Oligomerization of GPCRs involved in endocrine regulation

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Abstract

More than 800 different human membrane-spanning G-protein-coupled receptors (GPCRs) serve as signal transducers at biological barriers. These receptors are activated by a wide variety of ligands such as peptides, ions and hormones, and are able to activate a diverse set of intracellular signaling pathways. GPCRs are of central importance in endocrine regulation, which underpins the significance of comprehensively studying these receptors and interrelated systems. During the last decade, the capacity for multimerization of GPCRs was found to be a common and functionally relevant property. The interaction between GPCR monomers results in higher order complexes such as homomers (identical receptor subtype) or heteromers (different receptor subtypes), which may be present in a specific and dynamic monomer/oligomer equilibrium. It is widely accepted that the oligomerization of GPCRs is a mechanism for determining the fine-tuning and expansion of cellular processes by modification of ligand action, expression levels, and related signaling outcome. Accordingly, oligomerization provides exciting opportunities to optimize pharmacological treatment with respect to receptor target and tissue selectivity or for the development of diagnostic tools. On the other hand, GPCR heteromerization may be a potential reason for the undesired side effects of pharmacological interventions, faced with numerous and common mutual signaling modifications in heteromeric constellations. Finally, detailed deciphering of the physiological occurrence and relevance of specific GPCR/GPCR–ligand interactions poses a future challenge. This review will tackle the aspects of GPCR oligomerization with specific emphasis on family A GPCRs involved in endocrine regulation, whereby only a subset of these receptors will be discussed in detail.

G-protein-coupled receptors

The importance of GPCRs

Physiological functions such as development, growth, behavior, learning, emotions, senses and aging are strongly dependent on the control of endocrine circuits. Hormonal signals exert their action via binding to nuclear receptors or membrane-bound receptors. The largest superfamily of membrane-bound receptors are G-protein-coupled receptors (GPCRs) (Kristiansen 2004), which are involved in the modulation of almost all physiological processes (Limbird 2004). The relevance of GPCRs is due to their fundamental role as information transducers (Raymond 1995, Wess 1998) by serving as hubs for signals transiting biological barriers (Rosenbaum et al. 2009).

Activation of GPCRs by different ligands, crosstalk of receptors within the membrane, and their interaction
with cellular or extracellular proteins determine the subsequently induced intracellular signaling pathway. The overall result of GPCR activation are specific physiological responses (Schwartz et al. 2006) and are based on various mechanisms such as induction of gene expression (Ho et al. 2009) or ion channel regulation (Veldhuis et al. 2015). Due to their key role in tuning physiological processes, approximately 50% of approved drugs modulateGPCRs (Hopkins & Groom 2002, Tyndall & Sandilya 2005, Schlyer & Horuk 2006, Mason et al. 2012).

How do GPCRs function?

GPCRs are activated by a wide variety of ligands including small peptides, nucleotides, ions, amines, or even large glycoprotein hormones, and they activate various signaling pathways (Kristiansen 2004). Based on different conserved amino acid motifs, the superfamily of GPCRs is subdivided into several families, whereby family A, also known as family 1 or rhodopsin-like GPCRs, constitute the family with most different members (Fredriksson et al. 2003, Fredriksson & Schioth 2005). They share a common structural architecture of seven transmembrane helices (TMHs) that are connected by three intracellular loops (ICLs) and three extracellular loops (ECLs). This architecture is confirmed by the inspection of solved GPCR crystal structures (reviewed in Koblika & Schertler 2008, Hanson & Stevens 2009, Lodowski et al. 2009). As a result of advanced experimental methods (Tate & Schertler 2009), many GPCR crystal structures have been published in the last decade (Zhao & Wu 2012, Piscitelli et al. 2015) and are useful tools to improve pharmacological approaches directed to GPCRs (Carlsson et al. 2011, Kontoyianni & Liu 2012, Mason et al. 2012, Shoichet & Koblika 2012).

Most of the endogenous and synthetic ligands of family A GPCRs are believed to bind within the transmembrane domain close to the second extracellular loop 2 (ECL2) (Surgand et al. 2006, Wichard et al. 2011), with the exception of glycoprotein hormone receptors (GPHRs) and leucine-rich repeat-containing G-protein-coupled receptors (LGRs) (Svensen et al. 2008, Kleinau & Krause 2009, Svensen et al. 2009, Kleinau et al. 2013). Based on a large amount of experimental data, a ‘global toggle switching’ mechanism is suggested to occur during ligand-induced activation (Schwartz et al. 2006, Smit et al. 2007). Correspondingly, activation is characterized by a spatial rearrangement of the TMHs relative to one another (Scheerer et al. 2008, 2009, Schertler 2008). This structural rearrangement is supported by amino acids acting as ‘micro-switches’ (Ahuja & Smith 2009, Hofmann et al. 2009, Nygaard et al. 2009). Different GPCR conformations are assumed to be related to different signaling activity states (Seifert & Wenzel-Seifert 2002, Koblika & Deupi 2007, Deupi & Standfuss 2011). Due to the capability for activation of G-proteins and several G-protein-independent signaling pathways (e.g. via arrestin), GPCRs share common ‘read-outs’, even if they bind different ligands (reviewed in Galandrin et al. 2007, Oldham & Hamm 2008).

GPCR oligomerization as a regulatory mechanism in signaling

How can an oligomeric GPCR constellation be defined, and is there a relevance for oligomerization?

Organisation of GPCRs as oligomers is an interesting feature under structural and functional prospective. It has been reported for numerous GPCRs (Tadagaki et al. 2012a), also in native tissue (Albizu et al. 2010a, Bouvier 2001, Rozenfeld & Devi 2011). Dimerization is a general term used to describe a GPCR-x/GPCR-x (homomer) or GPCR-x/GPCR-y (heteromer) constellation. The term oligomerization is used for dimeric, tetrameric or higher order complexes between GPCR protomers (Fig. 1). Such GPCR–GPCR interrelations should be characterized by the following parameters (for further details, see also (Gomes et al. 2016)): (1) physical interactions (side-chain interactions); (2) direct mutual functional modulation; or (3) a particular spatial distance to one another. It could be hypothesized that a relevant oligomerization is dependent on a functional significance (Tadagaki et al. 2012a). In case of heterodimerization, GPCR expression in the same cell type (Waldhoer et al. 2005, Pin et al. 2007) and simultaneous occurrence (Gonzalez et al. 2012) are prerequisites. Oligomerization has been reported for several GPCR subfamilies such as for the family A, family B or taste receptors (Li et al. 2002, Harikumar et al. 2008, Ng et al. 2012, Ng & Chow 2015).

Which general functional role may GPCR oligomerization have in physiology? To date, the functional significance of both homo- and heteromerization on the molecular level is related to the determination, enlargement and fine-tuning of signaling options such as signal amplification or modification (see below for details). Moreover, the GPCR capacity to form heteromers particularly provides an opportunity for their respective ligands to act in a synchronized manner and thereby to balance or coordinate related cellular responses and biological processes (such as metabolism or reproduction). As a hypothetical example, one ligand activates a specific
GPCR oligomerization

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Abstract: The rapid and effective system of counter-regulation in physiological signaling processes is mainly based on the ability of certain GPCR systems to interact with each other as homo- or heterooligomers. This flexibility of GPCR function is strongly supported by previous and recent findings, e.g. for the α1-adrenergic receptor (α1AR), whereby heterodimerization of α1AR with the chemokine receptor 4 controls blood pressure regulation (Tripathi et al. 2015). Moreover, the trace amine-associated receptor 1 (TAAR1) interacts, for example, with the dopamine-2 receptor (D2R) and, in turn, modifies dopaminergic neurotransmission (Hameier et al. 2015). Further examples include circadian-controlled heterodimers between the D4R and the β1- or α1B-adrenergic receptor, respectively, that are involved in the regulation of serotonin and melatonin syntheses (Gonzalez et al. 2012) and the interactome of melatonin receptors involved in circadian rhythm regulation (Benleulmi-Chaachoua et al. 2016).

What do we know about interactions between GPCR protomers constituting oligomeric interfaces?

Several GPCR-GPCR protomer interfaces have been reported under involvement of TMH4 (Carrillo et al. 2004, Hernanz-Falcon et al. 2004, Guo et al. 2005, 2008, Mancia et al. 2008), TMH1, and TMH5-6 (Hebert et al. 1996, McMillin et al. 2011, Yanagawa et al. 2011) or the extracellular N-terminal region (Uddin et al. 2012). The intermolecular interactions are constituted between single amino acids or between multitudes of side chains. Specific roles for such interactions or spatial distances between protomers are not defined as obligate for ‘protomer interrelations’ or cannot be determined as a standard, and may vary for different GPCRs. In conclusion, different putative interfaces and types of interactions for homo- and heterodimers can be assumed, whereby no universal interface property can be suggested. Several interfaces revealed by biophysical or biochemical methods have
been confirmed by determined crystal structures of GPCR dimers (Fig. 2). Furthermore, the GPCR-GPCR interfaces are most likely to be of dynamic character (Hu et al. 2013). GPCRs were described to be potentially expressed as a mixture of monomers and homomers, and the two forms were found to interconvert dynamically in an equilibrium (Hern et al. 2010, Kasai et al. 2011, Calebiro et al. 2013, Teichmann et al. 2014), reviewed in Lambert 2010).

Which functions or molecular properties of GPCRs are influenced by oligomerization?

Homo- and heterooligomerization is not a prerequisite for a general signaling capacity at family A GPCRs (Whorton et al. 2007), but it defines and widens the spectrum of fine-tuning options in signaling (White et al. 2007, Ciruela et al. 2010, Smith & Milligan 2010). Oligomerization can have a major influence on the
signaling properties of interacting protomers (Prinster et al. 2005) such as in ligand binding (Levoye et al. 2006a, Lohse 2010), G-protein coupling selectivity, and signal transduction mechanisms (reviewed in Bouvier 2001, George et al. 2002), or for cell surface expression (Uberti et al. 2005). Functional cross-regulation of G-protein activation may occur (Maurice et al. 2011b), despite the assembly of these GPCRs in heterooligomeric complexes (Nijmeijer et al. 2010).

Specifically, a particular GPCR in a specific cell type can function as a protomer, homomer, or in a heteromeric constellation (Fig. 3). Several functional scenarios of receptor property modifications dependent on oligomerization have been identified or have been suggested, as indicated in the following examples:

1. Constitution of homo- and/or heteromers:
   (i) The internalization of heteromers differs from that of homomers or monomers (Hanyaloglu et al. 2002, Kilpatrick et al. 2015).
   (ii) Signaling of the monomer is enhanced compared with the oligomer (Piechowski et al. 2013).
   (iii) Signaling of the monomer is weaker compared with the oligomer (Wilson et al. 2007, Magalhaes et al. 2010, Pellissier et al. 2011).

2. A homomer is capable of binding various ligands, and/or binding capacities differ compared with the monomer (Durroux 2005).

3. A heteromer, which may also be an orphan receptor (Levoye et al. 2006b), in a given cell type may:
GPCR oligomerization

(i) activate the same signaling pathways as the protomer(s) (neutral heterooligomeric) (Prinster et al. 2005);
(ii) modulate signaling pathways (e.g. strength of signaling) (AbdAlla et al. 2000, Terrillon et al. 2004);
(iii) activate pathways that differ from both protomers (Hague et al. 2006, Rashid et al. 2007, Gomes et al. 2013);
(iv) bind ligand(s) that differ (e.g. also at allosteric binding sites) (Durroux 2005);
(v) bind artificial ligands that modify signaling pathways such as bivalent ligands (Shonberg et al. 2011, Mohr et al. 2013, Yuan et al. 2013).

How is oligomerization detected experimentally?

In the last two decades, various methods have been applied to detect GPCR–GPCR interactions (Table 1). Each of these techniques exhibits both advantages and disadvantages when compared with one another and with regard to their aim; however, this field is characterized by strong development and rapid advancement. Because of previous thorough reviews of methods for the detection of oligomers (e.g. Persani et al. 2007, Kaczor & Selent 2011, Bonomi & Persani 2013, Gomes et al. 2016), we will not describe the particular methods in detail, but will merely provide a brief overview of commonly used approaches. Currently applied techniques can be subdivided based on their methodological principles or experimental conditions as follows: (i) antibody-based and antibody-independent; (ii) dynamic and nondynamic systems; (iii) analyses of living, fixed or lysed cells; or (iv) in vitro cell culture or an in vivo model. Antibody-based techniques, such as co-immunoprecipitation, co-immunolocalization, sandwich-ELISA or the proximity ligation assay, primarily recognize epitope tags (Table 1). Due to the lack of specific GPCR antibodies, these techniques are often limited to in vitro cell culture overexpression conditions. Homo- or heteromer detection by antibody-based assays are mostly used with differentially epitope-tagged GPCRs and are effective in fixed or lysed cells (reviewed in e.g. Skieterska et al. 2013, Gomes et al. 2014). In the case of antibodies that exist for a specific GPCR or GPCR

Table 1 Methods that have been used to determine di- and oligomerization of GPCRs.

<table>
<thead>
<tr>
<th>Method</th>
<th>Application</th>
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<tbody>
<tr>
<td>Antibody-based</td>
<td>In vitro</td>
</tr>
<tr>
<td>Co-immunoprecipitation (Co-IP)</td>
<td>+</td>
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<tr>
<td>Co-immunolocalization</td>
<td>(+)</td>
</tr>
<tr>
<td>Sandwich-ELISA</td>
<td>+</td>
</tr>
<tr>
<td>Proximity ligation assays (PLA)</td>
<td>mostly antibody-independent</td>
</tr>
<tr>
<td>Proximity biotinylation</td>
<td>+</td>
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<tr>
<td>Resonance energy transfer (RET)</td>
<td>+</td>
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<tr>
<td>FRET (F, fluorescence), e.g. via fluorescent ligands or based on SNAP- and CLIP-tag technology</td>
<td>+</td>
</tr>
<tr>
<td>BRET (B, bioluminescence), SRET (S, sequential), TR-FRET (TR, time-resolved)</td>
<td>+</td>
</tr>
<tr>
<td>Protein complementation assays (PCA)</td>
<td>+</td>
</tr>
<tr>
<td>e.g. based on fluorescent proteins, enzymes, ubiquitin</td>
<td>+</td>
</tr>
<tr>
<td>Photoactivated localization microscopy (PALM)</td>
<td>+</td>
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<tr>
<td>Fluorescence cross-correlation spectroscopy (FCCS)</td>
<td>+</td>
</tr>
<tr>
<td>Allosteric crosstalk e.g. via radioligand binding</td>
<td>+</td>
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</table>

Dimer detection using antibody-based and antibody-independent techniques. Antibody detection primarily requires tagging of GPCRs. Due to the availability of several primary GPCR antibodies and the possibility of tagged GPCR expression in animal models, in situ detection by antibody-based methods may be possible (indicated by bracketed plus symbol). The most promising technique used to detect GPCR–GPCR interactions in the physiological in vivo background is by analyzing allosteric crosstalk. The limitation of this method is the knowledge of adequate ligands, their detailed effects, and tissue specificity (also indicated as bracketed plus symbol).
oligomer, expression and detection of labeled GPCRs are also possible in animal models.

Elaborate enhancement of methods and constantly refined techniques even allow for the detection of GPCR protomers and oligomers in living cells or tissue samples by some antibody-independent methods. Of these methods, the application of resonance energy transfer (RET) and protein complementation assay (PCA) (for review see e.g. Vidi & Watts 2009, Lohse et al. 2012, Ng et al. 2012, Bonomi & Persani 2013, Ciruela et al. 2014, van Unen et al. 2015) have been particularly used to detect GPCR homo- or heteromerization.

Moreover, photo-activated localization microscopy (PALM) and fluorescence fluctuation spectroscopy techniques (FFCS) have been developed (for review see Herrick-Davis et al. 2013, Hink & Postma 2013). These straightforward methods permit a quantified interaction monitoring of fluorescent-labeled GPCRs at a nanomolar concentration level even as single molecules without a distance limit for interactions compared with RET.

The most promising methods to detect GPCR homo- and heteromerization in in vivo systems are via the analysis of allosteric crosstalk (Durroux 2005, Teitler & Klein 2012). This method is based on the idea that the dimerization process may lead to crosstalk between interacting GPCRs and may result in cooperative binding of ligands to these protomers. As a prerequisite, sufficient knowledge of the relevant GPCR-ligand interdependency (agonist, antagonist, inverse agonist effects) is required to analyze interactions with this method.

**Oligomerization of receptors in endocrine regulation**

Endocrine regulatory circuits consist of releasing hormones secreted from the hypothalamus. These hormones activate their cognate receptors on specialized pituitary cells, which subsequently lead to pituitary hormone release. The anterior pituitary hormones function at their end organs, which may be an endocrine gland. These hormones exert their action or further modulate end-organ hormone secretion, which is regulated in a negative feedback manner to the hypothalamus and the pituitary as it is known for the regulation of growth, thyroid function, fertility-reproduction and adrenal functions. Furthermore, many other endocrine functions such as energy metabolism or glucose homeostasis are tightly regulated by hormone/receptor feedback systems.

**What is known about oligomerization of GPCRs involved in endocrine regulation?**

Many receptors for releasing hormones and tropic hormones have been suggested to comprise of homo- or heterooligomers, whereby these findings are primarily based on in vitro studies. Although oligomerization of GPCRs is known for several of GPCR subfamilies (Young et al. 2007, Harikumar et al. 2008, Ng et al. 2012, Ng & Chow 2015), we will focus on several examples from the largest GPCR family, family A, to provide insights into linked aspects.

**GPCRs of the pituitary-thyroid axis**

In specific cells, the pituitary thyrotropin-releasing hormone (TRH) binds to the TRH receptor (TRHR) (Sun et al. 2003). In addition to TRHR expression in the pituitary, extrapituitary TRHR localization has also been found in various areas of the brain (Cao et al. 1998, Heuer et al. 1999, Bilek 2000). The TRHR may form homomers (Kroeger et al. 2001) in the absence of the ligand; however, ligand stimulation is suggested to further increase homooligomerization (Kroeger et al. 2001, Zhu et al. 2002). TRHR dimerization was found to affect trafficking but not signaling (Song & Hinkle 2005) and potentiated hormone-dependent receptor phosphorylation (Song et al. 2007).

There are two different subtypes of TRHR in rodents. These receptor subtypes are able to heterooligomerize, which has been found to change their functional properties in terms of β-arrestin recruitment (Hanyaloglu et al. 2002).

The secreted hormone thyrotopicin (TSH), in turn, activates the thyroid gland via binding to the thyrotopicin receptor (TSHR), which induces the production of thyroxine (T 4) and triiodothyronine (T 3). The TSHR has been previously reported to homooligomerize (Latif et al. 2001, Davies et al. 2002), which is most likely independent from TSH stimulation (Bonomi & Persani 2013; Urizar et al. 2005) or constitutive activation by mutation (Zoenen et al. 2012, Biebermann et al. 2012b). In regards to the TSHR, interaction between the receptor protomers occurs at the transmembrane bundle (Urizar et al. 2005), but an additional role of the extracellular domain on the oligomer constitution is proposed based on BRET experiments (Urizar et al. 2005). In the same study, further experimental evidence for negative cooperativity with respect to ligand binding was demonstrated and supports a functional relevance of homomers. This is of general
importance to further understand endocrine regulation at a wide range of ligand (hormone) concentrations with the highest level of sensitivity occurring at lower hormone concentrations (Urizar et al. 2005). This is true for physiological TSH concentrations in serum that span one order of magnitude (Bianco et al. 2014). Moreover, a recent study leads to the observation that two TSH molecules bound at a TSHR homodimer are necessary to activate Gq (IP) (Allen et al. 2011). Further support for a functional role of TSHR oligomerization is provided by studies showing dominant negative effects by pathogenic mutations (Calebiro et al. 2005), which can be best explained by mutual interrelations between TSHR protomers.

The receptors of the hypothalamic-pituitary-gonadal (HPG) axis

The first step in the production and release of the gonadotropins lutropin (LH) and follicitropin (FSH) is binding of gonadotropin-releasing hormone (GnRH) to its receptor (GnRHR) in the pituitary. Different studies revealed that the GnRHR is able to form microaggregates (oligomers) due to GnRH stimulation (Conn et al. 1982a,b, Cornnea et al. 2001, Kroeger et al. 2001). Evidence of human GnRHR dimerization derives from mutant receptors that inhibit ligand binding and second messenger production of wild-type via a dominant negative effect (Brothers et al. 2004). Interestingly, in the protochordate Ciona intestinalis, which lacks a hypothalamus-pituitary-gonad axis, four different subtypes of GnRHR exist (reviewed in Satake et al. 2013). One of the receptor subtypes (R4) is an orphan receptor and modulates the functionality of a further particular GnRHR receptor subtype in terms of intracellular Ca2+ elevation and ERK phosphorylation (Sakai et al. 2008). Moreover, GnRHR-mediated cAMP signaling is decreased in the heterodimeric constellation between this orphan GnRHR and another paralog due to a shift from Gs to Gi signaling (Sakai et al. 2012). However, how GnRHR oligomerization influences the expression and secretion of the gonadotropins LH and FSH has not yet been thoroughly investigated (Satake et al. 2013).

The receptors for FSH (FSHR) and LH/CG (LHCGR) play a crucial role in reproductive physiology (Themmen & Huhtaniemi 2000). First indications for LHCGR oligomerization were achieved from studies where co-expression of binding-deficient (with full signaling capacity) and signaling-inhibited (with diminished binding capacity) receptor fragments partially restored ligand-induced signal generation (Osuga et al. 1997). This was the first indication of a functional interrelation between LHCGR monomers. For the FSHR, transactivation was found to most likely induce biased signaling in terms of the generation of only one of two potentially induced hormone signals, but not both simultaneously (Ji et al. 2004).

In 2004, constitutive and agonist-dependent self-association of the LHCGR was demonstrated (Tao et al. 2004, Fanelli 2007) as well as the negative effects of inactive LHCGR mutants on wild-type receptor signaling in oligomeric constellations (Tao et al. 2004). Moreover, the functional rescue of two loss-of-function mice LHCGR mutants, a ligand binding defective and a signaling defective that both suffer from infertility in the homozygous state, was proven (Rivero-Muller et al. 2010), which pointed to a physiological relevance of LHCGR oligomerization and receptor–protomer interplay. Recently, a sophisticated method of single molecule analysis has revealed contacts of interacting LHCGR variants (Jonas et al. 2015).

Furthermore, heteromerization of FSH and LHCGR co-expressed in in vitro cell systems have been observed (Feng et al. 2013, Mazurkiewicz et al. 2015). In granulosa cells, LHCGR and FSHR are co-expressed and LHCGR/FSHR interaction is speculated to play a role during granulosa cell differentiation.

Besides the classical form of endocrine feedback regulation comprising the hormone/receptor systems in the hypothalamus, pituitary, and end-organs, and their feedback regulation, many primary nonendocrine tissues also express and secrete hormones such as leptin and adiponectin (adipocytes), ghrelin (stomach), and PYY cholecystokinin (gut). Action of these hormones is tightly regulated and their receptors are reported to constitute and function as oligomers.

One prominent example is body weight maintenance. The most important pathway of weight regulation is the leptin–melanocortin pathway that communicates peripheral signals to the hypothalamus, where information is integrated to an orchestrated reaction resulting in the maintenance of a constant body weight (Cone 2005, Oswal & Yeo 2007, Biebermann et al. 2012a, Farooqi 2014). From the periphery, anorexigenic hormones, such as the adipocyte-derived leptin or the pancreatic β-cell-derived leptin, affect their receptors on neurons of the arcuate nucleus in the hypothalamus (ARC). As a result, the expression of orexigenic peptides, such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), is repressed. Moreover, the expression of pro-opiomelanocortin (POMC) is enhanced, which gives rise to the expression of a
that are expressed in the PVN (Fig. 4), although these results have only been indicated in *in vitro* studies (Rediger *et al.* 2009). *In vivo* studies confirming these preliminary results are lacking.

An interesting study on MC4R oligomerization revealed receptor determinants that are involved in the constitution of protomer interfaces. Inhibition of MC4R oligomerization could be achieved by domain substitution using the noninteracting cannabinoid-1 receptor (Piechowski *et al.* 2013). Substitutions of the MC4R ICL2 and adjacent regions of TMH3 and TMH4, with respective regions of the cannabinoid-1 receptor, lead to partial MC4R dimer dissociation. Most importantly, the signaling capacity was increased in monomeric MC4R variants, which indicates a link between receptor dimerization and the signaling capacity.

In the hypothalamus, the MC3R is expressed on POMC and AgRP/NPY neurons (Barsh *et al.* 2000, Rediger *et al.* 2011). The MC3R is known to be embedded in a network of GPCR interactions with e.g. the ghrelin receptor (GHSR) (Fig. 4). Interaction of MC3R with GHSR leads to the upregulation of α-MSH-stimulated signaling and the downregulation of basal- and ghrelin-induced GHSR signaling (Rediger *et al.* 2011). Both the GHSR and the MC3R have been reported to have further diverse GPCR interaction partners (Borroto-Escuela *et al.* 2014) and also interact with orphan GPCRs such as GPR83 (Muller *et al.* 2013b). This, in turn, makes predictions of any physiological/functional impact due to modifications at the MC3R, the MC4R, or further interacting partners difficult.

The GPR83 was recently identified as a new determinant involved in body weight regulation (Muller *et al.* 2013b). So far this orphan receptor is expressed e.g. in the thymus and hypothalamus (Harrigan *et al.* 1989, 1991, Brezillon *et al.* 2001). Most importantly, GPR83 has been previously found to be involved in the control of circulating adiponectin levels (De Moerlooze *et al.* 2000), and Gpr83 expression is decreased in obese mice when compared with lean mice (Muller *et al.* 2013b). Moreover, GPR83 constitutes homodimers but also has the capacity for heteromerization, as has been demonstrated by heteromerization with the ghrelin receptor (Muller *et al.* 2013a). Interaction between both receptors leads to inhibition of the GHSR signaling capacity.

The signaling output of the previously mentioned GPCR interaction network (e.g. MC3R, GHSR and GPR83) is most likely crucial for the integration of all peripheral and central signals that are involved in energy metabolism. Future elucidation of combinatorial possibilities will be a matter of experimental confirmation and discussions (Fig. 4).
Further examples of GPCRs involved in endocrine regulation, which have been reported to oligomerize, are the prokineticin-receptor 2 (PROKR2), the oxytocin receptor (OTR) (Table 2), or dopamine receptor (D2R). Several examples will be provided to pinpoint the complexity and common occurrence of oligomerization.

The OTR and vasopressin receptors (AVPRs: V1aR, V1bR, V2R) belong to a subclass of family A GPCRs. The OTR and OT are involved in memory and learning, stress regulation and anxiety, and aggressive, sexual and maternal behaviors (e.g. Argiolas 1992, Malik et al. 2012, Ebner et al. 2013). Moreover, the OTR is involved in thermoregulation and brain development (Gimpl & Fahrenholz 2001, Kasahara et al. 2013). In the periphery, the OTR is expressed in the uterus and in the myoepithelial cells of the mammary gland at the end of pregnancy, and plays an important role during labor and milk ejection in a positive feedback manner (Viero et al. 2010). Several diseases or pathogenic conditions are associated with the function of this receptor including autism and schizophrenia (Gimpl & Fahrenholz 2001). In the brain, the OTR is expressed in neurons, which points to a functional significance of dimerization (Albizu et al. 2006). It has been suggested that constitutive homodimerization and heterodimerization between the OTR and the V1aR and V2R is established early in the endoplasmic reticulum (ER) and that ligand binding at the plasma membrane does not modify the dimerization state (Terrillon et al. 2003). Heterodimerization of V1aR and V2R vasopressin receptors determines the interaction with β-arrestin and their trafficking patterns (Terrillon et al. 2004). Of note, heteromerization may be of physiological relevance in tissues where the aforementioned receptors are co-expressed, such as heterodimers with the D2 dopamine receptor in the striatum (Romero-Fernandez et al. 2013). Heterodimerization was also reported for OTR and β-2 adrenergic receptors. This interaction modifies ERK1/2-activation allosterically in dependency of ligand treatment (agonists or antagonists) (Wrzal et al. 2006). It has been suggested that constitutive dimerization of the CRH receptor 1 and the corticotropin-releasing hormone receptor 1 were recently described (Murat et al. 2012). Via costimulation, this heterodimer mediates a synergistic catecholamine secretion compared with single ligand stimulation. Moreover, vasopressin potentiated CRH-induced cAMP accumulation and CRH potentiated AVD-induced inositol phosphate production. Vasopressin and CRH are both involved in the regulation of adrenocorticotropic hormone (ACTH) and insulin release (Murat et al. 2012).

Four major dopaminergic pathways exist in the central nervous system (CNS). The nigrostriatal system is critical for movement and the mesolimbic and mesocortical neurons play a major role in cognitive functions including feeding, affect, reward, sleep, attention, memory and

<table>
<thead>
<tr>
<th>Homomers</th>
<th>Heteromers</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Weight and appetite regulation and/or metabolism</td>
<td>MC3R-MC3R</td>
<td>(Rediger et al. 2011, 2012)</td>
</tr>
<tr>
<td></td>
<td>MC4R-MC4R</td>
<td>(Muller et al. 2013a,b)</td>
</tr>
<tr>
<td>GHSR-GHSR</td>
<td>GHSR-MC3R, GPR83, SSTR5, DRD1/2, SHTR2C</td>
<td>(Schelshorn et al. 2012, Ng &amp; Chow 2015)</td>
</tr>
<tr>
<td>GIPR-GIPR</td>
<td>GIPR-GLPR</td>
<td>(Harmeier et al. 2015, Dinter et al. 2015b)</td>
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<tr>
<td>TAA1-TAA1</td>
<td>TAA1-ADRA2A, D2R</td>
<td>(Dinger et al. 2003, Rediger et al. 2009)</td>
</tr>
<tr>
<td>NPY2R-NPY2R</td>
<td>NPY2R-MC3R</td>
<td>(Dinger et al. 2003)</td>
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<td>NPY1R-NPY1R</td>
<td>Reroduction</td>
<td>(Marsango et al. 2011)</td>
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<td>FSHR-FSHR</td>
<td>LHCGR-LHCGR</td>
<td>(Cornea et al. 2001, Kroeger et al. 2001)</td>
</tr>
<tr>
<td>LHCGR-LHCGR</td>
<td>GnRHR-GnRHR</td>
<td></td>
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</table>

References

This overview represents examples of GPCRs that are involved in endocrine regulation, which have been reported to comprise homo- or heteromers. The examples in brackets are confirmed in their interaction but have not yet been functionally characterized.
GPCRs in interaction with non-GPCR proteins

In addition to the above-described interactions between GPCRs in homomeric or heteromeric constellations, GPCRs are also able to associate with proteins that are capable of modifying signaling properties. They may impact trafficking or act as allosteric modulators (Maurice et al. 2011a). Furthermore, GPCRs also act as scaffolding proteins that link the receptor to downstream effectors (Walther & Ferguson 2015). Interacting proteins may interfere with the receptor either on the intracellular site or on the transmembrane spanning proteins and interact with the transmembrane helices (Kristiansen 2004).

Intracellular interacting proteins

Many interacting proteins exist, modifying the efficacy or mode of signaling and localization of the GPCRs (reviewed in Ritter & Hall 2009, Maurice et al. 2011a, Walther & Ferguson 2015). Examples of intracellular proteins can be subdivided into arrestin, PDZ (postsynaptic density 95/disc large/zona occludens-1)-containing proteins, and non-PDZ-containing proteins (Walther & Ferguson 2015), which are a large group of scaffolding proteins modifying GPCR functions. PDZ-containing proteins are a diverse group of proteins such as the Na+–H+ exchange regulatory group of proteins such as the Na+–H+ exchange regulatory group of proteins such as NHERF1, ADRB2 is degraded (Hall et al. 2011). Furthermore, GPCRs also act as scaffolding proteins that are capable of modifying signaling properties. They may impact trafficking or act as allosteric modulators (Maurice et al. 2011a). Furthermore, GPCRs also act as scaffolding proteins that link the receptor to downstream effectors (Walther & Ferguson 2015). Interacting proteins may interfere with the receptor either on the intracellular site or on the transmembrane spanning proteins and interact with the transmembrane helices (Kristiansen 2004).

Interacting transmembrane proteins

Identification of receptor activity modifying proteins (RAMPs), the single transmembrane-spanning proteins, underline a new mode of the functional regulation of GPCRs. It was initially identified as an obligatory factor for calcitonin gene-related peptide (CGRP) to activate its receptor, the calcitonin receptor-like receptor

learning (Perreault et al. 2014). Dopamine receptors have been suggested to be divided into two classes related to their signaling capacities (Beaulieu & Gainetdinov 2011): D1-class receptors (D1 and D5 subtypes) activate the adenylyl cyclase and increase cAMP levels; and D2-class receptors (D2, D3 and D4 subtypes) inactivate the adenylyl cyclase. Regions where the D2R is expressed include the striatum, nucleus accumbens, olfactory tubercle and hippocampus (Beaulieu & Gainetdinov 2011). The D2R comprises homomers (Guo et al. 2003, 2005) and a multitude of heteromers (Perreault et al. 2014), e.g. with the D1R, D4R, D5R, adenosine A2 receptor (A2R), serotonin 2A receptor (SHT-2AR), OTR, and the histamine H3 receptor (H3R). A functional relevance for the D1R/D2R heterodimer has not yet been confirmed (Frederick et al. 2015). Recently, in vivo relevance for GHSR/D1R interrelation was found to be involved in hippocampal behavior and memory (Kern et al. 2015). Dopaminergic pathways play a critical role in diseases such as Parkinson’s disease (nigrostriatal system), schizophrenia, and addiction (mesolimbic and mesocortical system) (Li & Ma 2013). In schizophrenia, cortical D2Rs may be hypersensitive to dopamine (Seeman et al. 2005, 2007) or present in increased amounts in some regions.

The prokineticin system is related to several physiological and pathological conditions (Maldonado-Perez et al. 2007, Negri et al. 2007, Zhou et al. 2012). Knockout mice models for both ligand and receptor revealed the role of PROK2 signaling in olfactory bulb morphogenesis and sexual maturation, and suggested PROK2 and PROKR2 as strong candidate genes for human GnRH deficiency (Ng et al. 2005, Matsumoto et al. 2006). The PROKR2 is involved in the pathogenesis of hypogonadotropic hypogonadism (Dode et al. 2006, Bonomi et al. 2012). Pathogenic germline PROKR2 mutations associated with central hypogonadism have been identified to cause modulation of distinct intracellular pathways (biased signaling modulation) (Libri et al. 2014, Sbai et al. 2014), whereby the PROKR2 wild type activates different G-protein subtypes (Chen et al. 2005). PROKR2 variants have been further described in patients with idiopathic combined pituitary hormone deficits (CPHD) including gonadotropin deficiency (Raivio et al. 2012, Reynaud et al. 2012). The PROKR2 also forms constitutive homomers in vivo (Marsango et al. 2011), and specific protomer–protomer interfaces for the oligomer have been suggested (Sposini et al. 2015). Future investigation of the relationship between oligomerization and pathogenic biased signaling modulation at this receptor is of interest.
(CL-R) (McLatchie et al. 1998). Different members of RAMP modify ligand specificity such as RAMP1 associated with CL-R to facilitate CGRP binding; however, association with RAMP2 to CL-R enables adrenomedullin to bind to CL-R (McLatchie et al. 1998). Since then, different members of the RAMP family have been identified. All RAMPs interact with CL-R, the calcitonin and the VPAC1 receptor; however, other GPCRs, such as glucagon or the PTHR, function together with distinct RAMPs (Hay et al. 2006).

The importance of interacting proteins for GPCR function is further evidenced by various examples. Previously, investigation of the functional aspects of the ACTH receptor (MC2R, melanocortin 2 receptor) in vitro was inconceivable as cell surface expression in different cell types was impossible. This changed with the identification of the melanocortin receptor-associated protein (MRAP) (Metherell et al. 2005), a single transmembrane-spanning protein that escorts the MC2R to the cell surface. Other melanocortin receptors (MC1R, MC3R, MC4R and MC5R) are able to traffic to the cell surface in the absence of interacting proteins; however, melanocortin receptor-associated protein 2 (MRAP2), which is primarily expressed in the hypothalamus, has been identified to influence the function of these MCRs (Cooray & Clark 2011, Jackson et al. 2015). Moreover, prokineticin receptor-1 (PROKR1) is the first non-melanocortin GPCR that is regulated by MRAP2 (Chaly et al. 2016). This suggests a new pathway by which MRAP2 regulates energy homeostasis by inhibition of PROKR1.

**Existence of direct interactions between GPCRs/ion channels and GPCRs/transporters**

Direct protein–protein interactions have also been reported for GPCRs and ion channels such as KIR channels or voltage-gated calcium channel Cav 2.2 that form a macromolecular ion channel/GPCR signaling complex (Douplik 2008, Benleulmi-Chaachoua et al. 2016). In turn, this leads to the idea of the possibility for a direct interaction and functional association between GPCRs and further transmembrane-spanning proteins. Channel types such as Ca2+ ion transporting transient receptor potential channels (TRPs) (Veldhuis et al. 2015) or substrate transporters, such as members of the major facilitator superfamily (MFS) (Law et al. 2008), are known to be closely linked to the function of GPCRs or vice versa. Specific signals from GPCRs located on sensory neurons converge on members of the TRP family, leading to channel sensitization and activation, which amplify e.g. pain, itch and neurogenic inflammation (Veldhuis et al. 2015). On the other hand, several GPCR ligands are transported out of the cell by members of the MFS with 12-transmembrane helices (e.g. dopamine transporter). Furthermore, transporter substrates, such as glucose for cell metabolism, are transported into the cell by MFS members (e.g. glucose transporter (GLUT)). There are few examples that support the notion of a direct GPCR/channel (Yekkirala 2013) or GPCR/transporter interactions, e.g. the observed interplay between the ADRB2, GLUT4 and melatonin 1 receptor/Cav 2.2 (Dehvari et al. 2012, Benleulmi-Chaachoua et al. 2016), or modulation of monoamine transporters by common biogenic amines via the TAAR1 (Xie et al. 2008). Altogether, this raises the possibility that an association between different proteins and GPCRs should be of importance due to a fast mutual or synergistic influence on functional properties. This hypothesis requires further and solid confirmation and validation.

**GPCR oligomerization in endocrine dysfunctions**

Is there a direct link between GPCR oligomerization and pathophysiology?

Malfunctions of approximately 100 GPCRs are associated with various human diseases (Hutchings et al. 2010) including cancer, viral infections, inflammation, infertility, and metabolic and neurological disorders (Seifert & Wenzel-Seifert 2002, Dorsam & Gutkind 2007, Garcia-Jimenez & Santisteban 2007, Schoneberg et al. 2004, Vassart & Costagliola 2011). Almost 20% of tumors harbor mutations in GPCRs and approximately 10% of cancer-related mutations have been identified in G-proteins (O’Hayre et al. 2013). An overview of the diverse roles of G-protein-coupled receptors (GPCRs) in the pathophysiology of various human diseases, subdivided into associations with obesity, diabetes, cardiovascular diseases, allergies or cancers, can be found in the excellent review by Heng and coworkers (2013). The pathogenic triggers at GPCRs vary from hormonal dysregulation, virus infections, mutations, or interactions with autoantibodies. Interestingly, it was recently reported that 365 human GPCRs are potential drug targets (Garland 2013).

As described in the numerous examples above, oligomerization is a common property for GPCRs (Ferre & Franco 2010) and can also be addressed to receptors.
involved in endocrine regulation such as the TSHR, LHCGR or MC4R (Table 2). Secondly, oligomerization is of functional relevance, although it has/could not have been rendered more precisely for all the GPCRs described here so far. By combining these two facts, that the correct function of GPCRs correlates with a specific protomer/protomer arrangement, makes it very likely that a dysfunction of GPCR signaling can also be due to modified oligomeric properties, e.g. by mutants in the protomer interfaces or in the ligand binding sites.

Despite the huge and increasing amount of in vitro findings and potentially artificial data, the relevance of GPCR oligomerization in vivo has been confirmed by several examples (Overton & Blumer 2000) including cases of pathophysiological importance (Tadagaki et al. 2011, 2012b, Tschische et al. 2011, Kern et al. 2015). GPCRs, in relation to neurophysiological processes, are one of the best studied subjects in this field (Gomes et al. 2000, 2013, Prinster et al. 2005, Fuxe et al. 2012). For example, a serotonin/glutamate receptor complex implicated in psychosis has been identified in previous studies and a role for GHSR in modulation of the D1R function in memory and behavior (Gonzalez-Maeso et al. 2008, Kern et al. 2015). Heteromerization between the dopamine receptor subtypes, D1R and D2R, are likely to be implicated in depression (Rashid et al. 2007). The D1R–D2R heterodimer was detected at higher levels in the postmortem striatum of patients than in normal subjects (Pei et al. 2010). Furthermore, the pathophysiological relevance of oligomerization was indicated in a specific case for angiotensin receptor II (AT1R) and bradykinin receptor-2 (B2R). The AT1R–B2R heterodimer is supposed to be functionally correlated with preeclampsia, as this heteromer was observed to be more abundant on platelets from pre-eclamptic women than on platelets from normotensive pregnant women (AbdAlla et al. 2001). Moreover, for the GABAB receptors, signaling activity is inhibited due to ligand-induced monomerization of the obligatory dimers, which has an impact on neuropathic pain sensitization (Laffray et al. 2012).

For GPCRs involved in endocrine regulation, which has omni-relevance in physiology and medicine, the elucidation of heteromeric forms have only recently started to become systematic (e.g. Rediger et al. 2009), with the motivation to investigate the entire ‘interactome’ and ‘signalosome’ of particular receptors (Table 2). The homomeric arrangement, even in an oligomer–monomer equilibrium (Lambert 2010), defines e.g. the LHCGR or TSHR per se as complexes. To date, there have been no difficulties in adequately combining or incorporating this property into current and previous mechanistical explanations of activation or inactivation, with few exceptions (Zoenen et al. 2012).

In the GPCRs described here, one particular feature potentially highlights a direct link between oligomerization and loss-of-function phenotypes: the dominant negative effect of reported heterozygote mutants or distinct receptor splice variants. Such dominant negative effects have been observed for numerous GPCRs such as rhodopsin (Kurada & O’Tousa 1995), AVPR2 (Zhu & Wess 1998), CCR5 (Benkirane et al. 1997), LHCGR (Tao et al. 2004), TSHR (Calebiro et al. 2005), MC1R (Beaumont et al. 2005), GnRHR (Grosse et al. 1997, Brothers et al. 2004), MC4R (Biebermann et al. 2003, Tarnow et al. 2008), or the prostacyclin receptor (Ibrahim et al. 2010). Dominant negative AT2R receptor oligomers induce G-protein arrest and symptoms of neurodegeneration (AbdAlla et al. 2009). For the aforementioned examples, it is generally assumed that the negative effect of receptor variants is transferred to the wild-type receptor by multimerization with the modified and nonfunctional receptor variant.

For the GHSR, MC3R, MC4R, PROKR2 or GPHRs, the occurrence of loss-of- or gain-of-function mutants in patients with hypo- or hyperfunction have been described (Themmen & Huhtaniemi 2000, Schoneberg et al. 2004, Tao 2006, 2010, Vassart & Costagliola 2011, Rediger et al. 2012, Troppmann et al. 2013, Libri et al. 2014, Vassart & Kleinau 2014). In particular, the mutations may cause different mechanisms at the receptor on the molecular level (excellently reviewed in Vassart & Costagliola 2011). In case of gain-of-function, constitutively activating mutations (CAMs) or substitutions leading to a loss of ligand selectivity have been described. The interrelated combination of both mechanisms caused by one single side-chain substitution such as that reported for the TSHR (Smits et al. 2003) is also feasible. Inactivating mutants accompanied by a loss-of-function may influence many individual or linked parameters at the receptor or ligand such as decreased expression level or loss of ligand binding capacity. In TSHR, pathogenic mutations lead to diseased conditions such as congenital hypothyroidism or nonautoimmune hyperthyroidism, whereas in the LHCGR, male-limited precocious puberty (by CAMs) or hypogonadism may be associated with inactivating variants, although rare. CAMs of the TSHR have been proven on their impact on oligomerization and a direct modification of oligomeric states has not yet been confirmed (Zoenen et al. 2012, Biebermann et al. 2012b).
This does not exclude the possibility that natural mutations in other receptors may indeed modulate oligomerization. Moreover, the opportunity that inactivating mutations, or a specific entity, interferes with a proper oligomer function should be considered and systematically proven in future studies.

**Open aspects and future perspectives**

Oligomerization is widely accepted to be a pivotal aspect of GPCR function regarding transport, signal transduction, signaling regulation and pharmacology. A certain number of examples for relevant oligomers have been reported and will further increase with the use of advanced methods and by directed in vivo searches for GPCR oligomers. Therefore, it is necessary to take GPCR oligomers into consideration in comprehensively understanding their physiological roles, and to explain interrelated processes such as pathogenic dysfunctions and clinical disorders, and in endocrine regulation. This perspective opens fascinating avenues for potential directed and selective pharmacological interventions. The incorporation of oligomeric receptor models into strategies for GPCR drug discovery and the targeting of homo- or heteromers are both challenging and exciting for the identification of new perspectives on the mechanisms of established or new therapeutic agents. Therefore, studying GPCR oligomerization and related structural–functional consequences, including the physiological relevance, is of importance. This includes the identification of the particular GPCR interaction partners (interactome analyses), which is dependent on physiological parameters such as cell type or developmental stage.

A further major effort in future studies is the dissection of functional effects induced by either monomers or oligomers. This requires forced monomerization and oligomerization of GPCRs, which is currently difficult to achieve. Only a comparison of the properties of monomers, homodimers, and those of wild-type receptor ‘mixtures’ present in a monomer/oligomer equilibrium will lead to definitive answers for the functionality of the different assembly states (Song et al. 2007, Lambert 2010, Teichmann et al. 2012, 2014, Calebiro et al. 2013). Of specific note, at the cell surface with a high number of expressed GPCRs (likely to range between 10 and 100s, e.g. Regard et al. 2008) or many other transmembrane spanning proteins such as transporters, it must be considered that many different heteromers may indeed exist. Unfortunately, current measurement methods do not simultaneously detect quantitative numbers of homomers, heteromers and monomers. This leaves open the possibility of multiheteromers and multiple equilibriums for different oligomers, expecting diverse oligomers of a particular GPCR at the same time point. Finally, these questions must be tackled in future studies, in line with the importance of elucidating proteins expressed at specific cell types and the further development of methods to study functional oligomers in in vivo systems.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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