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Neuropeptides as Ligands for GPCRs

Burcin Duan Sahbaz and Necla Birgul Iyison

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Abstract

Neuropeptides constitute an important part of the nervous system, since the simple nerve nets (i.e. of *Hydra*). The assigned functions of these peptides vary enormously. For instance, besides inhibiting or stimulating the release of some hormones, they can be responsible for tentacle contraction of the *Hydra*, dropping the tail of the lizard, postnatal care of the beetles and also aggressiveness of humans. They perform these tasks via activating their cognate GPCRs, which are hypothesized to be coevolved with their ligand neuropeptides. In this chapter, we will introduce the concept of neuropeptide, its intracellular maturation process, characteristics of some typical neuropeptide families and the common properties of their cognate GPCRs. At last, we will try to give information about the widely used methods for studying GPCR-neuropeptide interactions.

Keywords: neuropeptide, GPCR, peptide hormone, interaction

1. Introduction

Neuropeptides are polypeptides expressed in and secreted from neurons. They are produced as propeptides, cleaved into smaller fragments and matured via posttranslational modifications, differing from classical neurotransmitters in size, concentration and secretion mechanisms. They are expressed everywhere in the nervous system, take role in synapsis and can have distal target organs, as do the hormones.

Neuropeptides constitute the most diverse class of molecules in the body. They have various roles in development, reproduction, physiology and behavior of the animals. There are at least 70 known genes coding for neuropeptide precursor proteins, called prepropeptides, in

mammals and 42 genes in *Drosophila*. Secreted forms are processed from these prepropeptides and can vary from 5 to 80 amino acids in length. They coexist with classical neurotransmitters; for instance, neurotensin is released with dopamine [1] or vasoactive intestinal peptide with acetylcholine [2]. They mostly act on G-protein coupled receptors (GPCRs) and transduce their signals via intracellular secondary messenger systems.

2. Evolution of neuropeptide signaling

Simple nerve nets have evolved since the earliest animals like cnidarians (such as *Hydra*, sea anemones and jellyfish). Even they have no real brain, their nervous system includes secretory vesicles and produces different types of neuropeptides [3]. Neuropeptides constitute an important part of their nervous system. Therefore, neuropeptide signaling should have been evolved before the divergence of cnidarians and bilaterians, which means more than 550 million years of evolution.

Secretory vesicles of cnidarian nervous system are accumulated at the synapses [4, 5]. This may help for directed-signal transmission such as tentacle contraction of *Hydra* [6]. However, in vertebrates, the peptide secretory vesicles are not localized only to the synapse but distributed also along the nerve body and soma. Because neuropeptides interact mostly with GPCRs, their action mechanism is slower than classical neurotransmitters. This fact should be disadvantageous for the peptidergic nervous system of the *Hydra*. However, it was found that they have evolved different receptor-binding mechanisms to overcome this problem. For instance, mammalian RFamide neuropeptides activate different GPCRs and this activation leads to a slow response. On the other hand, *Hydra*-RFamide I and II act through a so-called peptide-gated ionotropic receptor, which is a trimeric complex of ion channels [7]. This system results in an advantage like faster transmission than that of classical neuropeptide-GPCR system, in absence of classical neurotransmitters.

In evolution of neuropeptide signaling, echinoderms are the second most important because they constitute an intermediate step between Protostomia (which include fruit fly) and Deuterostomia (which include both the vertebrates and echinoderms). Echinoderm neuropeptides are suggested to be involved in unusual mechanisms such as autotomy (dropping the tail of the lizard) and regeneration, or control of stiffness of connective tissue [8].

The major assumptions behind the diversity of neuropeptide genes are tandem duplications and following substitutions. Neuropeptide sequences are conserved in most cases (such as oxytocin family). However, some neuropeptide sequences show variations, and these variations can lead to differences in half-life, receptor affinity or expression profiles. Finally, these changes can generate a pressure in the direction of neuropeptide-receptor coevolution. Additionally, the mature peptides that are processed from the same gene can have sequence variations. These variations cannot be explained by gene duplication. An example for this is 37 peptide products of metamorphosin A prepropeptide of sea anemone [9]. All of these peptides can show functional redundancy, which means that they can be coexpressed,

coreleased and activating the same receptor. In a study on *Drosophila* genus, multiple copies of peptides from the same prepropeptide were analyzed [10], and it was found that they were highly conserved and under stabilizing selection. The numbers of peptide copies were the same within the genus (except FMRFamides). This conservation is important for receptor selectivity, affinity or the final response. Additionally, the researchers showed that the most conserved peptides were the most potent ligands for their receptors. Finally, these results on *Drosophila* neuropeptides supported the idea of evolutionary pressure of peptide-receptor coevolution on neuropeptide selection. This idea was proposed also for the vertebrate neuropeptides. Some regions of the vertebrate peptides are conserved, and these regions are thought to be the most important parts for functioning. For instance, the C-terminal residues of tachykinins are strictly conserved within vertebrates, and this region has roles in binding with tachykinin receptors. However, these similarities between neuropeptides of different species do not have to mean cross-reactivity with the receptors of different species [11, 12]. And this fact would be a support for the discussion of peptide-receptor coevolution.

3. Processing and trafficking of neuropeptides

Neuropeptides are the gene products that range from 5 to 80 amino acids in length. They born like prepropeptides, which contain an N-terminal signal sequence (between 15 and 40 residues in length). A typical signal sequence contains a positively charged region, a hydrophobic region and some polar but uncharged amino acids until the cleavage site, in the order [13]. This signal sequence is responsible for the anchorage of prepropeptide to the endoplasmic reticulum (ER) membrane via a complex called translocon, where folding and signal peptide cleavage occur. In some prepropeptides, the N-terminal region includes a signal anchor instead of a signal sequence. This signal anchor is responsible for the anchorage of precursor protein to the ER membrane but not cleaved. An example for this signal anchor can be given for the precursor of Allatostatin CC peptides of insects [14]. These signal anchors produce single-pass membrane proteins, which can act as juxtacrines in nervous system.

Cleaved propeptides are exported to the Golgi for further processing. Mainly, two types of “trypsin-like” endopeptidases are responsible for the cleavage of propeptides. These enzymes are called proprotein convertase 1 (PC1/3) and 2 (PC2). Seven PC types are expressed in mammals, but only three PCs in fruit fly (Amontillado, Dfurin1 and Dfurin2). PCs recognize and cleave the C-terminal site of dibasic residues such as KR or RR, especially of R-X-(R/K/X)-R motif on propeptides [15]. However, cleavage preferences differ within organisms. For instance, if valine or leucine is placed in place of X, the site will become resistant to cleavage by vertebrate PC (furin) but will be efficiently cleaved by insect PCs (Dfurin) [16]. In processing of neuropeptides, mammalian PC1 and PC2 and fly Amontillado are widely expressed in neurons, whereas furins have ubiquitous expression [17, 18].

Cleaved propeptide contains a basic C-terminus, which is further cleaved by carboxypeptidase E. In order to stabilize peptide structure against degradation, C-terminal glycine of most of

intermediate propeptides is amidated. This amidation is a multistep process of two enzymes in invertebrates, while vertebrates have a multifunctional enzyme to perform this task, called as peptidylglycine alpha-amidating monooxygenase (PAM).

Mature peptides are transported in large dense core vesicles (DCV), which are different from small vesicles delivering classical neurotransmitters. Furthermore, posttranslational modifications occur in DCVs. These modifications may include acetylation, sulfation, glycosylation, phosphorylation and cyclization. Some peptides can be processed even after secretion to the extracellular space. For instance, it was found that CPA6 of A/B family of carboxypeptidases is secreted to the extracellular matrix, cleaves hydrophobic C-terminal residues of neuropeptides and can lead to activation of Angiotensin I while degradation of some other peptides [19].

The engagement of DCVs to the nerve terminals is a very rare event. This is because DCVs respond to the changes in Ca^{+2} content and hundreds of spikes are needed to stimulate a DCV to release its content [20]. Even in these rare events, very large amounts of neuropeptides are released to the synaptic cleft where they are enzymatically cleaved and degraded. On the other hand, unlike neuropeptides, classical neurotransmitters are very rapidly transported to the membrane, easily released and recycled from the synaptic cleft.

Finally, one precursor protein can generate more than one neuropeptide and these peptides can be distinct or the same. Additionally, a precursor molecule can be alternatively spliced to yield different mature neuropeptides in different cells [21].

4. Types, cognate GPCRs and functions

As the simplest nervous system, cnidarians express at least 17 different neuropeptides, which can be grouped in three: FMRFamide-like peptides (FLPs), GLWamides and Hym-355 [3]. The neuropeptides expressed in worm *C. elegans* are also classified in three major groups, depending on their structural and functional similarities [21]. These groups are called as insulin-like peptides (ILPs), FLPs and neuropeptide-like peptides (NLPs). Vertebrate neuropeptides can be clustered in a wide range of families according to sequence similarities [22]. However, in human, neuropeptides expressed from 96 different genes were clustered in 22 distinct families together with the no-family peptides and deposited in neuropeptide databases [23].

In a study of metazoan (all animals) propeptides, neuropeptides of 10 phyla were taken and clustered in about 80 families according to their similarities within propeptide sequences [22]. Twenty-two of these families showed high similarity with each other. These included FMRFamides, LWamides, myoinhibitory peptide (MIP), neuropeptide FF and gonadotropin inhibitory hormone (GnIH).

In this chapter, we will introduce some of the neuropeptides that show conservation within species (as reviewed from the study of Jékely [22]) or that are specific examples for vertebrates and exclude the ligands interacting with non-GPCR targets. Summary of all mentioned neuropeptide families is given in **Table 1**.

Peptide family	Examples for active peptides	Expressed in	Structural similarity	Function(s)	Cognate GPCR
FMRFamide-like peptides	FMRFamide	Mollusks	Tetrapeptide FMRFamide	Cardioacceleration	<i>nd</i>
	FLP peptides	Nematodes	C-terminal FMRFamide	Control of feeding, reproduction, sensation	Various NPRs
	CCK/gastrin-type peptides		C-terminal QFamide	Control of feeding behavior, energy homeostasis	
	FMRFamide-like peptides	Arthropods and Cnidaria	C-terminal RFamide	Cardioacceleration	FMRFamide and sulfakinin receptors
	Gonadotropin-inhibitory hormone	Vertebrates		Inhibition of gonadotropin release	GPR147
	Neuropeptide FF			Nociception	NPFFR1, NPFFR2
	Pyroglutamylated RFamide peptide			Control of feeding	GPR103
	Prolactin-releasing peptide			Stimulation of prolactin release	PrRP receptors
	Kisspeptin			Reproductive development	Kisspeptin receptor
Tachykinins	Substance P-like tachykinins	Cnidaria, nematodes, arthropods	C-terminal FXGXRamide	Promotion of aggression, sexual activity and fecundity	NK-1
	Locustatachykinins	Invertebrates		Stimulation of muscle contractions	NKRs
	Eledoisin	<i>Eledone</i>	C-terminal F(Y/I)GLMamide	Vasodilation, hypotension	NK-1
	Sialokinin I and II	Arthropods		Vasodilation	NKRs
	Skin tachykinins	Amphibians	C-terminal FXGLMamide	Ion transport	
	Brain/gut tachykinins	Submammalian vertebrates		Vasodilation, smooth muscle contraction	
	Neurokinin A	Mammals		Regulation of inflammation and pain responses	NK-2
	Neurokinin B			Regulation of reproduction, secretion of gonadotropin-releasing hormone	NK-3
	Neuropeptide K			Regulation of sensation	NK-2
	Neuropeptide γ			Regulation of reproduction	NK-2
	Substance P				NK-1

Peptide family	Examples for active peptides	Expressed in	Structural similarity		Function(s)	Cognate GPCR
					Regulation of inflammation and pain responses, promotion of aggression	
Vasopressin/ oxytocin	Conopressins	Invertebrates	Sequence similarity and disulfide bridge between the 1st and 6th residues		Regulation of reproduction	Conopressin receptor
	Diuretic hormones				Regulation of water balance	Diuretic hormone receptor
	Mesotocin	Submammalian vertebrates				Mesotocin receptor
	Vasopressin	Vertebrates			Antidiuretic activity	V1A, V1B and V2
Myoinhibitory peptide/ GWamides	Oxytocin				Contraction of the uterus, lactation	OXTR
	Sex peptide	From cnidarians through annelids	C-terminal W(X)8 W–amide		Increasing egg laying, reduction in the female's receptivity, stimulation of juvenile hormone synthesis	SPR
	Myoinhibitory/allatostatin-B peptide		C-terminal W(X)6 W–amide and disulfide bridge		Inhibition of contractions of hindgut and oviduct, ecdysteroid synthesis and juvenile hormone synthesis	MIPR
	Prothoracicostatic hormone				Regulation of reproduction, release of ecdysone hormone	Torso (not GPCR), also activates SPR
Orexin/ allatotropin	APGWamides	Mollusks	Tetrapeptide		Sex organ growth, regulation of reproduction	<i>nd</i>
	Allatotropin	Arthropods (except <i>Drosophila</i>), mollusks, annelids	N-terminal GFK residues	No structural similarity	Stimulation of juvenile hormone synthesis, cardioacceleration, myostimulation	AT receptor
	Orexin A	Mammals	Sequence similarity		Sleep and wakefulness	OX1 and OX2
	Orexin B					
GnRH/ corazonin/ AKH	Corazonin	Arthropods	N-terminal pyro-glutamate		cardioacceleration, melanization and developmental processes	Corazonin receptor
	Adipokinetic hormone	Arthropods		Phe in 8th position	Mobilization of carbohydrates, lipids and proteins from the fat body	AKH receptor
	GnRH-like peptides	Annelids and mollusks		Following HWS residues	Stimulation of testosterone and progesterone synthesis	GnRHR
	GnRH	Vertebrates			Stimulation of gonadotropin release	

Peptide family	Examples for active peptides	Expressed in	Structural similarity		Function(s)	Cognate GPCR
Neuropeptide Y	Neuropeptide Y	Vertebrates and invertebrates	C-terminal amidation and a pancreatic polypeptide fold structure		Regulation of blood pressure and feeding behavior	GnRH1 and GnRH2 receptors
	Peptide tyrosine tyrosine	Vertebrates			Inhibition of gastric motility and electrolyte secretion	Y1,2,4,5 and y6
	Pancreatic polypeptide	Vertebrates			Inhibition of pancreatic exocrine secretion	
Somatostatin/ allatostatin C	Allatostatin C	Arthropods	C-terminal PISCF and a disulfide bridge	No structural similarity	Inhibition of juvenile hormone synthesis	AlstR-C
	SST14	Vertebrates	Sequence similarity		Inhibition of growth hormone release	SSTR1, SSTR2A, SSTR2B, SSTR3, SSTR4 and SSTR5
	SST28					
Galanin/ allatostatin A	Allatostatin A	Arthropods	C-terminal FGLamide	No structural similarity	Inhibition of juvenile hormone synthesis, regulation of food intake	AST-A receptors
	Galanin	Vertebrates	N-terminal similarity and C-terminal amidation		Nociception, feeding and osmotic regulation	GalR1, GalR2 and GalR3
VIP/PACAP	α -Pigment dispersing factor	Invertebrates	C-terminal amidation	No structural similarity	Regulation of circadian clock	PDF receptor
	β -Pigment dispersing factor					
	VIP	Vertebrates	β -turns and α -helical structures			VPAC1 and VPAC2
	PHI					
	PHM					
	PHV					
	PACAP27					
	PACAP38					
						PAC1, VPAC1 and VPAC2

Table 1. Summary of the neuropeptide families and the similarities within these families.

4.1. FMRFamide-like peptides

Genome searches and mass spectrometry-based methods on nematode *C. elegans* yielded around 30 genes encoding for FLPs. These peptides share a common C-terminal motif like FMRF residues. RNAi studies on these genes showed that FLPs can have roles on different processes such as hyperactivity, timing of egg laying, number of laid eggs, fat metabolism and acetylcholine signaling [24–26]. In mollusk *Macrocallista nimbosa*, this neuropeptide takes role in cardioexcitatory activity [27]. FLPs are expressed in all of the animal species. However, the conserved C-terminal residues may become FMRFamide, QFamide or RFamide. In arthropods, sulfakinins, myosuppressins, RFamides and other extended FMRFamides have the common C-terminal amidated RF residues. Myosuppressins seem to be restricted to crustaceans and insects and have a role in inhibiting contractions of the hindgut, cardiac muscle and release of adipokinetic hormone [28, 29]. Extended FMRFamides of arthropods affect respiration, heart rate, gut motility and muscle contractions. *Drosophila* sulfakinin (drosulfakinin) was shown to regulate locomotor behavior [30], feeding behavior [31] and smooth muscle contraction [32]. FMRFamides act through two types of receptors. Most of them activate GPCRs. However, FMRFamides of snail *Helix aspersa* lead to an excitatory response in amiloride-sensitive Na⁺ channels [33].

4.2. Tachykinins

Vertebrate tachykinins are one of the largest groups of neuropeptides expressed in both invertebrates and vertebrates. They contain conserved C-terminally amidated motifs such as FXGLM residues, while some of arthropod tachykinins show FXGXRamide conservation. These five residues are very conserved but not vital for receptor activation, instead phenylalanine at the fifth position and the C-terminal amidation are essential for their activity. They can be localized both to the brain and the gut of various organisms, as well as the skin of amphibians. They can be secreted from the enteroendocrine cells of mammals as paracrine or as true hormones.

Human tachykinin family includes neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK), neuropeptide γ (NP γ) and substance P (SP), which are expressed from two genes. These peptides activate three types of GPCRs: NK-1, NK-2 and NK-3. SP interacts with NK-1, while NKA with NK-2 and NKB with NK-3. Higher concentrations of SP in patients with personality disorders were correlated with aggressive behavior [34]. It was also shown that *Drosophila* tachykinins have aggression-promoting functions [35] and control systemic lipid homeostasis [36]. Tachykinin-like natalisin peptide regulates sexual activity and fecundity of arthropods [37]. Another tachykinin family peptide, eledoisin, was identified from the salivary glands of mollusk *Eledone* in 1962 [38]. And, eledoisin and kassinin were shown to be expressed and stimulated ion transport in the frog skin [39].

4.3. Vasopressin/oxytocin

Vasopressin (VP) and oxytocin (OXT) are members of the same family due to their sequence similarity. They are conserved from arthropods to mammals. Vertebrate VP/OXT peptides are

expressed from different genes. Processing of propeptides of vasopressin gene produces three peptides called VP, neurophysin II and copeptin, while processing of oxytocin gene produces only OXT and neurophysin I peptides. OXT and VP bind with their corresponding neurophysins, OXT with neurophysin I and VP with neurophysin II. These neurophysins are responsible for the storage of VP and OXT inside DCVs. In physiological pH, VP and OXT do not bind with neurophysins and circulate freely in the plasma. Both mature VP and OXT are nine amino acids in length, eight of which are identical and contain a disulfide bridge between the first and sixth residues [40], while neurophysins have seven bridges. The first cysteine and the following tyrosine residues play the major role in neurophysin binding [41]. Although VP and OXT show sequence similarity, their functions differ from each other. VP has antidiuretic activity and released as a response to increased blood plasma osmolarity, while OXT has roles in contraction of the uterus and in lactation and is stimulated with suckling movement of the newborn. VP and OXT receptors constitute a big family of GPCRs. There are three types of vasopressin receptors: V1A, V1B and V2. However, only one type of oxytocin receptor was identified: OXTR [42].

Invertebrate homologous peptides also contain the disulfide bridge at the same position and five or six amino acids of the peptides are well conserved. The invertebrate homologs of vertebrate VP/OXT peptides are conopressins and diuretic hormones (DH) [43].

4.4. Myoinhibitory peptide/GWamides

These peptides are expressed from *Cnidaria* to Annelids but not present in vertebrates. This family of peptides shares a common motif like $W(X)_6W$ and includes various similar peptides such as myoinhibitory/allatostatin-B peptide (MIP/AST-B), sex peptide (SP), prothoracicostatic hormone (PTTH) and GWamides (of mollusks).

The first AST-B peptide is identified in *Locusta migratoria* as an MIP. It inhibits contractions of hindgut and oviduct, as well as ecdysteroid synthesis. It has a $W(X)_6W$ amide motif on its C-terminus and is widely expressed in the central nervous system. Similar peptides are identified in *Gryllus bimaculatus* and found that they inhibited juvenile hormone synthesis in *corpora allata*. Therefore, they are called as allatostatins.

SP is found in *Drosophila* male accessory glands and regulates mating behaviors of the females. During mating, SP is released from male's ejaculatory duct and acts on the corresponding receptor on the female reproductive duct, increases egg laying and reduces the female's receptivity. This peptide is 36 amino acids in length. N-terminal eight residues are responsible for sperm binding and stimulation of juvenile hormone synthesis. Following 12 amino acids have roles in innate immune responses against bacteria. And the C-terminal 16 amino acids have role in postmating responses. A disulfide bridge is localized to the C-terminal part of the peptide. In addition, there is an internal $W(X)_8W$ motif instead of $W(X)_6W$ amide of the others. Therefore, the tryptophan residues on both peptides seem to be important for receptor binding [44]. Sex peptide receptor (SPR) of *Drosophila* is CG16752, and this receptor is expressed in female reproductive organs and in the central nervous system of both genders. It is proposed to be $G\alpha_i$ -coupled. MIP and SP both activate SPR, but MIP has lower affinity for this receptor [45].

APGWamide is a mollusk tetrapeptide. It is mostly correlated with sex organ growth and reproduction of the animal.

PTTH is a homodimer of two identical peptide chains that are held together by disulfide bridges [46]. It regulates the reproduction and release of ecdysone hormone. The target receptor of PTTH is Torso, which is a kind of receptor tyrosine kinase [47]. However, it can also activate SPR [48].

4.5. Orexin/allatotropin

Allatotropin (AT) was first identified in *Manduca sexta*, stimulating juvenile hormone synthesis [49]. These peptides show conservation on their N- and C-termini. They include glycine, phenylalanine and a following basic residue in the order in their N-termini (for instance, GFK residues). This N-terminus is important for biological activity of the peptide. On their C-termini, aromatic amino acids are conserved, followed by an amide group (such as R-amide or Y-amide). These peptides are not identified in *Drosophila*, *Apis mellifera* and *Nasonia vitripennis*. However, *Drosophila* expresses sex peptide for the same function. Therefore, it seems that different peptides may work for the same function in different species. Additionally, AT has other roles such as cardioacceleration, stimulation of muscle contractions and myostimulation in the gut [50–52].

Orexin receptors (or hypocretin receptors) are found to be orthologs of AT receptors, via similarity on their C-terminus [22]. However, their peptide ligands are not structurally or functionally related. Orexin peptides are about 28–33 amino acids in length. They are hypothalamic neuropeptides and have roles mainly in sleep and wakefulness [53].

4.6. GnRH/corazonin/AKH

Gonadotropin releasing hormone (GnRH) is the peptide-stimulating gonadotropin release in vertebrates. However, invertebrates, such as annelids and mollusks, also express GnRH-like peptides. Octopus GnRH induces synthesis of testosterone and progesterone in the ovary and testis, respectively. From tunicates to mammals, GnRH sequence shows a high conservation. It is a decapeptide that has an N-terminal pyroglutamine and following HWS residues and C-terminal PGamide residues [54].

Insects express corazonin and adipokinetic hormone (AKH), instead. Corazonin is 11 amino acids in length and has a cardioacceleratory effect in cockroaches. However, other actions are defined in other insects, such as melanization in locusts and developmental pathways in other insects such as *M. sexta* and *Bombyx mori*. AKH is generally 8–10 amino acids in length. It has an N-terminal pyroglutamate, C-terminal amidation and at least two aromatic residues in between. These aromatic residues (at positions 4 and 8) are important for receptor binding. Its structure exhibits a β -turn between these positions. It regulates mobilization of carbohydrates, lipids and proteins from the fat body. Additionally, it has roles in cardioacceleration as corazonin.

Vertebrate GnRH receptors and insect AKH receptors are closely related. *Drosophila* corazonin receptor is clustered in the same family of AKH and VP receptors. However, corazonin receptor is highly selective for corazonin peptide [55].

4.7. Neuropeptide Y

Neuropeptide Y (NPY) family of vertebrate neuropeptides includes NPY, peptide tyrosine tyrosine (PYY) and pancreatic polypeptide (PP). These peptides are C-terminally amidated and show a hairpin-like structure called pancreatic polypeptide fold (PP-fold). This fold was composed of one polyproline helix and one α -helix running antiparallel to each other [56]. Five types of Y receptors (for NPY family) are expressed in mammals ($Y_{1,2,4,5}$ and y_6). It is proposed that hydrophobic surface of the PP-fold is responsible for receptor binding. NPY is localized to the brain, while PP and PYY are localized to the gastrointestinal tract. NPY is a highly conserved peptide from frog to human. Circulating NPY acts on regulation of blood pressure and eating behavior [57].

A mollusk NPY was identified in *Lymnaea stagnalis* via activation assays on its corresponding NPY receptor homolog [58]. This peptide was 39 amino acids in length and very similar to the vertebrate NPYs. Invertebrate NPY prepropeptides lead to two peptides, one is NPY and the other one is C-terminal peptide of NPY (CPON). The important residues that are responsible for the PP-fold of vertebrate NPYs are conserved in mollusk NPYs, but only some of them are conserved in *Drosophila* NPYs. Additionally, C-terminal four residues and amidation, which are essential for the activity of the peptide [59], are conserved between vertebrate and invertebrate NPYs. *Lymnaea* NPY has role in regulation of energy consumption processes, while the other invertebrate NPYs mostly affect food intake of the animal [60].

4.8. Somatostatin/allatostatin C

Allatostatin C (AST-C) is the arthropod homolog of vertebrate somatostatin (SST). SST is found as the inhibitor of growth hormone release from the pituitary gland. And AST-C is the inhibitor of juvenile hormone synthesis in *corpora allata*. From the same SST propeptide, one peptide with 14 amino acids and another with 28 amino acids are released, which are secreted from and acting on different tissues such as central and peripheral nervous system, as well as gastrointestinal tract. Both SST and AST-C peptides exhibit a disulfide bridge, which is important for receptor affinity [11]. The pharmacophore of SST is defined with FWKT residues. And it functions for the inhibition of pituitary hormones such as growth hormone, thyroid stimulating hormone and adrenocorticotrophic hormone. SST acts on six different subtypes of SST receptors (SSTRs), SSTR1, SSTR2A, SSTR2B, SSTR3, SSTR4 and SSTR5.

On the other hand, AST-C has highly conserved C-terminal PISCF amino acids. In addition to juvenile hormone inhibition, it inhibits heart muscle contraction in *Drosophila*.

4.9. Galanin/allatostatin A

Galanin peptide is first identified in porcine intestine. Human galanin propeptide produces two peptides, galanin (30 amino acids) and galanin-message associated peptide (GMAP), after cleavage. N-terminal residues and a C-terminal amidation (except in human) of galanin are highly conserved. These peptides are expressed in both central and peripheral nervous systems and have roles in nociception, feeding and osmotic regulation, via acting on three GPCRs; GalR1, GalR2 and GalR3.

Allatostatin A (AST-A) exhibits a conserved C-terminal FGLamide group, which is not similar to galanin peptide. However, these two peptides activate ortholog receptors of vertebrates and arthropods. AST-A peptides are mainly expressed in brain and gut and serve for the inhibition of juvenile hormone synthesis and regulation of food intake, as similar to other AST types.

4.10. Vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide

Expression of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) is restricted to vertebrates. They belong to the glucagon/secretin superfamily that also includes glucagon, secretin, growth hormone releasing hormone (GHRH) and gastric inhibitory peptide (GIH). VIP and PACAP show structural similarity on their N-terminal 27 amino acids. VIP is expressed in both central and peripheral nervous system, while PACAP in hypothalamus, central nervous system, respiratory and gastrointestinal tract. Mature VIP peptide is very well conserved in both mammals and nonmammalian vertebrates. Short PACAP (27 amino acids in length) is restricted to mammals, but the longer form (38 amino acids in length) can be found also in nonmammalian vertebrates [61]. It exhibits an α -helical structure on binding to the receptor but can fold into different secondary structures in different solutions. PACAP is responsible for the release of growth hormone, luteinizing hormone, adrenocorticotrophic hormone, follicle-stimulating hormone and prolactin from the pituitary gland, acts on testis and ovary and stimulates insulin and glucagon release [62]. VIP was discovered due to its vasodilatory effects [63]. It can act as both a paracrine or a hormone. However, its half-life is very short when compared to classical hormones [64]. VIP gene produces other forms of peptides such as peptide histidine isoleucine (PHI), peptide histidine methionine (PHM) and peptide histidine valine (PHV), in different organisms. However, the information about the functions of these peptides is limited.

High similarity between PACAP and VIP peptides make them to activate the same receptors, but with different affinities. Three different PACAP receptors are identified (PAC1, VPAC1 and VPAC2). And two types of PACAP selectivity were detected in tissues. In one type, PAC1 receptor has high affinity for PACAP peptides (PACAP27 and PACAP38) and expressed in anterior pituitary and hypothalamus. For the second selectivity, VPAC1 and VPAC2 receptors showed affinity for both PACAP and VIP peptides, and this was detected in peripheral organs. All of these receptors are known to activate adenylate cyclase, leading to cAMP stimulation. In other circumstances, they can stimulate Ca^{+2} levels and phospholipase D.

Pigment dispersing factor (PDF) receptors are homologs of VPAC2 in invertebrates. They regulate circadian clock. In nematodes, they regulate locomotion, but in crustaceans, they regulate pigment movements in the retina.

There are other additional neuropeptide families that interact with GPCRs, such as proopiomelanocortin (POMC) family, which is typical for its precursor complexity and others. However, we will not go into details of other families in this chapter.

5. Common features of neuropeptide GPCRs

Neuropeptides activate various receptors most of which are GPCRs. Some neuropeptides as given in Section 4.4 can bind to membrane receptors that couple with receptor tyrosine kinases (i.e. insulin receptors and Torso for PTTH). Some small neuropeptides do not have defined receptors but are ligands for other peptides or enzymes (i.e. 7B2 binding to PC enzymes and neurophysins binding to VP or OXT). Most of the others interact with their cognate GPCRs from the extracellular region and activate a downstream signal transduction pathway. Peptide GPCRs belong to either Class A (rhodopsin-like) or Class B1 (secretin-like) receptor.

Class A GPCRs exhibit two types of ligand-binding pockets. In one type, the hydrophobic ligand interacts with the transmembrane (TM) region, and the N-terminal region together with the second extracellular loop (ECL2) forms a closed lid-like structure (i.e. rhodopsin and S1P receptors that have highly hydrophobic ligands). However, in the second type, ECL2 folds over the extracellular region of the receptor and forms a pocket-like vacancy, which is exposed to the soluble environment. Peptide GPCRs show the characteristics of this latter binding pocket. Here, ECL2 comprises sheets, instead of β -hairpin loops of rhodopsin or helices of adrenergic receptors. Another feature of Class A GPCRs is the presence of a disulfide bridge between transmembrane domain 3 (TM3) and ECL2. This bridge is important for the stability of the receptor and serves as a barrier against conformational changes in this region, which is important for the ligand affinity. In a review on the defined 3D structures of Class A GPCRs, the depths of bound ligands were compared with regard to positioning of TM4 [65]. Within the Class A GPCRs that exhibit open binding pockets, amines (i.e. doxepine) were interacting deeply, while peptides and nucleoside ligands were closer to the extracellular environment. Three TM regions (TM3, TM6 and TM7) of Class A GPCRs were proposed to have consensus binding residues. These consensus amino acid positions are 3.32, 3.33, 3.36, 6.48, 6.51 and 7.39 (Ballesteros-Weinstein numbering). However, peptide receptors such as neurotensin receptor (NTSR) and allatostatin C receptor (AlstR-C) were shown to have different interactions within the TM regions. For instance, neurotensin forms salt bridges and hydrogen bonds with the Y3.29, R6.54, R6.55, F6.58 and Y7.35 residues of NTSR1 [66]. Additionally, AST-C was binding with proposed AlstR-C model from the extracellular site, except for the two amino acids of TM6 (I6.59 and F6.60 residues) [67]. In addition to these consensus residues, Venkatakrishnan et al. proposed that the positions 6.48 and 6.51, which were conserved within Class A GPCRs, might be responsible for the structural folding of the binding pocket, forming a scaffold consensus [65]. However, the evidences for these consensus residues of binding pockets and scaffold interfaces of peptide GPCRs are limited.

Secretin-like neuropeptide GPCRs include the receptors for VIP/PACAP, PDF (in invertebrates), calcitonin, insect DHs, corticotropin releasing factor (CRF), GHRH and parathyroid hormone (PTH) peptides. There is less information about the structures of secretin-like neuropeptide receptors than that of rhodopsin-like receptors. Within the receptors mentioned above, the only solved full-length structures come from CRF1 receptor (PDB entry: 4Z9G) and calcitonin receptor (PDB entry: 5UZ7). Additionally, there are ligand-bound structures of glucagon

receptors of which the ligand is not a neuropeptide. Because glucagon receptor has the most well-known structure and secretin receptor is the most studied in this class, we will use them as examples to understand ligand binding of secretin-like neuropeptide GPCRs, even though they are not neuropeptides. Additional information comes from the N-terminal region of ligand-bound structures of PAC1 (PDB ID: 2JOD) and PTH receptor (PDB ID: 3C4M), together with the free forms of V2 receptor (PDB ID: 2X57) and GHRH receptor (PDB ID: 2XDG).

In order to understand ligand-binding features of this class, we need to look at their ligands. Neuropeptide ligands that couple with secretin-like GPCRs have a common secondary structure of at least one α -helix. As that of glucagon peptide, PACAP and CRF exhibit two α -helices. VIP, PTH and calcitonin peptides have only one helical structure. On the other hand, the common feature of these family receptors is that they have a long and complex N-terminus that may include three disulfide bridges forming an α - β - β - α fold [68]. This N-terminal region of the receptors is shown to be important in ligand binding. Provided by the experimental structure of human glucagon receptor, another region on the N-terminus was identified as “stalk” at the top of TM1. And mutagenesis studies on this stalk region proved that it was important for ligand binding, by providing a defined conformation of N-terminal loop with regard to TM1 [69, 70]. As another hypothesis, Dong et al. proposed an endogenous agonism for the N-terminal region of secretin receptor [71]. Here, binding of C-terminus of the ligand to the N-terminus of the receptor results in a conformational change that results in movement of a hidden tripeptide region and becomes an endogenous agonist for the receptor itself. This tripeptide region consists of WDN residues (inside one of the N-terminal helices) on secretin receptor, which are also conserved for calcitonin and VPAC1 neuropeptide receptors.

According to FRET study by Harikumar et al., C-terminal part of secretin peptide was in proximity to the groove above the β -hairpin of receptor N-terminus, while N-terminal part of it was in proximity to ECL3 and TM6 [72]. This model of secretin binding is proposed as a general mechanism for all secretin-like GPCRs. N-terminus of the peptide ligands was shown to be important for receptor activation (i.e. for CRF, calcitonin, glucagon and VIP) [73–76]. Deletion of this region revealed antagonism for the receptor. And C-terminus of the peptide was shown to be involved in ligand binding to the receptor (i.e. VIP, PTH and CRF) [77–79]. This binding includes hydrophobic residues of the helical structures on receptor N-terminus, as well as hydrogen bonds or salt bridges formed between the ligand and polar receptor residues. In this model, the ligand adopts an α -helical structure upon binding to the receptor. This is supported by the soluble structures of glucagon, PTH or PACAP in aqueous solution and their helical structures in organic solvents. Only calcitonin did not change in either media, due to stabilization by disulfide bridges. The salt bridges between the ligand and the receptor are thought to be responsible for the helix formation. After forming a binding helix, this structure is covered by two β -sheets of the receptor N-terminus. Exceptionally, in case of PACAP binding, the peptide wraps around the helical structures of receptor N-terminus [80].

All the details proposed for ligand binding to secretin-like GPCRs add up to a common model of “two-domain” binding. The C-terminus of the peptide is responsible for receptor binding, mostly to the N-terminus of the receptor, producing a conformational change here. And N-terminus of the peptide enters to the TM region and produces a second conformational change that will lead to signal transduction.

6. Methods to study neuropeptide-GPCR interactions

Studying the ligand interaction properties of GPCRs is an essential concept in pharmacology. Neuropeptide GPCRs contribute to the majority of drug targets in central nervous system disorders. Also, insect neuropeptide GPCRs are valuable targets for pesticide designs. Finding the binding sites, discovering agonists, antagonist and even allosteric modulators, understanding the binding affinities and thermodynamic properties and measuring retention times produce a need for case-specific types of GPCR-ligand interaction studies. These may require direct, indirect or *in silico* methods, or a combination of these.

Direct methods for studying GPCR-ligand interactions involve nuclear magnetic resonance (NMR) spectroscopy, X-ray diffraction and surface plasmon resonance (SPR) techniques. The information coming from these studies are deposited in Protein Data Bank (PDB) and increasing every day. However, the increase in deposition of GPCR structures is not as fast as that of soluble protein structures. For instance, most of the data coming from NMR studies include only partial GPCR structures bound with their ligands. Obtaining pure crystals of GPCRs is a challenge in X-ray analysis. And studying with hydrophobic ligands is difficult in SPR method. Therefore, we will not go in detail of these direct methods in this chapter, due to their challenges in working with membrane-bound proteins.

Indirect methods for studying GPCR-ligand interactions include fluorescent-based methods, radioligand binding, photoaffinity labeling, luminescence-based methods, force spectroscopy and activity-based assays.

In silico approaches do not yield direct or indirect evidence for GPCR-ligand interactions, but they reduce the problem space, facilitate the following assays and qualitative comparisons between molecules and can mimic the assay conditions, so that they are highly valuable tools for drug design studies.

In this chapter, we will only focus on the indirect methods that are widely used for GPCR-ligand interaction.

6.1. Radioligand-binding assays

In principle of radioligand-binding assays, the ligand is previously radiolabeled and added onto the receptor, and its binding is measured quantitatively. The first study of radioligands on GPCRs is that of Lefkowitz and his collaborators where they used I^{125} -labeled adrenocorticotrophic hormone (ACTH) against ACTH receptor [81]. Since then, modifications on the method made use of membrane patches and also whole cells [82]. With the help of radioligand saturation binding, indirect binding or kinetic-binding assays can be performed and result in calculation of EC_{50} , K_d values together with the retention time of the ligand on the receptor. Also, they show if the ligand binding is reversible or not. The major challenges of this method are the cost and half-lives of radioligands and the health issues in regard to exposure to them. Agonists cannot be distinguished from antagonists with these assays. Additionally, optimizations should be performed to minimize nonspecific binding (i.e. to the cell, to the plastic ware).

6.2. Photoaffinity labeling

In the study of GPCR-ligand interactions, photoaffinity labeling (PAL) is one of the oldest methods. Here, the ligand is bound with a photoreactive group (PRG). Upon binding with the receptor, PRG is activated by UV light and forms an irreversible covalent bond with the closest residues on the receptor. This approach can be combined with immunoprecipitation and mass spectrometry to sequence the amino acids that are in proximity to the ligand-binding pocket. An example can be given as the study of Ceraudo et al. for the interaction between VIP and VPAC1 receptor. They first labeled the C-terminus of VIP with a photoreactive p-benzoyl-p-phenylalanine (Bpa) group. Then they followed by cleavage and Edman sequencing. Finally, they found that the C-terminus of VIP was interacting with the N-terminus of VPAC1 [83].

In another study, Grunbeck et al. have performed site-directed mutagenesis on some proposed residues of CXCR4. These mutant residues were producing amber stop codons, which can be engineered to incorporate photocrosslinkers (i.e. BzF and azF). They have transfected HEK cells with these mutant constructs and treated the cells with the ligand. After UV activation, lysis and immunopurification of the receptor-ligand complexes, they saw that 189F residue of the receptor was in close proximity to the ligand during binding [84].

6.3. Fluorescence-based methods

The use of fluorescently labeled ligands has many advantages when compared to radioactively labeled ligands. For instance, detection efficiency is higher in fluorescent ligands, and health safety issues are easier to handle for the methods utilizing fluorescent ligands. Additionally, fluorescence-based methods can generate quantitative data as given by radioligand assays (i.e. EC₅₀, etc.). For instance, microscopy and flow cytometry can be used in real-time experiments; they can measure the amount of fluorescence that is interacting with or within the cells [85]. Dissociation rate constants (K_d) of fluorescently labeled ligands can be calculated in various approaches. First, physical separation of bound ligand from free ligand in different fractions can be measured by means of concentrations. Second, the emission intensity of the ligand changes upon binding with the receptor and this change can be measured. Third, diffusion rates of bound and free ligands differ. In an approach called fluorescence correlation spectroscopy (FCS), diffusion rate of labeled ligand can be measured on a highly sensitive confocal microscope. Another approach depends on anisotropy, which means that polarization of the molecule changes between bound and free ligands. As a fifth approach, flow cytometry can be used to detect presence of labeled ligands on receptor carrying cells or beads. At last, the most frequently used sensitive approach is called as fluorescence resonance energy transfer (FRET).

There are other methods such as fluorescence recovery after photobleaching (FRAP) that is similar to FCS in principle. However, this method is used only for GPCR oligomerization or G-protein coupling until now [86, 87], but no study was performed on GPCR-ligand interactions yet. Another complex approach combines two-photon excitation microscopy with FCS and quantum dot technology (TPE-XCS), which seems very promising for the following days [88]. In this chapter, we will give some more detail on FRET experiments performed on GPCR-ligand studies that are widely preferred by the researchers.

FRET is based on the energy transfer between two different fluorophores when they come close to a defined distance (typically between 10 and 100 Å). In principle, emission of first fluorophore (donor) should excite the second fluorophore (acceptor). In case of GPCR-ligand interactions, different approaches can be used. First, the ligand and an extracellular domain of the receptor can be expressed in fusion with different fluorescent proteins. When the ligand is in proximity to the receptor, two fluorophores also come close to yield an energy transfer. The difference between the FRET signals of interacting and noninteracting GPCR-ligand couples gives an information about the presence of interaction. FRET can also be time-resolved so that information on kinetics of ligand binding can be achieved. This method was used for various types of receptors such as M1 muscarinic acetylcholine receptor, PTH receptors, neurokinin NK2 receptor, cholecystokinin receptor and secretin receptor [89–93]. In another approach, mapping of the ligand-binding region is possible. Here, cysteine residues can be added to different locations of the proposed binding pocket of GPCR via site-directed mutagenesis. These cysteine residues can bind with small fluorophores which would not interfere ligand binding. Additionally, the environment of the ligand-binding pocket can be assessed, via accessibility of aqueous solution and changes in quenching and polarity upon ligand binding. In a technique by Hoffman et al., tetracysteine residues were added to ICL3 and C-terminus of the GPCR. These residues can bind with FLAsH reagent, which is a small fluorophore. When used in combination with cyan fluorescent protein (CFP), conformational changes upon binding of the ligand were made possible to detect [93].

6.4. Bioluminescence resonance energy transfer (BRET)

In principle, BRET is similar to FRET by using a bioluminescent donor on one molecule and a fluorescent acceptor on the target molecule. Generally, a luciferase (i.e. Rluc8) is used as the donor. It can be performed real time, giving quantitative information about ligand binding. It is advantageous over FRET, because it does not require an initial illumination of the donor molecule. As an example, Stoddart et al. performed BRET on beta adrenergic receptor 2 (β_2 AR) with an antagonist in live cells. They generated the N-terminus of the receptor with luminescent donor and used a fluorescently labeled ligand [94].

6.5. Atomic force microscopy (AFM)

AFM is based on the principle of single-molecule force spectroscopy, in which binding force of two single molecules is measured as a difference in laser deflection. One molecule is bound on the tip of a cantilever and the other molecule stays on a rigid surface (or on cell surface). If interaction occurs, the laser deflection from the cantilever tip differs from the state of no-interaction events. Here, the receptors can be in lipid bilayers, as performed by Pfreundschuh et al. [95] and Alsteens et al. [96], and binding the ligand to the cantilever tip. Also, the method can utilize the receptors on live cells directly as performed by our group for AlstR-C receptor [67].

In AFM, direct measurements can be obtained from single molecules, and the controls can be designed to exclude nonspecific-binding events. There is no need for fluorescent, luminescent or radiolabeling of the molecules, which may interfere with the binding sites. In most of the cases, peptide ligands are much smaller than fluorescent proteins. However, in AFM, the peptides can

be utilized in their native forms, or they can be functionalized from defined terminal sites. The method in our study also provides the native environment of the receptor as in FRET experiments. And this makes it more advantageous than the methods analyzing purified receptors. AFM setup is suitable for working on adherent cancer cells for long hours, so that the performer can take hundreds of data points from the same cell. And ectopic expression produces enough saturation of the receptor on the surface to detect at each approaching step. Therefore, AFM seems a promising and easy way to study GPCR-ligand interactions on live cells.

6.6. Activity-based assays

Activity-based assays depend on the previously known downstream effects of the GPCR in cells. The advantage of these assays is that they allow discrimination of agonists from antagonists and also partial agonists. Quantification of EC₅₀ values is possible, so that they can also be used in high-throughput pharmacological studies. Examples can be given as GTPγS (guanosine 5'-O-[gamma-thio]triphosphate) binding assays, cAMP (cyclic adenosine monophosphate) assays, IP₃ (inositol triphosphate) and Ca⁺² assays, TGF-α (transforming growth factor alpha) shedding assay, β-arrestin recruitment and internalization assay, dimerization assays and voltage-clamp experiments. These assays can be coupled with fluorescent techniques or site-directed mutagenesis of the receptor when required.

7. Conclusion

There are at least 80 genes encoding for neuropeptide precursors in human. These precursors give rise to at least 150 mature neuropeptides. And until now, at least 109 of these peptides were shown to signal via GPCRs. All these peptides and their cognate GPCRs are still being studied against the neurological disorders, which range from the simplest stress and pain relief cases to the complex schizophrenia and Alzheimer's disease. Therefore, understanding the kinetics, interactions and transduction pathways of GPCR-neuropeptide signaling systems will remain crucial for the human wealth.

Author details

Burcin Duan Sahbaz* and Necla Birgul Iyison

*Address all correspondence to: burcin.duan1@boun.edu.tr

Molecular Biology and Genetics Department, Bogazici University, Istanbul, Turkey

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