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# Accepted Manuscript

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## Understanding the common themes and diverse roles of the second extracellular loop (ECL2) of the GPCR super-family.

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*Abbreviated title:* ECL2 of the GPCR super-family

### *Abbreviations*

|        |   |
|--------|---|
| 5-HT1B | Serotonin 5-HT1B receptor                 |
| 5-HT2B | Serotonin 5-HT2B receptor                 |
| A2AR   | Adenosine A2A receptor                    |
| AT1R   | Angiotensin II type 1 receptor            |
| AT2R   | Angiotensin II type 2 receptor            |
| β1AR   | β1 adrenergic receptor                    |
| β2AR   | β2 adrenergic receptor                    |
| CCR5   | C-C chemokine receptor type 5             |
| CB1    | Cannabinoid receptor 1                    |
| CLR    | Calcitonin receptor-like receptor         |
| CRF    | Corticotropin-releasing factor            |
| CRFR1  | Corticotropin-releasing factor receptor 1 |
| CXCR   | C-X-C chemokine receptor                  |
| D3R    | Dopamine 3 receptor                       |
| ECD    | Extracellular domain                      |
| ECL    | Extracellular loop                        |
| ET-1   | Endothelin-1                              |
| FFAR1  | Free fatty acid receptor 1                |
| GCGR   | Glucagon receptor                         |
| GIP    | Gastric inhibitory polypeptide            |
| GIPR   | Gastric inhibitory polypeptide receptor   |
| GLP1   | Glucagon-like peptide-1                   |
| GLP1R  | Glucagon-like peptide-1 receptor          |
| GPCR   | G protein-coupled receptor                |
| H1R    | Histamine H1 receptor                     |
| ICL    | Intracellular loop                        |
| LB     | Lobe                                      |
| mGLUR  | metabotropic glutamate receptor           |
| M2R    | M2 muscarinic acetylcholine receptor      |
| M3R    | M3 muscarinic acetylcholine receptor      |
| NMR    | Nuclear magnetic resonance                |
| N/OFQ  | Nociception/orphanin FQ peptide           |
| OR     | Opioid receptor                           |
| PAR1   | Proteinase-activated receptor 1           |

RAMP1 (R1) Receptor activity modifying protein  
S1P1R Sphingosine-1-phosphate 1 receptor  
SCAM Scanning cysteine accessibility method  
TM Transmembrane  
VFT Venus fly trap

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## **Abstract**

The extracellular loops (ECLs) of G protein-coupled receptors (GPCRs) can bind directly to docked orthosteric or allosteric ligands, they can contain transient contact points for ligand entry into the transmembrane (TM) bundle and they can regulate the activation of the receptor signalling pathways. Of the three ECLs, ECL2 is the largest and most structurally diverse reflecting its functional importance. This has been shown through biochemical techniques and has been supported by the many subsequent crystal structures of GPCRs bound to both agonists and antagonists. ECL2 shares common structural features between (and sometimes across) receptor sub-families and can facilitate ligand entry to the TM core or act directly as a surface of the ligand-binding pocket. Structural similarities seem to underpin common binding mechanisms; however, where these exist, variations in primary sequence ensure ligand-binding specificity. This review will compare current understanding of the structural themes and main functional roles of ECL2 in ligand binding, activation and regulation of the major families of GPCRs.

## **Keywords**

GPCR; Extracellular loop; ligand-binding; receptor activation; signalling; family B.

## **1 Introduction**

G protein-coupled receptors (GPCRs) are a super-family of receptors. There are over 800 different GPCRs in humans and they are involved in almost every physiological process (Fredriksson et al., 2003, Bockaert and Pin, 1999, Rosenbaum et al., 2009). This makes them ideal targets for drugs and thus between 25-50% of therapeutics interact with these receptors (Salon et al., 2011). GPCRs undergo conformational changes that result in active and inactive conformations (Cherezov et al., 2007, Rasmussen et al., 2011b). Both drugs and natural agonists function by stabilising a particular conformation (Jazayeri et al., 2015). For most ligands to bind to a specific receptor propagating the signalling response, the extracellular regions of the receptor (specifically the extracellular domain (ECD) and the extracellular loops (ECLs)) are particularly important. It is known that the ECLs of GPCRs can permit or facilitate ligand-entry into the TM bundle, hold ligands within the TM domain, direct ligands towards their binding site, change the shape of the binding pocket or bind directly to orthosteric or allosteric ligands (Wheatley et al., 2012).

GPCR crystal structures and functional studies have shown that ECL2 is particularly important. ECL1 and ECL3 are often short loops that run parallel to the membrane connecting TM 2/3 and TM 6/7 respectively, however ECL2 is longer and shows much greater variation with its structural features (Venkatakrisnan et al., 2013). As well as this structural diversity, ECL2 is vital for ligand binding and the subsequent activation of the receptor (Wheatley et al., 2012).

The importance of ECL2 (connecting TM4 and TM5) was first identified through chimeric receptor experiments and mutagenesis studies of this domain (Olah et al., 1994, Walker et al., 1994, Fitzpatrick and Vandlen, 1994). Even in receptors with known TM ligand-binding pockets, the ECLs were implicated in early stage ligand-binding and presentation to the TM binding site (Colson et al., 1998, Perlman et al., 1997). In many cases ECL2 residues have been implicated in direct or indirect ligand-binding due to the location, flexibility and sequence variability of the loop (Kim et al., 1996, Wheatley et al., 2012).

Given the large recent increase in structural information, highlighting the importance of ECL2 in the extracellular region, this review will specifically focus on ECL2. The crystal

structures, together with a large amount of biochemical data, have implicated ECL2 in many key aspects of receptor function (e.g. ligand binding and activation) and this loop often has a role in the current “hot topics” of receptor function (e.g. biased signalling, allosteric modulation). This review will start by summarising the structural information known for this loop. This will be followed by a comparison of the functional characteristics of ECL2 in the super-family. Finally, we will discuss whether this loop acts as a molecular “gatekeeper” controlling the course of activation of the receptor.

## ***2 Structure of ECL2***

A number of GPCR crystal structures have now been published. These have provided important information with respect to both structure and contact sites with the bound ligand. In this section these structures are illustrated with ECL2 specifically highlighted. Key structural information has also been summarised. The crystal structures often include additional, useful ‘non-structural’ information (e.g. contact sites with a bound ligand), which will be discussed later in the review. For clarity the receptors have been grouped according to phylogenetic similarity or in some cases a shared structural or functional property.

### ***2.1 An ECL2 $\beta$ -hairpin structure forms an extracellular cap over the TM bundle in receptors with hydrophobic or covalently attached ligands***

The unique structural properties of ECL2 were first highlighted in the crystal structure of bovine rhodopsin (see figure 1 and table 1), which found ECL2 to be an antiparallel  $\beta$ -sheet in a hairpin formation ( $\beta$ -hairpin) extending deep into the centre of the TM bundle (Palczewski et al., 2000). ECL2 makes extensive contacts between both the retinal ligand and the extracellular regions. It also associates with the extracellular N-terminus and ECLs to form an extracellular cap over the TM bundle, closing off the ligand-binding pocket to the extracellular side (the “closed” ECL2 position).

This closed extracellular cap involving ECL2 is a feature that is also present in the sphingosine-1-phosphate 1 (S1P1) receptor (see figure 1 and table 1) and the GPR40 (free fatty acid receptor 1, FFAR1). In both these structures, ECL2 contains a  $\beta$ -sheet structure. In the S1P1R the N-terminal ECD folds over and packs tightly with ECL1 and ECL2 forming a cap over the receptor. This creates the top surface to a ligand-binding pocket (Hanson et al., 2012). In GPR40 the  $\beta$ -sheet structure is preceded by an auxiliary loop (Srivastava et al., 2014). The GPR40 ECL2 has low flexibility and functions as a cap over the binding site, similar to that observed with the S1P1R.

### ***2.2 The ECL2 $\beta$ -hairpin structure is “open” in the family A GPCR peptide, opioid and chemokine receptor sub-types***

The ECL2  $\beta$ -hairpin structure is common in many GPCRs and is a feature shared across receptor sub-families. However, compared to its “closed” position in the extracellular capped receptors (with hydrophobic or covalently attached ligands) the loop is open for receptors with soluble ligands. “Open” means that the loop “allows” or mediates extracellular access to the TM bundle. These include the peptide-binding receptors as well as the opioid and chemokine receptors (both in the same sub-family ( $\gamma$ ) of the family A receptors; see figure 1 and table 1) (Wu et al., 2010, Park et al., 2012, Wu et al., 2012, Granier et al., 2012, Tan et al., 2013, Manglik et al., 2012). The nociceptin/orphanin FQ (N/OFQ) peptide receptor shares high sequence homology with the opioid GPCRs but has distinct pharmacology. ECL2 has the same  $\beta$ -sheet hairpin structure as the opioid and chemokine receptors (Thompson et al., 2012). The peptide binding endothelin ET<sub>B</sub> receptor also shares this  $\beta$ -hairpin structure (Shihoya et al., 2016).

The  $\beta$ -hairpin structure is also present in the protease activated receptor 1 (PAR1). This is part of the  $\delta$ -subfamily of the family A GPCRs, which include the glycoprotein receptors, the purinergic receptors and the olfactory receptors (Zhang et al., 2012). However PAR1 has been included in this section due to its activation through a (tethered) peptide ligand and a similar ECL2 structure.

### ***2.3 There are a variety of ECL2 structures within the aminergic receptors***

The aminergic receptors of the histamine H1 receptor (H1R), dopamine D3 receptor (D3R), muscarinic (M1, M2, M3, M4) receptors, serotonin receptors,  $\beta$ 1AR and  $\beta$ 2AR all have an open ECL2 conformation with this loop forming the top of a ligand-binding pocket (see figure 2 and table 1). There are some structural differences between the receptors in this group.  $\beta$ 1AR and  $\beta$ 2AR have an  $\alpha$ -helical ECL2 structure (Warne et al., 2008, Cherezov et al., 2007). ECL2 of the  $\beta$ 2AR was exposed at the surface and has an intra-loop disulphide bond as well as the conserved disulphide bond with TM3. The loop also made contacts with ECL1 and had a glycosylation site at position N187. The D3R ECL2 lacks any secondary structure and is shorter than the  $\beta$ ARs ECL2s. Despite this, it still forms a binding pocket that interacts with various ligands that were studied and may be important for ligand specificity (Chien et al., 2010).

The four available muscarinic acetylcholine receptor structures (M1, M2, M3 and M4) have ECL2 domains that also lack secondary structure but adopt a conformation similar to the  $\beta$ -hairpin structure in many other GPCRs (see figure 2 and table 1); this is “open” allowing ligand entry to a deep binding pocket (Haga et al., 2012, Kruse et al., 2012, Thal et al., 2016). The H1R ECL2 also lacks secondary structure but partially covers the ligand-binding pocket (Shimamura et al., 2011). In the 5-HT1B (serotonin) receptor structure, ECL2 forms a loop that *partially* covers the ligand-binding pocket (Wang et al., 2013). Whereas in the 5-HT2B receptor structure, there is an additional helical turn at the extracellular end of TM5 (Wacker et al., 2013). This shortens the segment of ECL2 that is connected to TM3 through the conserved disulphide bond, causing an inward shift and a constraint on the position of the extracellular end of TM5. This may explain the differing conformations seen in the crystal structures of the 5-HT1B and 5-HT2B receptors in the presence of the same ligand.

### ***2.4 ECL2 within the adenosine receptors has different structural features depending on the crystal structure***

The ECL2  $\alpha$ -helix observed in the  $\beta$ 1AR and  $\beta$ 2AR is also present in the agonist-bound A2AR adenosine receptor (Lebon et al., 2011) but not in the antagonist bound structure (Jaakola et al., 2008), however part of the loop was missing in this structure (see table 1).

There is also a  $\beta$ -strand that forms hydrogen bonds with a second  $\beta$ -strand in ECL1; however the degree of hydrogen bonding varies between antagonist- and agonist-bound structures. In the antagonist-bound structure there is an extensive disulphide bond network with three bonds connecting ECL1 and ECL2 and an intra-loop disulphide bond in ECL3 that creates a rigid open structure exposing the ligand-binding cavity (Jaakola et al., 2008).

### ***2.5 ECL2 within the family B GPCRs is a loop (with no secondary structure) open to the TM bundle***

Crystal structures have been solved for two family B GPCRs (Hollenstein et al., 2013, Siu et al., 2013, Jazayeri et al., 2016). The corticotropin releasing factor receptor 1 (CRFR1) and the glucagon receptor (GCGR) lack the  $\beta$ -sheet or  $\alpha$ -helix present in so many of the family A



GPCRs (see figure 2 and table 1). Both receptors have a similar ECL2 structure, where the loop extends from TM4 towards the middle of the TM bundle before twisting 90° to cover the binding pocket and twisting back 90° to form TM5. This is more representative of some of the aminergic sub-family of the family A GPCRs (e.g. the muscarinic and dopamine receptors) (Chien et al., 2010, Haga et al., 2012, Kruse et al., 2012, Park et al., 2012).

### ***2.6 ECL2 within the family C GPCRs is a $\beta$ -hairpin in a different position to the family A $\beta$ -hairpin***

Both family C GPCR structures (mGluR1 and mGluR5) have a  $\beta$ -hairpin structure within ECL2 (Wu et al., 2014, Dore et al., 2014), however this is angled more towards TM6 and 7 (especially mGluR1) than the other  $\beta$ -hairpin receptors in family A (see table 1). Any role in receptor activation or ligand-binding is not known but ECL2 is not likely to be a major factor in agonist-interaction as the “venus fly-trap” process in the ECD of family C GPCRs is very well established.

### ***2.7 Structural differences in ECL2 between inactive and active structures***

A number of active structures of GPCRs have now been published. These are receptors bound to agonist and in some cases the G protein or G protein mimetic antibody.

Crystal structures and NMR analysis of the  $\mu$ OR have shown that the  $\beta$ -hairpin structure of ECL2 moves upon receptor activation however this movement is slight, creating subtle differences in the ligand binding pocket (Sounier et al., 2015, Huang et al., 2015). In the M2 muscarinic receptor active structure, there is an inward movement (towards the TM bundle centre) of both ECL2 and ECL3 that creates an orthosteric binding pocket too small for the binding of an inverse agonist (Kruse et al., 2013). Binding of the endothelin-1 (ET-1) agonist to the ET<sub>B</sub> receptor caused a small movement of the ECL2  $\beta$ -hairpin that together with the N-terminal tail, created a “closed” lid structure and almost irreversible binding of the ET-1 ligand (Shihoya et al., 2016). In the active  $\beta$ 2AR structures, there is very little movement of ECL2 (Rasmussen et al., 2011b, Rasmussen et al., 2011a).

### ***2.8 Summary of the ECL2 structures***

In the solved family A and family C crystal structures, the most common ECL2 conformation is a  $\beta$ -hairpin (see table 1). These include receptors for lipid soluble ligands and the family A peptide ligands (for example the opioid and chemokine receptors). Perhaps surprisingly, this is not a feature of the peptide binding family B receptor ECL2 structures. Instead these contain a loop structure more similar to some of the family A aminergic receptors (e.g. muscarinic receptors). There are exceptions to these categories, most obviously the  $\beta$ 1AR and  $\beta$ 2AR, which both contain an  $\alpha$ -helical ECL2 structure. Across the solved structures, the ECL2 domain can be open or closed to the orthosteric binding pocket in the TM bundle. Receptors with covalently attached or lipid ligands have ECL2 in a closed position over the top of the TM bundle. Receptors with soluble ligands often have ECL2 in an open position, however there is flexibility of the loop. The conformation changes that occur during GPCR activation are largest on the intracellular side (Rasmussen et al., 2011b). The movement of ECL2 is more subtle however this can result in large functional effects.

## **3 ECL2 and receptor function**

### ***3.1 Key features and motifs***



### *3.1.1 A conserved TM3-ECL2 disulphide bond across the GPCR superfamily forms a structural constraint*

There is a conserved ECL2 cysteine residue across the GPCR super-family as a whole, forming a disulphide bond with a cysteine residue at the top of TM3 (Wheatley et al., 2012). This has been thought to be necessary for the structural integrity of the loop and therefore receptor function (Venkatakrisnan et al., 2013). In some cases it has been shown to be essential for receptor function; With the C5aR, the double alanine substitution did not produce a signalling response upon ligand stimulation (however constitutively active substitutions remained active) (Klco et al., 2005). In the chemokine CCR8 receptor, the conserved disulphide bond between TM3 and ECL2 was essential for receptor function with the CCL peptide agonist but also small molecular antagonists that bind deeper in the TM bundle (Barington et al., 2016). However in other receptors the disulphide bond is functionally redundant; Double alanine mutations (to remove the bond and any free sulphhydryl groups) on the family A adenosine A2A receptor (Naranjo et al., 2015) and the family B CGRP and GLP1 receptors (Woolley et al., 2013, Mann et al., 2010) retained receptor function.

### *3.1.2 Conserved motifs in family B GPCRs*

In a number of the family B receptors (CGRP receptor, GCGR and GLP1R), several residues have consistently been shown to have importance in ligand binding and the subsequent activation of the receptors. These include a conserved basic residue at the start of ECL2, an acidic residue before the conserved cysteine and a tryptophan immediately after the cysteine (forming a conserved family B CW motif); (Woolley et al., 2013, Siu et al., 2013, Koole et al., 2012). This central ECL2 tryptophan residue was also important for ligand binding and activation of the CRF1R (Gkountelias et al., 2009).

## **3.2 The role of ECL2 in ligand binding and receptor activation in the GPCR super-family**

### *3.2.1 ECL2 can control ligand entry into the orthosteric binding pocket*

The extracellular region of GPCRs can be split into two categories; those with a closed orthosteric ligand binding pocket (to the extracellular side) and those with an open orthosteric ligand binding pocket (Venkatakrisnan et al., 2013).

The closed ligand binding pocket group are the receptors corresponding with the extracellular capped receptors in section 2.1. In these receptors ECL2 and the rest of the extracellular region cover the ligand binding pocket, preventing ligand access from the extracellular side. These receptors have either a unique, covalently attached retinal ligand in the TM bundle of the bovine rhodopsin receptor that makes contacts with ECL2 (Palczewski et al., 2000), or lipid ligands that gain access to the ligand-binding pocket through the lipid bilayer (S1P1R and GPR40) (Hanson et al., 2012, Srivastava et al., 2014). However with GPR40, a ligand-binding pocket was predicted that would require extracellular entry (Srivastava et al., 2014). This type of ligand entry (through the lipid bilayer) has also been proposed for lipophilic drugs (e.g. vorapaxar with the PAR1) (Zhang et al., 2012).

Those receptors with an open ligand-binding pocket bind to water soluble ligands, which gain entry to the TM bundle from the extracellular side (Venkatakrisnan et al., 2013).

In the case of the opioid, chemokine and family A peptide GPCRs, the ECL2 regions of these receptors have the same  $\beta$ -hairpin structure as the extracellular capped receptors with the closed pocket, however this is open to the orthosteric binding pocket allowing ligand entry to

the binding pocket from the extracellular side (Wu et al., 2010, Park et al., 2012, Wu et al., 2012, Granier et al., 2012, Tan et al., 2013, Manglik et al., 2012). Often ECL2 forms part of the binding pocket, interacting with the bound ligand. In a more unique case, activation of PAR1 occurs when thrombin cleaves the N-terminal ECD, exposing a tethered peptide ligand that binds to the TM bundle (that is speculated to occur following interactions with the ECLs) (Zhang et al., 2012).

### *3.2.2 ECL2 can function as part of the orthosteric binding site*

Residues in ECL2 can face the orthosteric binding site and be involved in ligand binding. These residues can be aromatic, hydrophobic, polar and charged. In the V1AR vasopressin receptor, aromatic residues that are highly conserved in the neurohypophysial GPCR sub-family were important for agonist binding and receptor activation (Conner et al., 2007). Tyrosine residues in the chemokine CCR8 receptor were important for agonist and antagonist binding (Barrington et al., 2016). Aromatic and hydrophobic residues of ECL2 line the orthosteric binding pocket of the ET<sub>B</sub> receptor (Shihoya et al., 2016). The C-terminus of the ET-1 ligand fitted into a hydrophobic binding pocket, which included an ECL2 phenylalanine. Hydrophobic residues of the ET-1  $\alpha$ -helix interacted with both aromatic and hydrophobic residues of ECL2, which tightly holds the ligand in place with the  $\beta$ -sheet structure. The importance of hydrophobic residues in ECL2 was shown in the dopamine D1 receptor (Mente et al., 2015). An ECL2 leucine residue is important for agonist binding to this receptor.

Both acidic and basic residues of ECL2 have been shown to be important in forming the orthosteric binding site. Mutation of these residues in family A receptors have had a detrimental effect on ligand-binding and receptor activation (Walker et al., 1994, Kim et al., 1996, Moro et al., 1999). Acidic residues have been shown to be important in the opioid receptors. An ECL2 aspartate residue (D187) in the D3R is important in the tolerance to different receptor agonists (Gil-Mast et al., 2013). The ECL2 of the N/OFQ peptide receptor is enriched with aspartate and glutamate residues making this loop acidic and a predicted site of docking with the peptide antagonist UFP-101 (Thompson et al., 2012). In the H1R an ECL2 lysine (K179) forms part of an anion-binding site at the entrance to the ligand-binding pocket, which is a feature unique to the H1R-doxepin (antagonist) complex. K179 and the preceding aspartate (D178) are also able to interact with specific antagonists (Shimamura et al., 2011).

### *3.2.3 ECL2 is involved in ligand selectivity*

ECL2 residues can be involved in the selective binding to agonists, antagonists and inverse agonists. Residues in the C-terminus of the cannabinoid receptor 1 (CB1) ECL2 were important for agonist binding but not inverse agonist binding (Ahn et al., 2009). Modelling also implicated ECL2 in inverse agonist binding in the cannabinoid receptor subtype CB2 (Hu et al., 2016). Analysis of the crystal structures of  $\beta$ 1AR and serotonin receptor crystal structures found ECL2 to be involved in the binding to different ligands to the same receptor ( $\beta$ 1AR) or an altered docking of the same ligand to different receptors (5-HT1B and 5-HT2B) (Shukla et al., 2014).

In the two angiotensin receptor subtypes (AT1R and AT2R), ECL2 showed flexibility and variability. This became a target for selective ligand design that results in the creation of an agonist analogue with 18,000 fold higher selectivity for AT2R (Magnani et al., 2014).

### *3.2.5 ECL2 affects receptor binding kinetics*

In the unliganded structure of ET<sub>B</sub> receptor, the receptor is “open” creating a large cavity (Shihoya et al., 2016). Following binding of ET-1 to the ET<sub>B</sub> receptor, ECL2 and the N-terminal tail form a lid which covers the orthosteric binding pocket. This “closed” structure would explain the incredibly slow dissociation rate of ET-1 from its receptor (Clozel et al., 1989, Fischli et al., 1989, Takasuka et al., 1994). The scanning cysteine accessibility method (SCAM) was used to investigate the exposure of the AT1R ECL2 domain to the aqueous environment in different receptor states (Unal et al., 2010). In the un-liganded receptor, a segment either side of the conserved disulphide bond was available, suggesting an open conformation. Upon binding to an agonist or inverse agonist, ECL2 acted like a lid albeit in different conformations. ECL2 was involved in slowing down the rate of dissociation of ligands but also adopted different conformations according to the functional state of the receptor. This dynamic effect of ECL2 was also observed with the M2R, where a reduction of loop flexibility caused by the addition of a disulphide bond between ECL2 and TM7 reduced the ligand binding affinity (Avlani et al., 2007).

In the M2 muscarinic receptor, the only ECL2 residue that formed interactions with the bound antagonist was a phenylalanine (F181). This phenylalanine is absent in the equivalent position in the M3 receptor (L225) and creates a larger binding pocket. In molecular dynamic simulations, the M2R binding pocket was found to be more mobile than M3R and the inverse agonist tiotropium dissociated from the M3 receptors more slowly. However reciprocal mutagenesis of these ECL2 residues suggested these residues alone were not sufficient to explain the different off rates (Haga et al., 2012, Kruse et al., 2012).

### *3.1.2 ECL2 is important for ligand binding in the family B GPCRs*

Cross-linking experiments identified contact sites between the CRFR1 and radiolabelled sauvagine (Assil-Kishawi and Abou-Samra, 2002). Disuccinimidyl suberate (DSS) was used to form cross-links between  $\epsilon$  amino groups of lysine residues that have a molecular distance of 11.4 Å. This technique revealed that K257 in the ECL2 domain was in close proximity to K16 of the ligand sauvagine. Cysteine trapping experiments with secretin and its receptor also showed that the N terminus of the ligand was in close proximity to ECL2, with position 2 of the peptide closer to the N-terminal half of ECL2 and the C-terminal half of ECL2 closer to positions 5, 6 and 7 (Dong et al., 2012, Dong et al., 2016).

### *3.3 ECL2 is a proposed site of allosteric modulation*

There is evidence that residues of ECL2 are required for allosteric enhancement of agonist signalling (Kennedy et al., 2014, Peeters et al., 2012) in the family A GPCRs. In the M2R crystal structure with a bound positive allosteric modulator (LY2119620), the allosteric site was situated above the orthosteric binding pocket and involved interactions with the ECLs. ECL2 interactions were hydrogen bonding with E172 and stacking interactions with Y177 (Kruse et al., 2013). Allosteric modulation involving ECL2 has been shown for the M1R through interactions with another tyrosine residue (Y179) and a phenylalanine residue (F182) (Abdul-Ridha et al., 2014). ECL2 in the somatostatin receptors has been used as a successful antibody target to induce an agonist-like response (Leu and Nandi, 2010).

It is likely that ECL2 can facilitate binding to allosteric modulators across all GPCR families. For example, in the family C mGluR1, ECL2 forms the top of a ligand binding pocket to the negative allosteric modulator FITM (Wu et al., 2014) and ECL2 was involved in the binding of negative allosteric modulators in the family C mGluR2 (Lundstrom et al., 2011). It is interesting to speculate that this site is similar to the orthosteric binding pocket in the other

GPCR families and may reflect a loss of direct ECL2/ligand interaction in this specific sub-family.

It is well established that the affinity of a ligand can change based on the activation conformation of the receptor. As different intracellular interactions (such as beta-arrestin or G protein promiscuity) can create “different” conformations, it is not unreasonable to speculate that this will have a subsequent effect on the stability of ECL2 interactions with the ligand and subsequent modulation of signalling. This effect can lead to allostereism or in some cases, biased agonism.

### ***3.4 ECL2 may mediate biased agonism in the two major GPCR families.***

Biased agonism is a phenomenon of growing interest in receptor biology research. Biased agonism means that two ligands of the same receptor activate different signalling pathways or have different preferences for activation of multiple pathways (Shukla et al., 2014). Activation of particular signalling pathways has been linked to the development of certain diseases (Luttrell, 2014). Understanding the physiological effects of biased agonism may provide a platform for the design of drugs that target only the relevant signalling pathway; thereby increasing efficacy and reducing off-target effects.

The involvement of ECL2 in biased agonism has been shown in family B GPCRs using alanine-substitution experiments. Three GLP-1R ligands (GLP-1, exendin-4 and oxyntomodulin) that demonstrated biased agonism at this receptor all showed some requirement for ECL2. GLP-1 and exendin-4 ligands required ECL2 for cAMP and Ca<sup>2+</sup> signalling but not for ERK1/2 signalling. Oxyntomodulin required ECL2 for the coupling of the ligand to the ERK1/2 and cAMP signalling pathways (Ca<sup>2+</sup> was not tested for oxyntomodulin) (Wooten et al., 2016, Koole et al., 2012). Mutagenesis and modelling studies in the family B CGRP receptor family show that the three receptor activity modifying proteins (RAMPs) can induce different ECL2 conformations with distinct binding pockets for CGRP and adrenomedullin, altering their signalling profile (Weston et al., 2016).

ECL2 has also been shown to be involved in biased agonism for family A GPCRs. In the PAR1 receptor, glycosylation of ECL2 stimulated a G<sub>12/13</sub> signalling response compared with a mutant receptor that lacked glycosylation, which produced a G<sub>q</sub> signalling response resulting in different cellular effects (Soto et al., 2015). Mutagenesis of the ECL2 region of the M1 and M2 muscarinic receptors produced receptors that both had biased signalling responses to different ligands. In the M1R, ECL2 was involved in signalling bias between Ca<sup>2+</sup> and ERK1/2 pathways (Keov et al., 2014) and in ERK1/2 signalling in the M2R (Gregory et al., 2010). This shows that there are inter-relationships between biased agonism and allosteric modulation discussed in section 3.3) as some of the muscarinic ligands demonstrating biased agonism were bitopic ligands (having both orthosteric and allosteric binding properties).

### ***3.5 A role for ECL2 on basal signalling***

Mutagenesis of two ECL2 phenylalanine residues in the human histamine 4 receptor (H4R) resulted in reduced constitutive *and* ligand-induced receptor activation showing a direct involvement in receptor activation for this domain (Wifling et al., 2015a, Wifling et al., 2015b). Conversely, constitutive activity resulting from mutagenesis has led to speculation that this domain may constrain the receptor in its inactive state through interactions with the TM domain (Klco et al., 2005). In the melanocortin receptor, ECL2 is much shorter and does

not interact with the TM core, leading to a higher constitutive activity (Holst and Schwartz, 2003).

### **3.6 Summary**

In the largest two families of GPCRs, ECL2 almost always forms part of the ligand-binding pocket. The variation in shape and physicochemical properties of ECL2 gives it a key functional role in ligand selectivity and binding kinetics. The location of ECL2 at the top of the receptor allows it to function as one side of the orthosteric binding pocket (on the underside) but also as a site for allosteric modulation (the top side). ECL2 is also at the interface of ligand binding and receptor activation, allowing it to control aspects of signalling, such as biased signalling and constitutive activity.

## **4 Discussion**

ECL2 is arguably the most structurally diverse and functionally important ECL of the GPCR super-family. This has been shown in the solved crystal structures and also through biochemical analysis of the ECLs and is a common phenomenon across all the GPCR sub-families. ECL2 has a more complex structure than the other ECLs broadly forming a surface of the ligand-binding pocket that can act as a “lid” in either an open or closed position. The conformation and physicochemical properties of the ECL2 domain can influence the size and shape of the ligand-binding pocket and determine ligand selectivity. ECL2 can form direct interactions with agonists, antagonists, inverse agonists and allosteric modulators and its conformation can change upon binding to these ligands. ECL2 can also have a substantial impact on ligand on/off rates. ECL2 has roles in the super-family in ligand binding, allosteric modulation, conformational activation or biased signalling. Some of these functions can be retained even if ECL2 has lost direct agonist contact (such as allostereism in the family C receptors).

ECL2 often functions as the “lid” of the “box” that is the ligand-binding pocket. As such it can often be thought of as one of the sides of the ligand-binding pocket. This ECL2 lid can be open or closed and can control the entry and exit of ligands to their receptors and the kinetics of this process. This is an exciting and important target for rational drug design.

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### *Conflict of interest*

Authors declare they have no conflict of interest.

### *Authorship contribution*

MW managed the literature searches, and wrote the first draft of the manuscript. AC organized the first draft and prepared the final version of the manuscript. All the authors contributed to and have approved the final manuscript.



**Figure legends**

Figure 1. Crystal structures of the extracellular capped, opioid and chemokine GPCRs. The crystal structures of bovine rhodopsin (PDB 1F88), S1P1R (PDB 3V2Y),  $\mu$ OR (PDB 4DKL) and CXCR1 (PDB 2LNL) are presented. Each row shows the receptor in two orientations. These are planar to the membrane (left box) and perpendicular to the membrane from the extracellular side (right box). ECL2 is highlighted red and the remainder of the receptor light blue.

Figure 2. Crystal structures of the aminergic and family B GPCRs. The crystal structures of  $\beta$ 2AR (PDB 2RH1), M2R (PDB 3UON), D3R (PDB 3PBL) and CRFR1 (PDB 4K5Y) are presented. Each row shows the receptor in two orientations. These are planar to the membrane (left box) and perpendicular to the membrane from the extracellular side (right box). ECL2 is highlighted red and the remainder of the receptor light blue.

Figure 1.

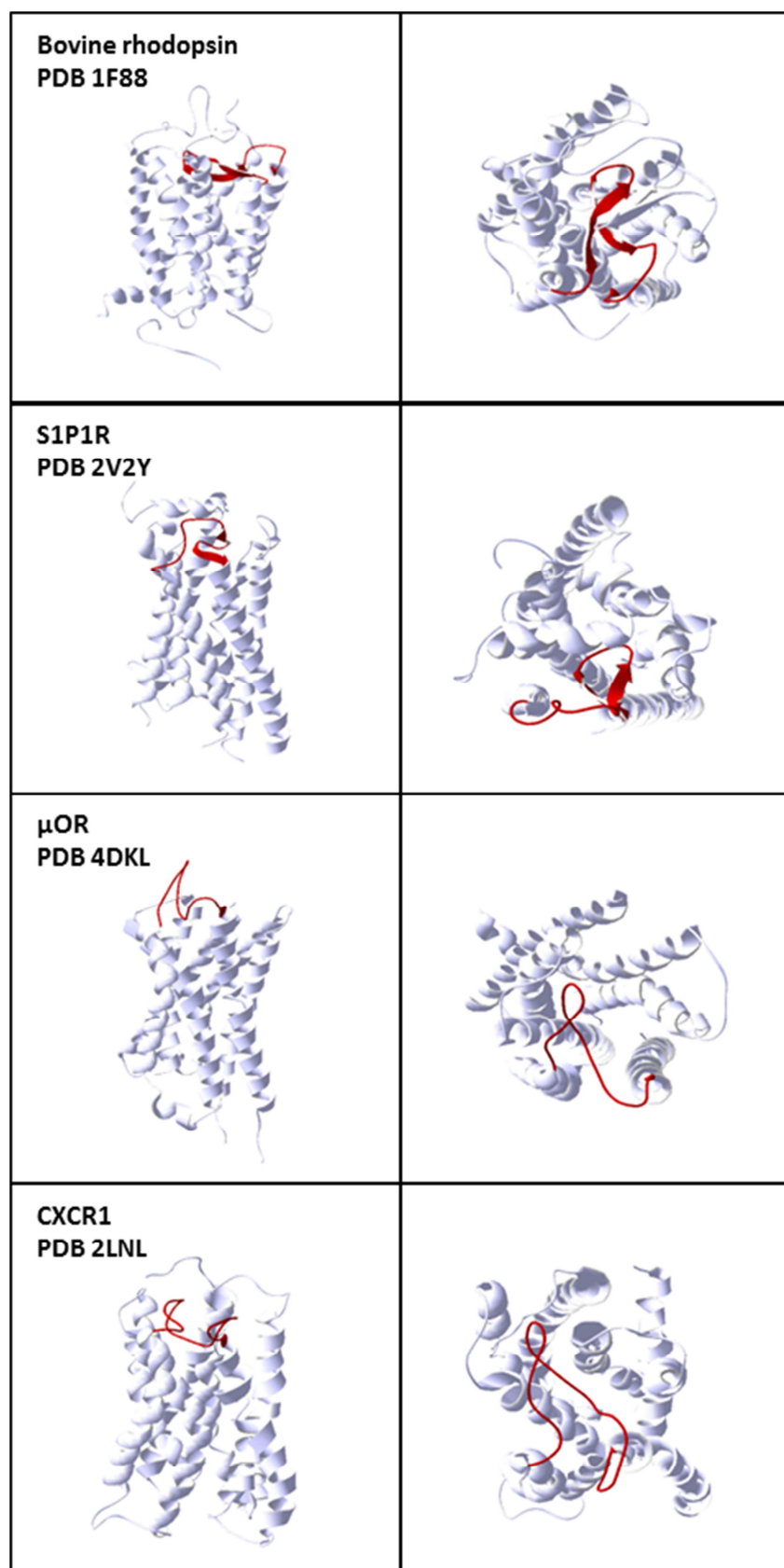




Figure 2.

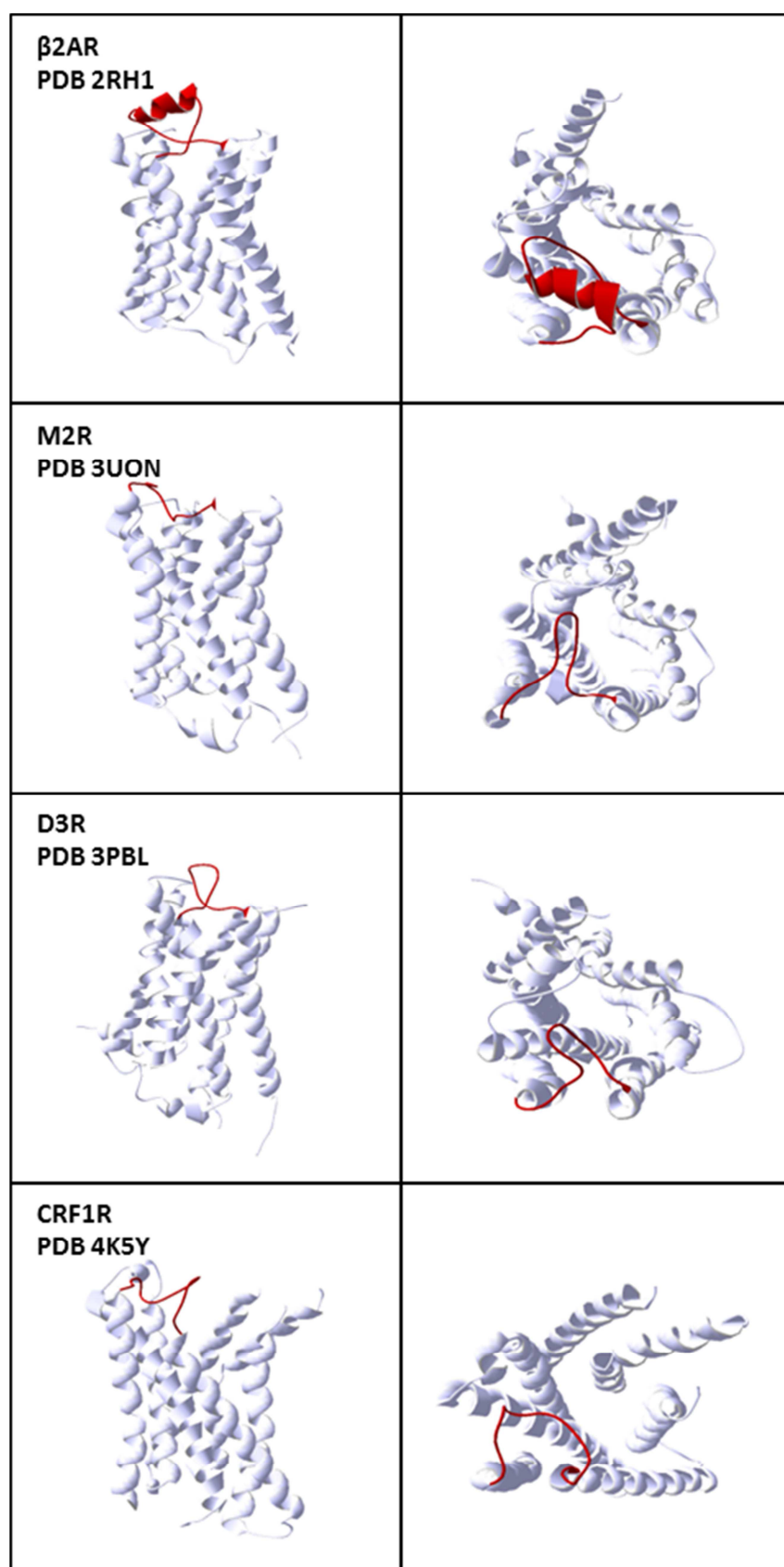


Table 1.

| GPCR              | PDB  | group                      | feature                                |
|-------------------|------|----------------------------|--|
| bovine rhodopsin  | 1F88 | covalently attached ligand | $\beta$ hairpin                        |
| S1PR              | 3V2Y | lipid receptor             | loop and $\beta$ sheet                 |
| GPR40             | 4PHU | lipid receptor             | loop and $\beta$ sheet                 |
| LPA1              | 4Z34 | lipid receptor             | loop, no secondary structure           |
| $\beta$ 1AR       | 2VT4 | Aminergic                  | $\alpha$ -helix                        |
| $\beta$ 2AR       | 2RH1 | Aminergic                  | $\alpha$ -helix                        |
| M1R               | 5CXV | Aminergic                  | (hairpin) loop, no secondary structure |
| M2R               | 3UON | Aminergic                  | (hairpin) loop, no secondary structure |
| M3R               | 4DAJ | Aminergic                  | (hairpin) loop, no secondary structure |
| M4R               | 5DSG | Aminergic                  | (hairpin) loop, no secondary structure |
| D3R               | 3PBL | Aminergic                  | loop, no secondary structure           |
| H1R               | 3RZE | Aminergic                  | loop, no secondary structure           |
| 5-HT1B            | 4IAR | Monoamine                  | loop, no secondary structure           |
| 5-HT2B            | 4IBR | Monoamine                  | loop, no secondary structure           |
| A2AR              | 2YDO | purine nucleoside          | $\alpha$ -helix and $\beta$ sheet      |
| P2Y1              | 4XNV | purinergic                 | $\beta$ hairpin                        |
| $\delta$ OR       | 4EJ4 | opioid (peptide)           | $\beta$ hairpin                        |
| $\kappa$ OR       | 4DJH | opioid (peptide)           | $\beta$ hairpin                        |
| $\mu$ OR          | 4DKL | opioid (peptide)           | $\beta$ hairpin                        |
| NORQ              | 4EA3 | opioid (peptide)           | $\beta$ hairpin                        |
| ET <sub>B</sub> R | 5GLH | peptide ligand             | $\beta$ hairpin                        |
| OX2               | 4S0V | peptide ligand             | $\beta$ hairpin                        |
| angiotensin       | 4YAY | peptide ligand             | $\beta$ hairpin                        |
| PAR1              | 3VW7 | cleaved peptide            | $\beta$ hairpin                        |
| CCR5              | 4MBS | chemokine (peptide)        | $\beta$ hairpin                        |
| CXCR1             | 2LNL | chemokine (peptide)        | $\beta$ hairpin                        |
| CXCR4             | 3ODU | chemokine (peptide)        | $\beta$ hairpin                        |
| CRFR1             | 4K5Y | family B                   | loop, no secondary structure           |
| GCCR              | 4L6R | family B                   | loop, no secondary structure           |
| mGLUR1            | 4OR2 | family C                   | $\beta$ hairpin                        |
| mGLUR5            | 4O09 | family C                   | $\beta$ hairpin                        |

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## Highlights

### **Understanding the common themes and diverse roles of the second extracellular loop (ECL2) of the GPCR super-family.**

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- Extracellular loop 2 (ECL2) is a key GPCR domain.
- ECL2 is usually a  $\beta$ -hairpin but can form other structures.
- ECL2 can be directly involved with the ligand-receptor functions.
- ECL2 is also involved in as biased agonism and allosteric modulation.
- These properties of ECL2 make it an important site for rational drug design.