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REVIEW ARTICLE

GPCR signalling from within the cell

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Traditionally, signal transduction from GPCRs is thought to emanate from the cell surface where receptor interactions with external stimuli can be transformed into a broad range of cellular responses. However, emergent data show that numerous GPCRs are also associated with various intracellular membranes where they may couple to different signalling systems, display unique desensitization patterns and/or exhibit distinct patterns of subcellular distribution. Although many GPCRs can be activated at the cell surface and subsequently endocytosed and transported to a unique intracellular site, other intracellular GPCRs can be activated *in situ* either *via de novo* ligand synthesis, diffusion of permeable ligands or active transport of nonpermeable ligands. Current findings reinforce the notion that intracellular GPCRs play a dynamic role in various biological functions including learning and memory, contractility and angiogenesis. As new intracellular GPCR roles are defined, the need to selectively tailor agonists and/or antagonists to both intracellular and cell surface receptors may lead to the development of more effective therapeutic tools.

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Abbreviations

Ang II, angiotensin II; CCR2, chemokine receptor 2; CXCR4, cysteine (C)-X-C receptor 4; ER, endoplasmic reticulum; ET-1, endothelin 1; ET_B, endothelin receptor B; GnRH, gonadotropin-releasing hormone; GRK, GPCR kinase; IP_3, inositol 1,4,5-trisphosphate; LPA, lysophosphatidic acid; NLSs, nuclear localization signals; NM, nuclear membrane; OA1, ocular albinism I; PACAP, pituitary adenylate cyclase activating polypeptide; PM, plasma membrane

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Intracellular GPCRs

From their position on the cell surface, **GPCRs** are known to transform external stimuli into a broad range of signalling pathways within the cell. GPCRs have also been found on the endoplasmic reticulum (ER), where they are synthesized, folded, modified and assembled, as well as in sorting vesicles on their way to the cell surface, or on endosomes that have just come off the membrane. Emerging studies, however, indicate that certain intracellular membranes may serve as alternate destinations or even the preferred location for a number of GPCRs where they may couple to different signalling systems and exhibit distinct patterns of subcellular distribution (Irannejad *et al.*, 2013, 2017; Jong *et al.*, 2014; Branco and Allen, 2015; Calebiro *et al.*, 2015; Campden *et al.*, 2015; Joyal *et al.*, 2015). One example of an exclusively intracellular GPCR is that of the **ocular albinism I** (OA1) GPCR, **GPR143** (Goshima *et al.*, 2014; De Filippo *et al.*, 2017). GPR143 localize to melanosomes and late endosomes/lysosomes in pigmented and non-pigmented cells, where they appear to act as 'sensors' regulating organelle biogenesis and maturation (Schiaffino *et al.*, 1996; d'Addio *et al.*, 2000; Samaraweera *et al.*, 2001). Other early examples of intracellular GPCRs include **prostaglandin EP_3** and **EP_4 receptors** that signal from endothelial, brain and/or liver nuclei (Bhattacharya *et al.*, 1999) or metabotropic glutamate receptor, **$mGlu_5$**, signalling from neuronal ER or nuclear membranes (NMs) (O'Malley *et al.*, 2003). GPCRs have also been found on vesicles, mitochondria (Benard *et al.*, 2012), NMs (Gobeil *et al.*, 2006; Calebiro *et al.*, 2010; Tadevosyan *et al.*, 2012; Joyal *et al.*, 2014) and even within the nucleoplasm on nuclear bodies and/or nuclear invaginations (Lee *et al.*, 2004; Morinelli *et al.*, 2007; Wright *et al.*, 2012). The diversity of the various intracellular destinations has raised many questions such as (i) what are the signals responsible for trafficking or retaining GPCRs in these unique locations; (ii) how are these intracellular GPCRs activated; (iii) what are the functional consequences of activating an intracellular GPCR; and importantly, (iv) what are the pathophysiological consequences of intracellular GPCRs. Since over 30 GPCRs have been described at the NM, here, we will primarily summarize current findings regarding nuclear trafficking, activation and location-dependent signalling of this expanding class of intracellular receptors (Irannejad *et al.*, 2013, 2017; Jong *et al.*, 2014; Branco and Allen, 2015; Calebiro *et al.*, 2015; Campden *et al.*, 2015; Joyal *et al.*, 2015). Where sufficient information exists, we have included additional information on GPCRs functioning on other intracellular membranes such as endosomes and mitochondria (Vilardaga *et al.*, 2014; Irannejad *et al.*, 2017).

Trafficking of nuclear GPCRs

There appear to be many mechanisms associated with nuclear GPCR translocation. These range from lateral diffusion through peripheral channels between the nuclear pore complex and the pore membrane to movement through the nuclear pore complex using linkers, carrier proteins and even components of the soluble transport machinery (Lusk *et al.*, 2007; Zuleger *et al.*, 2012; Katta *et al.*, 2014). For example, certain GPCRs such as the **apelin**, **angiotensin AT_1**, **adenosine A_1** and A_{2B} and **bradykinin B_2 receptors** use canonical nuclear localization signals (NLSs), that is, short stretches of basic amino acids that are subsequently recognized by specific members of the karyopherin superfamily for nuclear import (Lee *et al.*, 2004; Morinelli *et al.*, 2007; Wright *et al.*, 2012; Branco and Allen, 2015). In many instances, mutating various residues within the NLS prevents karyopherin recognition and hence nuclear localization of these GPCRs (Branco and Allen, 2015). Some GPCRs contain multiple NLS motifs including the proteinase-activated receptor **PAR2** [also known as F2R-like trypsin receptor 1 (F2rl1) and coagulation factor II receptor-like 1], which has two NLS motifs (Joyal *et al.*, 2014). After ligand-mediated receptor internalization, these motifs are recognized by importin β1, which allows PAR2 to translocate from the plasma membrane (PM) to the NM *via* sorting nexin11 and dynein transport along microtubules (Joyal *et al.*, 2014). In a similar fashion, agonist stimulation of the **oxytocin receptor** leads to its β-arrestin-mediated internalization followed by binding of the karyopherin, transportin-1 to NLS sequences within intracellular and C-terminal domains followed by transport to the nucleus (Di Benedetto *et al.*, 2014). The chemokine receptors **CCR2** and **CXCR4** are also trafficked to the nucleus *via* transportin-1 (Favre *et al.*, 2008; Don-Salu-Hewage *et al.*, 2013). Other GPCRs like the **platelet-activating factor (PAF) receptor** traffic to the nucleus *via* a process involving the small **GTPase**s, Rab11a and importin-5 (Bhosle *et al.*, 2016; 2017). Thus, there is no one single karyopherin or one preferred pathway that is involved in this process.

Not all nuclear GPCRs contain canonical NLS sequences. Some like the metabotropic glutamate receptor, $mGlu_5$, contain previously unidentified targeting sequences that are critical for the receptor's nuclear localization (Sergin *et al.*, 2017). Although the transport proteins responsible for this movement have not been identified, the sequences themselves are necessary and sufficient for $mGlu_5$ nuclear localization (Sergin *et al.*, 2017). Interestingly, the $mGlu_5$ C-terminal targeting sequence contains several kinase structural motifs, including motifs for **PKA**, **casein kinase II**, **PKC** and **Ca^{2+}/calmodulin-dependent protein kinase II** (CaMKII; Mao *et al.*, 2008). Conceivably, phosphorylation of this NM targeting sequence might influence binding of the transport protein (s) responsible for redistributing $mGlu_5$ receptors to the NM.

As described above, many GPCRs are first trafficked to the cell surface, activated, internalized and subsequently transported to the nucleus. Interestingly, many of the receptors trafficked from the cell surface are not associated with NMs but rather appear within the nucleoplasm itself (*via* unknown mechanisms). These include the apelin, CCR2, **vasopressin V_{1A} receptor**, **sphingosine 1-phosphate receptor, $S1P_1$**, oxytocin and CXCR4 receptors (Lee *et al.*, 2004; Kinsey *et al.*, 2007; Favre *et al.*, 2008; Estrada *et al.*, 2009; Verzijl *et al.*, 2010; Don-Salu-Hewage *et al.*, 2013; Di Benedetto *et al.*, 2014; Sun *et al.*, 2016). Some GPCRs, however, are trafficked directly to the NM. The **endothelin ET_B receptor**, **angiotensin II (Ang II)** and $mGlu_5$ receptors all seem to fit this model (Bkaily *et al.*, 2006; Jong *et al.*, 2014; Sergin *et al.*, 2017). In these cases, a simple

diffusion–retention model has been proposed since the outer NM is contiguous with the ER (Zuleger *et al.*, 2012; Katta *et al.*, 2014). This model suggests that proteins synthesized in the ER rapidly diffuse along the outer NM before passing through peripheral channels located between the nuclear pore complex and the pore membrane to become tethered on the inner NM *via* interactions with nuclear lamins or chromatin (Lusk *et al.*, 2007; Hinshaw *et al.*, 1992; Zuleger *et al.*, 2012). For example, the $mGlu_5$ receptor NM motif interacts with chromatin *via* a basic region (pI > 9.8) that may promote its nuclear retention (Sergin *et al.*, 2017). Because most transmembrane proteins are tagged with N-linked oligosaccharides as they are translocated through the ER and Golgi, differential glycosylation can be used to monitor protein trafficking. At least one nuclear GPCR appears to be directly transferred to NMs without going through the Golgi, the ET_B receptor (Merlen *et al.*, 2013). In contrast, $mGlu_5$ and PAF receptors exhibit glycosylation patterns consistent with a more dynamic diffusion model, one in which $mGlu_5$ at least is cycled back from the Golgi to the ER and then undergoes either lateral diffusion or a facilitated process to reach the inner NM (Sergin *et al.*, 2017). Most recently, it was reported that **$VPAC_1$**, a class B GPCR shared by **pituitary adenylate cyclase activating polypeptide (PACAP)** and **vasoactive intestinal peptide**, was trafficked to the NM *via* palmitoylation of its most N-terminal cysteine (Cys^{37}) in the extracellular domain (Yu *et al.*, 2017). Taken together, there seems to be many different signals and types of processes by which nuclear GPCRs arrive at their destination (Figure 1).

Ligand activation of nuclear receptors

Peptide receptors are amongst the earliest GPCRs to be described in the nucleus and/or on NMs (Gobeil *et al.*, 2006;

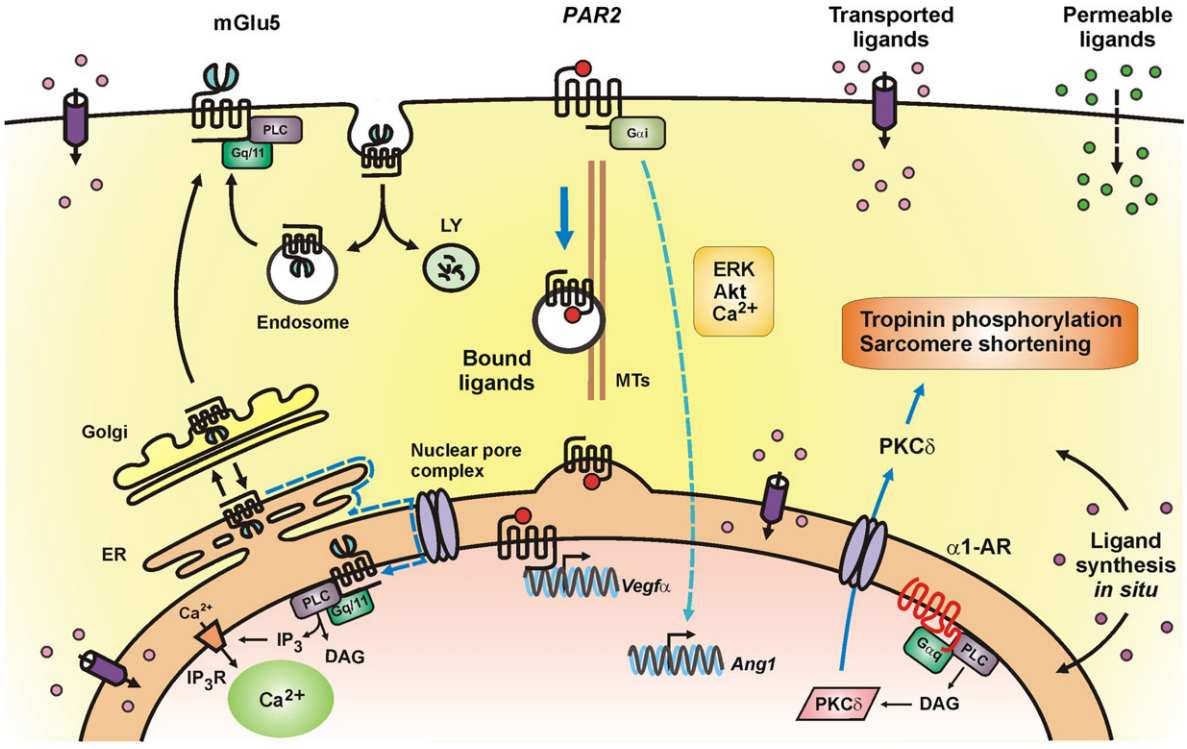

Figure 1

Schematic representation of selected intracellular GPCRs and ways in which they are activated. Left: proposed model of $mGlu_5$ receptor trafficking in neurons in which >90% of $mGlu_5$ receptors traffick through the golgi (Sergin *et al.*, 2017). From there, between 15 and 40% (Hubert *et al.*, 2001; López-Bendito *et al.*, 2002; O'Malley *et al.*, 2003) go to the cell surface where they undergoe a cycle of constitutive endocytosis and recycling (Trivedi and Bhattacharyya, 2012). Alternatively, 60–85% of $mGlu_5$ receptors are retrogradely trafficked back to the ER and *via* lateral diffusion (dotted blue line) reach the NM (Vincent *et al.*, 2016; Sergin *et al.*, 2017). Middle: ligand-bound PAR2 can translocate from the retinal ganglion cell surface to the nucleus *via* importin-β, Snx11 and dynein; nuclear PAR2 activates *Vegfa* expression. In contrast, signalling from cell surface PAR2 results in angiopoietin 1 expression (Joyal *et al.*, 2014). Right: the α_1-adrenoceptor (α1-AR) is localized to the inner NM of adult cardiac myocytes where it is activated by noradrenaline transported *via* the **organic cation transporter 3 (OCT3)** located on the cell surface and ER membranes. The nuclear α_1-adrenoceptor couples to $G_{\alpha q}$ and PLC, which activate nuclear PKCδ. PKCδ is then transported out of the nucleus through the nuclear pore complex. PKCδ induces phosphorylation of troponin 1 to regulate contractility and ERK to regulate survival signalling (Wu *et al.*, 2014; Wu and O'Connell, 2015). Intracellular GPCRs can be activated *via* (i) channels, transporters or exchangers recognizing specific ligands such as glutamate in the case of $mGlu_5$ receptors or OCT3 for noradrenaline activation of α_1-adrenoceptors; (ii) alternatively, ligands sufficiently permeable can freely diffuse across cell membranes; (iii) as in the case of PAR2, receptor-bound ligands can simply be internalized with a given GPCR; and (iv) ligands can be synthesized within the cell and either diffuse or be trafficked to a given cellular compartment.

Boivin *et al*., 2008). Frequently, the same receptors are also abundantly expressed on the PM. Although some peptide ligands are known to be destroyed in early endosomes, a process that allows receptors to be resensitized and recycled to the cell surface (Poole and Bunnett, 2016), in other cases, the cognate peptide ligand triggers internalization of the peptide GPCR and its subsequent trafficking to the nucleus (e.g. Figure 1). For example, both PAR2 and the PAF receptor appear to internalize with their ligands bound to the receptor (Joyal *et al*., 2014; Bhosle *et al*., 2016). The oxytocin receptor also moves to the NM after ligand binding in osteoblasts, breast cancer cells and primary fibroblasts (Kinsey *et al*., 2007; Di Benedetto *et al*., 2014). In the latter two cases, the authors used iodinated oxytocin and fluorescently labelled receptors to unequivocally demonstrate that cells internalize both the receptor and its ligand (Kinsey *et al*., 2007). In addition, peptide ligands can also activate endogenous peptide GPCRs already present within the nucleus or on NMs. The mechanism underlying this is not clear although ligand application shows radiolabelled co-localization of nuclear receptors followed by appropriate functional outcomes. Thus, application of radiolabelled **gonadotropin-releasing hormone (GnRH)** can be found in the nucleus along with the nuclear GnRH receptor in rat, hamster and human tissue whereupon it triggers histone H3 acetylation and phosphorylation within minutes of stimulation (Re *et al*., 2010). Re *et al*. (2010) speculate that GnRH is either transported intracellularly *via* an active uptake mechanism or that after peptide processing it is somehow transported back to the nucleus where GnRH receptors are activated. Intracrine signalling has also been proposed for CXCR4, which is abundantly expressed in the nuclei of various cancer cells especially metastatic prostate cancer cells. CXCR4 receptors are activated by **CXCL12α** that can be made by the same cells that express CXCR4 on NMs (Don-Salu-Hewage *et al*., 2013). Although the exact mechanisms underlying peptidergic intracrine signalling are not yet known, mounting data suggest that it does indeed occur.

Besides binding ligand at the cell surface and subsequent trafficking to an intracellular site, GPCRs can also be activated at subcellular locations in a variety of ways (Figure 1). Ligands can enter cells *via* diffusion or be made *in situ*, endocytosed and/or transported through channels or pores (Boivin *et al*., 2008; Barlow *et al*., 2010; Tadevosyan *et al*., 2012). Since ligand binding sites would be within the vesicle or luminal region of the ER or nucleus, extracellular ligands would have to cross both the PM as well as the intracellular membrane to activate intracellular GPCRs (O'Malley *et al*., 2003). A highly permeable ligand might freely cross such membranes, whereas a less permeable, charged ligand might require an active transport process. For example, in order to activate $mGlu_5$ receptors, at least two uptake systems are responsible for transporting glutamate into a neuron: the **sodium-dependent excitatory amino acid transporters** and the **cystine/glutamate exchanger** (Jong *et al*., 2005; 2007). Conditions that block either type of transporter reduce agonist uptake in cortical, hippocampal and striatal neurons (Jong *et al*., 2005; 2007; Purgert *et al*., 2014). Uptake of radiolabelled ligand can also be observed in isolated nuclei; nuclear ligand uptake can be blocked with sodium or chloride-free buffers or by inclusion of transporter blockers, such as **L-cystine** or **threo-β-benzyloxyaspartate**. Thus, for nuclear $mGlu_5$ receptors, 90–95% of all ligand-induced nuclear responses can be accounted for by these transporters (Jong *et al*., 2005; 2007; Purgert *et al*., 2014). Direct demonstration of the 'intracrine' effects of glutamate can be better achieved using microinjection of caged glutamate into $mGlu_5$-expressing neurons and then uncaging *via* restricted photoactivation (Jong and O'Malley, 2017). Only photoactivated neurons expressing caged glutamate exhibit $mGlu_5$-mediated Ca^{2+} responses, showcasing the spatial and temporal resolution of intracellular GPCRs (Jong and O'Malley, 2017). Similarly, Tadevosyan *et al*. (2015) prepared soluble caged Ang II compounds, which upon photoactivation can also stimulate nuclear angiotensin receptors to increase nuclear Ca^{2+} and, in turn, transcription.

In contrast to ligand transport, ligands might also be made *in situ via* localized biosynthetic machinery. For example, a large number of GPCRs such as the prostaglandin, platelet-activating factor and lysophosphatidic acid (**LPA) receptors**, whose ligands are bioactive lipids derived from membrane hydrolysis, are also located on NMs (Zhu *et al*., 2006). As ligand-generating enzymes are present on NMs and because such ligands readily diffuse through lipid bilayers, **PGE_2**, **PAF** and **LPA** can easily activate their cognate receptors. A variation on this paradigm is exemplified by the **LTC_4** pathway. This pathway is regulated both by induction of its synthetic components and by their translocation and co-localization at the nucleus. This allows for the effective production of LTC_4 followed by its binding to the nuclear receptors, **$CysLT_1$** and **$CysLT_2$**. In turn, receptor activation leads to the nuclear translocation of NOX4, generation of ROS, subsequent DNA damage and apoptosis (Pedruzzi *et al*., 2004; Weyemi *et al*., 2012; Dvash *et al*., 2015). Alternatively, activation of nuclear-localized GPCRs may not need ligands. Many GPCRs exhibit constitutive ligand-independent activity that might allow nuclear receptors to function (Chidiac *et al*., 1994; Boivin *et al*., 2008). For example, proteins like Homer1a can lead to agonist-independent $mGlu_5$ receptor activation (Ango *et al*., 2001). Agonist-independent activation of the **pituitary adenylate cyclase-activating polypeptide PAC_1 receptor** also occurs due to a close association with the insulin-like growth factor 1 receptor and subsequent transactivation by **Src (**Delcourt *et al*., 2007). Collectively, these studies emphasize the notion that nuclear GPCRs are fully functional even when their ligand binding domain is inside the cell and even within an intracellular lumenal domain. Thus, as long as a ligand is either made *in situ* or transported to the site of action, an intracellular receptor can be activated (Boivin *et al*., 2008; Vaniotis *et al*., 2011; Tadevosyan *et al*., 2012; Figure 1). Whether nuclear GPCR desensitization occurs does not appear to have been specifically addressed to date. Since nuclei are reported to express PKCα, δ and ε (Wu *et al*., 2014) and striatal nuclei at least express GPCR kinase (**GRK)2** and **GRK5** as well as arrestins 2 and 3 (Bychkov *et al*., 2012), some desensitization machinery is present in nuclei. However, confirmation or resolution of this issue awaits more definitive studies.

Signalling of intracellular GPCRs

Signalling molecules that have traditionally been considered to be associated with the PM or in the cytosol have also been demonstrated in the nucleus or on NMs. Both monomeric G proteins (e.g. Ras, Rab, Rho and Ran) and heterotrimeric G proteins (Gi, Gs, Gq, G_{11}, G_{12}, G_{13} and G_{16}) (Campden *et al.*, 2015) have been found in the nucleoplasm along with other effector molecules such as **adenylyl cyclase**, **PLA**, **C** and **D**, **PDEs** (Branco and Allen, 2015), DAG kinase, **phosphatidylinositol (PI) 3-kinase**, β-arrestin-1 and **GRKs** (Branco and Allen, 2015; Campden *et al.*, 2015). Many other regulatory proteins (PKA, PKC and **regulators of G-protein signalling** proteins) and critical channels [inositol 1,4,5-trisphosphate (**IP_3) receptors** and **ryanodine receptors**] are present (Campden *et al.*, 2015). Luminal Ca^{2+} is refilled at least in part by the nuclear **Ca^{2+}-ATPase**s (Nicotera *et al.*, 1989; Petersen *et al.*, 1998) located on the outer NM. Thus, although signals originating at the PM may be transmitted to the nucleus (Power and Sah, 2002), the presence of specific signalling machinery on the NM or in the nucleus argues for a nuclear regulatory system independent of the PM. For example, the nuclear $mGlu_5$ receptor couples to $G_{q/11}$ and PI-PLC in striatal nuclei to generate IP_3-mediated release of Ca^{2+} *via* IP_3 and ryanodine receptors in the nucleus (Kumar *et al.*, 2008). Further, nuclear **α_{1A}-adrenoceptors** on cardiac myocytes also signal through $G_{q/11}$ leading to PKCδ activation. The latter then translocates into the cytoplasm leading to troponin phosphorylation and sarcomere shortening (Wu *et al.*, 2014; Wu and O'Connell, 2015; Figure 1). Mislocalization of either the receptor or PKCδ blocked the effects of these α_{1A}-adrenoceptors (Wu *et al.*, 2014; Wu and O'Connell, 2015). **Endothelin-1 (ET-1)** also increases nuclear Ca^{2+} in isolated cardiac nuclei, cardiomyocytes and whole heart (Branco and Allen, 2015). Similarly, intracellular release of a caged ET-1 analogue also evokes an increase in Ca^{2+} that is attenuated by the IP_3 receptor blocker, 1,3-dicyclohexylcarbodiimide (Merlen *et al.*, 2013). Conversely, a caged cell-permeable endothelin ET_B receptor antagonist blocks the ability of intracellular ET-1 to increase nuclear Ca^{2+}, whereas extracellular ET_B receptor antagonists do not (Merlen *et al.*, 2013). Hence, acting as intracrine ligand, ET-1 is able to trigger an IP_3-mediated release of Ca^{2+} from perinuclear stores, which is involved in regulating transcription. Therefore, activated nuclear GPCRs can induce nuclear Ca^{2+} changes independent of cytosolic Ca^{2+}. Moreover, just as cell surface GPCR activation can trigger nuclear changes, activation of nuclear GPCRs can trigger profound cytoplasmic effects as well.

Despite the large number of GPCRs found on NMs, deducing their functional significance remains challenging because of the difficulty in probing the nucleus *in situ*, and because these same GPCRs are also present at the cell surface. Novel genetic and pharmacological strategies are overcoming some of these hurdles; several GPCRs have now been shown to play unique roles at the NM versus the cell surface – *in vivo*. For example, activation of the GPCR PAR2 anchored at PMs triggered the expression of the angiogenic gene, angiopoietin 1, whereas nuclear-activated PAR2 induced an increase in **VEGFA** in retinal ganglion cells providing *in vivo* evidence for a physiological function brought about by the receptor's subcellular localization (Joyal *et al.*, 2014). Similarly, ligand-stimulated, nuclear PARs activate angiogenic genes such as *Vegfa* and ***NOS3***, whereas cell surface PARs induce an up-regulation of pro-inflammatory cytokines *in vivo* (Bhosle *et al.*, 2016). Taken together, these data suggest that nuclear signalling may represent a versatile, independent system by which nuclear function is regulated.

Outside the nuclear membrane

Endosomal GPCRs

Desensitization and endosomal internalization of GPCRs is a well-known mechanism to regulate receptor number *via* degradation and/or resensitization. In addition, this process is emerging as a major mechanism by which internalized GPCRs generate unique signalling responses that are different from those initiated at the PM. For example, ligand-activated GPCRs are phosphorylated by GRKs, which, in turn, promote the recruitment of β-arrestins. The latter serve as scaffolding proteins that can themselves initiate a programme of G-protein-independent signalling (Shenoy and Lefkowitz, 2011). It is thought that while the cell surface GPCR might rapidly desensitize, its internalization as a receptor/G-protein/β-arrestin complex leads to a stable complex generating sustained endosomal signals (Bahouth and Nooh, 2017). Although initially arrestin-mediated signalling focused on the **MAPK/ERK1/2** cascade, many other signalling moieties can interact with the receptor/G-protein/β-arrestin complex such as **Akt**, **p38MAPK**, **JNKs** and activators of transcription, STATs (Reiter *et al.*, 2012). In turn, these proteins mediate downstream functions such as growth, cell survival, apoptosis, contractility, cell migration and cytoskeletal reorganization (Reiter *et al.*, 2012). While recognizing the ability of GPCRs to adopt distinct conformations contributing to the overall outcome of receptor activation, many drug discovery teams are searching for distinct ligands that can modulate these processes (Geppetti *et al.*, 2015; Rankovic *et al.*, 2016).

Besides G-protein-independent signalling, internalized endosomal GPCRs can also trigger G-protein-dependent signalling. For example, conformation-specific single-domain antibodies (nanobodies) were used to directly assess activation of the **β_2-adrenoceptor**. This approach verified *bona fide* G-protein signalling from early endosomes (Irannejad *et al.*, 2013, 2017). Using nanobody tools, an activated conformational state of the β_2-adrenoceptor was first detected at the PM seconds after ligand application. Shortly thereafter, a second activation phase was detected on the endosomes, which lasted long after the PM signals had diminished (Tsvetanova and von Zastrow, 2014). Interestingly, although both the PM receptor and the endosomal receptor generated cAMP, the G-protein-dependent response of the latter induced unique cAMP-generated transcriptional responses compared to those generated by the cell surface receptor (Tsvetanova and von Zastrow, 2014). In addition to $G_{\alpha s}$ endosomal signalling (Vilardaga *et al.*, 2014; Irannejad *et al.*, 2017), Jensen *et al.* (2017) recently demonstrated that

internalized **neurokinin NK_1 receptors** contributed to sustained endosomal signalling *via* $G_{\alpha q}$. Endosomal NK_1 receptor/$G_{\alpha q}$ signalling but not cell surface NK_1 receptors induced an increase in cytosolic cAMP, PKC and nuclear ERK resulting in neuronal excitation and nociception. As described below, only compounds preventing internalization or blocking the endosomal receptor were effective in blocking pain transmission.

Mitochondrial GPCRs

As with nuclear and endosomal GPCRs, there is a growing list of mitochondrial GPCRs. These include the purine, **$P2Y_1$** and **$P2Y_2$ receptors** (Belous *et al.*, 2004), **5-HT_4 receptor** (Wang *et al.*, 2016), angiotensin (Ang II) types 1 and 2 receptors (Abadir *et al.*, 2011), **melatonin MT_1 receptors** (Gbahou *et al.*, 2017) and **cannabinoid CB_1 receptors** (Benard *et al.*, 2012; Ma *et al.*, 2015: Hebert-Chatelain *et al.*, 2014a,b, 2016; Xu *et al.*, 2016; Melser *et al.*, 2017). To date, activation and/or inhibition of mitochondrial GPCRs appears to affect processes such as mitochondrial Ca^{2+} uptake, ATP production, ROS production and even apoptosis. For example, several studies have shown that activated CB_1 receptors trigger a cascade of events mediated by intra-mitochondrial $G_{\alpha i}$, which inhibits soluble adenylate cyclase leading to decreased cAMP, decreased PKA phosphorylation of various complex 1 proteins and a subsequent decrease in mitochondrial activity (Benard *et al.*, 2012; Hebert-Chatelain *et al.*, 2016). Short-term consequences of mitochondrial CB_1 receptor signalling include loss of mitochondrial mobility and synaptic depression; long-term consequences can include memory loss (Hebert-Chatelain *et al.*, 2016), metabolic defects and apoptosis (Xu *et al.*, 2016). Tools to determine the contribution of mitochondrial verse cell surface receptors will be critical in developing new drug targets and/or targeted therapeutics going forward.

Pathophysiological consequences of intracellular GPCR signalling

Emerging evidence furthers the notion that GPCR localization also plays an important role in various disease conditions. As described above, several chemokine receptors, such as CXCR4, are found on NMs in cancer cells where they have been shown to be ligand-responsive. Their activation leads to increased nucleoplasmic Ca^{2+} levels, which appear to enhance and extend signals initiated in the cytoplasm. Since increased amplitude and/or duration of nuclear Ca^{2+} is known to differentially activate unique transcription factors, nuclear chemokine receptors may be critical in regulating the process of tumourigenesis in given cell types. If so, impermeable or non-transported antagonists targeting cell surface receptors may be ineffectual for nuclear receptors. Another intracellular receptor playing a role in a disease process is spinal cord dorsal horn $mGlu_5$. In the spinal cord, the $mGlu_5$ receptor is a key mediator of neuroplasticity underlying persistent pain. Recently, we showed that the expression of $mGlu_5$ receptors is increased on spinal NMs following nerve injury in a model of neuropathic pain (Vincent *et al.*, 2016). Biochemical, pharmacological and ultrastructural studies all support the notion that increased $mGlu_5$ receptor levels are observed on spinal nuclei following nerve injury, whereas decreased numbers of receptors are present on the cell surface or intracellular membranes (Vincent *et al.*, 2016). Amazingly, blockade of spinal nuclear $mGlu_5$ receptors inhibits pain behaviours, whereas blockade of cell surface $mGlu_5$ receptors has little effect. Moreover, inhibition of a glutamate transporter mimics the effects of intracellular $mGlu_5$ antagonism by preventing intracellular uptake of the ligand. The re-localization of $mGlu_5$ receptors away from the cell surface onto NMs reinforces the importance of considering location in the drug discovery process: drugs designed to antagonize cell surface $mGlu_5$ receptors or unable to pass through the lipid bilayer or be transported into the nuclear lumen would be ineffectual in this chronic pain model. Location-dependent signalling of receptors redistributed to endosomes also contributes to disease pathology. As described above, Jensen *et al.* (2017) discovered that substance P/NK_1 receptor-mediated nociception is mediated by internalized, endosomal NK_1 receptors, not cell surface receptors. Inhibition of endocytosis (dynamin, clathrin or β-arrestin inhibitors) prevented pain signalling and promoted antinociception as did targeting NK_1 receptor antagonists to the endosomes (Jensen *et al.*, 2017). These findings reinforce the notion that where the receptor is signalling from (location-dependence) plays a critical role in whether a given ligand can modulate its receptor: conventional NK_1 receptor antagonists are clinically ineffective for the treatment of chronic pain because the receptor is no longer on the cell surface but rather is signalling from the early endosome promoting nociception (Jensen *et al.*, 2017). Taken together, GPCR localization can strongly contribute to receptor function and even disease pathology, underscoring the importance of designing drugs to block or enhance a given biological output in the context of where the receptor is signalling from.

New opportunities

Just as biased agonism led to a paradigm shift in GPCR research and drug development, emerging data documenting G-protein-dependent signalling from intracellular GPCRs should result in a similar marked change. Although for some GPCRs like the PAR2, receptor activation and/or inhibition may occur at the cell surface; for others, such as the $mGlu_5$ receptor and/or the α_{1A} adrenoceptor, whether a ligand gets across a given cellular membrane may change a its functional response (Figure 1). Thus, drugs with a desirable pharmacokinetic outcome might be further optimized for a desirable cell surface and/or intracellular response. In the latter case, the same key parameters associated with drug development for cell surface receptors such as efficacy, potency and specificity are still essential for intracellular GPCR drug design, with the added requirement that the preferred outcome might necessitate the drug accessing the cell's interior without perturbing its cell surface counterpart.

What are the ways in which an intracellular receptor can be activated or inhibited without blocking cell surface function? Furthermore, can intracellular modulators be targeted to a given subcellular organelle? Nuclei? Mitochondria? Lysosomes? Ways in which to address these issues are emerging as new challenges and opportunities for intracellular drug delivery. The *in vitro* and/or *ex vivo* applications have often used so-

called caged ligands in which the biologically active molecule is protected by a functional group, which, upon cellular uptake, can be activated by intracellular enzymes, pH, light and so forth. Indeed, caged compounds have been shown to activate intracellular receptors *in vitro* (Tadevosyan *et al.*, 2016; Jong and O'Malley, 2017). Although caged compounds are useful for a given set of *in vitro* applications, *in vivo* applications would be limited. Fortunately, a wave of new techniques drawn from chemistry, materials science and nanotechnology are creating a plethora of novel delivery strategies that can safely get a drug or a biomolecule into the cell and even to the appropriate organelle in the cytoplasm (Stewart *et al.*, 2016). For example, to prevent NK_1 receptor endosomal signalling, Jensen *et al.* (2017) synthesized tripartite compounds composed of cholestenol to promote membrane insertion, a polyethylene linker for flexibility and a membrane impermeable NK_1 receptor antagonist. This strategy successfully delivered NK_1 receptor antagonists into the endosome, blocking further NK_1 receptor signalling and promoting antinociception (Jensen *et al.*, 2017). Other new techniques include polymer-based nanocarriers, which can be tailored to display a given charge or combined with other biomolecules such as drugs, antibodies, proteins and oligonucleotides in order to more effectively deliver the desired entity to the particular intracellular location (Cohen and Granek, 2014; Wang *et al.*, 2014; Ye *et al.*, 2016). Collectively, it seems clear that next-generation technologies will allow an unprecedented ability to deliver therapeutics to every part of the cell.

In summary, current efforts to develop allosteric modulators and biased ligands for GPCRs might be further enhanced by recognizing that intracellular GPCRs are functional. Given the 'drugability' of GPCRs, the need to selectively tailor agonists and/or antagonists to both intracellular and cell surface receptors is critical. This would be highly significant for translational applications, since developing the most highly effective and minimally toxic drugs is the long-term goal for any effective treatment.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c,d,e).

Conflict of interest

The authors declare no conflicts of interest.

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