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Review

Alternative splicing of G protein-coupled receptors: Physiology and pathophysiology

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Running title. Alternatively spliced GPCRs

Abstract. The G protein-coupled receptors (GPCRs) are a superfamily of transmembrane receptors that have a broad distribution and can collectively recognize a diverse array of ligands. Activation or inhibition of GPCR signalling can affect many (patho)physiological processes and consequently they are a major target for existing and emerging drug therapies. A common observation has been that the pharmacological, signalling and regulatory properties of GPCRs can vary in a cell- and tissue-specific manner. Such “phenotypic” diversity might be attributable to post-translational modifications and/or association of GPCRs with accessory proteins, however, post-transcriptional mechanisms are also likely to contribute. Although, approximately 50% of GPCR genes are intronless, those that possess introns can undergo alternative splicing, generating GPCR subtype isoforms that may differ in their pharmacological, signalling and regulatory properties. In this Review we shall highlight recent research into GPCR splice-variation, and discuss the potential consequences this might have for GPCR function in health and disease.

Key words. G protein-coupled receptor (GPCR), alternative splicing, exon, isoform, signalling properties, pathophysiology

Introduction

Despite estimates of over 100,000 human genes being made in the “pre-genomic” era, a surprising outcome of the human genome project [1] was the finding that we are apparently less complex than we had imagined, with perhaps only 30-40,000 genes being sufficient to make us who and what we are. This discrepancy can be partially explained by the fact that a single gene can potentially encode a number of protein products due to alternative mRNA splicing. Alternative mRNA splicing is an important mechanism for generating protein diversity. Indeed, recent microarray analyses suggest that around 40% of *Drosophila* genes [2] and >90% of human genes undergo alternative splicing [3].

Alternative splicing is the process by which exons (or even introns) can be either included or excluded from a precursor-mRNA (pre-mRNA) resulting in multiple, mature mRNA variants. This process is carried out by a large complex, the spliceosome, which is a supramolecular assembly consisting of 5 small nuclear ribonucleoprotein particles (snRNPs) [4]. The process is governed by: (i) various *cis*-acting elements encoded in the pre-mRNA sequence itself (the 5' and 3' splice sites, branch points, exonic and intronic enhancer/silencer sites (ESE/ESS, ISE/ISS)); (ii) *trans*-acting elements, specific proteins that recognize particular ESE/ESS or ISE/ISS and bind to them (serine/arginine-rich (SR) proteins and heterogeneous ribonucleoproteins (hnRNPs)) and; (iii) cell-type-specific regulatory mechanisms. The final outcome of alternative splicing is translation of isoformic variants of proteins, encoded by the same gene, but differing in sequence and therefore potentially in their biomolecular and cellular properties. In addition, if alternative splicing of mRNA leads to the introduction of a premature stop codon some of these mRNAs are not translated to proteins, but undergo nonsense-mediated decay (NMD) [5]. Proteins affected by alternative splicing might have unaltered function, altered function, or no function at all. Splicing that generates non-functional isoforms might have a significant impact on susceptibility to, and development of a range of illnesses (e.g. muscular dystrophy, cystic fibrosis, asthma, insulin-dependent (type I) diabetes, cancer [6]). In addition, mutations that lead to alterations in the splicing of various proteins, including G protein-coupled receptors (GPCRs), have been identified in a variety of cancers, including melanomas, breast, ovarian, prostate, liver and gastrointestinal cancers [7, 8, 9].

GPCRs constitute the largest family of membrane-associated receptors. A key feature of the GPCR family is that different receptor subtypes have evolved that can specifically detect very small (e.g. H^+ , Ca^{2+}) or very large (e.g. thyroid-stimulating hormone) ligands and everything in-between. In addition, these receptors, through G protein-dependent and -independent mechanisms, can link to an array of intracellular signalling pathways that can in turn regulate a plethora of physiological functions. Not surprisingly therefore, GPCRs are almost universally expressed in cells/tissues and specific manipulation of their activity is the primary target of approximately 50% of presently available drugs [10, 11, 12]. It is also becoming increasingly apparent that GPCRs sometimes exhibit cell- and tissue-specific signalling, regulatory and/or pharmacological properties [13]. Genetic variation (single nucleotide polymorphisms), post-translational modification (including phosphorylation, glycosylation and lipidation) as well as association with accessory proteins may contribute to such “phenotypic” diversity [11, 13]. However, much accruing evidence also indicates that alternative splicing of GPCRs can profoundly affect signalling, regulatory and/or pharmacological properties. In addition, it is worth noting that alternatively spliced GPCR isoforms can differ in their abilities to undergo post-translational modification and/or to interact with accessory proteins. For example, a splice-variant form of the parathyroid hormone (PTH)/PTH-related peptide receptor expressed only in kidney is not post-translationally glycosylated [14].

For a long time it was assumed that human GPCRs that undergo alternative splicing to form different mRNA variants and protein isoforms were rare. In 1999, based on data available from GeneBank, Gentles and Karlin estimated that more than 90% of human GPCRs are intronless [15]. This estimation was based on 120 mammalian GPCRs and 82 primate GPCRs. Since then, many more GPCR entries were created. An interrogation of 365 human GPCR entries from the IUPHAR database (<http://www.iuphar-db.org/GPCR/ReceptorListForward>) using the CCDS sequence data available from the NCBI web site (<http://www.ncbi.nlm.nih.gov/CCDS/CcidsBrowse.cgi>) showed that 52% of these GPCRs contain at least two exons in their open reading frame (ORF), suggesting the existence of an intron (see Figure 1). All members of family B (secretin and adhesion) and family C (glutamate) GPCRs have multiple exons; while only 58% of rhodopsin (family A) (N.B. olfactory receptors were not included) and 64% of frizzled family GPCRs are

intronless. Thus, the assumption that more than 90% of human GPCRs are intronless needs to be revised [15]. An important role in postulating the existence of novel splice variants is played by bioinformatics studies. This approach sets in motion fast identification of novel splice variants as demonstrated for adhesion GPCRs [16].

Although a large proportion (~50%) of GPCRs are intronless (see Figure 1), those encoded by more than a single exon can potentially undergo alternative mRNA splicing. Almost a decade ago, Kilpatrick and colleagues [17] and Minneman [18] thoroughly reviewed GPCR splice variants known at the time and their (altered) signalling and functional characteristics; thus in this manuscript we will focus on what has happened subsequently in this area. A list of newly identified GPCR variants/isoforms is given in Table 1. However, we cannot move forward without first mentioning two classic examples. The D₂ dopamine receptor was one of the first GPCRs identified as being expressed in two variant forms generated by alternative splicing [36]. The isoforms, termed short (D_{2S}) and long (D_{2L}), differ by 29 amino acids in the third intracellular (i3) loop, and this change in amino acid sequence results in isoforms that are differentially expressed in the CNS and have subtly distinct signalling properties [37]. Thus, the D_{2L} receptor is highly expressed post-synaptically in the regions that receive dopaminergic input, while the D_{2S} receptor isoform is more abundant in regions of the hypothalamus and mesencephalon known to synthesize and release dopamine [36, 37]. Another well-established example of a GPCR that is expressed as multiple isoforms is the rat type 1 pituitary adenylate cyclase-activating peptide receptor (PAC₁ receptor). This receptor has been reported to be expressed in five splice variant forms with the variation existing within the i3 loop (variants termed “hip”, “hop1”, “hop2”, “hip-hop1”, and null). In this case, protein isoforms differ with respect to their regulation of adenylate cyclase and phospholipase C activities [85].

In general, alternative splicing of GPCRs can lead to the expression of proteins with (see Figure 2):

(a) Isoforms in which the *N*-terminus differs due to distinct promoters (e.g. corticotropin-releasing hormone receptor type 2 (CRH-R2), PTH-R), or deletion of exons (e.g. calcitonin receptor (CT-R), corticotropin-releasing hormone receptor type 1 (CRH-R1), secretin receptor). In most cases these isoforms have impaired or abolished ligand binding properties.

(b) Isoforms in which the C-terminus varies in length (e.g. prostaglandin EP₃ receptor, prostaglandin F_{2α} receptor, α_{1A}-adrenoceptor, γ-aminobutyric acid type B (GABA_B) receptor, metabotropic glutamate (mGlu) receptors, μ-opioid receptor, 5-hydroxytryptamine (5HT) receptors, somatostatin receptors, thyrotropin-releasing hormone (TRH) receptor type 1, neurokinin-1 receptor (NK₁)) may have distinct signalling and regulatory (desensitization/internalization) properties. In some cases retention of an intron or deletion of an exon can introduce a premature stop-codon, leading to expression of a truncated receptor. In a structural sense these receptors can be missing the original C-terminus, parts of the 7th transmembrane domain (TM7), and even several transmembrane regions. Some of these receptor variants can exhibit a “dominant-negative” effect, acting to nullify signalling by the wild-type receptor (e.g. for the gonadotropin-releasing hormone receptor [86]). A unique example where alternative splicing changes the nature of a receptor is follicle-stimulating hormone (FSH) receptor. A full-length isoform of FSH receptor is a GPCR which couples to the Gα_s protein; in contrast, a truncated isoform of FSH receptor (generated by exclusion of exons 9 and 10 and use of additional exon 11) belongs to the superfamily of growth factor receptors [41].

(c) Isoforms in which an intracellular loop varies in size (e.g. CRH-R1, D₂ dopamine, H₃ histamine, cholecystokinin-B (CCK_B) and PAC₁ receptors). Alternative splicing within the i3 (and perhaps also i1/i2) loops can affect G protein coupling preference and/or coupling can be altered or even abolished.

(d) Isoforms in which TM7 is shortened by 14 amino acids (e.g. CRH-R1, CT-R, PTH-R, VPAC₂ receptor) can result in poor receptor expression at the plasma membrane and potential dominant-negative effects on wild-type receptor function. To date, these isoforms have been reported only for secretin (B1 family) GPCRs [27, 69, 87, 88, 89]; however, some members of the newly classified family of adhesion GPCRs which have exon-intron organization similar to the secretin family [90] could potentially give rise to similar splice variants.

(e) Isoforms with insertions or deletions within the extracellular loops of GPCRs are much less common compared to intracellular loop variants and to date only a small number have been reported (e.g. CT-R, D₃ dopamine, orphanin FQ/nociceptin receptors [17]). In the case of the CT-R, an insertion of 37 amino acids within the first extracellular (e1) loop alters

ligand specificity and binding kinetics, while wild-type receptor signal transduction properties appear to be preserved [91].

(f) Soluble isoforms of GPCRs are severely truncated spliced forms usually composed of only all or part of the extracellular *N*-terminal domain (e.g. luteinizing hormone receptor-LHR [54], mGluRs [58], CRH-Rs [92]). In many cases these proteins can bind their ligand, but do not transduce a signal, and thus behave as “decoy” receptors.

Although many members of the rhodopsin (family A) and frizzled/taste2 (frizzled and family C) sub-families are expressed as multiple isoforms, all members of the secretin (family B), adhesion (family B) and glutamate (family C) sub-families studied to date appear to undergo extensive alternative splicing due to their unique exon-intron organization [58, 89, 90]. A fascinating example of the importance of splicing in GPCR biology and hormone actions is the CRH-R1 receptor, which orchestrates mammalian responses to stressful stimuli [33]. In humans the CRH-R1 gene spans over 50 kb and contains 14 exons, and at least 14 alternatively spliced mRNAs for CRH-R1 variants have been detected to date [33, 34]. The complete gene product incorporating all 14 exons encodes a 444-amino acid GPCR, termed CRH-R1 β , which displays weak agonist binding and impaired signalling properties [93-95]. Omission of exon 6 from the mature mRNA results in expression of a 415 amino acid GPCR, termed CRH-R1 α , which encodes the major functional CRH-R1 isoform. Deletion of exon 3 results in CRH-R1c, which lacks 40 amino acids within the *N*-terminus and no longer binds CRH [96]. Various exon deletions or insertions of “cryptic” exons (exons that are absent in the normal form, but occur in an alternative form) result in frame-shifts and the potential introduction of a premature termination codon, leading to the expression of soluble or severely truncated membrane proteins (termed CRH-R1e-h) [34, 97]. Another splice variant, termed CRH-R1d, contains a 42 base-pair exon deletion resulting in a 14 amino acid deletion from TM7 [87]. It is interesting to note that at least 3 other members of the secretin family of GPCRs (CT-R, PTH-R and VPAC₂ receptor) can undergo similar splicing to produce isoforms with shortened TM7 domains [27, 69, 88, 89]; and in each case this sequence modification causes signalling impairment, at least partly due to endoplasmic reticulum retention and poor plasma membrane expression of the spliced isoform [27, 98, 99]. At present, the biological relevance of expression of these different splice variants is unknown;

however, a recent study had suggested that over-expression of CRH-R1d might lead to impairment of type 2 CRH-R signalling [98, 100], and similar results have been obtained for the calcitonin receptor variant, CTR Δ 13 [27, 99] and the VPAC₂ receptor variant, SD-VPAC₂ [101].

Mechanisms of Alternative Splicing of GPCRs

Although what governs GPCR alternative splicing remains largely unknown, there are a number of indications that changing (patho)physiological conditions can influence the way in which GPCR pre-mRNAs are processed. Thus, it has been reported that activation of protein kinases (PKA and PKC) and environmental insults (UV irradiation) can lead to modulation of CRH-R1 mRNA expression in keratinocytes and melanoma cells [34]. In addition, physiological changes, such as the onset of labour, can alter the relative expression of CRH-R1 variants in the uterus [102]. An increasing body of evidence points towards steroid hormones as potential modulators of alternative splicing. Oestrogen can bring about the up-regulation of important regulators for splicing, such as SR-protein (SC35) and hnRNPA1 [103] and can regulate transcription and mRNA splicing directly through CAPER molecules [104]. Oestrogen and other steroid hormones can also affect the ratio of D_{2S} and D_{2L} dopamine receptor variants in various target cells both *in vitro* and *in vivo*, without affecting total receptor mRNA expression, suggesting that steroid sex hormones exert their effects, at least in part, at the level of alternative splicing [105, 106]. In addition, in myometrial smooth muscle, progesterone treatment can regulate the ratio of CRH-R1 α/β mRNA expression [33].

During normal development the expression profile of some of GPCR splice variants changes. A study published recently by Bovolin *et al.* [57] showed that mice embryonic mitral cells express mainly the mGlu1b receptor variant, but by one week of age, this splice variant has become a minor component and mRNA for the mGlu1a receptor variant predominates. The authors speculate that the two splice variants, which have different trafficking properties and sub-cellular localizations [56], might encode proteins that have distinct roles in embryonic mitral cell maturation (mGlu1b receptor) and *post partum* in synaptic refinement (mGlu1a receptor) [57]. The differential expression patterns of the wild-type gonadotropin-releasing hormone receptor and its splice variant forms in neonatal and adult tissues also suggest that different isoforms of this receptor are involved in early development of ovary and testis [107].

In many cancers, proteins involved in regulation of alternative splicing are down-regulated resulting in expression of differently spliced GPCR isoforms; such is a case with CCK-B and secretin receptors in gastrointestinal, colonic and pancreatic cancers [31, 76].

All of the examples of GPCR isoforms described so far are products of *cis*-splicing, that is, a single molecule of pre-mRNA is processed to generate mRNA. However, *trans*-splicing, alternative splicing of the 5'-untranslated region (5'-UTR) and single nucleotide polymorphisms (SNP) have been also identified as important mechanisms in alternative splicing of GPCRs.

Trans-splicing: Numerous members of the adhesion GPCR sub-family are subject to undergo alternative splicing to generate functionally unique isoforms [16, 108]. Although, in mammals the process of *trans*-splicing (occurring between two separate mRNAs [109]), is rare compared to *cis*-splicing, adhesion GPCRs undergo this type of event. The epidermal growth factor-seven transmembrane spanning (EGF-TM7) receptors, which are mostly restricted to leukocytes, undergo extensive *cis*- and *trans*-splicing in order to expand the range of functional adhesion GPCRs [40], including diversification of ligand repertoire and cellular functions.

5'-UTR alternative splicing: Even in the case of GPCRs that have only one intron, or are intronless in the ORF, alternative splicing of the 5'-UTR could play a crucial role in their post-transcriptional regulation, including the modulation of translational efficiency, message stability and subcellular localization. For example, Kreth and co-workers [22] have reported that alternative splicing of the 5'-UTR of the A_{2A} adenosine receptor gene can alter receptor protein expression, and this could be of potential importance in how these receptors contribute to the physiological response to sepsis. It is established that adenosine, via activation of A_{2A} adenosine receptor, reduces phagocytosis, increases secretion of anti-inflammatory cytokines and induces lymphocyte apoptosis [110]. Thus, the reduced expression of the receptor might be beneficial in the treatment of infection and sepsis [110].

Single nucleotide polymorphisms (SNPs): SNPs, as providers of molecular diversity, are responsible for approximately 90% of human DNA variation. The SNPs in the coding regions of genes can be synonymous (silent) or non-synonymous, with the latter causing an amino acid change leading to potential phenotypic variation [111]. However, SNPs in the non-

coding regions of genes can have an effect on gene expression and splicing. Recently, it has been reported that several SNPs within the D₂ dopamine receptor gene can affect the receptor variant expression profile [112]. One of these SNPs is an upstream promoter polymorphism and two intronic SNPs are able to affect D₂ receptor splicing, by generating a novel transcription factor binding site and new *cis*-acting sites to which splicing factors (e.g. SC35 and SRp40) could bind [112]. A physiological consequence of these SNPs is that the genetically-driven changes in D₂ dopamine receptor splicing appear to affect behaviour and working memory in humans [112].

Aberrant Transcription versus Physiologically-Relevant Processing

The majority of GPCR splice variants have initially been identified at the mRNA level and the physiological relevance of alternative mRNA splicing to generate GPCR isoforms might be questioned or even dismissed as the result of aberrant or “leaky” transcription. A general lack of isoform-specific antibodies has often made it very difficult to demonstrate that mRNA variants are translated into mature proteins in endogenous systems. However, in recent years increasing numbers of reports suggest potential physiological consequences of GPCR alternative splicing. For example, it has been established that alternatively spliced GPCRs can have distinct (and perhaps physiologically desirable), signalling characteristics (e.g. PAC₁ receptor [85, 113], mGlu1 and mGlu5 receptors [114-116], NK₁ receptor [63]), impaired signalling properties and/or dominant-negative effects on wild-type receptors (as described above for CT-R and CRH-R1), and constitutive activity (e.g. mGlu1, prostaglandin EP₃, and 5HT₄ receptors (reviewed in [18])). Perhaps more importantly, hormone responsiveness can be fine-tuned by regulating the relative expression of GPCR isoforms (e.g. angiotensin II type 1 (AT₁) [24, 25] and CRH [102] receptors). A recent study by Einstein and co-workers [21] has shown that the GPCR complement within human airway smooth muscle undergoes frequent alternative splicing, resulting in unexpected receptor isoforms which might be of potential significance in the development of future drugs for respiratory diseases.

Alternative Splicing of GPCRs in Cancer

A large number of molecular changes, including genetic instability, insufficient DNA mismatch repair, increased oncogene expression and defects in several splicing mechanisms

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have been described in cancer cells relative to their healthy counterparts [7, 117]. Alternatively spliced mRNAs of many GPCRs have been detected in various cancers. However, whether this is simply a consequence of cancer cell biology, or the cancer-associated GPCR isoforms have an effect on cancer growth and metastasis is poorly understood. Nevertheless, the role of GPCRs and their ligands in cancer progression has been quite widely investigated.

Proliferation of tumour cells is promoted by neuropeptides and growth factors, which are either synthesized locally by the cancerous cells or derived from the systemic circulation. One such peptide is growth hormone-releasing hormone (GHRH). Many cancers, including ovarian, endometrial, prostate, small cell lung carcinoma and various sarcoma cell-lines have the ability to produce GHRH, which might serve as an autocrine growth factor for these cancers [44, 118]. Until recently [44], full-length GHRH-receptor (pGHRH-R), which is abundant in the anterior pituitary gland, was not detected in many GHRH-responsive cancers and human cancer cell-lines; instead four alternatively spliced, truncated mRNAs for GHRH-R have been detected [45, 46]. Among these truncated isoforms of the GHRH receptor, SV1, which is primarily detected in a majority of GHRH-responsive tumours, shows the greatest similarity to full-length, pGHRH-R. This isoform differs only in the first 3 exons, encoding a part of the extracellular domain of the receptor. In the SV1 isoform this region is replaced by a fragment of intron 3, which has a new putative in-frame start codon [46]. It has been demonstrated that when expressed in MCF-7 breast cancer cells, that do not possess either pGHRH-R or SV1, SV1 is more potent than pGHRH-R in inducing ligand-dependent cell proliferation [47]. Additionally, MCF-7 cells transfected with SV1 proliferate more quickly than non-transfected counterparts, even in the absence of GHRH, suggesting the existence of ligand independent SV1 activity [47]. Moreover, *in vitro* studies utilizing human oestrogen-independent breast cancer cell lines (MDA-MB-468 and MDA-MB-435), as well as mouse mammary carcinomas, showed that GHRH antagonists can directly inhibit cell proliferation [119, 120]. Thus, an antagonist of GHRH-R might be effective in the treatment of some oestrogen-independent cancers, and it might be desirable to selectively target the SV1 isoform, potentially sparing normal GHRH-R function [48, 49, 121]. In 2005, Havt and co-workers employed a sensitive real-time PCR technique, combined with Western blotting and a receptor ligand-binding assay to show that malignant and non-malignant human tissues

express the full-length pGHRH-R and its splice variants [44]. These findings demonstrated for the first time the co-existence of pGHRH-R and its splice variants in human tumours, but further studies are needed to dissect any (patho)physiological significance of this co-expression.

Thromboxane synthesis has been reported to be elevated in bladder cancers and inhibition of the synthesis or action of these prostanoid mediators can decrease rates of cell proliferation and migration [122]. In the late 1990s it was demonstrated that two isoforms of the thromboxane receptor (TP- α and TP- β), alternatively spliced at the C-terminus, have distinct agonist-induced internalization properties and abilities to bind β -arrestins [80], with only the TP- β isoform able to internalize in a ligand-dependent manner. Thromboxane receptor splicing could, as a consequence of each variant giving rise to distinct cellular responses, differentially affect key processes, such as mitogenesis. In this context it is interesting to note that the TP- β isoform has been shown to be over-expressed in some patients with bladder cancer and this expression is associated with a poorer prognosis. Moreover, this isoform alone is able to transform SV-HUC (immortalized human uroepithelial cells) into highly proliferative and motile cells and is highly expressed in various cancer-derived cell-lines, including prostate, breast, colon and renal cell carcinoma [81], suggesting an important role of this isoform in determining the progression of the disease.

Another group of GPCRs that has been heavily implicated in cancer pathophysiology are the gastrointestinal peptide hormone receptors, including the gastrin/cholecystokinin-B (CCK-B) [31], secretin receptors [76, 77] and somatostatin [78]. An alternatively spliced isoform of the CCK-B receptor that retains the fourth intron might play an important role in the development and progression of colon and pancreatic carcinoma [8, 31]. Thus, it has been demonstrated that in a pancreatic carcinoma cell-line (MIA PaCa-2) the low expression of U2AF35 (a small unit of the U2AF complex, that specifically recognizes the 3'-end dinucleotide AG which serves as an important splicing signal for pre-mRNA processing) is responsible for the retention and incorporation of the fourth intron into the mature CCK-B receptor protein [31], and this isoform plays a role in stimulating tumour growth [8].

Detecting the expression and understanding the significance of the various GPCR isoforms up-regulated in cancer could be of pivotal importance for future *in vivo* targeting of tumours both diagnostically and therapeutically.

Alternative Splicing of GPCRs in Reproduction

Much effort has been made to identify genes that are up-regulated or down-regulated in pregnancy, and especially in pathophysiological conditions, such as pre-term labour and pre-eclampsia. Here too, alternative pre-mRNA splicing has been shown to play a major role in smooth muscle myogenesis and contractility. The role of myometrial processes in regulating the activity of the uterus during gestation and parturition are linked to the differential expression and function of specific genes including: cyclooxygenase-2, oxytocin receptors, progesterone receptors, specific prostaglandin receptor subtypes, CRHR1, and $G\alpha_s$ protein [123]. Most of these, and many other proteins, are expressed as distinct isoforms generated from alternate pre-mRNA splicing. These isoforms have a potentially dominant role in the normal progression of pregnancy.

CRH and CRH-related peptides are expressed in placental and intrauterine tissues during pregnancy and labour. However, the precise biological function of CRH during human pregnancy is still an enigma [124]. There is a wealth of evidence, including myometrial contractility studies, suggesting that CRH plays a “protective” role for the pregnant uterus, by regulating intracellular signalling pathways that can maintain the uterus in a state of relaxation and prevent contraction [124]. On the other hand, increased maternal plasma CRH levels are a predictor of the onset of labour [125]. It is evident that human pregnancy is linked with changes in CRH-R isoformic expression in reproductive tissues [102, 126]. Two CRH-R1 mRNA variants, R1 α and R1c, have been identified in syncytiotrophoblasts (the outermost syncytial layer of the foetal component of the placenta) and amniotic epithelial cells [127]. Additionally, the presence of CRHR1d mRNA has been found in foetal membranes (chorion and amnion tissues) [87]. Seven mRNA splice variants (R1 α , R1 β , R1c, R1d, R2 α , R2 β and R2 γ) of the CRH receptors have also been reported in the human pregnant myometrium immediately prior to onset of labour, whereas only three isoforms (R1 α , R1 β and R2 β) are routinely detected in the non-pregnant myometrium [87, 128]. These findings suggest that CRH, acting via different receptor variants, may be able to exert distinct actions on the human

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4 myometrium in pregnant versus non-pregnant states [129]. More recent evidence indicates
5 that as pregnancy progresses towards labour (either term or pre-term) transcription of the
6 CRH-R1 gene, and its alternative splicing, is increased [102]. The precise mechanism
7 underlying this event is unknown, but *in vitro* studies of myometrial cells originating from
8 pregnant subjects identified interleukin-1 β as a potential regulator of CRH-R1 gene
9 transcription and splicing [102]. However, due to a lack of CRH-R isoform-specific
10 antibodies it has not been possible to establish which of these alternatively spliced mRNAs
11 are translated to mature proteins. Therefore, the physiological importance of these alternative
12 splicing events is presently unclear. However, it is tempting to speculate that the expression
13 of various CRH-R isoforms might allow activation of alternative signalling cascades (or
14 dampen wild-type receptor activity), facilitating the transition from relaxed to contractile
15 uterine states [102, 129].
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19 Another hormone involved in reproductive physiology is relaxin, which binds to the relaxin
20 family peptide receptors (RXFP1 and RXFP2) [130]. Several splice variants of RXFP1
21 (formerly known as LGR7 (leucine-rich-repeat-containing G protein-coupled receptor 7))
22 have been identified. Some of them produce a soluble, truncated and secreted protein which
23 binds to relaxin and antagonizes its actions *in vivo* [73]. Several splice variants, cloned from
24 human foetal membranes [74, 75], human uterus and brain [130], encode RXFP1 isoforms
25 with different lengths of the extracellular domain. These *N*-terminally truncated isoforms can
26 exert dominant-negative effects on the wild-type receptor, which could be of potential
27 functional significance in dampening the responsiveness of reproductive tissues to relaxin,
28 especially since animal studies have indicated that these truncated RXFP1 isoforms are
29 prevalent in pregnancy [75].
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33 Luteinizing hormone (LH) and its receptor (LHR) play a pivotal role in female and male
34 gonadal function, pregnancy and foetal sex differentiation. Mutations that lead to loss-of-
35 function in the male are linked with severe phenotypes, such as female external genitalia,
36 micropenis and oligospermia; while in women primary and secondary sexual characteristics
37 develop normally, but infertility is common [131, 132]. Bruysters and colleagues have
38 recently described a family that carries a homozygous mutation G \rightarrow A at position -1 at the
39 intron 10/exon 11 boundary of the LHR gene, resulting in alternative splicing of LHR and an
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eight amino acid deletion [53]. *In vitro* studies have shown that receptor expression is not affected, but the potency of LH (but not human chorionic gonadotropin) is reduced; thus, this LHR isoform shows impaired hormone responsiveness [53]. A male patient and his three sisters showed reproductive impairment. The male patient had delayed puberty, micropenis and oligospermia; while two of his sisters were infertile and the third sister had three spontaneous miscarriages [53]. Another splice variant of LHR that results in a frame-shift in the reading frame and creates a truncated receptor composed of the *N*-terminal ectodomain (responsible for high-affinity ligand binding), causes an alteration in pituitary-gonadal function, and morphological changes in the adrenals and kidneys of transgenic mice [54]. This variant also appears to control the cell surface expression of the wild-type receptor by misrouting newly synthesized receptors in the ER [54].

Alternative Splicing of GPCR in Other Pathophysiological Conditions

Obesity - Insulin secretion from pancreatic β -cells is potentiated by binding of “incretin” peptides, including gastric inhibitory polypeptide (GIP), to their receptors. The 460 amino acid wild-type GIP receptor, a GPCR of the secretin family, is expressed in mouse β -cells along with a GIP receptor splice variant that retains intron 8, which introduces a premature stop-codon resulting in a truncated C-terminus [43]. GIP activation of this latter isoform does not lead to production of cyclic AMP, and inhibits the ability of the wild-type receptor to activate this pathway on agonist addition. This is achieved through interactions between truncated and wild-type GIP receptors in the ER, which reduce trafficking of the wild-type GIP receptor from the ER to the cell-surface [43]. Mice fed a high-fat diet exhibit a relative reduction in the expression of the truncated isoform, suggesting that loss of this isoform might be involved in the hypersensitivity to GIP and hyperinsulinaemia evident in this diet-induced model of obesity [43].

Immuno-modulation - The neurokinin-1 (NK_1) receptor, which binds substance P, an important neuropeptide involved in pain perception and the modulation of both inflammatory and immune responses, occurs as two variants: a full length 407 amino acid receptor, and a truncated, 311 amino acid isoform [60-62, 133]. These isoforms have distinct signalling properties, with only the wild-type receptor being able to increase intracellular Ca^{2+} concentration and activate the transcription factor NF κ B, leading to increased expression of

mRNA for interleukin-8 [59]. In addition, compared to the wild-type receptor, the truncated NK₁ receptor variant may have an increased ability to interact with different G proteins, other GPCRs, or G protein-independent signalling pathways [59, 63]. This could be of considerable importance in cells and tissues that have distinct expressions of the NK₁ receptor isoforms. For example, in undifferentiated THP-1 cells (a human monocytic cell-line), only the truncated NK₁ receptor isoform is expressed. However, differentiation of these cells towards a macrophage phenotype with a phorbol ester leads to the expression of both isoforms [64], which could modulate cellular activity. Different brain regions appear to exhibit different patterns of NK₁ receptor pre-mRNA alternative splicing, with expression of the truncated NK₁ receptor often being greater than that of the full-length variant [64], which could result in effects on pain perception.

Histamine receptor-associated pathophysiological states - Drugs that target different histamine receptors have been widely used to treat various conditions, including allergies, gastric-related disorders and migraine. In contrast to the H₁ and H₂ histamine receptor genes, the genes encoding H₃ and H₄ histamine receptors include several intronic sequences. This can result in expression of alternatively spliced isoforms and several H₃ histamine receptor spliced variants have been described (see [50] for review). For example, novel isoforms lacking TM7 and containing an alternate C-terminus (6TM-H₃ isoforms) have been isolated and characterized from rat brain [51]. When recombinantly expressed, these isoforms are retained within intracellular compartments, and thus they are unlikely to bind ligand or couple to signalling pathways [51]. However, co-expression studies have demonstrated that 6TM-H₃ can selectively interfere with the membrane expression of the wild-type H₃ receptor and affect its ability to signal normally; thus, 6TM-H₃ acts as a dominant-negative isoform. It is therefore again tempting to speculate that functional (and constitutive) activity of the H₃ receptor can be “fine-tuned” through expression of both 6TM-H₃ and wild-type, 7TM isoforms in a given system [51].

Recently, van Rijn and co-workers have identified two novel, alternatively spliced H₄ histamine receptor isoforms from CD34+ cord blood-cell-derived eosinophils and mast cells [52]. One splice variant encoded a 302 amino acid protein that contained a deletion of 88 amino acids between predicted TM2 and TM4 domains; while a second splice variant

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4 encoded only the first 67 amino acids of the whole receptor. Both of the receptor variants,
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6 when over-expressed in HEK293 cells, had primarily an intracellular localization and failed to
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8 bind ligand. However, when co-expressed with the wild-type H₄ histamine receptor, both
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10 isoforms had a dominant-negative effect via hetero-oligomerization [52]. Again, more
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12 research is needed to assess whether splice variations in H₄ receptor expression are relevant to
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14 any pathophysiological aspects associated with the normal functioning of this receptor
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16 subtype *in vivo*.

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18 **Respiratory diseases** - Susceptibility to asthma and related respiratory diseases has in many
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20 cases a genetic component. A genome-wide scan suggested that one of the contributory
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22 factors to asthma and other IgE-mediated diseases could be GPRA (also known as GPR154
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24 and the neuropeptide S receptor) [134]. Several splice variants of the receptor have been
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26 described [67]. Two splice variants that encode GPRA isoforms with distinct C-termini
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28 (termed A and B isoforms) are full-length isoforms that traffic to the plasma membrane.
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30 Interestingly, another isoform, the short one that is expressed in intracellular compartments
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32 does not affect the cell surface expression of the full-length receptors when co-expressed [67].
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34 Immunohistochemical studies showed that the A isoform is primarily expressed in smooth
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36 muscle cells, while the B isoform is principally detected in epithelial cells. When these
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38 authors compared airway smooth muscle (ASM) of asthmatic and healthy individuals they
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40 found that the B isoform was more strongly expressed in samples from asthmatic patients
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42 [134], suggesting that the neuropeptide S receptor could be a strong candidate for
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44 involvement in the pathophysiology of asthma and respiratory atopy in Caucasian
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46 populations. However, pharmacological studies did not find any substantial differences in the
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48 binding and signalling properties of the two GPRA isoforms [135]. Further work on knockout
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50 GPRA^{-/-} mouse model failed to detect differences in the development of allergic lung disease
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52 in these animals, despite the demonstrable loss of functional GPRA [136]. Additionally,
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54 studies conducted in a Mexican population showed that in contrast to Caucasian and Asian
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56 populations, GPRA variants do not appear to be important contributors to childhood asthma
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58 susceptibility in Mexicans [137].

59
60 Work by Einstein and co-workers [21] highlighted the complexity of GPCR expression and
alternative splicing in human airway smooth muscle (ASM). The authors hypothesized that

sometimes complex and contradictory pharmacological responses of ASM GPCRs might be due to variable GPCR expression and the frequent alternative splicing of the receptors leading to a “highly diversified receptor milieu”. This study highlighted the fact that potentially 353 different GPCRs are expressed in airways smooth muscle, of which 192 GPCRs have on average five alternatively spliced variants. One GPCR expressed in multiple splice variants is the leukotriene B₄ (LTB₄) receptor, a receptor known to contribute to abnormal responsiveness in lung disease [21]. High-affinity LTB₄ receptors, expressed on neutrophils, eosinophils and T-lymphocytes, on activation by its endogenous agonists facilitate leukocyte migration to the lung [138]. Moreover, expression of the LTB₄ receptor in ASM could contribute to bronchoconstriction, due to coupling of the receptor to G_q and G_i proteins [139]. The work from Einstein and co-workers [21] raises a possibility that discrepant bronchoconstrictive effects of LTB₄ receptor antagonists might be due to the presence of different receptor isoforms in ASM.

Neuronal function – The mammalian CNS shows the greatest diversity of GPCR expression of any organ in the body. Neuronal GPCRs are presently targeted directly (GPCR agonists and antagonists) or indirectly (through the use of drugs that alter endogenous neurotransmitter levels) to treat a variety of neurological and psychiatric disorders. As already stated, an array of important neurotransmitter/neuromodulator GPCRs are expressed in a number of alternatively spliced variant isoforms, including dopamine, opioid, 5HT₄ and 5HT₇, V₂ vasopressin, metabotropic glutamate (mGlu), and GABA_B receptors [17, 19, 20, 68, 83, 140, 141]. Although some of the better characterized GPCR splice variants (e.g. D_{2S} and D_{2L} dopamine receptors) exhibit only quite subtle differences in their signalling properties, data are beginning to appear that at least hint at how such splice variation might affect GPCR function both physiologically and pathophysiologically.

For example, C-terminal μ -opioid receptor variants differ in their ability to be recycled to the plasma membrane (“resensitized”) following agonist-induced internalization [142]. As described above, mGlu1 receptor isoforms (a and b) are differentially expressed during neuronal development and differentiation, and the mGlu1a and 1b isoforms appear to have distinct cellular localizations and constitutive activities, as well as exhibiting potency and/or efficacy differences with respect to agonists [56, 57, 140]. Similarly, alternative splicing of

the D₃ dopamine receptor can produce seven variants, including a non-dopamine binding D3nf isoform [38, 143]. Quaternary interaction of D3nf with full-length D₃ (or D₁) dopamine receptors to form dimers can reduce trafficking of the dopamine-binding isoform to the plasma membrane [143]. Altered splicing efficiency with respect to the D₃ dopamine receptor and the consequent alteration in the proportion of full-length and truncated forms generated in neuronal populations has been hypothesized to be linked to schizophrenia [18, 38, 143].

Closing Remarks

In this short review we have not sought to cover all GPCRs that are expressed from alternative splicing of pre-mRNAs, but we have attempted to provide an overview of GPCR alternative splicing with an emphasis on newly emerging aspects. In Table 1 we provide an additional summary of some recent findings not mentioned above (and see [17, 18] for the developments before 2001). Additionally, we have aimed to show that many more GPCRs than was initially thought (or perhaps has become a widely accepted misconception), possess introns (Figure 1). This does not mean that all multi-exon-containing GPCRs will undergo alternative pre-mRNA splicing. For GPCRs that are encoded by only two exons (e.g. some members of the rhodopsin and frizzled families), it is very likely that alternatively spliced mRNA would be subject to nonsense-mediated decay, and the message is never translated into a protein product.

The interest in alternative splicing of GPCRs is increasing, and novel variants are being identified on a regular basis. In 2005, two companies launched GPCR SpliceArray™ for the identification of alternatively spliced GPCR mRNAs (<http://www.exonhit.com>). Other manufacturers, including Affimetrix and Jivan Company, also provide GPCR arrays. The arrays can be custom-made in order to investigate particular (sometimes rare) splicing events within a sub-group of GPCRs, but also broader arrays can be designed to study the usage of alternative splice donor/acceptor sites, insertion of novel exons, exon skipping, intron retention, or a partial internal exon deletion in a broad number of GPCRs. This new high-throughput approach based on microarray technology uses several probes (usually six) that are specific for an exon and span over the exon junctions allowing robust and specific detection of the two possible mRNAs resulting from each splicing event. The data obtained from these arrays have to be validated using traditional experimental methods (usually RT-

PCR and western blotting) which confirm the presence of particular alternatively spliced mRNAs and protein isoforms. Bioinformatics studies that predict the existence of mRNA variants and analyse whether potentially transcribed protein isoforms would be functional are of great importance in this task [16].

More often than not, isoforms of a particular GPCR have distinct pharmacological and signalling properties. In order to start understanding the link between these diverse pharmacological and signalling characteristics on one side and physiological consequences of the activation of various GPCR isoforms on the other side, comprehensive studies that dissect the regulatory mechanisms at the molecular level of GPCRs alternative mRNA splicing are crucial. These studies could be of pivotal importance in understanding and treating pathophysiological conditions that might favour expression of isoforms with altered signalling.

Interestingly, signalling cascades, including the Ras/extracellular signal-regulated kinase and Ca^{2+} /calmodulin-dependent protein kinase pathways, which are often activated as a consequence of ligand binding to various GPCRs, have been implicated in regulation of alternative splicing of CD44, a cell-surface receptor that is also involved in cell adhesion and migration [144] and the large conductance, voltage- and Ca^{2+} -activated (SLO) K^{+} channel pre-mRNA splicing [145]. Moreover, signalling via D_1 dopamine receptors activates a pathway that results in the phosphorylation of key transcriptional and splicing factors, including Ania-6 (human homologue = cyclin-L), RNA polymerase II, SC35 and CDK^{p110} [146], and could potentially regulate the alternative splicing of an array of proteins. It is well established that the majority of GPCRs are desensitized, internalized and down-regulated by constant or repeated exposure to agonist. Is it possible that expression of spliced isoforms allows fine-tuning of this process? Is it possible that activation of a GPCR “auto-regulates” expression of its own spliced isoforms, or regulates the expression of other GPCR isoforms? These and many other questions remain to be answered.

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TABLE 1 Splice variation and potential physiological roles of receptor isoforms. Numerous splicing events that affect the expression of GPCRs mRNA and protein have been described since 2001 (for splice variants known before 2001, see [17, 18]). Over-expression studies provide valuable insights into potential physiological relevance of these events; although in many cases the real physiological roles in endogenous systems are unknown.

Receptor	Event description	Potential physiological relevance	References
5-HT receptors	Several	Various	Reviewed in [19] and [20]
Muscarinic acetylcholine receptor	Novel intron/exon	Unknown	[21]
Adenosine receptors	5'-UTR splicing; Novel exons	Differential translation	[21, 22]
α_1 -adrenoceptors	Several	Various	Reviewed in [23]
Angiotensin-II receptor	Exon inclusion/exclusion	Distinct signalling properties	[24, 25]
Calcitonin receptor	Exon exclusion-headless variant	Abolished ligand binding/dominant-negative effect.	[26, 27]
Cannabinoid receptor type 1	Alternative donor/acceptor site	Altered pharmacology	[28, 29]
Chemokine receptor (CXCR3)	Novel acceptor site-distinct N-terminus	Distinct signalling properties	[30]
Cholecystokinin receptor (CCK-B)	Intron inclusion	Constitutively active, ligand-independent Src activation	[8, 31, 32]
Corticotropin-releasing hormone receptors (type 1 and 2)	Several	Various	[33- 35]
Dopamine receptor (D ₂)	Exon inclusion/exclusion	Distinct signalling properties and cellular localization	[36, 37]
Dopamine receptor (D ₃)	Premature stop codon-lacks TM6 and TM7	Distinct cellular localization	[38]
Endothelin B receptor	Exon exclusion	Not transcribed	[39]
Epidermal growth factor-seven TM receptor	Trans-splicing	Diversification of ligand repertoire	[40]
Follicle-stimulating hormone receptor	Exon exclusion and used of additional exon	Not GPCR, but growth factor receptor	[41]
GABA _B receptor	Several	Various	Reviewed in [42]
Gastric inhibitory polypeptide receptor	Intron retention-premature stop codon	Impaired signalling; dominant-negative effect	[43]
Growth hormone-releasing hormone receptor	Exon exclusion, intron retention	Impaired signalling; dominant-negative effect	[44-49]
Histamin receptors (H ₃ and H ₄)	Several	Various	[50-52]
Leukotriene B ₄ receptor	Alternative donor/acceptor sites, novel exons/introns	Unknown	[21]
Luteinizing hormone receptor	Mutation at the intron 10/exon 11, resulting in 8 aa deletion	Abolished reproduction	[53, 54]
Melanocortin 2 receptor	5'-UTR splicing	Adipogenesis?	[55]
Metabotropic glutamate receptors	Alternative C-terminal exons	Distinct signalling; role in development	[56-58]
Neurokinin receptor 1	Exon exclusion- truncation	Distinct signalling; role in immunomodulation	[59-64]
Neurokinin receptor 2	Exon exclusion	Impaired ligand binding and signalling	[65, 66]
Neuropeptide S receptor (GPRA)	Alternative C-terminal exons, truncation	Intracellular localization of truncated isoform	[67]
Opioid receptors	Several	Various	Reviewed in [68]

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Parathyroid hormone (-related peptide) receptor	5'-UTR; exon exclusion	Lack of post-translational glycosylation;	[14,69]
Prostaglandin receptors	Several	Various	[70-72]
Relaxin receptors	Several	Various	[73-75]
Secretin receptor	Exon exclusion	Unknown - cancer biology?	[76,77]
Somatostatin receptor (sst2)	Exon exclusion- truncation	Altered desensitization	[78], reviewed in [79]
Thromboxane receptors	Alternative C-terminal exons	Distinct internalization/interaction with arrestins	[80, 81]
Vasopressin V ₂ receptor	Alternative splice site	Dominant-negative effect	[82, 83]
VIP and PACAP receptors	Several	Various	Reviewed in [84]

For Review Only

Figure Legends

Figure 1 Overview of GPCRs encoded by more than one exon. In the “pre-human genome era” it was assumed that more than 90% of human GPCRs were intronless [15]. However, analysis from various databases and bioinformatic studies indicate that the number of intron-containing GPCRs has been under-estimated. Using a list of over 350 GPCRs from the IUPHAR database and examining their ORF sequences for the presence of alternate exons (using CCDS data from NCBI), it is obvious that a large number of GPCRs contain more than one exon (52%). This does not mean that all of them can be expressed as alternatively spliced variants (mRNA) or isoforms (proteins), but that they are not intronless. These data are also likely to be an under-estimate, because if the CCDS data are not available for a gene, we would assign the GPCR as intronless.

Figure 2 Schematic representation of GPCR structure summarizing potential splice variants. The 7TM domains are shown as blue cylinders; the *N*-terminus is facing the extracellular side of the plasma membrane, while the *C*-terminus is intracellular. The events that lead to translation of alternatively spliced isoforms are outlined in the boxes.

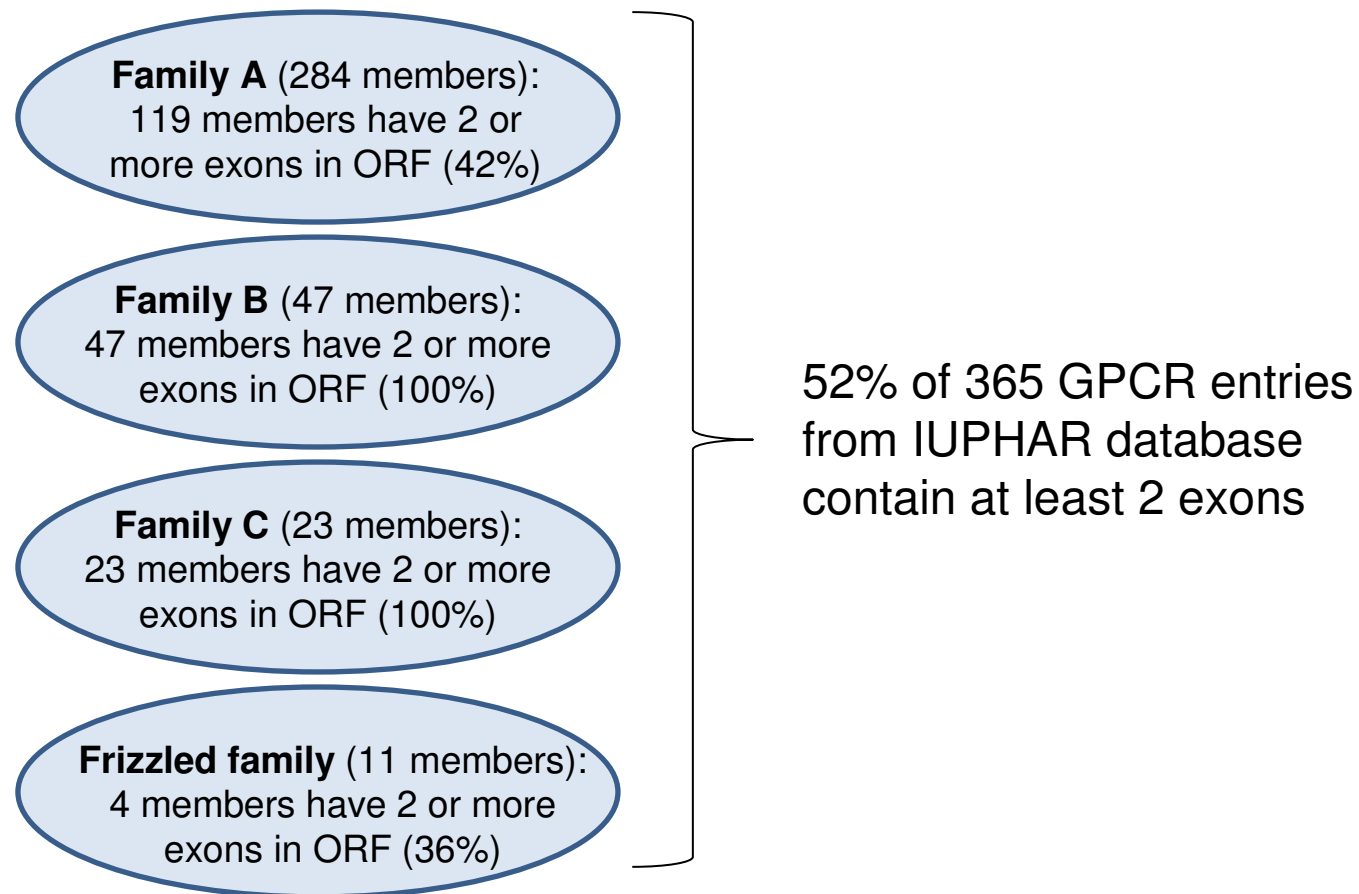
FIGURE 1 (Markovic & Challiss)

FIGURE 2 (Markovic & Challiss)

