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AT-A-GLANCE ARTICLE

Expanding neuropeptide signalling by multiplying receptor functional states and sub-cellular locations

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Abstract

Neuropeptide signalling is primarily based on activation of G protein-coupled receptors (GPCRs), the largest family of membrane receptors. GPCRs are involved in multiple physiological processes and are important drug targets for many human diseases. In this at a glance review, we focus on the recent advances in GPCR signalling related to the different structural and functional features of complexes involved in G protein- and arrestin-mediated signalling, receptor dimerization and oligomerization, modulation and transactivation of other signalling proteins and receptor compartimentalization. Our goal is to highlight the astonishingly complex and diverse network of signal transduction events that could arise from the activation of neuropeptide receptors.

Keywords GPCR signalling · Biased agonism · Receptor dimerization · Intracellular signalling · G protein

More than 100 neuropeptides known to be present in the nervous system play various roles by acting on neuronal and glial receptors (van den Pol 2012). Most neuropeptide receptors belong to the seven transmembrane-spanning G protein-coupled receptor (GPCR) family, although the few but important exceptions include the peptide/protein hormones that bind to receptor tyrosine kinases (RTKs), such as nerve growth factor, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, brain-derived neurotrophic factor and insulin, whose ligand-induced activation stimulates RTK protein-tyrosine kinase activity that subsequently induces intracellular signal transduction cascades with a wide range of functions.

However, this review will be limited to the neuropeptides acting at GPCRs with the aim of identifying currently "hot" areas of research. A number of recent outstanding reviews have considered every single aspect of GPCR structure and function in detail and, while thanking some of their authors for expressing the thoughtful and thought-provoking views on which this review is based, I must apologise to others whose work could not be specifically mentioned because of the limited number of references allowed.

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Structure and classification

GPCRs share a common counter-clockwise bundle structure of seven transmembrane (TM) helices and associate with heterotrimeric guanine nucleotide-binding proteins (G proteins) (Rosenbaum et al. 2009). They have been classically classified into six classes (A-F) on the basis of their amino acid sequences and functional similarities (Lagerstrom and Schioth 2008): class A, also known as the "rhodopsin-like family", is the largest group and includes hormones, neurotransmitters and light receptors; class B, also called "the secretin receptor family" contains about 70 receptors; class C includes the metabotropic glutamate family, GABA receptors, calcium-sensing receptors and taste receptors; class D are fungal-mating pheromone receptors; class E are cAMP receptors; and class F consists of frizzled/smoothened receptors. Neuropeptide GPCRs are widespread throughout class A and B.

Knowledge of the structure of GPCRs has increased exponentially over the last 10 years (Salon et al. 2011). The crystal structures of more than 20 peptide-binding GPCRs have now been solved: these include more than 50 structures of various active/inactive states bound to ligands with agonist/antagonist activity and thus provide new insights into GPCR functioning. Wu et al. (2017) reviewed the common architecture consisting of seven TM domains connected by intra- and extra-cellular loops, an extra-cellular N-terminus and an intra-cellular C-terminus. Most ligands bind to GPCRs in an ancestral binding cavity located between the TM domains and the extracellular

loops. In the case of class A and B GPCRs, this cavity is an orthosteric pocket divided into a major pocket (delineated by TM regions 3, 4, 5, 6 and 7), a minor pocket (delineated by TM regions 1,2,3 and 7) and an extra-cellular vestibule (involving the extracellular loops, the N-terminus domain and the tops of the TM regions). Small endogenous ligands belonging to "fast neurotransmitters" such as adrenaline bind in the major pocket, or at the interface between the major and the minor pocket but the larger size of endogenous peptides means that they simultaneously occupy both pockets, and the residues targeting the deep orthosteric pocket are usually critical in triggering receptor activation. Interestingly, the small synthetic agonists and antagonists targeting neuropeptide receptors interact with the receptors in a wide range of binding modes that are often distinct from those of endogenous ligands. Elucidating the structural determinants of bindings for endogenous and synthetic ligands is providing the ground for the successful design and development of new drugs.

A particularly important aspect of receptor pharmacology is receptor subtype selectivity and, given the close similarities of the peptides and receptors belonging to the different subfamilies, the development of receptor subtype-specific analogues has been a daunting task. However, as subtle differences in the size and shape of the binding site may affect receptor subtype selectivity, solving the structural puzzle at the molecular level will eventually lead to the design of more selective drugs.

Fundamental functional features

Neuropeptide receptors have some very well-known key features that distinguish them from the receptors for "fast-acting" neurotransmitters and are due to the particular release of neuropeptides. Unlike "fast-acting" neurotransmitters, which are packed in small clear synaptic vesicles (SCSV) located at synaptic terminal, neuropeptides are stored in large dense core vesicles (LDCV), which are present in pre-synaptic terminals and the soma and dendrites of neuronal cells (Zupanc 1996). Synaptic vesicles are released at the synapse in response to even a single action potential, but LDCV release occurs in response to high-frequency stimulation both at and outside the synapse. This means that neuropeptide receptors can be located at the synapse or all along axons, dendrites and cell bodies, where they are involved in mediating different physiological responses.

Unlike with fast neurotransmitters, which are rapidly degraded or re-uptaken from the synaptic cleft, neuropeptides are more slowly degraded, which allows their diffusion over greater distances. However, their maximum local concentration is much lower than that of fast neurotransmitters and this is reflected by their greater receptor affinity, which is usually in the nanoM range whereas that of fast neurotransmitters is usually in the microM range. Finally, the absence of re-uptake mechanisms requires neuropeptides to be re-synthesised and transported to the site of release once they have been liberated into extra-cellular space.

The distinguishing features of neuropeptide receptors are therefore their relatively high affinity for endogenous ligands and their distribution in various parts of the cells of the nervous system and not only at synapses.

Neuropeptide GPCR signalling

The binding of a signalling molecule to a GPCR leads to G protein activation, which triggers the production of a number of second messengers. In classical GPCR signalling, the ligand-gated receptor undergoes a conformational change that allows the receptor to activate a specific G protein, which transmits and amplifies the signal to the intracellular compartments. However, various new paradigms of receptor activation and signalling have been proposed over the last few decades (Wang et al. 2018), the most important of which are biased signalling, receptor trans-activation, receptor dimerisation and receptor localisation in specific plasma membrane subdomains. Even more interesting is the fact that these new paradigms can influence each other in various ways, thus providing a vast repertoire of signal modulation. Finally, the canonical signalling of GPCRs assumed that their ligand-induced activation is restricted to the plasma membrane but recent evidence supports the view that GPCR activity also occurs at multiple intracellular locations (Eichel and von Zastrow 2018). The possibility that a single receptor may signal at multiple locations expands its ability to engage multiple signalling molecules and pathways and, even more interestingly, also allows the generation of "temporal patterns" of activation of signalling molecules (Grundmann and Kostenis 2017).

Delineating the functional role(s) of location- and temporal-based receptor signalling is an exciting challenge that opens up a new golden age for GPCR research. What follows is a summary of the molecular, spatial and temporal aspects of neuropeptide signalling that focuses on the plasma membrane and intracellularly mediated activation of neuropeptide GPCRs.

Classical plasma membrane signalling via G proteins

As mentioned above, the binding of a signalling molecule to a GPCR leads to G protein activation, which triggers the production of second messengers. The key players of GPCR signal transduction are G proteins, which are heterotrimeric complexes consisting of an α, a β and a γ subunit, with the α and γ subunits being tethered to the plasma membrane by lipid anchors. The α subunit binds the nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP) and has intrinsic GTPase activity. When the receptor is in a resting, unbound state, GDP is bound to the α subunit and the entire

G protein/GDP complex binds to the inactive GPCR. Upon ligand binding, a change in the GPCR conformation activates the G protein and GTP physically replaces GDP in the α subunit. Multiple lines of evidence suggest that, in the receptor/G protein complex, the release of GDP by the G alpha subunit (a key event in G protein activation) is allosterically triggered by the GPCR. This is consistent with crystal structures that suggest that the displacement of an α-helical domain of the G alpha subunit allows the opening of its nucleotide binding pocket for GDP–GTP exchange, as a result of which the G protein subunit dissociates into a GTP-bound α subunit and a $\beta\gamma$ dimer (Hilger et al. 2018). Still anchored to the plasma membrane, both the α subunit and the $\beta\gamma$ complex interact with the enzymes that produce second messengers (adenylyl cyclase, phospholipase C etc.), as well as with ion channels. The G protein remains active as long as GTP is hydrolysed back to GDP by its intrinsic GTPase activity. At this point, the three subunits again associate in an inactive heterotrimer and the G protein complex reassembles with the GPCR. In this cycle, G proteins are turned on or off by ligand-receptor interactions working as a molecular switch (Fig. 1.1).

However, there are a group of mechanisms limiting the number of activation/inactivation cycles that any single GPCR can experience in the presence of its activating ligand, thus ensuring strict control of receptor activation (Rajagopal and Shenoy 2018). These mechanisms include receptor phosphorylation, followed by β-arrestin binding and endocytosis. The phosphorylation of receptor residues located in intracellular domains is linked to a decrease in receptor affinity for its activating ligand, a process known as receptor desensitisation (Fig. 1.2). In addition, receptor phosphorylation leads to β-arrestin(s) binding (Fig. 1.3) followed by the physical removal of the receptor from the plasma membrane and to its sorting to intracellular endosomal compartments (Fig. 1.4). From there, depending on the nature of the receptor and/or the cell type, the receptor may be recycled back to the cell surface (Fig. 1.5) or degraded upon reaching the lysosomal compartments (Fig. 1.6). These events together are responsible for blunting receptor responses at the cell surface.

Intracellular signalling from endosomes

Upon agonist binding, phosphorylation and binding to β-arrestins, GPCRs are internalised by means of endocytosis and can then be targeted for degradation or recycled to the cell membrane for another round of stimulation. It has long been assumed that internalised GPCRs are inactive and that internalisation protects cells against excessive stimulation, whereas resensitisation protects them against hormone resistance. However, one of the most intriguing aspects of GPCR signalling is perhaps their ability to signal not only from the plasma membrane but also from intracellular compartments (Eichel and von Zastrow 2018) and there is increasing evidence that internalised GPCRs not only remain active but also show sustained signalling from endosomes (Fig. 1.4), where they participate in multi-protein complexes that can sustain a variety of signalling pathways with particular temporal and spatial resolutions (Grundmann and Kostenis 2017). Some of the key players in this process are the β-arrestins whose persistent receptor binding may allow the formation of scaffolding platforms for other cytoplasmic signalling partners. Signalling from the plasma membrane and endosomes is responsible for complex, long-lasting and often biphasic responses, as has been clearly described in the case of β-arrestin ERK activation in which a first transient phase is due to receptor signalling at the plasma membrane and a second and prolonged phase is due to intracellular signalling. Compartmentalised signalling may also lead to the generation of qualitatively different signalling pathways: one (or more) at the plasma membrane and one (or more) at the internalised GPCR in the signalosome. The challenge is to resolve the diverse signalling outcomes in time and space and, most importantly, their specific roles in living cells.

Intracellular signalling from the Golgi, mitochondria, melanosome and nuclear membranes

Endosomal signalling is also triggered by a ligand-dependent mechanism but it has been found that some GPCRs signal from intracellular compartments without the need for any activating ligand (Jong et al. 2017). GPCRs in the Golgi regulate Golgi trafficking by coupling to heterotrimeric G proteins and inducing the reorganisation of the Golgi apparatus itself (Fig. 1.8). In this case, the signal from the Golgi apparatus is completely independent of the signal at the plasma membrane. GPCRs are also located in melanosomes (where they regulate organelle biogenesis) and mitochondria (Fig. 1.7). Finally, approximately 30 different GPCRs have been identified in the nucleus (Fig. 1.9), where they can interact with chromatin to regulate events such as gene transcription, DNA synthesis, histone modification and chromosome remodelling.

Biased signalling

Over the last 20 years, it has become clear that GPCR signalling is pluridimensional and occurs via multiple G protein and non-G protein effectors (Smith et al. 2018; Zhou et al. 2017). The concept of biased agonism assumes that GPCRs must exist in several distinct conformations and that different ligands can stabilise different subsets of conformations, each of which can activate a specific subset of intracellular effectors (Zhou et al. 2017). As each receptor conformation can be optimally be engaged by one specific ligand, ligand structure plays a crucial role because of its capacity to bias the distribution of receptors across an active conformational ensemble.

GPCRs can be coupled to more than one G protein subtype (G*a*s, G*a*q, G*a*i1–3, G*a*o, etc.) and different ligands may create a bias towards different G proteins (Fig. 2.1). One example

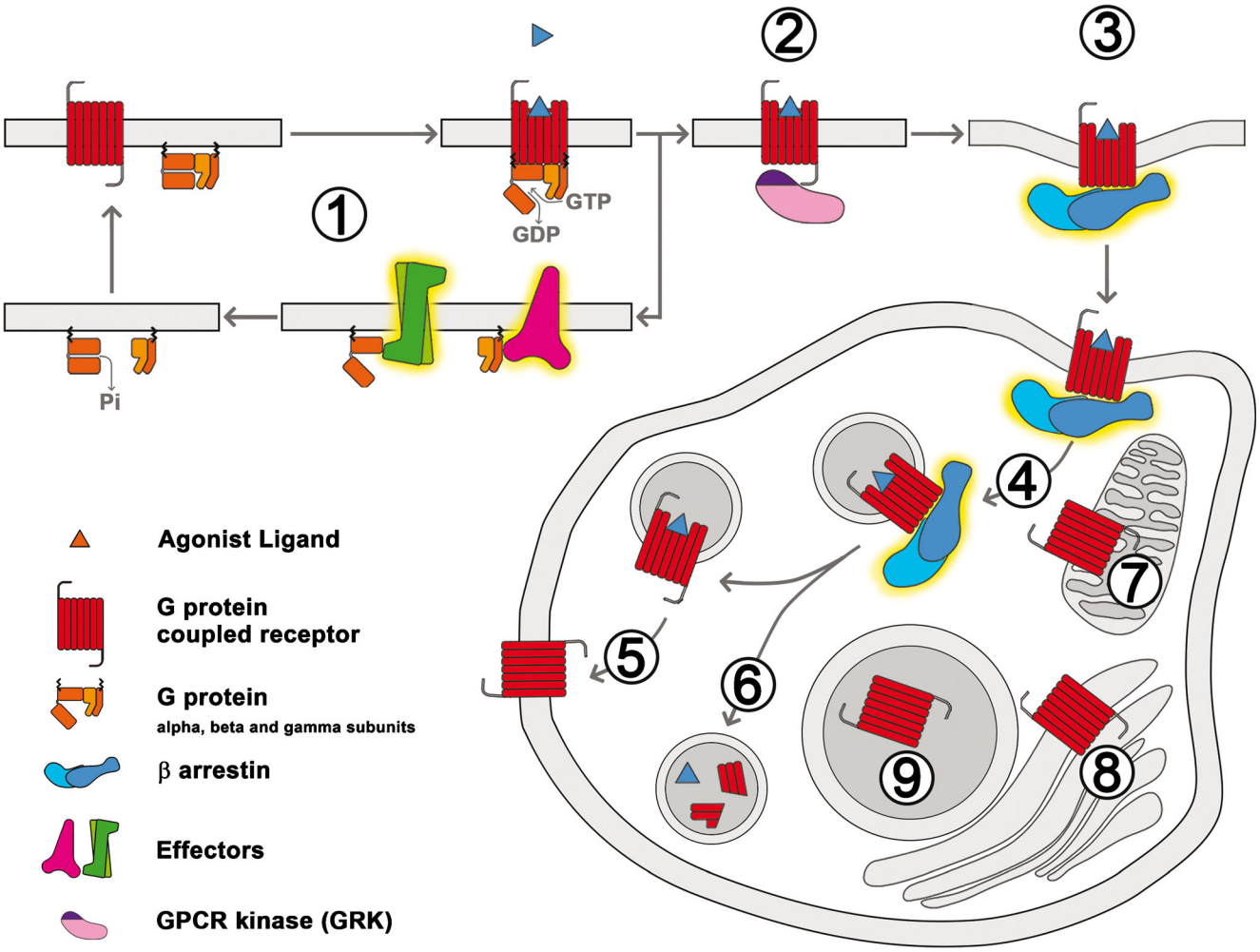

Fig. 1 GPCR signalling at the plasma membrane and in intracellular compartments. 1) *Classical G protein cycle;* in this cycle, G proteins are turned on or off by ligand-receptor interactions working as a molecular switch. 2) Receptor phosphorylation by GPCR kinases. 3) Recruitment of β-arrestins. 4) Receptor internalisation and signalling from endosomes. 5) Receptor recycling. 6) Receptor degradation in lysosomal compartments. 7) Receptor signalling in mitochondria. 8) Receptor signalling from Golgi compartments. 9) Receptor signalling in the nucleus

is the oxytocin receptor (OTR), which was one of the first GPCRs that was found to be characterised by biased agonism (Reversi et al. 2005). Oxytocin (the endogenous peptidic ligand of OTRs) promotes receptor coupling to G*a*q, G*a*i1–3 and G*a*o and the activation of the different G proteins depends on its concentration (Chini et al. 2017). Oxytocin-derived peptidic ligands with subtle modifications promote the specific coupling of the OTR to single G protein subtypes, thus providing evidence of ligand-dependent G protein bias (Busnelli et al. 2012).

A second type of biased signalling involves β-arrestins (Fig. 2.2). These adaptor proteins, which were originally shown to be involved in terminating receptor signalling by promoting receptor internalisation and down-regulation, were subsequently found to initiate G protein-independent signalling. This signalling mode was first reported in relation to the angiotensin AT1 receptor, in which the modified angiotensin peptide promotes β-arrestin-dependent signalling without any coupling to heterotrimeric G proteins (Wei et al. 2003). It is now widely acknowledged that ligands can selectively activate G protein pathways, β-arrestin pathways, or both (the last being the case for most endogenous ligands).

The implications of "functional selectivity" or "ligand bias" are substantial. First, although the biological response at the organ level is still determined by tissue receptor distribution, response at the cell level becomes a function not only of cell background and receptor structure but also of ligand structure. Secondly, G protein-mediated and β-arrestin-mediated effects, can lead to beneficial or detrimental outcomes depending on the tissues or organ and so the use of biased ligands may selectively promote therapeutic effects while preventing unwanted side effects. For example, knowing that the adverse effects of morphine at the μ-opioid receptor (MOR) are mediated by β-arrestin pathways, a biased MOR analogue capable of G

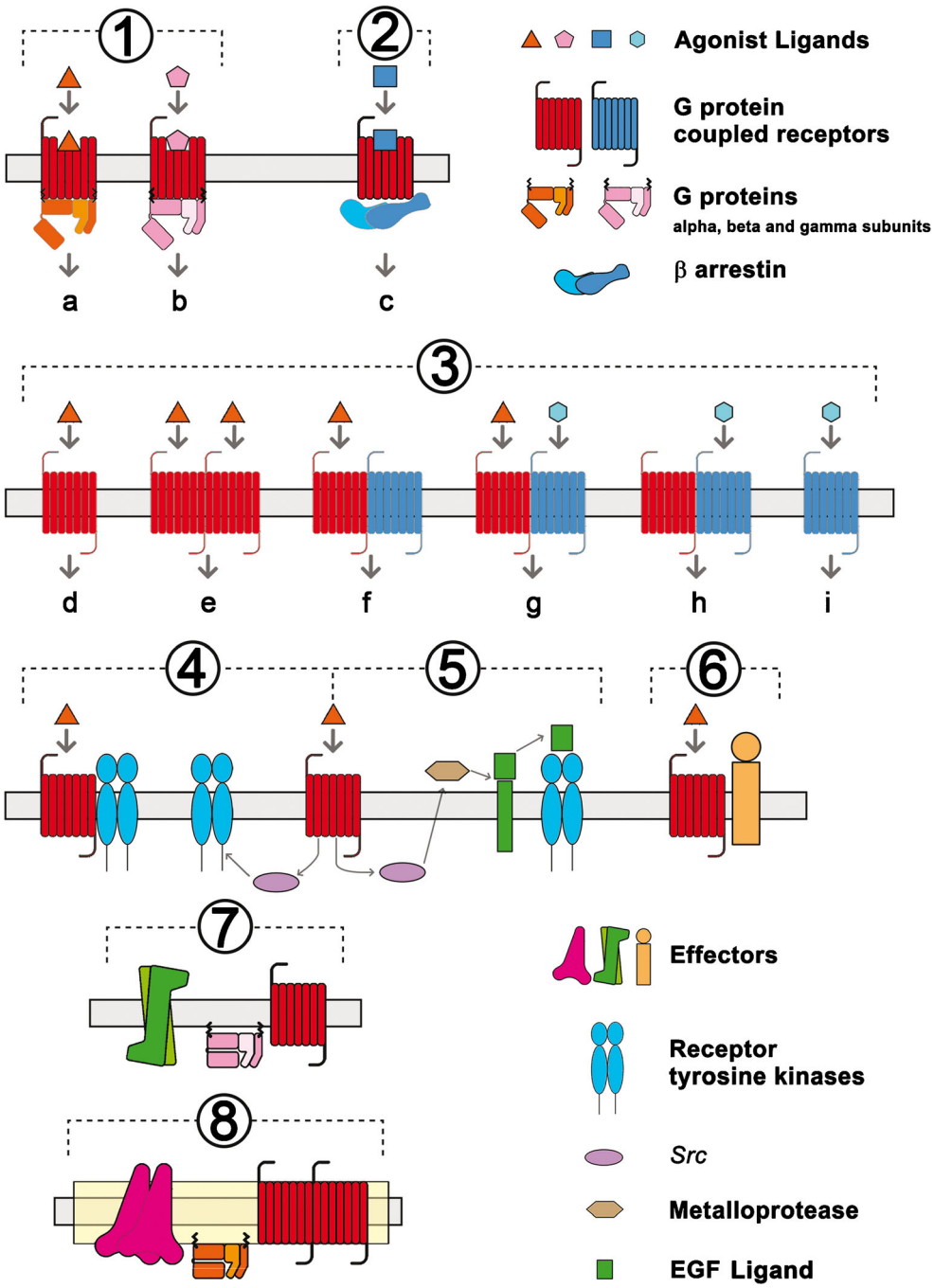

Fig. 2 Multiplying GPCR signalling at the plasma membrane. Various mechanisms are involved in generating the spatial complexity and diversity of GPCR signalling: 1) G protein-biased agonism. In this case, different ligands stimulate the coupling of the same GPCR to different G protein isoforms, thus leading to the activation of different intracellular signalling pathways (a,b). 2) β-arrestin biased agonism. β-arrestin-biased ligands promote the recruitment of β-arrestin in the absence of any previous G protein activation, thus, leading to the activation of alternative signalling pathways (c). 3) Homo- and hetero-dimerisation originate a number of possible combinations, each targeted by specific combination(s) of ligands and possibly recruiting different intracellular effectors (d-i). 4) GPCR-mediated transactivation of RTK receptors via Src. 5) GPCR-mediated transactivation of RTK receptors via a membrane metalloprotease that releases an extracellular ligand for RTK. 6) Direct activation of membrane effectors by GPCRs. 7) Compartmentalisation of GPCRs, G proteins and effectors outside membrane microdomains. 8) Compartmentalisation of GPCRs, G proteins and effectors inside membrane microdomains

protein coupling but devoid of β-arrestin coupling has been used in vivo to provide equivalent analgesia but with fewer adverse effects than morphine (Thompson et al. 2015). The existence of biased signalling has now been demonstrated for the vast majority of neuropeptide GPCRs and offers compelling evidence of the potential of biased signalling development.

Dimeric receptor signalling

It is now widely accepted that GPCRs associate with each other as homomeric or heteromeric dimers (Fig. 2.3) or higher-order oligomers (Ferre et al. 2014). GPCR subtypes and even the same receptor at different stages of its life cycle, may exist in different states of oligomerisation. GPCRs may even form dimers in the endoplasmic reticulum, where dimerisation allows for receptor transport to the plasma membrane. Dimers could also form at the plasma membrane as a result of a ligand-induced or dynamic ligand-independent, process. Advanced imaging techniques have been used to monitor the dynamics of monomer/dimer formation at the cell membrane (Calebiro and Sungkaworn 2018); it has been shown that some receptors are preferentially monomeric and

others preferentially dimeric; furthermore, the ratio of dimers/monomers is stable in some receptors and modulated by ligand binding in others. However, it is still unclear whether and how the oligomerisation of many GPGCs affects ligand binding, signalling or other receptor properties such as receptor trafficking. As shown in Fig. 2.3, different pathways may be activated by homo- or hetero-dimers, thus multiplying the repertoire of neuropeptide signalling.

Although there is strong evidence of oligomerisation in a number of experimental settings, the existence and relevance of receptor oligomers in native tissues and cells is still debated (Borroto-Escuela et al. 2017). Opioid receptor heteromerisation has been extensively investigated in the nervous system and many studies have described heteromerisation between the different types of opioid receptor and a wide range of GPCRs including adrenoceptors, cannabinoid, 5-HT, metabotropic glutamate and sensory neuron-specific receptors (Fujita et al. 2014) and μ and δ opioid receptor heteromers have been implicated in the development of tolerance to morphine (Moreno et al. 2018). Cannabinoid receptors also form complexes with a number of other GPCRs. Heteromers of the cannabinoid CB1 and adenosine subtype 2A (A2A) receptors have been reported in the dorsal striatum, a region that regulates motor activity, cognitive functions and mood. Co-activation of the two receptors in the dimer leads to a *reduction* in receptor signalling, accompanied by a switch in the activated intracellular signalling pathway and, most interestingly, CB1-A2A heteromers are selectively lost as Huntington's disease progresses to its later stages (Moreno et al. 2018). Evidence that dimerisation plays an important role in the physiopathology of the nervous system is starting to emerge.

Transactivation and direct modulation of other membrane proteins

Receptor-tyrosine kinases (RTKs) are a large group of membrane receptors that transduce various extra-cellular stimuli into intracellular signals in order to regulate almost all kinds of cell functions. RTKs can be transactivated by GPCRs in two different ways, as originally demonstrated for the epidermal growth factor receptor (EGFR) (Gavi et al. 2006). First, GPCRs induce the activation of a membrane metalloprotease capable of cleaving the EGF ligands released into the extracellular space, where they bind to and activate the EGFR (Fig. 2.4); the second way involves the GPCR activation of an intracellular protein tyrosine kinase such as Src, which phosphorylates tyrosine residues in the cytosolic domain of EGFRs, thus promoting their downstream signalling activity (Fig. 2.5). Although many studies suggest the relevance of RTK transactivation by GPCRs to various diseases (particularly cancer), this is supported by very few data.

Neurotrophic factors, including the neurotrophins/nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4, are a family of proteins that are essential for neuronal survival and plasticity. Neurotrophins signal through RTKs that can be transactivated by GPCRs and this transactivation has emerged as an important aspect of the biology of neurotrophin function (Jeanneteau and Chao 2006). Abnormal activity of the neurotrophin system has been found in a number of neurobiological conditions and, consequently, it has been suggested that neurotrophins may play a role in the treatment of neurodegenerative and psychiatric diseases. However, many of the attempts to do so have been unsuccessful because of unwanted side effects. Using GPCR ligands to transactivate neurotrophin receptors could be an alternative means of promoting trophic effects during neurodegeneration.

In addition to RTKs, GPCRs can interact with and modulate other membrane proteins (Fig. 2.6). It is becoming increasingly clear that GPCRs form physical signalling complexes with ion channels, including a number of calcium and potassium channels (Huang and Zamponi 2017). These interactions may occur directly via the binding of intracellular regions of the receptors with the proximal C-terminus region of the channel, or indirectly via a scaffolding protein. As a result, GPCRs could control channel opening and, consequently, calcium entry into cells. The fact that the receptor and channel can be internalised together after GPCR activation provides a means of acutely controlling channel density (and thus membrane excitability) in response to receptor activation.

Microdomain localisation

The plasma membrane of eukaryotic cells has been classically described as having a 'fluid mosaic' environment that randomly partitions proteins and lipids. However, this partitioning is not random but consists of ordered clusters of structural and signalling proteins embedded in membrane lipid rafts (MLRs) enriched in cholesterol, glycosphingolipids and gangliosides, which may exist as caveolae (morphologically observable flask-like invaginations enriched in caveolin proteins) or planar MLRs (Sezgin et al. 2017). Given their highly dynamic nature, it has proved to be extremely difficult to characterise the fundamental physico-chemical and biological nature of lipid rafts but recent studies using advanced techniques have begun to provide a picture of the organisation within these domains by mapping receptor dimerisation, clustering and lateral diffusion (Briddon et al. 2018). The current view is that MLRs act as scaffolds for many molecular entities, including signalling receptors and ion channels and regulate their assembly into active or inactive complexes in response to extra-cellular events. Specific subsets of receptors, G proteins and effectors may be enriched outside (Fig. 2.7) or inside the membrane microdomains (Fig. 2.8), leading to the compartmentalisation of receptor signallling. MLRs play functional roles in healthy and

pathological states of the nervous system and accumulating evidence indicates that the pre- and post-synaptic proteins essential for neuronal communication localise in them. It has been shown that GPCRs, RTKs, ion channels, G proteins, adenyl cyclases and members of the Rho GTPase family are clustered in MLRs upon scaffolding with proteins such as Cav-1, flotillin-2 (Flot-2) and tetraspanin-7 (TSPN-7), thus contributing to the organisation of the neuronal signalling components that regulate synaptic function and plasticity.

Nanodomains with diameters of approximately 80–100 nm and characterised by high receptor density have been described within excitatory synapses in the hippocampus (Biederer et al. 2017) and these "nanoclusters" are probably generated by membrane-associated guanylate kinase (MAGUK) scaffold proteins such as PSD-95. Interestingly, the number of PSD-95 molecules per nanodomain and the number of nanodomains per synapse each vary considerably. Nanodomains of differerent sizes and enriched in different proteins are present in various hippocampal cell types and even in different regions of the same neuron, thus suggesting that they may contribute to the functional diversity of excitatory synapses. The mechanisms underlying the clustering of MAGUKs are unclear but may involve palmitoylation, a common post-translational modification that can be involved in regulating receptor segregation in different specialised regions of a cell (Tortosa and Hoogenraad 2018). More than 70% of all known GPCRs contain potential palmitoylation site(s) downstream of their seventh transmembrane domain, which strongly suggests that palmitoylation may be a general feature. Most importantly, palmitoylation is a reversible process and repeated palmitoylation/depalmitoylation cycles may modulate different functions of proteins in a highly dynamic manner.

Conclusions and final remarks

The complexity of neuropeptide receptor signalling is becoming increasingly fascinating as technical advances in structural, cellular and genetic research open up new means of investigating their multifaceted features in live cells, tissues and organisms. As shown in Figs. 1 and 2, multiple stations of receptor signalling modulation have been identified at the cellular level, each possibly contributing to the diversification and/or amplification of specific signalling pathways. All of these possibilities may be exploited differently by different receptors and even by the same receptor depending on the cells in which they are expressed and/or the particular developmental/environmental conditions to which each cell is exposed.

Receptor signalling investigations now need to examine single living cells in different tissues and under specific physiological/pathological conditions. Future analyses will also have to consider systematically how the different mechanisms and factors regulating receptor signalling interact and influence each other. It is highly likely that localisation in specific microdomains influences receptor dimerisation/oligomerisation and biased signalling in a receptor-dependent manner and vice versa. Similarly, cell type-specific internalisation may influence the type(s) and kinetics of the activated signalling pathways.

Defining the rules governing neuropeptide receptor signalling in such a complex network of cross-platform interchange is the next challenge that lies ahead.

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