebrates) and in

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**Fig. 7.** A potential scenario of the evolution of GnRH functions in metazoa. (a) In primitive organisms, like corals or sea pansy with a diffuse nervous system, a network of GnRH producing neurons is likely in close proximity to the gonads on which they can act directly or indirectly to stimulate gamete release or other gonadal functions, like gametogenesis or steroidogenesis. The activity of such neurons is modulated by environmental factors (light, ...) or internal factors (steroids?). This ensures synchronization of the individuals and the sexes together with spawning at the best time for survival of the progeny which are basic requirements for the success of reproduction. (b) As complexity increases, a primitive nervous system develops under the form of ganglia gaining specialized roles in controlling the various functions and organs. Progressively, some of these ganglia take the lead over others to start building a central nervous system. The GnRH neurons form a network receiving inputs from both external and internal factors, but some neurons keep direct connections with the gonads. (c) As complexity further increases, together with size, there is need for an amplification of the central gonadotropic signal. This is achieved by development of intermediate endocrine glands such as the optic gland in octopus or the pituitary in vertebrates. Central GnRH neurons are submitted to multiple regulations, but remain the final integrators of both external and internal factors. They loose their direct connections with the gonads and specialize in the control of intermediate endocrine glands. However, an intragonadal GnRH/GnRH receptor system remains to achieve GnRH crucial functions on the gonads.
spiralia (worms, leeches, molluscs, ...), and less in ecdysozoa (arthropods, nematods, nematomorphs, ...).

Given the available information, one can suggest three main steps in the evolution of the GnRH systems in the control of reproductive functions of metazoan (Fig. 7). In primitive organisms–like corals or sea pansy with a diffuse nervous system, GnRH producing neurons are likely in close proximity to the gonads on which they can act directly or indirectly to stimulate gamete release or other gonadal functions, like gametogenesis or steroidogenesis (Fig. 7a). Activity of such neurons would be modulated by environmental factors (light, ...) or internal factors (steroids). In these animals, we cannot exclude that GnRH acts as a pheromonal signal to ensure synchronization of the individuals and mass spawning.

As complexity increases, a primitive nervous system develops under the forms of ganglia gaining specialized roles in controlling the various functions and organs. Progressively, some of these ganglia take the lead over others to start building a central nervous system. This is typically the situation in marine bivalves and may be in tunicates. In these species, the GnRH neurons integrate more and more information to synchronize gonadal functions with the environment or the energy status of the individuals (Fig. 7b). These neurons are still in more or less direct contact with the gonads on which GnRH acts directly through GnRH receptors (Kanda et al., 2006; Pazos and Mathieu, 1999; Rodet et al., 2005; Tello et al., 2005).

As complexity further increases together with size, there is need for an amplification of the central gonadotrophic signal. This is achieved by development of intermediate endocrine glands such as the optic gland in Octopus or the pituitary in vertebrates (Fig. 7c). The GnRH neuron is submitted to multiple regulations, but remains the final integrator of both external and internal factors. It looses its direct connection with the gonads, but an intragonadal GnRH/GnRH receptor system remains to achieve GnRH crucial functions at the gonads.

8. Conclusions

Nothing is truer than the famous and beautiful sentence proposed by Theodosius Dobzhansky: “In Biology nothing makes sense except in the light of evolution”. This is certainly the case of the GnRH systems. If one only looks at the tip of the iceberg, i.e. GnRH as a hypophysiotropic peptide, it is hard to understand how such a neuroendocrine system has emerged and what were the driving forces over 650 millions years of evolution. One-hundred fifty years after Maestre de San Juan’s first report (1856), we certainly made significant advances in our understanding of GnRH biology and evolution. This is thanks to the fact that new discoveries and concepts have moved back and forth between models to help us building a comprehensive view. It is very likely that before serving hypophysiotropic functions in vertebrates, GnRH peptides were involved in modulation of cell functions such as proliferation or apoptosis in different organs, notably the gonads, according to mechanisms that remain to be detailed. However, whereas GnRH agonists and antagonists have a wide range of applications based on the modulation of pituitary gonadotropic activities, the fact that GnRH agonists are now more and more used to directly inhibit tumour cell proliferation and stimulate apoptosis, notably in prostate cancers, is likely linked to these early functions. Many black boxes remain which will require attention in the future, but we can predict that the current sequencing of multiple genomes will bring further advances in our understanding of the evolution of GnRH and GnRH receptor genes. However, there is still a great need of detailed physiological and anatomical studies to identify in details which form is expressed where and what are its functions.

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