Roles of copper in neurokinin B and gonadotropin-releasing hormone structure and function and the endocrinology of reproduction.

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Abstract

Copper is a metal ion present in all organisms, where it has well-known roles in association with proteins and enzymes essential for cellular processes. In the early decades of the twentieth century copper was shown to influence mammalian reproductive biology, and it was subsequently shown to exert effects primarily at the level of the pituitary gland and/or hypothalamic regions of the brain. Furthermore, it has been reported that copper can interact with key neuropeptides in the hypothalamic-pituitary-gonadal axis, notably gonadotropin-releasing hormone (GnRH) and neurokinin B. Interestingly, recent phylogenetic analysis of the sequences of GnRH-related peptides indicates that copper binding is an evolutionarily ancient property of this neuropeptide family, which has been variously retained, modified or lost in the different taxa. In this mini-review the metal-binding properties of neuropeptides in the vertebrate reproductive pathway are reviewed and the evolutionary and functional significance of copper binding by GnRH-related neuropeptides in vertebrates and invertebrates are discussed.

1. Introduction

Copper is an essential trace element that is associated with a diversity of protein types, ranging from enzymes to neuropeptides. The metal's role in mammalian reproductive endocrinology was first documented over 80 years ago, but it is only relatively recently that details are emerging of how copper affects the structure and function of neurohormones and neuropeptides involved in reproductive pathways, particularly gonadotropin-releasing hormone (GnRH) (Fevold et al., 1936; Michaluk and Kochman, 2007) and the tachykinin neurokinin B (Russino et al., 2013; Gul et al., 2018). Orthologues and paralogues of mammalian GnRH are present across bilaterian animals and many of these peptides have sequence motifs that are known to coordinate copper, suggesting the metal has a role in their biological functions. Here we explore how copper influences mammalian reproductive signalling and highlight how metal-binding may be an evolutionarily ancient feature that has been variously retained or lost in GnRH-related neuropeptides.

2. The inorganic biochemistry of copper

Copper can act as a structural and/or regulatory cofactor in a wide range of proteins, but is predominantly used in enzymes that exploit the metal's redox ability. *In vitro*, copper has a number of accessible oxidation states ranging from Cu^+ to Cu^{4+}, but at biologically relevant redox potentials the chemistry is largely limited to the $Cu^{2+/+}$ redox couple. The redox cycling that is often a key role for copper is also potentially dangerous, and biological systems have developed sophisticated mechanisms to control uptake, delivery and use of the metal and avoid spurious redox activity (Inesi, 2017). Cu^+ is the relevant oxidation state in the reducing intracellular conditions whereas it is likely that Cu^{2+} is prevalent in the more oxidising extracellular environment. Cu^+ and Cu^{2+} are chemically different species; for

instance, when binding to proteins Cu^+ preferentially coordinates to sulfur atoms (e.g. cysteine/methionine amino acids) with low coordination numbers whereas Cu^{2+} preferentially coordinates to nitrogen/oxygen ligands with higher coordination numbers (Frausto da Silva and Williams, 1991). A key nitrogen donor in peptides and proteins is the histidine imidazole group and binding is driven by the pK_a of the imidazole group being approximately 6.4, which allows coordination at biologically relevant pHs. Histidine imidazole coordination is observed in proteins such as Cu-Zn superoxide dismutase (SOD), but Cu^{2+} is rare in that it is also able to deprotonate peptide nitrogens and thus coordinate to backbone nitrogens. This type of coordination is usually observed in protein regions that lack structure, given the backbone structuring and rearrangement required to allow binding, and is thus quite relevant to neuropeptides/neurohormones that are often natively unstructured. A common copper-binding motif utilising histidine and backbone nitrogens is the amino-terminal Cu(II) and Ni(II) (ATCUN) motif. The key to the motif is the presence of a histidine at position 3 in the primary sequence, and Cu^{2+} coordination occurs via the histidine imidazole Nε, the histidine amide nitrogen, the amide nitrogen of the amino acid at position 2 and the N-terminal amine to give a four-nitrogen complex (Fig. 1) (Sovago et al., 2016). This site usually has high thermodynamic stability ($K_d < 10^{-9}$ M) that can be enhanced by the presence of bulky, hydrophobic amino acids at positions 1 or 2 in the sequence (Trapaidze et al., 2012; Miyamoto et al., 2016). The ATCUN motif is observed in proteins such as serum albumin and in neuropeptides such as neuromedin C, and the motif is likely an evolutionarily ancient metal-binding site given its presence in a variety of protein types that occur in phylogenetically diverse species (Harford and Sarkar, 1995; Jones et al., 2016).
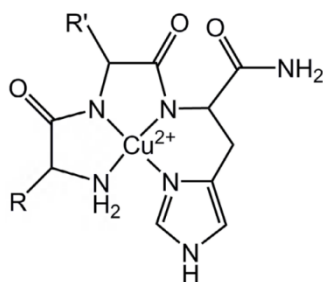
Fig. 1. General structure of a Cu(II)-bound Amino Terminal Copper and Nickel binding motif (ATCUN) in a tripeptide of general sequence XXH (X is any amino acid) at physiological pH. R refers to the side chain of amino-acid one, R' is the side chain of amino-acid two.

3. Copper in nervous system physiology

Despite representing only ~ 2% of the total body mass, the brain has the highest oxidative metabolism of all body organs and utilises ~ 20% of the total oxygen uptake. Due to the high metabolic demand, significant bound copper is present in mitochondrial cytochrome c oxidase, the multi-copper oxidase that is the terminal electron acceptor in the mitochondrial electron transport chain, and Cu-Zn SOD, which catalyses the disproportionation of harmful superoxide radicals (Rubino and Franz, 2012). These cuproproteins are found in all cells, but there are also brain-specific cuproproteins, such as dopamine β-monooxygenase which catalyses the oxidative hydroxylation of dopamine to noradrenaline, and a homologue of ceruloplasmin (Jeong and David, 2003). High demand for copper is also due to the synthesis of peptide neurotransmitters and neurohormones, many of which are C-terminally amidated to protect them from degradation by carboxypeptidases (Lutsenko et al., 2010). The cuproenzyme that catalyses C-terminal amidation is peptidylglycine α-amidating monooxygenase present in the secretory pathway of neuronal cells (Gaier et al., 2013). The adult brain in humans contains approximately 7% of the total copper present in the body. Although copper levels vary across different brain regions and

between species, relatively high concentrations are generally observed in the hypothalamic region, hippocampus and cortex (Merriam et al., 1979; Dobrowolska et al., 2008; Hare et al., 2012).

Intriguingly, some neuronal cells are able to release copper that appears to be 'free' or at least not bound to high-molecular weight species, such as proteins (Schlief et al., 2005; Schlief and Gitlin, 2006). Copper can accumulate in synaptosomal and endosomal fractions and be released by membrane depolarisation leading to synaptic concentrations that can be high, with some estimates near 100 μM although these estimates remain contentious and may more likely be in the low μM range (Kardos et al., 1989; Hopt et al., 2003; Lutsenko et al., 2010). The role of copper once released into the synapse is not clear but its interaction with receptors, neurotransmitters and signalling pathways suggests a very diverse role in the extracellular milieu of the brain (Badrick and Jones, 2010; Lutsenko et al., 2010; Gaier et al., 2013; Grubman and White, 2014; D'Ambrosi and Rossi, 2015).

4. The role of copper in the reproductive axis

Although the hypothalamus and pituitary gland are rich in copper, there is no clear understanding of how copper may influence the function of these brain regions. In humans and other vertebrates, the hypothalamus and pituitary gland are key players in neuroendocrine control of reproduction. Notably, follicle stimulating hormone (FSH) and luteinizing hormone (LH) are peptide hormones released from gonadotropic cells in the anterior pituitary gland and release of these hormones occurs in an orchestrated manner as part of the hypothalamic-pituitary-gonadal axis (Fig. 2). FSH is an essential follicular growth hormone that regulates the development and maturation of reproductive organs in the body. In women FSH stimulates the production of the sex steroids oestradiol and progesterone as well as the

growth of the ovarian follicle. LH is released in a pulsative manner and stimulates the

maturation of the ovarian follicle and the formulation of the corpus luteum thus stimulating

ovulation (Brinkley, 1981; Lunenfeld and Buhler, 2018).

A key breakthrough in reproductive biology was made in the early 1970s with the

identification of a neuropeptide, gonadotropin-releasing hormone (GnRH,

pQHWSYGLRPG-NH$_2$), that triggers pituitary release of LH and FSH (Amoss et al., 1971;

Schally et al., 1971). The primary effect of GnRH is to stimulate the synthesis and release of

LH and FSH and thus GnRH has a key role in vertebrate reproduction. Subsequently, a

neuropeptide related to GnRH was discovered in mammals and this is now known as GnRH-

II (pQHWSHGWYPG-NH$_2$), and the original peptide as GnRH-I (Sherwood et al., 1993;

White et al., 1998). GnRH-I and GnRH-II have been identified in most vertebrates and it is

likely that whilst GnRH-II does have some role in reproductive function, it also has other

non-reproductive roles (Desaulniers et al., 2017; Sakai et al., 2017). Additionally, a third

form of GnRH has been identified, but appears to be restricted to fish and amphibians (Sakai

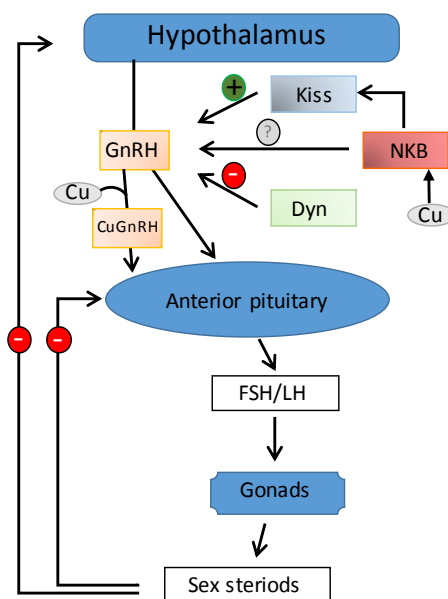et al., 2017). In this review we focus primarily on GnRH-I and its metal interactions.

Fig. 2. Schematic of signalling molecules associated with the hypothalamic-pituitary-gonadal
axis in humans. Gonadotropin-releasing hormone (GnRH) acts on the gonadotrope cells in
the anterior pituitary which release FSH and LH leading ultimately to release of sex

hormones from the gonads. GnRH release is inhibited by negative feedback from the sex hormones. Pulses of GnRH release are triggered by kisspeptin (Kiss) and supressed by Dynorphin (Dyn). Kisspeptin release is regulated by neurokinin B (NKB) which may also have a direct action on GnRH releasing neurons. Copper can bind to GnRH and/or to NKB to modulate the HPG axis.

In humans and some other vertebrates GnRH-I release is regulated by the actions of the kisspeptin, dynorphin and neurokinin B signalling systems. Kisspeptin triggers release of GnRH-I, dynorphin inhibits release and the tachykinin neurokinin B (NKB) regulates release of kisspeptin, at least at the initiation of puberty (Fig. 2) (Topaloglu et al., 2009). Thus, NKB has an indirect effect on GnRH-I release, although it may also have direct effects on release given that GnRH-expressing hypothalamic neurons also express the NKB receptor, NK3R (Gaskins, 2013). A relationship between the HPG-axis and copper has been known for many years. In 1936 Fevold et al. showed that administration of inorganic copper salts to rats increased the action of FSH and LH and induced ovulation. Subsequent studies showed that ovulation in rabbits and rats was increased when copper sulphate was injected into the median eminence, suggesting that the effect of copper was occurring at the level of the hypothalamus rather than at the gonadotropes or the ovaries themselves (Hiroi et al., 1965; Merriam et al., 1979). Several studies have since recapitulated this early work, going on to show that ovulation was likely induced through the ability of extracellular copper to promote GnRH-I release, especially when the metal was delivered as a complex with amino acids (Tsou et al., 1977; Rice and Barnea, 1983; Barnea and Colombani-Vidal, 1984; Schvartz and Hazum, 1986). Given the release of copper from synaptosomes derived from the median eminence, a plausible hypothesis is that copper released from hypothalamic neurons can affect LH/FSH release *in vivo* (Kardos et al., 1989). Thus, in line with the studies of exogenously added Cu(II), endogenous Cu(II) may interact with receptors and other extracellular components to influence LH/FSH release. One of these components may be GnRH-I itself. Indeed, investigation by (Kochman et al., 1992) showed that GnRH-I was

able to bind copper and there was a pronounced increase in the release of LH and FSH when metal was bound to GnRH-I compared to the metal-free peptide or copper alone. The enhanced release of LH and FSH was considered to be due to an increased binding affinity of the metal-bound peptide to the GnRH receptor. A more recent study provided another perspective on how the copper-GnRH complex may enhance LH/FSH release by showing that it modified the intracellular signalling pathway compared to GnRH alone. The complex appears to activate the cyclic-AMP/protein kinase A (cAMP/PKA) pathway in preference to the inositol triphosphate/protein kinase C pathway triggered by the binding of metal-free GnRH to the GnRH receptor (Kochman et al., 2005; Gajewska et al., 2016). Activation of the cAMP/PKA pathway is thought to occur via binding of the complex with the GnRH receptor, however interaction of Cu-GnRH with the pituitary adenylate cyclase-activating polypeptide type 1 receptor was not discounted (Gajewska et al., 2016). Given that the Cu(II) coordinating amino acids of GnRH-I are $pGlu_1$ and His_2 and both these residues have been shown to be important for activating the receptor (*vide infra*) (Sealfon et al., 1997), it is plausible that modifying the structure of the N-terminal region could influence downstream intracellular signalling pathways.

In addition to GnRH, another peptide in the HPG pathway, neurokinin B, has also been shown to coordinate copper, but the impact of copper-bound NKB on downstream GnRH activity and/or LH/FSH release has not been investigated (Fig. 2) (Russino et al., 2013). The affinity of Cu(II) for NKB is higher than that of GnRH, and NKB can strip GnRH of bound metal (Gul et al., 2018). The physiological consequence of this is not clear, but it might suggest that NKB not only shapes GnRH release via its effect on kisspeptin signalling but also fine-tunes or modulates the activity of GnRH by regulating the binding of Cu(II). At the level of the hypothalamus/pituitary copper appears to have a multimodal effect in vertebrate reproductive physiology, acting on GnRH receptors not only as 'free' or ionic

copper, but in complex with amino acids and also neurohormones/neuropeptides directly involved in the HPG-axis. Indirectly, copper can mediate GnRH release because it is a cofactor in dopamine β-monooxygenase, the enzyme that generates noradrenalin which is an important neurotransmitter involved in GnRH secretion (Herbison, 1997; Han and Herbison, 2008). Later in the HPG axis, copper appears to have complex dose dependent effects in the gonads and, as an example, in experiments with immature male rats, it influences serum testosterone levels and spermatogenesis (Roychoudhury et al., 2016; Ogorek et al., 2017). Further, the effects of copper on gamete formation and reproduction in marine organisms is being widely studied as a result of anthropogenic changes to copper levels in the marine ecosystem (Biandolino et al., 2018; Phillips and Rouchon, 2018; Lettieri et al., 2019).

5. Copper binding to GnRH-I and NKB modifies the structure and function of these neuropeptides

The relationship between the sequence of the vertebrate GnRH-I peptide (pQHWSYGLRPG-NH$_2$) and receptor binding and activation has been studied for many years. The sequences of the N- and C-terminal domains of the peptide are highly conserved in vertebrates and are essential for receptor binding and activation. Critically, the aromatic nature of His$_2$ appears important for both receptor binding and activation because loss of this residue significantly reduces the activity of the peptide (Sealfon et al., 1997). The glycine in position 6 is highly conserved in vertebrates and is considered important for the folding of the peptide and its bioactivity. The presence of the glycine is thought to allow the formation of a type-II β-type turn that stabilizes the peptide conformation and increases receptor binding affinity (Sealfon et al., 1997). These types of turns are rare but not unprecedented in peptides, even in aqueous solution, and may also be adopted on interaction with the receptor (Jen et al., 2013). The arginine in position 8 is essential in mammals as it is critical for high

affinity binding to mammalian GnRH receptors through the interaction with an aspartate present in the third extracellular loop of the receptor (Fig. 3A) (Petry et al., 2002; Millar et al., 2004). The binding of copper to GnRH-I is thought to require the amide of $pGlu_1$, the His_2 imidazole N_δ and the His_2 amide nitrogen to form a 3-nitrogen complex with a solvent molecule as a fourth ligand (Gerega et al., 1988; Nakamura et al., 2005). This binding can be predicted to disturb the ability of His_2 to interact with the receptor in the manner it does in metal-free GnRH-I and could account for the differential receptor signalling observed for the metal complex compared to the metal-free peptide. This would be further supported by N-terminal copper-binding not affecting the β-turn, with the overall structure remaining similar to the metal-free peptide, particularly in the C-terminal, receptor-binding region, Arg_8 – Gly_{10}. The copper-complex will therefore bind to the GnRH receptor, but the activation profile will be different, as is observed experimentally (Kochman et al., 1992; Gajewska et al., 2016). A benefit of metal coordination to the N-terminal region is that the complex appears more resistant to degradation by peptidases, suggesting that enhanced LH/FSH release may be not only due to modified receptor binding properties, but also due to an increased half-life of the peptide (Herman et al., 2012).
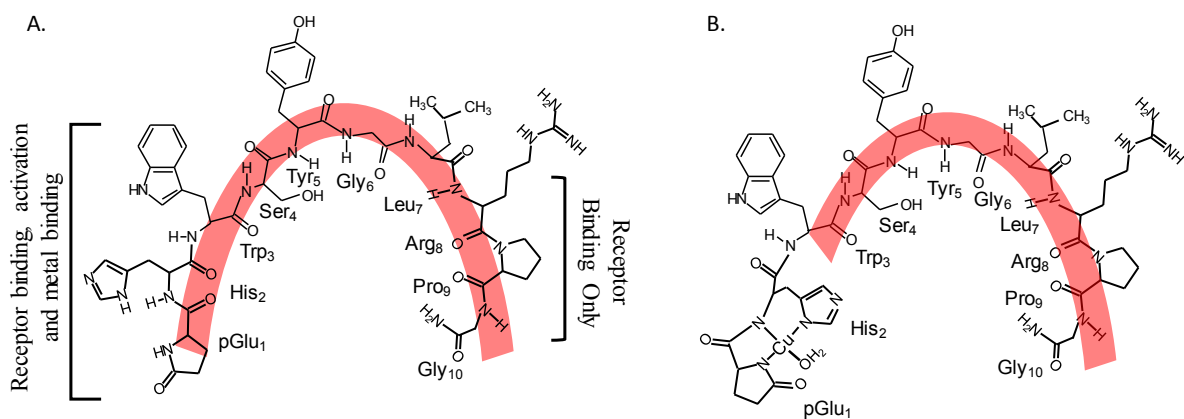

Fig. 3. Schematic of mammalian GnRH-I decapeptide in the absence and presence of copper. (A) The N-terminal amino acids are thought to promote receptor binding and activation, whereas the C-terminal residues allow high affinity binding. The presence of Gly_6 is thought to support the presence of a type-II β-turn in the peptide. Figure adapted from (Sealfon et al.,

1997). (B) Cu(II) coordination requires $pGlu_1$ and His_2 and alters the structure of the N-terminal region of the peptide in comparison with copper-free peptide shown in (A).

As discussed above, NKB contributes to the shaping of GnRH-I pulses by regulating the release of GnRH-I either indirectly via kisspeptin, or directly by activating neurokinin B receptors on GnRH-I releasing neurons (Fig. 2) (Corander et al., 2010). NKB is a decapeptide (DMHDFFVGLM-NH$_2$) containing the classic N-terminal ATCUN motif XXH predicted to bind copper (Fig. 1). Accordingly, this peptide was shown to bind copper in both the Cu^{2+} and Cu^+ oxidation states, although the Cu(II) coordination is less well established than the Cu(I) coordination (Russino et al., 2013; Grosas et al., 2014). It was proposed that NKB forms a mononuclear dimer ($[Cu^{II}(NKB)_2]$), with the dimer formation driven mostly by the hydrophobic nature of the C-terminal region of the peptide (F_5FVGL_9) (Russino et al., 2013). Spectroscopic evidence suggests that the Cu(II) ion in NKB is in a four-nitrogen environment that includes the histidine imidazole and the amino nitrogen and it is most likely that the Cu(II) ion resides in the ATCUN motif of one of the peptides. The four-nitrogen ATCUN site is predicted to have a higher Cu(II) affinity than the three-nitrogen site seen in GnRH-I and this is observed experimentally because both albumin, which contains a known ATCUN site, and NKB can remove metal from copper-bound GnRH-I (Gul et al., 2018). NKB can form a functional amyloid which allows high concentration packing into dense core vesicles in pituitary secretory cells (Maji et al., 2009). A physiological role for copper binding to NKB has recently been identified because copper coordination can trigger disassembly of the amyloid structure to generate monomeric or dimeric metal-bound species that are capable of activating the NK3 receptor (Jayawardena et al., 2019). Although it remains to be shown *in vivo*, we postulate that the structure, and thus activity, of NKB is dependent on the presence of copper in the extracellular environment. Therefore, for both GnRH-I and NKB copper modifies the structure of the peptide leading to molecules having

different functionality compared to the metal-free peptide. This provides a mechanism for how exogenously applied metal could lead to modification of the HPG axis and promote LH/FSH release (Hiroi et al., 1965).

6. Copper binding to GnRH-type peptides across the Bilateria

GnRH is a member of an evolutionarily ancient peptide superfamily with paralogues and orthologues present across the Bilateria, in both deuterostomes and protostomes. Homologs of GnRH have been identified in invertebrates and, although the most prominent role for mammalian GnRH is in reproductive biology, studies on invertebrates indicate that in these animals GnRH-type peptides regulate a wide range of physiological/behavioural processes along with, or instead of, reproductive processes (Kavanaugh and Tsai, 2016; Sakai et al., 2017; Tian et al., 2017). Orthologues of GnRH in protostomian invertebrates include adipokinetic hormone (AKH) and AKH/CRZ-related peptide (ACP), whilst corazonin (CRZ) is considered to be a paralogue of GnRH/AKH/ACP-type neuropeptides (Staubli et al., 2002; Hauser and Grimmelikhuijzen, 2014; Kavanaugh and Tsai, 2016; Zandawala et al., 2018). The presence of His at position 2 is conserved in vertebrate GnRH-type neuropeptides (Fig. 4) suggesting that they all bind Cu(II) with chemistry and structures closely resembling that of human (*H. sapiens*) Cu(II)-bound GnRH-I (Nakamura et al., 2005). Moving away from vertebrate GnRH-I, invertebrate chordates such as tunicates, which are a sister class to the vertebrates, also have His at position 2. However, GnRH-type neuropeptides in another deuterostome phylum, the Echinodermata, have a histidine residue in position 3. As described above, the move to position 3 generates an ATCUN motif known to chelate copper and nickel with high affinity (Trapaidze et al., 2012). Indeed, recent studies have explored the metal-binding ability of a GnRH-type peptide from the starfish *Asterias rubens* (ArGnRH, pQIHYKNPGWGPG-NH$_2$). These studies showed that the presence of a histidine

at position 3 results in ArGnRH having a Cu(II) dissociation constant several orders of magnitude lower than *H. sapiens* GnRH-I, suggesting it has a much higher metal affinity (Tran et al., 2019). Interestingly, the sea urchin *Strongylocentrotus purpuratus* also has His$_3$, consistent with other echinoderms, but it also has a His$_4$ (Fig. 4). Adjacent histidine residues tend to lower thermodynamic stability, so it can be predicted that this peptide does not have as high a copper affinity as ArGnRH, although it is likely to still be higher than *H. sapiens* GnRH (Csire et al., 2017). The movement of histidine from position 2 to position 3 and the consequent increase in affinity indicates the effects of copper on bioactivity of GnRH-type neuropeptides in echinoderms and in vertebrates may be different. In any case, the high degree of conservation of a histidine in the N-terminal region of deuterostomian GnRH-type neuropeptides suggests that the ability to bind copper has been retained in these animals over evolutionarily time as a biologically advantageous feature. Indeed, His in position three, and high affinity copper-binding, may be an ancestral feature that has been retained in echinoderms but lost (His moved to position two) during chordate evolution because GnRH-type neuropeptides with His in position three are also present in some protostomian invertebrates (Sakai et al., 2017; Tran et al., 2019). This may reflect the environment the peptide is released into and the bioavailability of copper. Indeed, the bioavailability of copper in seawater is very low so high affinity binding is required for secreted peptides to become metal-loaded. In contrast, the relatively copper rich pituitary in other vertebrates (e.g. *H. sapiens*) means very high affinity is not necessary for the peptide to become copper-bound.

When viewed through the lens of copper coordination, there are some anomalies observed in GnRH-type peptides of some species. For instance, GnRH in the coelacanth *Latimeria chalumnae*, a sarcopterygian (lobe-finned) fish, has no His in its sequence and contains a tyrosine at position 2 (Fig. 4) and therefore this peptide will have no

physiologically relevant copper binding ability. Further, in the cephalochordate *Branchiostoma floridae* two GnRH-like peptides were identified, pQILCARAFTYTHTW-NH$_2$ and pQEHWQYGHWY-NH$_2$. The first peptide was shown to act as ligand for a CRZ-type receptor in *B. floridae*, albeit with low potency (Roch et al., 2014b) and it has been suggested subsequently that a C-terminally truncated analogue of this peptide (FTYTHTW-NH$_2$) may be the natural ligand (Tian et al., 2016; Zandawala et al., 2018). The second peptide appeared non-functional, at least in terms of activating known *B. floridae* GnRH receptors (Roch et al., 2014b). This occurs despite having high sequence similarity to other vertebrate GnRH-type peptides and notably containing the position 3 His and likely retaining metal-binding ability. Whether the copper complex of pQEHWQYGHWY-NH$_2$ can activate a *B. floridae* GnRH receptor is not currently known.

In the protostomian branch of the Bilateria, GnRH-type peptides have been identified although many do not contain a histidine residue in their N-terminal region (Zandawala et al., 2018). Notably though, GnRH-type neuropeptides in some molluscan species do have an N-terminal His residue as exemplified by pQIHSFSPDWGT-NH$_2$ in the sea slug *Aplysia californica* and pQIHFSPTWGS-NH$_2$ in the sea snail *Lottia gigantea*. These peptides are also referred to as adipokinetic hormone (AKH)-type neuropeptides but it has been proposed that they are more appropriately referred to as GnRH-type peptides (Zandawala et al., 2018). Nonetheless, these molluscan GnRH-type peptides are predicted to exhibit high-affinity copper binding similar to that observed with the starfish GnRH-type neuropeptide ArGnRH (Tran et al., 2019), but this is not a consistent feature across the lophotrochozoan GnRHs (Hauser and Grimmelikhuijzen, 2014; Zandawala et al., 2018; Tran et al., 2019). The presence of a histidine residue in molluscan GnRH-type neuropeptides suggests that copper binding may be an evolutionarily ancient property that was a feature of GnRH-type peptides

before the divergence to protostomes and deuterostomes 640 - 760 million years ago (Douzery et al., 2004).

If copper-binding ability was present in an ancestral GnRH-type peptide, then it may also be a feature of the paralogous corazonin (CRZ)-type neuropeptides, which also predate the divergence of protostomes and deuterostomes (Tian et al., 2016; Zandawala et al., 2018). In the echinoderm *Asterias rubens*, a corazonin-type peptide and its receptor have been identified coexisting with the GnRH-type neuropeptide ArGnRH and its receptor (Tian et al., 2016). The *Asterias rubens* corazonin-type peptide (ArCRZ) has the sequence HNTFTMGGQNRWKAG-NH$_2$ and preliminary data suggests this peptide binds Cu(II), in keeping with other peptides that have a histidine at position 1 (Jones and Elphick, unpublished data; Kowalik-Jankowska et al., 2010). The hemichordate *Saccoglossus kowalevskii* peptide pQPHFSLKDRYRWKP-NH$_2$ (Fig. 4), which has been identified as a corazonin-type neuropeptide (Zandawala et al., 2018), has a high-affinity His$_3$ site. Furthermore, peptides identified as corazonin-type neuropeptides in molluscs, including the octopus *Octopus vulgaris* (pQNYHFSNGWHPG-NH$_2$), the sea slug *Aplysia californica* (pQNYHFSNGWHA-NH$_2$) and the oyster *Crassostrea gigas* (pQNYHFSNGWQP-NH$_2$), have a histidine residue at position 4 (Bose et al., 2017; Zandawala et al., 2018). The presence of a histidine at position 4 will allow metal binding with an affinity approaching the ATCUN motif (Sovago et al., 2016), although direct evidence of metal binding to molluscan corazonin-type peptides has not been obtained. Nevertheless, it appears that following the gene duplication event that gave rise to the GnRH-type and corazonin-type peptides in a common ancestor of the Bilateria there was retention of metal-binding ability in at least some members of both neuropeptide families (Zandawala et al., 2018). In contrast, the duplication of a GnRH-type neuropeptide gene that gave rise to AKH-type and ACP-type neuropeptides

in the Arthropoda seems to have invariably given rise to peptides that lack a histidine in the

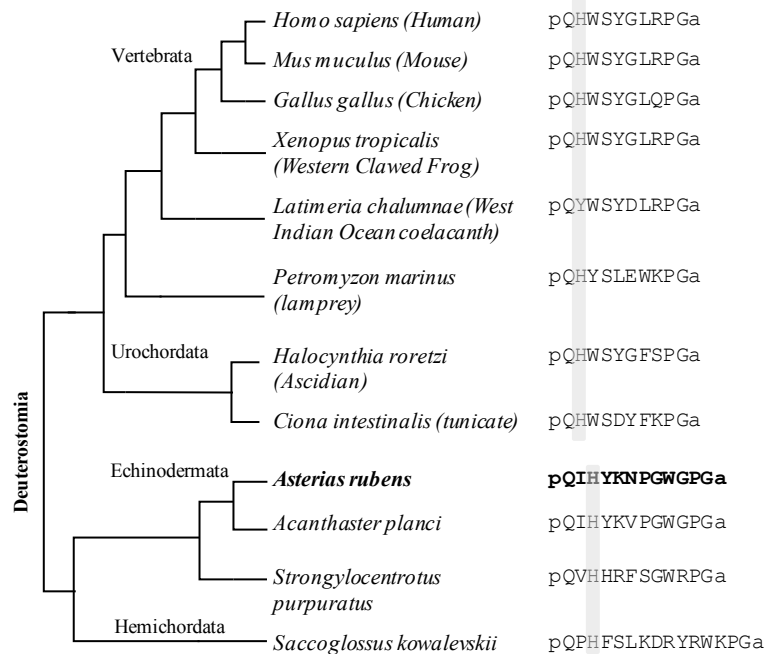N-terminal region and therefore a loss of metal binding.

Fig. 4. Diagram comparing the sequences of GnRH-type neuropeptides in selected deuterostomes with reference to a tree that shows phylogenetic relationships. Histidine is predominantly located at position 2 in the Vertebrata and Urochordata, with GnRH-type peptides in these taxa predicted to have Cu(II)-binding characteristics similar to human GnRH(I) (Kochman et al., 2005). In the Echinodermata and Hemichordata the histidine is located in position 3, forming a high-affinity Cu(II) and Ni(II) site, and these peptides are likely to have metal-binding characteristics similar to the prototypic peptide in *Asterias rubens* (highlighted in bold) (Tran et al., 2019). Peptide sequences derived from (Adams et al., 2003; Rowe and Elphick, 2012; Roch et al., 2014a, 2014b; Tian et al., 2016; Smith et al., 2017).

7. Conclusions

Despite recognition that copper can influence mammalian reproductive signalling,

there still remains uncertainty around the nature of the underlying mechanisms. Many

vertebrate species contain a GnRH-type peptide having sequence similarity to human GnRH-

I suggesting that metal-binding may be a feature of these peptides. Confirmation that a

GnRH-type peptide in an echinoderm can coordinate copper not only indicates broad

retention of this feature but also suggests it is a feature that may have been present very early on in the evolution of GnRH-type neuropeptides. Further investigation of the coordination chemistry of copper in GnRH-type neuropeptides in both vertebrates and invertebrates will allow exploration of how the peptides have evolved their sequence and structure to use the metal in different environments. Additionally, the presence of GnRH isoforms, notably GnRH-II, in many species raises the question of whether these have metal-binding ability and how it impacts on the function of the peptides when they may have roles beyond reproduction. The cellular and subsequent physiological consequences of metal-binding to GnRH peptides is an exciting area of study with many questions to be resolved, especially if the metal-bound peptide can lead to different signalling pathways compared to the apo-peptide.

Acknowledgements

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