

# Gonadotropin releasing hormone in the primitive vertebrate family Myxinidae: reproductive neuroanatomy and evolutionary aspects

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## Abstract

The family Myxinidae embraces all hagfish species, and occupies an evolutionary niche intermediate between ancestral vertebrates and the gnathostomes (jawed vertebrates). Gonadotropin releasing hormone (GnRH) modulates neuroendocrine activity in vertebrates and works in the context of the hypothalamic-pituitary (H-P) axis. The appearance of this neuroendocrine axis marks one of the most crucial developmental achievements in vertebrate evolution, because it enabled further diversification in general growth, metabolism, osmoregulation and reproduction as jawed vertebrates evolved. GnRH studies in hagfish draw attention because such work may be considered as providing proxy data for similar investigations conducted upon long extinct species. Indeed, the fossil record reveals little anatomical difference between those hagfish living 300 million years ago and their modern descendants. Accordingly, the hagfish can offer important evolutionary lessons as they have some highly unusual characteristics not seen in any other vertebrate; they retain many representative features of an ancestral state from which all vertebrates originated. Indeed, because central control of reproduction is perhaps the most basic function of the vertebrate H-P axis, and given the importance of GnRH in this network, research on GnRH in hagfish can help elucidate the early evolution of the H-P system itself. Like all vertebrates, hagfish have a functional hypothalamic area and a pituitary gland, constituting a basic H-P axis. But what role does GnRH play in the reproductive system of this “living fossil”? How can understanding GnRH in hagfish help advance the knowledge of vertebrate neuroendocrinology? Here, information on neuroendocrine function and the role of GnRH specifically in this very basal vertebrate is reviewed.

## DESCRIPTION AND TAXONOMY

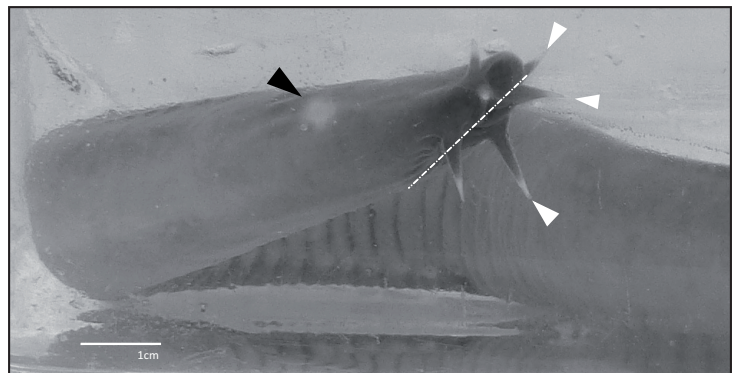
Hagfishes are eel-like jawless vertebrates that occupy the deepest levels of the ocean (Figure 1). They are highly efficient benthic scavengers, but may also occasionally hunt for live prey. Hagfish defensively secrete copious amounts of thick mucus, presumably to clog the gills of an attacker, when threatened (Winegard & Fudge 2010). Having teeth but no jaw, the hagfish mouth is especially peculiar. It consists of a retractable cartilaginous strip with two pairs of comb shaped keratin-based teeth (a “rasping tongue”), which attaches to food and conveys it toward the pharynx. The mouth is not involved in the intake of respiratory water, but instead water is siphoned through a single nasopharyngeal duct (nostril) at the head’s anterior central aspect. Hagfishes are included in the clade Cyclostomi with lampreys, notably because of their tongue apparatus, teeth, and gill configurations.

The term Agnatha (“no jaw”) was first used in the 19<sup>th</sup> century to describe a group that included the Cyclostomes (hagfish and lampreys) as well as several fossil groups where jaws were developmentally absent. While this classification has been generally useful, the precise position of hagfish within the Agnatha (*e.g.*, even the presence of true vertebrae in hagfish has been disputed) remains challenging (Janvier 2011). All living vertebrates may be divided into two major monophyletic groups, the Cyclostomata and the Gnathostomata (jawed vertebrates). The finding that gills were of endodermal origin in the cyclostomes, but were of ectodermal origin in gnathostomes, was thought to validate this schema. Refinement in evolutionary concepts led to searches for gnathostome ancestors, causing some armored fossil agnathans (especially the “ostracoderms” – Anaspida, Thelodonti, Heterostraci, Galeaspida and Osteostraci) to be regrouped more closely with gnathostomes than to cyclostomes. These fossil agnathans, although clearly jawless, are thus currently regarded as stem gnathostomes. Stensiö (1927) suggested that living cyclostomes were “diphyletic”, with hagfishes and lampreys arising separately (from two fossil groups of “ostracoderms”) but forming a single clade. This view is

no longer widely held, and the cyclostomes are regarded as having diverged before all the vertebrates that are able to produce bone; that is “ostracoderms” and gnathostomes. Fossil, non-skeletonized agnathans are exceptional, and lamprey and hagfish anatomy are so different that it is difficult to reconstruct a single ancestral morphotype to fit both forms; interestingly, there is currently no fossil taxon that is neither a fossil hagfish nor a fossil lamprey which is more closely related to the cyclostomes than to gnathostomes (Janvier 2008). The discovery of likely fossil hagfish, *Myxinikela* and *Myxineidus*, found in about 300 million year-old sediments, have placed the evolutionary significance of the hagfish into sharper focus (Bardack 1991; Poplin *et al.* 2001). Hagfish frustrate easy biological classification because they display numerous characteristics found in no other vertebrate, including the absence of radial muscles in unpaired fins, no cardiac innervation, and rudimentary optic organization (no lens or extraocular musculature), which are now regarded as secondary losses of phenotypic characters.

The fossil record suggests the first appearance of possible early chordate and even vertebrates during the Cambrian Period, about half a billion years ago (Shu *et al.* 2003; Brysse 2008). Although the still controversial, conodonts are regarded as possible vertebrates and even considered as possible cyclostome relatives; undoubted vertebrates represented by the earliest “ostracoderms”, or armored agnathans appear soon after in the fossil record (*e.g.*, late Ordovician Period – 450 million years ago), and subsequently the ancestral gnathostomes (Silurian Period – 420 million years ago) (Dores 2011). Although many agnathans existed in the Ordovician Period, the Myxiniformes (hagfish) and the Petromyzontiformes (lampreys) are the only two that have survived to the present. The chordate phylum displays several unifying themes in terms of overall somatic organization, compared to other metazoan phyla. The essential phylum designation requires a dorsal nerve cord, a notochord, and pharyngeal gills slits at some point in the developmental life history of the organism. All hagfish are assigned to the family Myxinidae, which is further split into two primary groups: *Myxine* and

**Fig. 1.** The hagfish eye does not have an iris, lens, or cornea, and is devoid of any extraocular musculature. Its “eye spot” is demarcated by unpigmented, translucent skin (black arrow), and includes a retina with only two main nuclear layers without bipolar or amacrine cells. Photoreceptors connect directly to output neurons (ganglion cells) which project predominantly to the hypothalamus. Three barbell pairs (2 oral + 4 nasal) are innervated by >5,000 afferent fibres (trigeminal) via ophthalmic nerves, bilaterally. Free nerve terminals populate the epidermal layer particularly localized to the barbell tip (white arrows), and enable a high level of mechanosensitivity and stereognostic perception. The rasping tongue (retracted) operates in a longitudinal axis for feeding (dashed line).



Eptatretus. The genus *Myxine* has approximately 20 species and is found in the Atlantic Ocean, while *Eptatretus* contains nearly 40 species having a Pacific Ocean distribution (Martini *et al.* 1998).

## ANCESTRAL GnRH, HAGFISH, AND THE EVOLUTION OF VERTEBRATES

The anatomical/structural differences which appeared gradually during vertebrate evolution were accompanied by some functional change of GnRH over time, and this appears to represent an adaptive evolutionary response. For example, the functional menu of GnRH peptides may have enlarged during the time when vertebrate brains became larger and thicker, placing greater distances between hypothalamus and pituitary. Kavanaugh *et al.* (2008) have hypothesized that the ancestral GnRH that gave rise to lamprey GnRH-II and Gnathostome GnRH may have originally been a hypothalamic GnRH in some extinct vertebrate, and after the duplication event (Fujimoto *et al.* 2013) the GnRH-II in Gnathostomes was “co-opted” by other cells in the brain (midbrain, olfactory region) with diverse function. In the Gnathostomes, GnRH-II predominates in extrahypothalamic regions and may act as a neuromodulator/neurotransmitter (Kavanaugh *et al.* 2008). However, there are species in which GnRH-II is found in the hypothalamic regions with pituitary action to stimulate gonadotropin function (King & Millar 1995).

In 1995, chromatographic investigations using antisera directed against mammalian GnRH and lamprey GnRH-I, and immunocytochemical data on *Myxine glutinosa* (Atlantic hagfish) revealed that the brain contains a gonadotropin-releasing hormone (GnRH)-like molecule structurally similar to lamprey GnRH-III. When lamprey GnRH-I antiserum was used, an early eluting GnRH form did coelute on HPLC with lamprey GnRH-III standard, and an unknown form coeluted with the chicken GnRH-II standard. GnRH immunoreactivity was identified in hagfish brain only when the lamprey GnRH-I antibody was used. These experiments by Sower and colleagues (1995) were the first to provide compelling evidence that hagfish possess a GnRH-like molecule; this new GnRH was further characterized as more structurally allied to lamprey GnRH-III than to any other known vertebrate GnRH molecule.

The degree of molecular variation in GnRH structure is somewhat surprising, given the relative constancy of its physiological role in vertebrates. Yet considerable evidence now exists to show that most vertebrates produce multiple GnRH isoforms (Dubois *et al.* 2002; Silver *et al.* 2004; Guilgur *et al.* 2006; Kah *et al.* 2007). Investigations mapping GnRH primary sequences and studies of their respective cDNAs have facilitated a better understanding of the phylogenetic relationships among the various GnRHs (Fernald & White 1999; Parhar 2002; Morgan & Millar 2004; Silver *et al.* 2004; Kavanaugh *et al.* 2008; Zhang *et al.* 2008).

Thus far, every vertebrate species studied has demonstrated the presence of at least two GnRH forms coexisting in the central nervous system. However, it is now known that three forms of GnRH exist in bony fish. Specifically, cGnRH-II is expressed by midbrain neurons, a species-specific GnRH is present mainly in the preoptic area and hypothalamus, while sGnRH is localized to the terminal nerve ganglion (Somoza *et al.* 2002). One interesting conceptual model proposes that if three GnRH ligands (and their corresponding receptors) are expressed in the central nervous system of a given species, then it is possible to identify three different GnRH lineages expressed by distinct brain areas in vertebrates. These lineages would include the conserved cGnRH-II or mesencephalic lineage, the “releasing” hypothalamic lineage with a primary structure diverging by specific point mutations, and a telencephalic form (Somoza *et al.* 2002).

More than a decade ago, a previously unknown hypothalamic transmitter was identified in quail, and this dodecapeptide (GnIH) directly inhibited release of pituitary gonadotropins (Tsutsui *et al.* 2000). GnRH neurons are a target of GnIH and seem to function in the hypothalamus via a novel G protein-coupled receptor for GnIH. As its name implies, GnIH opposes GnRH action and inhibits gonadal development by squelching gonadotropin production and output. This hypothalamic interplay between GnRH and GnIH seems consistent among numerous vertebrate species, including reptiles, amphibians, teleosts and mammals. Parallel with GnRH, GnIH and its homologs also share a common structural motif. A mammalian GnIH homolog inhibited gonadotropin release in mammals, as GnIH action originally was observed to do in birds. But unlike higher vertebrates, hypophysiotropic activities of GnIH homologs were not the same in lower vertebrates and required further study. A better understanding of the evolutionary origin of GnIH and its homologs was gained by research on RFamide peptides from hagfish and sea lamprey brain. Of note, the RFamide peptide and its cDNA were identified only in the brain of hagfish (Tsutsui & Osugi 2009).

## NEUROENDOCRINE AND REPRODUCTIVE CORRELATIONS

While the overall morphological design of vertebrates derives from several common somatic themes, there are aspects of chordates which show considerable evolutionary modification. One of these features is the anatomical connection (and physiological interaction) between pituitary and brain. For example, the capillary network of the median eminence is absent in teleosts, and hypophysiotropic neurons innervate the anterior pituitary directly. These neurons stimulate specific hormone-producing cells localized to the teleost anterior pituitary. For hagfish, although they do have a pituitary gland, a median eminence capillary network and evi-



dence for direct innervation of the pituitary by putative hypophysiotropic neurons are both lacking (Dores 2011).

The vertebrate hypothalamus coordinates many vital processes, including governance of endocrine-mediated circadian rhythms and multiple complex neuroendocrine outputs. One of these transmissions involves release of GnRH, which originates in the medial preoptic nucleus. This structure is laterally bounded by the lateral preoptic nucleus and is medially bounded by the preoptic periventricular nucleus. In mammals, magnocellular neurosecretory axons from the paraventricular nucleus and the supraoptic nucleus (containing ADH and oxytocin) merge with the posterior pituitary. Parvocellular neurons of the paraventricular nucleus have neurons which release hormones into the hypophyseal portal system, subsequently connecting to the anterior pituitary. Hagfish neuroanatomy features a poorly differentiated hypothalamus and forebrain (Kusunoki *et al.* 1981). A very limited ventricular system exists in the hagfish brain, and there is a “primordium hippocampi” in the telencephalon of unresolved homology (Kusunoki & Amemiya 1995). Perhaps not surprising for a nearly blind animal living in deep sea darkness where light, time, and seasonal variation would be largely irrelevant, the pineal body is absent in hagfish (Vernadakis *et al.* 1998). Of note, while basic organization of the hagfish brain including prosencephalon, mesencephalon, and rhombencephalon shares features with the Gnathostomata, important variations in developmental variation are apparent in sensory centers and higher information processing centers (Kusunoki & Amemiya 1995).

Immunolabeling studies specific for GnRH in hagfish brain have been helpful in localizing GnRH cell bodies in two systems: the infundibular hypothalamus (a neuromodulatory nucleus with diffuse projections to most areas of the brain), and a primarily preoptic system of putatively hypophysiotropic neurons projecting to the neurohypophysis (Braun *et al.* 1995). This finding in hagfish was parallel to reports in other Craniates where a preoptic-hypophysiotropic system had been established, thus supporting the general homology of this system in hagfish and vertebrates. The homology of the distributed hypothalamic system is more dubious, and some investigators (Braun *et al.* 1995) have proposed the two systems have distinct functions in hagfish. While GnRH and GnRH-like molecules in ancestral Craniates may have had both neuromodulatory and hypophysiotropic roles, the hagfish brain-pituitary axis is more similar to that of vertebrates than initially suggested (Braun *et al.* 1995).

The hagfish adeno-hypophysis consists of only a grouping of cells separated from the neurohypophysis by connective tissue, with no clear cytological distinction between the pars distalis and the pars intermedia (Uchida *et al.* 2010). It had been uncertain if hagfish pituitary even contained any tropic hormones (Matty *et al.* 1976) but subsequent research localized immu-

noreactive gonadotropic hormones to the adeno-hypophysis, and also demonstrated that cells containing such hormones predominated in adults with developing gonads (Miki *et al.* 2006). For jawed vertebrates, these gonadotropins are released in response to hypothalamic GnRH, and even lampreys have a functional HPG axis (Sower *et al.* 2009). In contrast, knowledge of the hypothalamic-pituitary-gonadal axis in hagfish has accumulated much more slowly. The initial dearth of information on hagfish pituitary function was due the difficulty in ascertaining reliable data from typical cytological and physiological studies (Uchida *et al.* 2010). For example, complete hypophysectomy in *Eptatretus stouti* did not provide clear evidence for pituitary gonadotropic activity (Matty *et al.* 1976). In contrast, observations from *Eptatretus burgeri* showed impairment of gonadal development and spermatogenesis following hypophysectomy (Patzner & Ichikawa 1977). These conflicting reports fostered an early belief that the pituitary-gonadal axis in hagfish was probably either absent, or minimally functional.

Their primordial follicles (early oocytes) appear to form in cyst-like cell nests, yet later oocytes may be derived directly from germinal epithelium. For more than 100 years, “brown bodies” have been observed in hagfish gonads without a full understanding of their function. Modern microscopic investigations of these structures have determined that hagfish gonads have progesterone-producing corpora lutea (Powell *et al.* 2006). In larger specimens, where testicular development occurs in the posterior gonad, the anterior gonad can regress, leaving a thin structure without germ cells. However, considerable variation has been noted in this schema which implies plasticity of the hagfish gonadal region such that it could undergo either male or female differentiation. Indeed, hermaphroditic specimens have been described where a well-differentiated testis existed posteriorly with a well-differentiated ovary (vitellogenic eggs) positioned anteriorly (Gorbman 1990). The developmental sequence of hermaphroditism in hagfish, their apparent lability of sex determination, and putative epigenetic factors associated with these phenomena were extensively reviewed by Gorbman (1990). It may be that GnRH plays some role in modulating these key maturational events during the hagfish's reproductive career.

Hagfish produce 12–45 prolate ellipsoidal eggs, each about one inch in length. These have a thick chorion, a large chorion:egg width ratio, a sizable micropylar cup featuring a unique substructure, and appendage tufts interspersed with adhesive substances derived not only from the follicular epithelium but also from the slime glands (Koch *et al.* 1993). Although the fertilization process in hagfish remains incompletely characterized, hagfish sperm do include an acrosome. Indeed, in hagfish sperm undergoing acrosomal exocytosis the outer acrosomal membrane and the overlying plasma membrane vanish and are replaced by a vesicular array,

resembling an early stage acrosome reaction in spermatozoa of higher vertebrates where no formation of an acrosomal process occurs (Morisawa & Cherr 2002).

## GENES ENCODING FOR GNRH AS EVIDENCE FOR EVOLUTIONARY CHANGE

Considering the entire spectrum of animal phyla, a great number of exceptions from the rule of equal segregation of chromosomes during cell division exist in numerous animal species. The most diversified is the process of chromosome re-arrangement during specification of somatic vs. germ-line cell fate in embryos, a process also evident in hagfish (Kloc & Zagrodzinska 2001). Many taxa demonstrate chromatin diminution (loss of selected chromosomal regions during differentiation of early embryonic cells into somatic cells), including the hagfish (Drouin 2006). However, this process does not appear uniform for all hagfish species. For example, while chromosome reduction occurs in cells of *Myxine glutinosa* (Sweden/Baltic Sea), *Eptatretus cirrhatius* (New Zealand/Pacific Ocean), and *E. stoutii* (Canada/Pacific Ocean), the amount of DNA eliminated from presumptive somatic cells averaged 43.5%, 48.7%, 54.6% and 52.8% respectively (Nakai *et al.* 1995).

Thus far, only two repetitive DNA segments have been found in chromosomes eliminated from somatic cells of *E. okinoseanus* (Japanese hagfish). Using molecular analyses, a second germ line-restricted and highly repetitive DNA family has been detected in another hagfish of Japan, *Paramyxine atami*. While the patterns and intensities of hybridization signals suggest that this DNA segment has undergone concerted molecular evolution (Kubota *et al.* 1997), other research favors chromosome terminus elimination (and entire chromosome elimination) as the mechanism of somatic chromosome differentiation. Telomere-FISH further showed that chromosome fragmentation and subsequent *de novo* addition of telomeric repeats could help explain chromosome terminus elimination. Such findings make further study of reproduction in general – and molecular processes of chromosome elimination specifically – among hagfish important (Kojima *et al.* 2010).

In the lancelet, a complete set of genes for sex steroid hormone-producing enzymes exists plus genes for steroid hormone receptors, one more closely related to vertebrate estrogen receptors (Holland *et al.* 2008). This observation is in contrast to Ciona (sea squirts), where only a limited number of genes exist for enzymes synthesizing steroid hormones, suggesting tunicate-specific losses (Campbell *et al.* 2004). Interestingly, neither amphioxus nor tunicates have homologs of anterior pituitary hormones (i.e., gonadotropins, growth hormone, ACTH), and genes encoding for their corresponding receptors are also absent from the amphioxus and tunicate genome (Uchida *et al.* 2010).

Prior to (or at some point nearly simultaneous with) early Agnathan evolution, two functionally distinct pituitary elements evolved to establish the complex operation of the brain-pituitary axis: (a) the posterior pituitary (neurohypophysis), a ventral hypothalamic out-pouching, and (b) the adenohypophysis, developing from endoderm in hagfish (but of ectodermal origin in all other vertebrates) (Gorbman 1983). Thus, studies on hagfish do provide evidence to support a crucial reproductive role for hypothalamic GnRH via its capacity to stimulate gonadotropin release from the adenohypophysis (Uchida *et al.* 2010). For vertebrates, the pituitary gland has therefore evolved as a physiological conductor of neural signals (from the hypothalamus) to hormonal information (to the gonad). It is plausible that concomitant with emergence of the pituitary gland at an early stage of vertebrate evolution, some vertebrate ancestor had already acquired a fundamental pituitary-gonadal axis using GnRH and ancestral gonadotropins.

## CONCLUSION

The curious and contentious phylogenetic classification of hagfish is certain to make them a subject of ongoing interest to anatomists and zoologists, especially in the context of vertebrate neuroendocrine evolution (Ota & Kuratani 2006; Herzig *et al.* 2012). Research on GnRH in hagfish provides a rare insight into a primordial neuroendocrine milieu which could approximate what may have been present in animals living more than 300 million years ago. As the basal exemplar of all vertebrates, hagfish are well suited to help clarify how GnRH and other signal mediators evolved among all vertebrates (Uchida *et al.* 2010). Their reproductive cycle remains poorly understood; thus far no specimens have been successfully mated in captivity (Powell *et al.* 2005). To be sure, progress in neuroendocrinology depends on the systematic investigation of potentially active hypothalamic neuropeptides in various animal models. Information about hagfish is growing slowly, but unfortunately remains limited. Commercial interest in the hagfish has increased as traditional fishery stock harvests have receded in some regions. While attention has recently turned to the hagfish as a potential source of edible meat and skin, there are no regulations on hagfish harvesting throughout America's eastern seaboard at present (Powell *et al.* 2005). As fascinating as hagfish may be in their abstract and conceptual role in supplying missing pieces to the vertebrate evolutionary puzzle, there are important practical reasons to study them, too. Considering that hagfish populations are being severely reduced secondary to overfishing in some locations (Powell *et al.* 2005), the need for improved understanding of its reproductive physiology, developmental history, age determination, growth rate and life span will take on renewed urgency.

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