

Themed Section: Molecular Pharmacology of GPCRs

REVIEW

The GPCR, class C, group 6, subtype A (GPRC6A) receptor: from cloning to physiological function

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GPRC6A (GPCR, class C, group 6, subtype A) is a class C GPCR that has been cloned from human, mouse and rat. Several groups have shown that the receptor is activated by a range of basic and small aliphatic L- α -amino acids of which L-arginine, L-lysine and L-ornithine are the most potent compounds with EC₅₀ values in the mid-micromolar range. In addition, several groups have shown that the receptor is either directly activated or positively modulated by divalent cations such as Ca²⁺ albeit in concentrations above 5 mM, which is above the physiological concentration in most tissues. More recently, the peptide osteocalcin and the steroid testosterone have also been suggested to be endogenous GPRC6A agonists. The receptor is widely expressed in all three species which, along with the omnipresence of the amino acids and divalent cation ligands, suggest that the receptor could be involved in a broad range of physiological functions. So far, this has mainly been addressed by analyses of genetically modified mice where the GPRC6A receptor has been ablated. Although there has been some discrepancies among results reported from different groups, there is increasing evidence that the receptor is involved in regulation of inflammation, metabolism and endocrine functions. GPRC6A could thus be an interesting target for new drugs in these therapeutic areas.

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Abbreviations

7TM, seven transmembrane; CaSR, calcium-sensing receptor; CRD, cysteine rich domain; CRP, C-reactive protein; GABA_B, GABA type B; GLP-1, glucagon-like peptide 1; GPRC6A, GPCR, class C, group 6, subtype A; GWAS, genome-wide association study; KO, knockout; mGlu, metabotropic glutamate; T1R, taste receptors; TRAMP, transgenic adenocarcinoma of the mouse prostate; VFT, Venus flytrap; WT, wild type

Molecular pharmacology*Cloning and sequence analyses*

Using the protein sequences of class C GPCRs as a query, we identified the GPRC6A (GPCR, class C, group 6, subtype A) sequence in the Ensembl human genome database and subsequently cloned it from a human kidney cDNA library in 2004 (Wellendorph and Bräuner-Osborne, 2004). The human and mouse genes consist of six exons, which can be alternatively spliced leading to truncations in the Venus flytrap (VFT) domain and presumably inactive variants of the recep-

tor (Figure 1) (Wellendorph and Bräuner-Osborne, 2004). Phylogenetic analyses revealed that the receptor indeed belongs to class C GPCRs, which consist of eight metabotropic glutamate receptors (mGlu₁₋₈), two γ -amino butyric acid type B receptors (GABA_{B1,B2}), the calcium-sensing receptor (CaSR), three taste receptors (T1R1-3) and seven orphan receptors (GPR156, GPR158, GPR179 and GPRC5A-D) (Figure 2) (Wellendorph and Bräuner-Osborne, 2004; Bräuner-Osborne *et al.*, 2007; Rondard *et al.*, 2011; Orlandi *et al.*, 2012) [receptor nomenclature according to (Alexander *et al.*, 2013)]. Expanding the homology search to non-mammalian species revealed that GPRC6A displays the

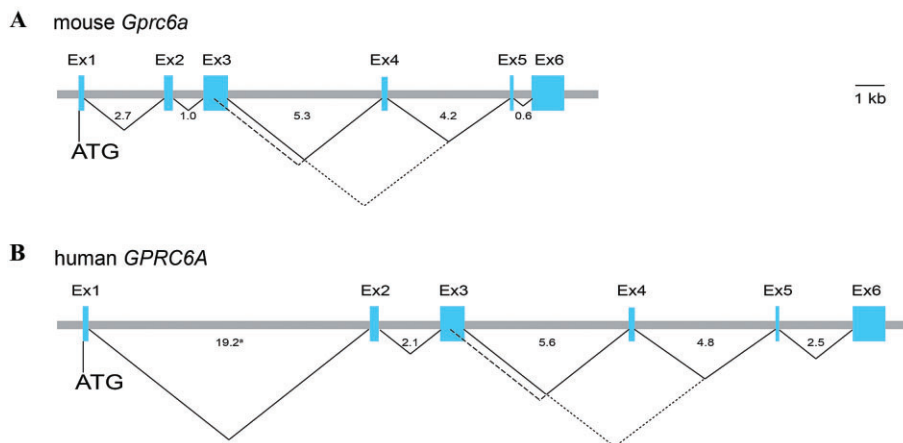


Figure 1

Genomic organization of the mouse and human GPRC6A genes. Exons (Ex1-6) are shown by boxes and introns by horizontal lines. The number of nucleotides in each intron is indicated (kb). Putative splicing to generate three receptor variants (Wellendorph and Bräuner-Osborne, 2004; Kuang *et al.*, 2005) is indicated by the connecting lines; isoform 1 (—), 2 (- - -), and 3 (· · ·).

*The size of intron 1 in human GPRC6A is 2 kb per unit due to its large size.

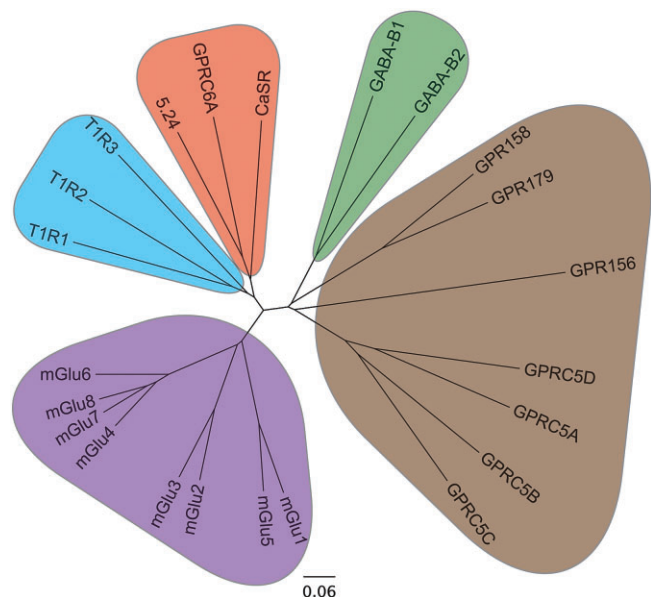


Figure 2

Phylogenetic analysis of GPRC6A with all presently known human family C receptors and the goldfish 5.24 odorant receptor. The predicted transmembrane spanning domains were used for generating a multiple alignment using ClustalW2 (<http://www.ebi.ac.uk/>), and a midpoint rooted tree was constructed using the program FigTree 1.4.0 (<http://tree.bio.ed.ac.uk/>). The scale bar is a function of amino acid substitutions based on the Blosum series substitution weight matrix.

highest amino acid sequence identity (44%) with the goldfish 5.24 receptor (Figure 2) (Specá *et al.*, 1999; Wellendorph and Bräuner-Osborne, 2004; Kuang *et al.*, 2005). Further analyses of the protein sequence revealed that the receptor contains a VFT domain, cysteine-rich domain (CRD) and seven trans-

membrane (7TM) domain (see modelled class C receptor in Figure 3A), which are hallmarks of class C GPCRs (Wellendorph and Bräuner-Osborne, 2004; Bräuner-Osborne *et al.*, 2007; Rondard *et al.*, 2011). The VFT domain bears homology to bacterial periplasmic binding proteins (O'Hara *et al.*, 1993) and has been shown to contain the orthosteric amino acid and calcium binding sites for mGlu, CaSR, GABA_{B1} and T1R1 receptors (Figure 3A) (Wellendorph and Bräuner-Osborne, 2009a).

The GPRC6A protein sequence has been cloned from human, mouse and rat and predicted in the genomes of dog, chicken, chimpanzee and several other simians. Mouse and rat receptor proteins display a notable higher degree of sequence identity (93%) than murine and human proteins (80%), based on alignments of the full-length sequences (Table 1). Instead human GPRC6A is more closely related to chimpanzee (98%) and dog (86%) orthologues (Table 1). GPRC6A has also been predicted to be conserved through evolution to goldfish, zebrafish and pufferfish (Luu *et al.*, 2004; Bjarnadóttir *et al.*, 2005; Christiansen *et al.*, 2006a), but not to the invertebrates fruit fly, mosquito and roundworm (Bjarnadóttir *et al.*, 2005). Within the different domains of the receptor, the sequence identity is highest in the VFT compared to the 7TM domain, for example human and mouse sequence identities are 82% between the VFTs but only 50% between the 7TMs (Wellendorph *et al.*, 2005). This suggests that the orthosteric VFT binding site is harder to discriminate between species than allosteric sites in the 7TM domain.

In 1996, it was shown by Romano *et al.* that the mGlu₅ receptor is a disulfide-linked dimer as a Western blot band, corresponding to the size of a dimer, was converted to a monomer size band upon reduction with dithiothreitol (DTT) (Romano *et al.*, 1996). A few years later, it was shown by mutagenesis that a cysteine residue in a loop in the VFT of mGlu₁ [C140, (Ray and Hauschild, 2000)], mGlu₅ [C129, (Romano *et al.*, 2001)] and CaSR [C129/C131, (Ray *et al.*, 1999)] makes this intramolecular disulfide bond. This

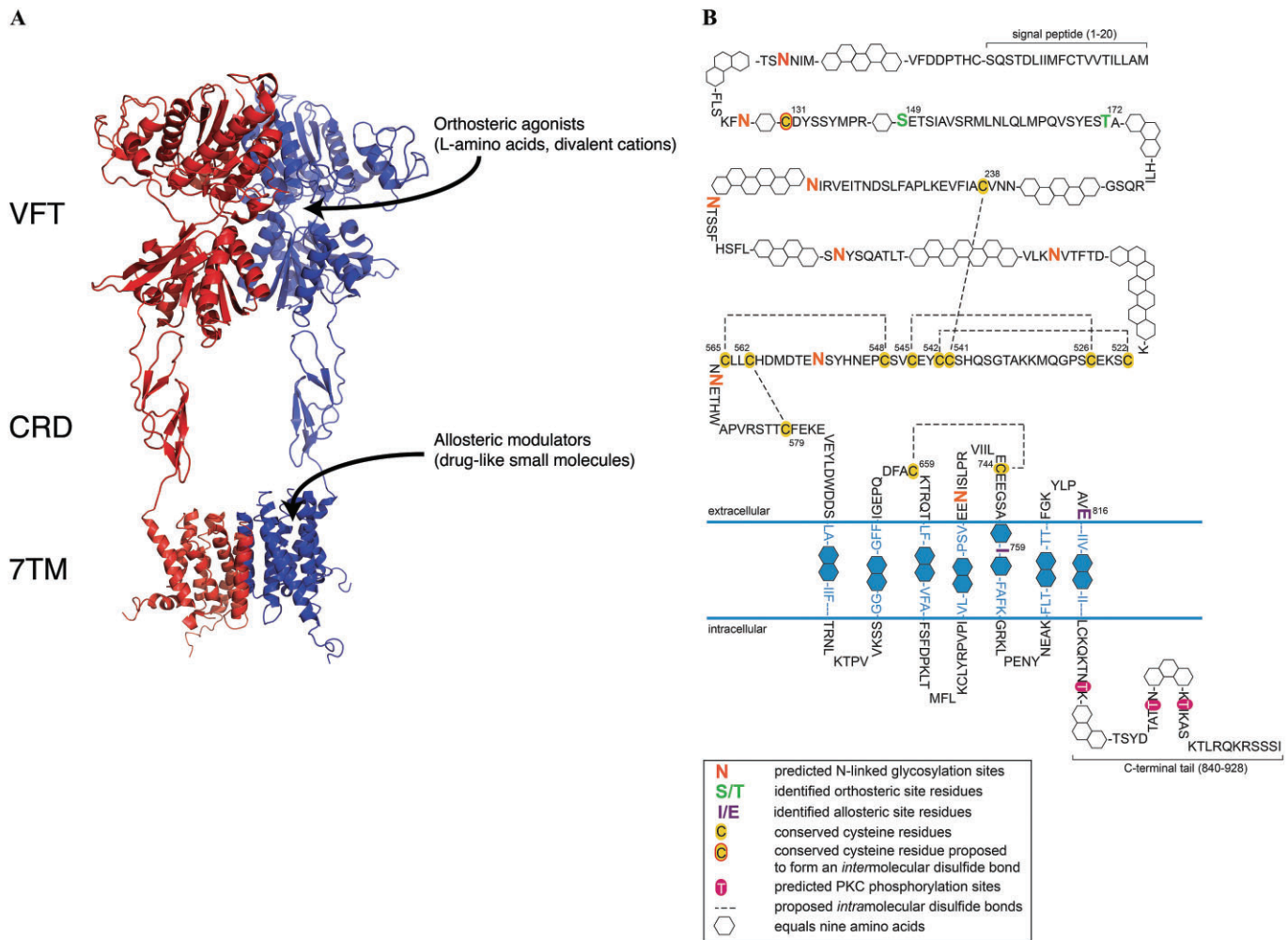


Figure 3

(A) Model of an active conformation of a dimeric family C GPCR based on 3D X-ray structures of domains from mGlu₁, mGlu₃ and β_2 -adrenergic receptors. The localizations of the Venus flytrap (VFT) domain, cysteine-rich domain (CRD) and seven transmembrane domain (7TM), orthosteric and allosteric ligand binding sites are indicated. The models were constructed with the program MacPyMol using coordinates from PDB files 1EWK (mGlu₁ open-closed/active VFT), 2E4U (mGlu₃ CRD) and 2R4S (β_2 -adrenergic receptor 7TM). (B) Schematic representation of key structural features of the predicted mGPRC6A protein. Symbols are provided in the key. Orthosteric and allosteric site residues were reported previously in human GPRC6A (Wellendorph *et al.*, 2005) and mouse GPRC6A (Faure *et al.*, 2009; Gloriam *et al.*, 2011) respectively. Cysteine residues predicted to be involved in intra- and intermolecular bond formation are conserved in class C ATDs (Bräuner-Osborne *et al.*, 2007), and in the CRDs of mGlu receptors (Rondard *et al.*, 2011). Predictions were made using the following servers at <http://www.cbs.dtu.dk>: Signal peptide, SignalP 4.1; transmembrane domains, TMHMM 2.0; N-linked glycosylations, NetNGlyc 1.0; kinase phosphorylation, NetPhosK 1.0.

cysteine is conserved in GPRC6A (C131 in mouse) and it is thus likely that this receptor is also a covalently linked dimer (Figure 3B). This is further substantiated by the observation of a dimer size Western blot band of mouse (Kuang *et al.*, 2005) and rat (Wellendorph *et al.*, 2007) GPRC6A expressed in HEK293 cells, which is converted to monomer size upon reduction with DTT (Kuang *et al.*, 2005). In addition, the VFT domain contains several potential N-glycosylation sites (Figure 3B) whereas no O-glycosylation sites are predicted (using NetOGlyc at <http://www.cbs.dtu.dk>). Albeit none of these glutamine residues have been confirmed experimentally to be glycosylated, we and the group of Hampson have previously shown by Western blotting that treatment with the N-linked deglycosylating enzyme PNGase F reduces

the size of receptor, strongly indicating the receptor is N-glycosylated (Kuang *et al.*, 2005; Wellendorph *et al.*, 2007).

The CRD contains nine cysteine residues, which are known to be 100% conserved in all class C receptors (except the GABA_{B1,B2} and the orphan receptors that do not contain a CRD) (Bräuner-Osborne *et al.*, 2007; Rondard *et al.*, 2011). Analyses of mGlu receptors have shown that eight of the cysteine residues form intra-CRD disulfide bonds (Muto *et al.*, 2007), whereas the ninth cysteine residue form a disulfide bond with a cysteine residue in the VFT (Rondard *et al.*, 2006; Muto *et al.*, 2007) thus promoting a very rigid connection between the VFT and CRD. All of these residues are conserved in the GPRC6A receptor and it is thus conceivable that the disulfide bonds are also present in this receptor subtype

Table 1

Amino acid sequence identities among GPRC6A orthologues from selected species. Numbers indicate percentages for the overall identities of amino acids between pairwise aligned sequences using the Blosum62 matrix. Protein sequences of chimpanzee, dog and chicken are based on the conceptualized translated mRNA from predicted gene products

	Human	Mouse	Rat	Chimpanzee	Dog	Chicken	Goldfish 5.24
Human	100	80	80	98	86	84	43
Mouse	–	100	93	79	80	63	42
Rat	–	–	100	79	80	63	42
Chimpanzee	–	–	–	100	85	65	43
Dog	–	–	–	–	100	66	44
Chicken	–	–	–	–	–	100	44
Goldfish 5.24	–	–	–	–	–	–	100

(Figure 3B). Furthermore, a disulfide bond between cysteine residues in the first and second extracellular loop, analogous to the disulfide bond shown in class A receptors (Cherezov *et al.*, 2007), is predicted (Figure 3B). Finally, the C-terminal contains three potential PKC phosphorylation sites, which might regulate receptor desensitization, internalization and/or interaction with scaffolding proteins (Figure 3B).

Activation mechanism, dimerization and cell surface expression

The activation mechanism of class C receptors including GPRC6A has not yet been fully elucidated, but the level of our current understanding has recently been reviewed in detail by the Pin group (Rondard *et al.*, 2011). Briefly, it is assumed that agonist binding to the cleft of the VFT promotes closure of either one or both of the clefts, which in turn leads to a twist of the dimer interface (Jensen *et al.*, 2002). This conformational twist is then relayed to the 7TM domains via the rigid VFT-CRD interaction, which leads to a reorientation of the 7TMs in the dimer and/or a conformation change within the 7TM domains (Rondard *et al.*, 2011). Most of this knowledge has been obtained using mGlu receptors as model systems, but it is reasonable to believe that the activation mechanism is conserved to the GPRC6A receptor given that it is also activated by L- α -amino acids like the mGlu model receptors.

It is evident from the information above, that the dimerization of class C receptors is essential for their function. mGlu and CaSR receptors form homodimers, whereas GABA_{B1} has to form a heterodimer with GABA_{B2} in order to traffic to the cell surface and become functional (Bräuner-Osborne *et al.*, 2007; Rondard *et al.*, 2011). Likewise, T1R1 and T1R2 have to form heterodimers with T1R3 to become functional (Nelson *et al.*, 2001; 2002). Recently, it was also shown by fluorescence resonance energy transfer that mGlu_{1,5} and mGlu_{2,3,4,6,7,8} receptors can form heterodimers within these two subgroups when expressed in HEK293 cells (Doumazane *et al.*, 2011), but whether they also form functional heterodimers *in vivo* remains to be demonstrated. In 2001, the CaSR was also suggested to be able to form heterodimers with mGlu₁ and mGlu₅ (Gama *et al.*, 2001), but the lack of

follow-up reports on this observation suggest that it is not of widespread importance compared to the homomeric receptors. It is worth noting that the highly confirmed GABA_B and T1R heterodimers and the proposed mGlu heterodimers each group together with their heterodimeric partner within branches of the phylogenetic tree (Figure 2). The closest mammalian homolog of GPRC6A is the CaSR. Both receptors are widely expressed and have overlapping expression patterns (Wellendorph *et al.*, 2009c; Rossol *et al.*, 2012). It is thus possible that the receptors could either directly form heterodimers or, if expressed as homodimers in the same cell, have synergistic effects on intracellular signalling. Future experiments will have to address these questions.

When we cloned the human GPRC6A receptor, we observed that albeit the protein was expressed intracellularly in tsA cells (HEK293 variant), only very low amounts trafficked to the cell membrane (Wellendorph and Bräuner-Osborne, 2004). Later, we and the group of Hampson demonstrated that both mouse (Kuang *et al.*, 2005; Wellendorph *et al.*, 2005) and rat (Wellendorph *et al.*, 2007) GPRC6A traffic to the cell membrane where they are able to form functional homomeric receptors. In analogy with the GABA_{B1} receptor, human GPRC6A contains a potential endoplasmic retention motif, RKR, in the C-terminal tail, which could cause the observed intracellular retention. However, mutation of the motif does not promote cell surface expression (Kuang *et al.*, 2005; Wellendorph *et al.*, 2005). It is also possible that activation of the receptor could lead to phosphorylation of the C-terminal tail (Figure 3B), which in turn could lead to internalization of the receptor. However, inactivating mutations of two highly conserved agonist binding residues in the VFT cleft did not lead to cell surface expression (Wellendorph *et al.*, 2005). It is possible that, for example, a scaffolding protein or a heterodimerization partner is necessary to promote cell surface expression of particularly the human orthologue, which is absent in the cell lines used for recombinant expression. The finding that endogenously expressed and functionally active GPRC6A receptor exist in the human 22RV1 and PC-3 cell lines (Pi and Quarles, 2012a), indicates that the human receptor can be functionally expressed at the cell surface and thus lacks a partner in the heterologous cell lines used.

Ligands and signalling

In early 2005, we were the first to deorphanize the GPRC6A receptor. Given the lack of cell surface expression and function of the human orthologue expressed in tsA, COS and CHO cells, we hypothesized that the intracellular retention was caused by the 7TM domain and/or C-terminal tail, and thus generated a chimeric receptor consisting of the VFT and CRD from human GPRC6A and the 7TM domain of the goldfish 5.24 receptor (Wellendorph *et al.*, 2005). This chimeric receptor trafficked to the cell surface and was functional in a Fluo-4 based intracellular calcium assay in agreement with the observed G_q pathway coupling of the 5.24 receptor (Specia *et al.*, 1999; Christiansen *et al.*, 2006a). These experiments showed that the receptor is a promiscuous L- α -amino acid receptor most potently activated by the basic amino acids L-arginine, L-lysine and L-ornithine (mid μ M EC₅₀ values) and less potently by small aliphatic amino acids (high μ M EC₅₀ values) (Table 2). We also expressed the chimeric human GPRC6A/goldfish 5.24 receptor and wild-type (WT) mouse GPRC6A in *Xenopus* oocytes where we could show that receptor activation by these L- α -amino acids led to increases in intracellular calcium and subsequent activation of an endogenously expressed calcium-activated chloride channel (Wellendorph *et al.*, 2005). A few months later these results were confirmed by the group of Hampson who also used two-electrode voltage clamp electrophysiology of mouse GPRC6A expressed in *Xenopus* oocytes to show that the receptor is activated by basic and small aliphatic L- α -amino acids (Kuang *et al.*, 2005). The activation of recombinantly

expressed GPRC6A by basic L- α -amino acid has later also been confirmed by the groups of Ruat (Faure *et al.*, 2009) and Quarles (Pi and Quarles, 2012a). Furthermore, using site-directed mutagenesis, we have shown that two highly conserved serine/threonine residues in the orthosteric VFT binding site are mediating L- α -amino acid recognition as has been observed for a number of other class C GPCRs (Figure 3B) (Wellendorph *et al.*, 2005; Wellendorph and Bräuner-Osborne, 2009a).

Whereas we did not observe any direct agonist activity of 4 mM Ca²⁺ on mouse GPRC6A expressed in *Xenopus* oocytes (Wellendorph *et al.*, 2005), the group of Hampson interestingly demonstrated that 5 mM Ca²⁺ potentiated L- α -amino acid responses (Kuang *et al.*, 2005) indicating that divalent cations could act as positive modulators as had previously been observed for mGlu and GABA_B receptors (Kubo *et al.*, 1998; Galvez *et al.*, 2000). At the end of 2005, this observation was further substantiated by the group of Quarles who reported that GPRC6A expressed in HEK293 cells could be directly activated by high concentrations of Ca²⁺ (> 5 mM) and other cations (Pi *et al.*, 2005). This report prompted us to return to test higher Ca²⁺ concentrations on mouse GPRC6A expressed in the *Xenopus* oocyte where we were able to confirm the positive modulatory effect observed by the group of Hampson, but unable to detect a direct agonist activity of up to 50 mM Ca²⁺ (Christiansen *et al.*, 2007). More recently, we have indeed confirmed an apparent direct activity of Ca²⁺ on mouse GPRC6A stably expressed in a CHO cell line (Jacobsen *et al.*, 2013). In both our and Quarles pharmacological

Table 2

Agonist potencies of L- α -amino acids at mouse, human and rat GPRC6A expressed in tsA cells compared to concentrations found in mouse plasma

L- α -amino acid	Mouse ^a EC ₅₀ (μ M) [% relative efficacy] ^d	Human ^b	Rat ^a	Concentration of L- α -amino acid (mean \pm SD) in mouse plasma ^c (μ M)
L-Orn	63.6	112	264 [100]	86
L-Lys	135	169	>1000 [44]	366
L-Arg	284 [100]	44.1	>1000 [51]	137
L-Cys ^e	356	>1000	>1000 [73]	Not determined
L-Ala	486	173	>1000 [50]	431
Gly	538 [49]	263	455 [74]	340
L-Ser	1160	623	859 [63]	181
L-Cit	>1000	287	615 [77]	106
L-Met	>1000	854	>1000 [72]	71
L-Gln	>1000	590	>1000 [76]	661

^aResults from mouse and rat GPRC6A were derived from agonist-induced inositol phosphate accumulation in tsA cells co-expressing G α_q (G66D) (Christiansen *et al.*, 2007; Wellendorph *et al.*, 2007).

^bResults from human GPRC6A were obtained using a chimeric receptor containing the human VFT/CRD domains and the goldfish 5.24 7TM domain using a Fluo-4 based intracellular calcium assay (Wellendorph *et al.*, 2005).

^cData from (Komarov and Reddy, 1998).

^dRelative efficacies were calculated as a percentage of maximum response to that observed for 10 mM L-Arg (mouse) or 10 mM L-Orn (rat) in the same experiment (set to 100%). For compounds with low activities (EC₅₀ values >1000 μ M), relative efficacies were calculated as a percentage of response at 10 mM for the compound divided by the response for L-Arg/L-Orn at 10 mM.

^eL-Cys displayed a small but significant effect on mock-transfected cells at high concentrations (\geq 1 mM). The EC₅₀ values might thus be overestimated.

experiments using endpoint assays with >1-h ligand incubations, it is likely that there will be a build up of amino acids released from the cells (Thomsen *et al.*, 1994). It is thus our hypothesis that the direct Ca^{2+} activity seen in these assays (in contrast to the electrophysiological experiments in *Xenopus* oocytes using continuous ligand perfusion) is in fact positive modulation of L- α -amino acids excreted from the cells during the experiment. However, regardless of whether Ca^{2+} , Mg^{2+} and other cations are direct agonists or positive modulators of basic/small aliphatic L- α -amino acids, it is evident that the GPRC6A receptor is able to sense fluctuations in concentrations of both ligand classes in physiological relevant concentrations (Table 2) and they could thus represent endogenous signalling molecules for the receptor.

More recently, the group of Quarles has suggested that GPRC6A mediates some of the observed physiological responses of the steroid testosterone (Pi *et al.*, 2010a) and the peptide osteocalcin (Pi *et al.*, 2011). The latter ligand has also been suggested by the Karsenty group who showed that osteocalcin elicited bell-shaped concentration-dependent cAMP increases, suggesting G_s coupling, but no osteocalcin-mediated activation of the G_q or ERK pathways in TM3 Leydig cells. These osteocalcin responses were, however, not shown to be specifically mediated by GPCR6A (Oury *et al.*, 2011) and the lack of ERK activation contrast findings in HEK293 cells expressing mouse GPRC6A reported by the Quarles group (Pi *et al.*, 2011). GPCRs are highly versatile proteins activated by a broad range of ligands (Granier and Kobilka, 2012). Nevertheless, it is not unprecedented that the same receptor should have evolved to be activated by four very different classes of endogenous ligands: cations, L- α -amino acids, a steroid and a 49 amino acid peptide. Phylogenetically, class C GPCRs are viewed as nutrient receptors derived from bacterial periplasmic binding proteins, which transport amino acids and other small nutrients (Conklin and Bourne, 1994; Kuang *et al.*, 2006). Accordingly, all other human class C GPCRs with a VFT domain (Figures 2 and 3) sense amino acids, cations and/or sugars and no other subtype in class C has previously been suggested to be activated by endogenous steroids or peptides of the size of osteocalcin (Bräuner-Osborne *et al.*, 2007). In analogy with this reasoning based on phylogenetic relationships, we have been unable to confirm the activity of either testosterone or osteocalcin on mouse GPRC6A stably expressed in CHO cells when measuring G_q , G_i , G_s and ERK pathways (Jacobsen *et al.*, 2013). More research is thus warranted to understand the molecular basis for the reported GPRC6A mediated effects of testosterone and osteocalcin.

All the previously mentioned putative endogenous ligands have multiple other targets in mammals, which severely limit their use as pharmacological tool compounds in tissue preparations and whole animals to investigate the physiological function and therapeutic prospect of the GPRC6A receptor. There is thus a great need for development of potent and selective agonists and antagonists. To this end, we initially screened a collection of L-Arg, L-Lys and L-ornithine analogues at the chimeric human GPRC6A/goldfish 5.24 receptor. Several compounds were equipotent with the natural basic amino acids as agonists at this receptor. However, these analogues are also known to be regulators of nitric oxide synthase and arginase and thus not sufficiently selective to be used as pharmacological tools to probe

GPRC6A function (Christiansen *et al.*, 2006b). The group of Ruat were the first to demonstrate that the CaSR allosteric modulators calindol and NPS2143 were antagonists at mouse GPRC6A ($\text{IC}_{50} \sim 10 \mu\text{M}$) (Faure *et al.*, 2009). However, both these compounds are ~ 30 fold more potent at CaSR and thus not very useful as pharmacological tools to probe GPRC6A function. This led us to use a chemogenomic approach to identify novel negative allosteric modulators based on a 2-phenyl-indole scaffold (Gloriam *et al.*, 2011). The resulting compounds are the most selective GPRC6A antagonists reported so far, but still need optimization of potency, solubility and selectivity for *in vivo* use. Using site-directed mutagenesis, it has been shown that calindol and the 2-phenyl-indole derivative 'compound 3' bind to E816 and I759 in transmembrane helix 5 and 7, respectively (Figure 3B) (Faure *et al.*, 2009; Gloriam *et al.*, 2011). This is in analogy with other class C GPCRs where most 'drug-like' allosteric modulators have been demonstrated to bind in the 7TM domain (Figure 3A) (Bräuner-Osborne *et al.*, 2007).

As previously mentioned, we and the group of Hampson initially demonstrated L- α -amino acid-mediated activation of a calcium activated chloride channel in *Xenopus* oocytes indicative of G_q coupling (Kuang *et al.*, 2005; Wellendorph *et al.*, 2005; Christiansen *et al.*, 2007). Furthermore, the Hampson group generated a chimeric receptor consisting of the VFT domain from the goldfish 5.24 receptor and the 7TM domain from mouse GPRC6A and demonstrated that this chimeric receptor expressed in HEK293 cells led to increased intracellular calcium levels upon activation by L- α -amino acids, which is also indicative of G_q coupling (Kuang *et al.*, 2005). Most recently, we have used a CHO cell line stably expressing mouse GPRC6A to show that L- α -amino acids and Ca^{2+} activate the G_q but not G_i or G_s pathways and that the former response can be blocked by the G_q inhibitor UBO-QIC (Jacobsen *et al.*, 2013). Using mGPRC6A-transfected HEK293 cells and pathway selective inhibitors, the Quarles group has shown that downstream serum-response element and/or ERK are activated by divalent cations [G_q and G_i pathways (Pi *et al.*, 2005; 2010a; Pi and Quarles, 2012a)], L-arginine [pathway not investigated (Pi *et al.*, 2011)], testosterone [G_i pathway, G_q not investigated (Pi *et al.*, 2010a)] and osteocalcin [G_q pathway, G_i not investigated (Pi *et al.*, 2011)]. In addition, the group has shown that all four agonist classes lead to cAMP accumulation in the GPRC6A-HEK293 cell line indicating G_s coupling (Dreaden *et al.*, 2012; Pi *et al.*, 2012b). It thus appears that GPRC6A can couple to several G protein pathways depending on the cell line and agonist used, albeit it is our opinion that the G_q pathway is the predominating signalling pathway. This is in analogy with the homologous CaSR, which is also preferentially coupled to G_q but also able to activate G_i , G_s and G_{12} pathways in some cells (Thomsen *et al.*, 2012).

Expression pattern

Information of expression pattern and tissue distribution of the GPRC6A receptor primarily originates from real time (RT)-PCR analyses that is qualitative/semi-quantitative mRNA expression levels. Several studies have examined the tissue expression in human, mouse and rat, and a number

of tissues evidently express the GPRC6A receptor, although with some discrepancies and differences between species (Wellendorph and Bräuner-Osborne, 2004; Kuang *et al.*, 2005; Pi *et al.*, 2005; Regard *et al.*, 2007; Wellendorph *et al.*, 2007; Bystrova *et al.*, 2010; Luo *et al.*, 2010; Haid *et al.*, 2011; 2012). Generally, these studies have shown that the receptor is widely expressed albeit at relatively low levels.

Human GPRC6A mRNA has been identified in brain, lung, liver, heart, kidney, pancreas, skeletal muscle, placenta, spleen, ovary, testis, prostate, leukocyte and monocyte (Table 3) (Wellendorph and Bräuner-Osborne, 2004; Kuang *et al.*, 2005; Pi and Quarles, 2012a; Rossol *et al.*, 2012;). So far, only a few studies have examined the mRNA expression of

GPRC6A in the rat (Wellendorph *et al.*, 2007; Harno *et al.*, 2008). We identified the receptor in rat kidney, lung, liver, brain and the so-called 'geschmacksstreifen' rich in taste buds, thus proposing a role for the receptor in taste sensation (Table 3) (Wellendorph *et al.*, 2007). Another research article demonstrated expression of GPRC6A in rat mesenteric artery endothelial cells and myocytes, suggesting a role for the receptor in regulating blood flow (Harno *et al.*, 2008). Several studies have examined expression pattern of GPRC6A in mice and the receptor has been identified in lung, pancreas, salivary gland, mammary gland, taste cells, aorta, heart, stomach, kidney, liver, skeletal muscle, eye, spleen, tongue, small intestine, large intestine, white adipose tissue, brown

Table 3

Expression pattern of the GPRC6A receptor in human, rat and mouse tissues

Species	Tissues/Cells	References
Human	Brain Lung Liver Heart Kidney Pancreas Skeletal muscle Placenta Spleen Ovary Testis Prostate Leukocyte Monocyte	(Wellendorph and Bräuner-Osborne, 2004; Pi and Quarles, 2012a; Rossol <i>et al.</i> , 2012;)
Rat	Kidney Lung Liver Brain Tongue ('geschmacksstreifen') Mesenteric artery	(Wellendorph <i>et al.</i> , 2007; Harno <i>et al.</i> , 2008)
Mouse	Lung Pancreas Salivary gland Mammary gland Taste cells Aorta Heart Stomach Kidney Liver Skeletal muscle Eye Spleen Tongue Small intestine Large intestine White adipose tissue Brown adipose tissue Thymus Testis Bone marrow Brain Embryonic tissue	(Kuang <i>et al.</i> , 2005; Regard <i>et al.</i> , 2007; Wellendorph <i>et al.</i> , 2009b; Bystrova <i>et al.</i> , 2010; Luo <i>et al.</i> , 2010; Pi <i>et al.</i> , 2010a; 2011; 2012b; Oury <i>et al.</i> , 2011; Oya <i>et al.</i> , 2013; Smajilovic <i>et al.</i> , 2013)

adipose tissue, thymus, testis, Leydig cells, bone marrow, brain and embryonic tissue (Table 3) (Kuang *et al.*, 2005; Regard *et al.*, 2007; Wellendorph *et al.*, 2009b; Bystrova *et al.*, 2010; Luo *et al.*, 2010; Pi *et al.*, 2010a; 2011; 2012b; Oury *et al.*, 2011; Oya *et al.*, 2013; Smajilovic *et al.*, 2013).

In addition, GPRC6A mRNA is endogenously expressed in the mouse insulin secreting cell line TC-6 as well as in three cell lines of human prostate cancer (Pi *et al.*, 2011; Pi and Quarles, 2012a).

However, discrepant reports about the presence of GPRC6A in islets of Langerhans from mice exist. Regard *et al.* reported in 2007, using a quantitative RT-PCR study, expression of GPRC6A mRNA in mouse islets of Langerhans, and proposed a possible role of the receptor in regulation of insulin secretion (Regard *et al.*, 2007). In contrast, a study of tissue expression of GPRC6A mRNA investigated by *in situ* hybridization showed the location of the expression to be mainly in the exocrine tissue of the pancreas and not in the islets of Langerhans (Luo *et al.*, 2010). However, using RT-PCR, we have recently confirmed the expression of GPRC6A in the islet of Langerhans (Smajilovic *et al.*, 2013).

Very recently, GPRC6A mRNA was detected in the intestinal L cell lines, GLUTag and STC-1 cells, and in the mouse small intestine (Oya *et al.*, 2013). Moreover, expression of the receptor was also detected in fluorescence-activated cell sorting (FACS)-purified mouse primary intestinal cells by quantitative RT-PCR, and was enriched in the L cell population compared with non-L cell population.

Identification of the GPRC6A receptor at the protein level has so far been hampered by the lack of specific and well-functioning antibodies. A few studies have reported GPRC6A protein expression and identified the receptor in rat mesenteric and porcine coronary artery, mouse kidney, 15-day-old mouse embryo, testis from both mouse and human monocytes (Harno *et al.*, 2008; Pi *et al.*, 2008; Oury *et al.*, 2011; Rossol *et al.*, 2012). However, only Pi *et al.* (2008) used GPRC6A deficient mice to validate antibody specificity (Pi *et al.*, 2008), hence well-founded knowledge on GPRC6A translation and protein expression remains to be established. Furthermore, there is an urgent need to obtain more detailed knowledge on which cells in the aforementioned tissues that express the GPRC6A receptor.

Physiology and pathophysiology

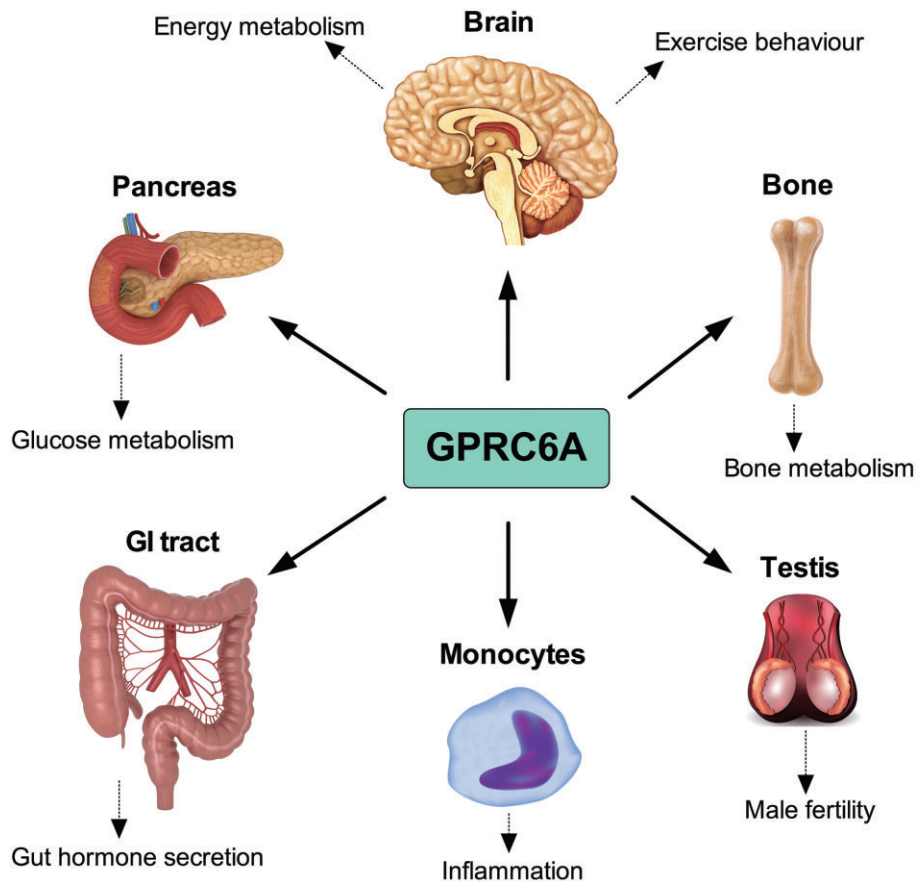
Knowledge pertaining to the expression pattern and ligand preferences of GPRC6A gave rise to early speculations on the physiological role of the receptor such as: monitor of the urea cycle, endocrine modulator, neuroreceptor and a detector of cell death (Civelli, 2005; Kuang *et al.*, 2005; Pi *et al.*, 2005; Wellendorph *et al.*, 2005; Christiansen *et al.*, 2006b). However, it required the engineering of genetically modified mouse models to make significant headway in understanding GPRC6A biology. Nonetheless, basic research has thus far only scratched the surface and truly uncovering the function of GPRC6A in physiology and pathophysiology requires a close synthesis of molecular and pharmacological approaches with whole-animal physiology. In the following, literature exploring the physiological function(s) of the GPRC6A receptor will be reviewed.

Role of GPRC6A in bone metabolism

By selectively deleting exon II of the mouse GPRC6A gene, which encodes for a minor part of the extracellular domain of the receptor, the group of Quarles generated in 2008 the first global GPRC6A knockout (KO) mouse model (Pi *et al.*, 2008). Phenotypic analyses of this GPRC6A KO model identified multiple physiological abnormalities associate with ablation of the receptor, including a disruption in bone metabolism (Pi *et al.*, 2008) (Figure 4). In a follow-up paper, these observations were further strengthened as direct function of GPRC6A in osteoblasts was reported along with an indication of a biological role for the receptor in humans (Pi *et al.*, 2010b). Thus, the authors described an association between two single-nucleotide polymorphisms in the vicinity of the GPRC6A gene and a reduction in bone mineral density and consequently proposed that GPRC6A polymorphisms may play a role in human osteopenia (Pi *et al.*, 2010b). By targeted disruption of the GPRC6A exon VI, containing the entire 7TM domain and C-terminal of the gene, we generated in 2009, another global GPRC6A mouse model. However, in contrast to the phenotype reported by the group of Quarles, we failed to identify bone abnormalities in our GPRC6A KO mouse model under standard physiological conditions (Wellendorph *et al.*, 2009b), thus leaving it unresolved if GPRC6A is causally involved in regulating bone metabolism. Whether the differences between the two KO models relate to their origin remains to be uncovered but these data indicate that a more subtle phenotype is associated with deletion of exon VI than with deletion of exon II of the GPRC6A gene.

Role of GPRC6A in male reproduction

It has been observed that ablation of GPRC6A in male mice results in feminization characteristics including reduced weight of the testis and decreased circulating testosterone levels (Pi *et al.*, 2008). These observations were recently supported, as a direct role for GPRC6A in male reproduction was discovered (Oury *et al.*, 2011) (Figure 4). GPRC6A is expressed in the Leydig cells of the testis where it serves to regulate testosterone secretion and subsequently spermatogenesis in male mice (Oury *et al.*, 2011). Further, the authors showed that GPRC6A^{Leydig} exon II conditional KO mice exhibit some of the same characteristics as the global GPRC6A exon II KO mice; hence ablation of GPRC6A in the Leydig cells results in reduced size and weight of the testis, decreased sperm counts and reduced circulating testosterone levels (Oury *et al.*, 2011). This was the first study to employ a tissue-specific GPRC6A KO model and to elegantly demonstrate that 75% knock-down of GPRC6A in a specific cell type potentially affects mouse physiology. Based on these findings, the group of Karsenty thus hypothesized a novel bone-testis endocrine loop in which bone-derived osteocalcin functions to regulate male reproduction via the GPRC6A receptor. Conversely, GPRC6A has also been proposed to act as a steroid sensor mediating non-genomic effects of testosterone (Pi *et al.*, 2010b). GPRC6A deficient mice administered with testosterone were reported to exhibit a dampened phosphorylation of the MAP kinase ERK and reduced mRNA levels of the early growth response protein 1 in bone marrow and testis relative to WT controls (Pi *et al.*, 2010b). Also, it was observed that testosterone highly induces luteinizing hormone secretion in GPRC6A KO but not in WT mice. Thus, despite that these

**Figure 4**

Cartoon of the major physiological systems in which the GPRC6A receptor plays a role.

data support a role for GPRC6A in male reproduction central questions concerning the biological mechanisms are left unanswered. Whereas the genetically modified mouse models clearly imply that GPRC6A is involved in fertility, the pharmacological evidence supporting that GPRC6A is a steroid and/or osteocalcin receptor is scarce, and further studies are therefore urgently needed to gain insight into the molecular details underlying GPRC6A's role in reproduction.

Role of GPRC6A in energy homeostasis

Several studies have shown that ablation of GPRC6A has no impact on body weight progression in mice with *ad libitum* access to a chow diet (Pi *et al.*, 2008; Wellendorph *et al.*, 2009b; Clemmensen *et al.*, 2013a), implying that the receptor is not vital for energy balance regulation under standard physiological conditions. This is underscored by the fact that parameters such as food intake, oxygen consumption and substrate metabolism are similar between GPRC6A KO and WT control mice (Clemmensen *et al.*, 2013a). Noteworthy, whereas we repeatedly have failed to identify any differences in body composition between chow fed KO and WT mice (Smajilovic *et al.*, 2013; Clemmensen *et al.*, 2013a,b), Pi *et al.* found increased adiposity in GPRC6A deficient mice on a chow diet (Pi *et al.*, 2008). However, we recently identified an increased sensitivity to high-fat diet induced hyperphagia in

GPRC6A deficient relative to WT mice (Clemmensen *et al.*, 2013b). The obese phenotype was associated with reduced locomotion, highly increased circulating leptin levels and multiple disruptions in glucose metabolic parameters (Clemmensen *et al.*, 2013b). Thus, despite that the different GPRC6A KO models exhibit differences in energy homeostatic sensitivity, the overall evidence points to a direct role for GPRC6A in energy metabolism (Figure 4). Worthy of note, we have very recently identified an exercise phenotype in our GPRC6A deficient mouse model. Ablation of the receptor results in a 50% increase in voluntary wheel running when compared to WT littermate mice (Clemmensen *et al.*, 2013a). We are currently working to uncover if the exercise phenotype and the susceptibility to diet-induced obesity are inter-related and thus controlled by a common biological pathway. Giving that eating palatable foods and exercising are both rewarding and evoke hedonic responses (Garland *et al.*, 2011), GPRC6A may serve a function in the mesolimbic system controlling rewarding behaviour. Important to note, the biological pathways involved in eating behaviour, reward and exercise are also highly interconnected to physiological stress responses (Adam and Epel, 2007; Garland *et al.*, 2011), hence the identified phenotypes may arise as a consequence of complex gene-by-environment interactions. Exercise is also known to differentially lower adiposity in diet-induced

obese animals versus diet-resistant animals (Levin and Dunn-Meynell, 2006), proposing that exercise behaviour may represent an energy homeostatic corrective response. Interestingly, it was recently discovered that hypothalamic orexin neurons, which are known to regulate reward and energy balance, are highly influenced by mixtures of dietary amino acids (Karnani *et al.*, 2011). Therefore, it should be interesting for future studies to explore if GPRC6A plays a role in the central orexin network and thus regulates foraging behaviour based on circulating amino acid availability.

Role of GPRC6A in glucose metabolism

The GPRC6A receptor is perhaps most studied for its potential involvement in regulating glucose metabolism (Figure 4) (Pi *et al.*, 2011; 2012b; Oya *et al.*, 2013; Smajilovic *et al.*, 2013; Clemmensen *et al.*, 2013b). But, despite a growing bulk of research on the subject, it is still elusive whether GPRC6A plays a direct role in glucose homeostatic control. Whereas the group of Quarles observes glucose intolerance and insulin resistance in GPRC6A deficient mice administered a chow diet (Pi *et al.*, 2008), our global GPRC6A KO mice neither suffer from glucose intolerance, nor from insulin resistance (Smajilovic *et al.*, 2013). However, disruptions in glucose metabolism surface when the animals are exposed to an obesogenic environment, and may thus appear secondary to the obese phenotype (Clemmensen *et al.*, 2013b). The metabolic phenotype identified by the group of Quarles also exhibits coexistence of adiposity and worsened glucose metabolism (Pi *et al.*, 2008); hence the disturbances in glucose homeostasis might likewise be a consequence of obesity and not directly linked to ablation of GPRC6A.

It has been known for decades that the GPRC6A agonist L-arginine impacts multiple glucose regulating hormones and that it is a potent insulin secretagogue (Floyd *et al.*, 1966; Palmer *et al.*, 1975). Further, it is known that dietary supplementation with L-arginine potentially improves glucose metabolism in mice (Clemmensen *et al.*, 2012). Interestingly, we and others have identified expression of the GPRC6A receptor in the endocrine pancreas (Regard *et al.*, 2007; Pi *et al.*, 2011; Smajilovic *et al.*, 2013), which, combined with the fact that the pharmacological data clearly classify L-arginine as a potent GPRC6A agonist, prompted us to explore the hypothesis that GPRC6A is mediating L-arginine-induced insulin secretion. We thus assessed a role for GPRC6A in L-arginine-induced insulin release *in vivo* and *ex vivo* (Smajilovic *et al.*, 2013). Both intravenous and oral administration of L-arginine potentially induced insulin release equally in our GPRC6A deficient and WT mice, implying that the receptor serves no key function in L-arginine-induced insulin release *in vivo*. These findings were confirmed in isolated islets of Langerhans incubated with L-arginine, as both genotypes responded similarly to the insulinotropic effects of the amino acid (Smajilovic *et al.*, 2013). The group of Quarles more recently explored a parallel hypothesis and found that isolated islets from GPRC6A KO mice exhibit a reduced response to multiple insulin secretagogues (L-arginine and glucose) due to an elevated basal insulin level in the KO mice compared to WT (Pi *et al.*, 2012b). Interestingly, the group of Quarles previously discovered that osteocalcin injected into the peritoneal cavity increase circulating insulin levels in WT but not in GPRC6A

KO mice (Pi *et al.*, 2011). Together, these data underline that the global GPRC6A mouse model, in which exon 2 from the GPRC6A gene is deleted, suffers from glucose metabolic disturbances in comparison with our exon 6 deleted KO mice (Figure 1). Nonetheless, additional data is required to confirm a specific and direct role for GPRC6A in amino acid- and/or osteocalcin-induced insulin release.

GPRC6A has also been proposed to serve a physiological relevant role as an amino acid sensor in the gastrointestinal tract (Haid *et al.*, 2011; Haid *et al.*, 2012; Janssen and Depoortere, 2013), which may consequently affect glucose homeostasis. In the intestinal GLUTag cell line, GPRC6A mediates L-ornithine-induced secretion of the glucagon-like peptide 1 (GLP-1) (Oya *et al.*, 2013) (Figure 4). Albeit these data show a direct role for GPRC6A in L-ornithine-induced GLP-1 secretion, the findings should be interpreted with caution, as GPRC6A was hardly detectable in FACS-sorted intestinal endocrine cells (but highly expressed in the GLUTag cells). Also, a role for the CaSR in amino acid-induced incretin hormone release *ex vivo* has been identified (Mace *et al.*, 2012), thus, follow-up studies are needed to uncover if GPRC6A – potentially in concert with the CaSR – is involved in amino acid-induced GLP-1 secretion *in vivo*.

Role of GPRC6A in inflammation

Recently, using our global exon VI GPRC6A KO mice, data from the group of Wagner elegantly illustrated a dual role for GPRC6A and the CaSR in mediating extracellular calcium-induced inflammatory responses (Rossol *et al.*, 2012). GPRC6A is expressed in monocytes, and primary cells isolated from our GPRC6A deficient mice exhibits a reduction in calcium-induced secretion of the proinflammatory cytokine IL-1 β . This discovery was corroborated *in vivo*, thus proposing a central role for GPRC6A in inflammation (Figure 4) (Rossol *et al.*, 2012). In line with this, a genome-wide association study (GWAS) identified GPRC6A as a novel loci associated with circulating C-reactive protein (CRP) levels (Dehghan *et al.*, 2011). CRP is a general biomarker for systemic inflammation and increased CRP levels are associated with an array of disorders, including human obesity (Visser *et al.*, 1999). Although not straightforward, it should be interesting for future studies to explore a potential link between the metabolic and the inflammatory phenotypes associated with GPRC6A deficiency and further down the road assess if this knowledge holds any human translational value.

GPRC6A in prostate cancer

Somewhat in line with the animal studies revealing a role for GPRC6A in male reproduction, GPRC6A has been associated with human prostate cancer. GWAS has identified GPRC6A as a genetic loci associated with prostate cancer in Japanese and Chinese populations (Takata *et al.*, 2010; Long *et al.*, 2012; Wang *et al.*, 2012). To study the role of GPRC6A in the progression of prostate cancer, the group of Quarles crossed the GPRC6A KO mouse model with the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model, which is known to mirror the pathogenesis of human prostate cancer (Pi and Quarles, 2012a). Deletion of GPRC6A in the TRAMP mouse retarded prostate cancer progression and improved survival relative to TRAMP null mice expressing

GPRC6A. Moreover, molecular biological experiments identified increased GPRC6A expression in cancer-derived prostate cells compared to healthy prostate tissue (Pi and Quarles, 2012a), supporting a role for the receptor in prostate cancer. Future studies may decipher if pharmacological tools designed to antagonise GPRC6A could be beneficial in halting the progression of and/or treating human prostate cancer.

Conclusion and future perspectives

Since the GPRC6A receptor was cloned in 2004, a lot of knowledge regarding its pharmacology and physiological function has been obtained. The data have shown that the receptor belongs to a growing family of promiscuous GPCRs (Wellendorph *et al.*, 2009c), albeit it remains a matter of debate which of the suggested ligands are the most important physiologically. Several studies have delineated that the receptor mRNA is widely expressed at the tissue level, but the lack of well-functioning antibodies have so far prevented a much needed global mapping of the expression pattern of the receptor protein at the subtissue level. Due to the lack of receptor-specific pharmacological tool compounds, the physiological function of the receptor has so far mainly been addressed by phenotype assessment of KO mice. Results from three different KO mice strains have been surrounded by discrepancies, but taken together suggest that the receptor is involved in regulation of inflammation, metabolism and endocrine functions. GPRC6A could thus be a new interesting target for novel drugs in these therapeutic areas. However, more studies and development of novel tools such as selective agonists, antagonists and antibodies are needed to fully uncover the physiological function and therapeutic potential of the receptor.

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Conflict of interest

None.

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