Molecular Pharmacology Fast Forward. Published on July 10, 2019 as DOI: 10.1124/mol.119.115915
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Understanding peptide binding in Class A G protein-coupled receptors

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Keywords: peptides, peptide GPCRs, peptide binding

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Running title page: Peptide Class A GPCRs

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Number of text pages: 10

Number of figures: 3

Number of references: 118

Number of tables: 2

Words in Abstract: 163

Words in Introduction: 503

Words in Concluding Remarks: 661

ABBREVIATIONS:

AT_1, AT_2, angiotensin receptors; APLNR, apelin receptor; C3a, $C5a_1$, $C5a_2$, complement peptide receptors; ET_A, ET_B, endothelin receptors; NTS1, NTS2, neurotensin receptors; RXFP1, RXFP2, RXFP3, RXFP4, relaxin receptors; B_1, B_2, bradykinin receptors; NPY_1, NPY_2, NPY_4, NPY_5, neuropeptide Y receptors; OX_1, OX_2, orexin receptors; V1A, V1B, V2, vasopressin receptors; OT, oxytocin receptor; CCK1, CCK2, cholecystokinin receptors; QRFP, pyroglutamylated RFamide peptide receptor; PrRP, prolactin-releasing peptide receptor; NPFF1, NPFF2, neuropeptide FF receptors; KISS1R, kisspeptin receptor; BB_1, BB_2, BB_3, bombesin receptors; TRH1, thyrotropin-releasing hormone receptor; NMU1, NMU2, neuromedin-U receptors; NK_1, NK_2, NK_3, neurokinin/tachykinin receptors; DOR, KOR, MOR, NOP, opioid receptors; NPBW1, NPBW2, neuropeptide B/W receptors; FPR1, FPR2, FPR3, N-formyl peptide receptors; MLNR, motilin receptor; GHSR, ghrelin receptor; MC1, MC2, MC3, MC4, MC5, melanocyte-stimulating hormone receptors; GNRHR, gonadotropin-releasing hormone receptor; GAL1, GAL2, GAL3, galanin receptors; NPSR1, Neuropeptide S receptor; UT, urotensin receptor; MCH1, MCH2, melanin-concentrating hormone receptors; SST1, SST2, SST3, SST4, SST5, somatostatin receptors; PAR1, PAR2, PAR3, PAR4, PAR5, proteinase-activated receptors; DIPP-NH2, H-Dmt-Tic-Phe-Phe-NH2; DAMGO, (H-Tyr-D-Ala-Gly-N(Me)Phe-Gly-OH).

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Abstract

Many physiological processes are controlled through the activation of G protein-coupled receptors (GPCRs) by regulatory peptides, making peptide GPCRs particularly useful targets for major human diseases such as diabetes and cancer. Peptide GPCRs are also being evaluated as next-generation targets for the development of novel anti-parasite agents and insecticides in veterinary medicine and agriculture. Resolution of crystal structures for several peptide GPCRs has advanced our understanding of peptide-receptor interactions and fuelled interest in correlating peptide heterogeneity with receptor binding properties. In this review, the knowledge of recently crystalized peptide-GPCR complexes, previously accumulated peptide structure-activity relationship (SAR) studies, receptor mutagenesis and sequence alignment are integrated to better understand peptide binding to the transmembrane cavity of Class A GPCRs. Using SAR data, we show that peptide Class A GPCRs can be divided into groups with distinct hydrophilic residues. These characteristic residues help explain the preference of a receptor to bind the C-terminal free carboxyl group, the C-terminal amidated group, or the N-terminal ammonium group of peptides.

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Introduction

Over fifty peptides released by endocrine and neuronal tissues play a role of circulating hormones, neurotransmitters, local regulators, or all at once, controlling human development, reproduction, physiology and behaviour. This regulatory peptide system is a major target of therapeutic intervention in the control of body function. Targeting the regulatory peptide system has been critical in oncology, endocrinology, neuroscience and many other areas (Lau and Dunn, 2018).

Biologically active peptides are produced from large precursor molecules via proteolytic cleavage and other post-translational modifications such as amidation, acetylation, cyclization, sulfation, glycosylation and phosphorylation. Although some peptides are characterized by a common motif (e.g. RFamide peptides are characterized by a Arg-Phe-NH2 sequence at their C-terminus), overall they have great diversity in size, chemistry and structure.

Most regulatory peptides elicit their biological responses by binding to over one hundred G protein-coupled receptors (GPCRs), also known as seven transmembrane-spanning receptors. Among the six known classes of GPCRs, receptors from Classes A and B bind regulatory peptides. In particular, a large number of regulatory peptides bind to Class A GPCRs.

A 'message-address' concept of peptide binding was introduced by Schwyzer in the 1970s following studies on adrenocorticotropin peptide truncation (Schwyzer, 1977). Thus, the adrenocorticotropin N-terminus – which is important for receptor activation – acts as a 'message', while the peptide C-terminus enhancing the activity represents an 'address'. This concept has been further explored in structure-activity relationship (SAR) studies of opioid peptide dynorphin (Chavkin and Goldstein, 1981) and design of opioid agonists and antagonists (Portoghese, et al., 1988; Portoghese, et al., 1993). From these studies, it was shown that there are separate recognition sites for the address and message moieties of a peptide in the receptor.

In recent years, remarkable progress had been made in the structural biology of GPCRs, mostly due to the determination of crystallographic structures of several family members, including peptide GPCRs. Currently, twelve peptide Class A and four peptide Class B GPCRs have been crystallized in complex with either peptides or non-peptide ligands. A comprehensive overview of the crystal structures of peptide GPCRs with regard to peptide and non-peptide ligand binding has been recently reviewed by Wu *et al.* (Wu, F., et al., 2017). The newly available structural information, in combination with peptide structure-activity studies accumulated over years, not only describes atomic details of peptide-receptor interactions for the crystalized GPCRs, but also help in understanding how these structurally

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diverse molecules bind within the peptide GPCR subfamily. In this review, using available crystal structures of peptide GPCRs in conjunction with peptide SAR studies, receptor mutagenesis and sequence analysis, we analyse binding of the C-terminus or N-terminus of regulatory peptides to the transmembrane cavity of the Class A GPCRs. We show that peptide SAR data has effectively subdivided the Class A GPCR binding site residues into different groups, which have distinct hydrophilic residues. These characteristic hydrophilic residues are linked to the preference of the receptor to bind the C-terminal free carboxyl group, the C-terminal amidated group, or the N-terminal ammonium group of peptides.

1. X-ray structures of GPCRs bound to peptides

The crystal structures of the following Class A peptide-GPCRs complexed with peptide agonists or antagonists are currently available – apelin (APLNR), angiotensin (AT_1), neurotensin (NTS_1), endothelin (ET_B), opioid (MOR and DOR), chemokine (US28 and CXCR4) and component peptide ($C5a_1$) receptors (Ma, et al., 2017; Asada, et al., 2018; White, et al., 2012; Shihoya, et al., 2016; Koehl, et al., 2018; Fenalti, et al., 2015; Qin, et al., 2015; Wu, B., et al., 2010; Burg, et al., 2015; Liu, et al., 2018). Furthermore, subtypes of the protease-activated (PAR1 and PAR2), chemokine (CXCR4, CCR2, CCR5 and CCR9), opioid (MOR, KOR, DOR and NOP), orexin (OX_1 and OX_2), angiotensin (AT_1 and AT_2), neuropeptide (NPY1), C5a and neurokinin (NK_1) receptors are crystallized in complex with non-peptide orthosteric and/or allosteric antagonists (Wu, F., et al., 2017; Yang, Z., et al., 2018; Liu, et al., 2018; Robertson, et al., 2018; Yin, et al., 2018; Schoppe, et al., 2019).

As expected, peptides tend to bind to GPCRs in many different conformations. However, in general, one part of the peptide is buried in the helical cavity, while another part forms interactions with the extracellular loops and the N-terminus of GPCRs. Peptides, including apelin, angiotensin, endothelin, neurotensin and cyclic PMX53, point the C-terminus inside the helical bundle, while peptides binding to the opioid and chemokine receptors (Figure 1) bind in the reverse orientation with the N-terminus pointing to the receptor helical side.

Polar interactions are the primary driving force for peptide recognition and binding. To identify the polar interactions, hydrogen atoms were added and optimized in the crystal structures; and hydrogen bonds and salt bridges between the peptide and the receptor were then calculated using the Maestro software (Schrodinger, LLC, New York, NY, USA, 2014) with default setting (Table 1). Given that the extracellular loops and the N-terminus vary in GPCRs while the helical bundle is conserved, we analysed interactions of the peptides with the helical bundle to compare the binding of the peptides. The peptides form multiple hydrogen bonds and salt bridges with the residues of the transmembrane helices via side chains, backbone and terminal groups (Table 1). Interestingly, the terminal charged group of the peptides

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located within the helical bundle is engaged in several polar interactions (Figure 1). Thus, the C-terminal carboxyl group of angiotensin, apelin, endothelin and neurotensin forms salt bridges and hydrogen bonds with positively charged lysine and arginine residues. The N-terminal ammonium group of the peptides in the opioid and chemokine receptors is engaged in an ionic interaction with an aspartate residue (Figure 1).

Table 1. Hydrogen bond (H) and salt bridge (SB) interactions between a peptide and the helical cavity from the available crystal structures of Class A GPCRs bound to a peptide.

Peptide	Receptor	Overall number of contacts	Number of contacts with peptide side chains	Number of contacts with peptide backbone	Number of contacts with the peptide terminal group	Number of peptide residues within the cavity	PDB code
Apelin	APLNR	8	2H+1SB*	2H	2H+1SB	6	5VBL
Neurotensin	NTS_1	6	1H	1H	3H+1SB	4	4GRV
Endothelin	ET_B	16	4H+1SB	6H	3H+2SB	16	5GLH
[Sar1, Ile8]AngII	AT_2	12	3H+1SB	6H	1H+1SB	6	5XJM
DIPP-NH2	DOR	4	1H		2H+1SB	3	4RWD
DAMGO	MOR	4	1H		2H+1SB	5	6DDF
CX3CL1	US28	5	1H	3H	1H	7	4XT3
CX3CL1/Nb7	US28	7	2H	4H	1H	11	4XT1
vMIP-II	CXCR4	13	6H+2SB	2H	2H+1SB	9	4RWS
CVX15	CXCR4	10	6H+3SB		1H	6	3OE0
cyclicPMX53	$C5a_1$	12	6H+1SB	5H		3	6C1R

*Hydrogen bond criteria: 3Å for the maximum distance between donor and acceptor atoms, 90° and 60° for donor and acceptor minimum angles, respectively. Salt bridge criteria: the maximum distance between atoms is 5Å.

Earlier SAR studies utilizing fragments and amidated or esterified analogues of angiotensin, endothelin and neurotensin have shown that the C-terminus together with the free carboxyl group is required for the activity of these peptides (Rioux, et al., 1975; Rovero, et al., 1990; Labbe-Jullie, et al., 1998). Indeed, the ionic interaction patterns observed in the crystal structures confirm the SAR studies.

The correlation between direct and indirect structural information of peptide binding suggests that SAR data of peptide terminal ends could be further linked with available structural information to gain insight into interactions of peptides with not yet crystalized GPCRs. A question then arises as to whether the large heterogeneity of peptide ligands and ability to activate different GPCRs can be linked or somewhat classified using binding characteristics of the peptide C- or N- terminal groups interacting with the transmembrane helices. In an attempt to address this question, we propose to integrate the knowledge of binding site residues gained from the crystal structures with peptide SAR studies and sequence analysis of the Class A GPCRs.

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2. Class A Receptor Grouping based on Peptide SAR and Hydrophilic Binding Site Residues

We collected SAR data from the literature for 32 peptides binding to Class A GPCRs to compare peptide binding properties. Table 2 shows a sequence of short active peptides, derived from peptide truncation studies and peptide end-terminal functional groups important for the activity, obtained from analogue studies. The peptides are grouped based on the importance of the peptide terminal end to the activity. The C-terminus of the peptide contains a free carboxyl or amide group. For the first six peptides in Table 2, the free carboxyl group is important for peptide activity, while for a large number of the peptides (17 in the table) amidation of the carboxyl group is favourable for activity. Amidation is the most common post-translational modification of peptides. For many peptides, this modification not only improves peptide stability and delivery but is also required for activity, as determined in the SAR studies (Table 2). The table also includes a number of regulatory peptides for which the N-terminus or the cyclic portion of the peptide structure is important for activity. Furthermore, we review available receptor mutagenesis and receptor-peptide modelling studies and show the peptide terminal end that is predicted to bind inside the helical bundle in Table 2. From available literature data, it appears that the terminal end of a peptide, which is important for the activity ('message' moiety) is pointed to the helical bundle.

Initially, 35 residues lining the helical cavity of the peptide Class A GPCRs were selected using the 'ligand binding pocket for Class A' option in the GPCRDB server (ww.gpcrdb.org) to relate the binding properties of the peptide GPCRs with the collected peptide SAR data. In addition to the human receptors, several other orthologs (bovine, chimpanzee, guinea pig, mouse, rat and rabbit) were considered in the amino acid residue alignment. The Ballesteros-Weinstein index (Ballesteros and Weinstein, 1995) was used for the residue selection and comparison among GPCRs. Next, taking into consideration the polar interactions in the crystalized peptide-GPCR complexes the number of residues for the analysis was reduced to 25, where only residue positions with a high occurrence of hydrophilic residues were considered. The alignment of 25 binding site residues was split into five groups based on the binding of the peptide terminal end that is important for activity (Figure 2). Figure 2 shows the alignment of binding site residues for the human receptors and the conservation score for hydrophilic residues in each residue position from the analysis of receptor orthologs. In the next sections, we will assess each of the receptor groups and highlight patterns of residues anchoring the 'message' peptide end.

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Table 2. Peptides binding to Class A GPCRs with key regions important for activity.

Peptide	Receptor	Sequence of short active peptides (Peptide region important for activity is highlighted in grey, cysteine involved in the disulphide bridges are in bold.)	End group important for activity	Peptide terminal end inside the helical bundle	Reference
Angiotensin	AT_1, AT_2	H2N-DRVYIHPF-COOH	-COOH	C-end	(Karnik, 2000; Rioux, et al., 1975; Asada, et al., 2018)
Apelin	APLNR	H2N-LVQPRGPRSGPGPWQGGRRKFRRQRPRLSHKGPMPF-COOH	-COOH	C-end	(Murza, et al., 2012; Ma, et al., 2017)
Bradykinin	B_1, B_2	H2N-RPPGFSPFR-COOH	-COOH	C-end	(Rhaleb, et al., 1990; Ha, et al., 2006; Jarnagin, et al., 1996)
Complement C	C3a, $C5a_1$,$C5a_2$	H2N-TLQKKIEEIAAKYKHSVVKK**CC**YDGA**C**VNNDET**C**EQRAARIS-LGPRCIKAFTE**CC**VVASQLRANISHKDMQLGR-COOH	-COOH	C-end	(Mollison, et al., 1989; Higginbottom, et al., 2005; Liu, et al., 2018)
Endothelin	ET_A ET_B	H2N-**C**S**C**SSLMDKE**C**VYF**C**HLDIIW-COOH	-COOH	C-end	(Rovero, et al., 1990; Shihoya, et al., 2016)
Neurotensin	NTS_1, NTS_2	H2N-LYENKPRRPYIL-COOH	-COOH	C-end	(Labbe-Jullie, et al., 1998; White, et al., 2012; Kleczkowska and Lipkowski, 2013)
Relaxin	RXFP1-4	Chain A: H2N-RPYVALFEK**CC**LIG**C**TKRSLAKY**C** Chain B: KWKDDVIKL**C**GRELVRAQIAI**C**GMSTWS-COOH		C-end of Chain B	(Hu, M. J., et al., 2017; Patil, et al., 2017; Bathgate, et al., 2013; Wong, et al., 2018)
Cholecystokinin	CCK1	H2N-RDY-SO3H-TGWLDF-CONH2	-CONH2	C-end	(Morley, et al., 1965; Jensen, et al., 1982; Black and Kalindjian, 2002; Archer-Lahlou, et al., 2005; Dufresne, et al., 2006)
Gastrin	CCK2	H2N-LEEEEEAYGWMDF-NH2			
Orexin	OX_1, OX_2	Orexin A: (pyroglutamoyl)Pyr-PLPD**CC**RQKT**C**S**C**RLYELLHGAGNHAAGILTL-CONH2 Orexin B: H2N-RSGPPGLQGRLQRLLQASGNHAAGILTM-CONH2	-CONH2	C-end	(Darker, et al., 2001; Ammoun, et al., 2003;

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Neuropeptide Y	NPY1-6	H2N-YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-CONH2	-CONH2	C-end	Lang, M., et al., 2004; Heifetz, et al., 2013) (Boublik, et al., 1989; Xu, B., et al., 2013; Xu, B., et al., 2018; Yang, Z., et al., 2018)
Prolactin-releasing peptide	PrRP	H2N-SRTHRHSMEIRTPDINPAWYASRGIRPVGRF-CONH2	-CONH2	C-end	(Boyle, et al., 2005; Findeisen, et al., 2011; Rathmann, et al., 2012)
Pyroglutamylated RFamide peptide	QRFP	H2N-TSGPLGNLAEELNGYSRKKGGFSFRF-CONH2	-CONH2	C-end	(Findeisen, et al., 2011)
Neuropeptide FF	NPFF1-2	H2N-SQAFLFQPQRF-CONH2	-CONH2	C-end	(Findeisen, et al., 2011)
Metastin/kisspeptin	KISS1R	H2N-YNWNSFGLRF-CONH2	-CONH2	C-end	(Kotani, et al., 2001; Findeisen, et al., 2011)
Vasopressin	V1A, V1B, V2	H2N-**C**YFQN**C**PRG-CONH2	-CONH2	Cyclic end	(Jard and Bockaert, 1975; Chini and Fanelli, 2000; Mouillac, et al., 1995)
Oxytocin	OT	H2N-**C**YIQN**C**PLG-CONH2	-CONH2	Cyclic end	(Jard and Bockaert, 1975; Chini and Fanelli, 2000; Gimpl and Fahrenholz, 2001)
Bombesin Gastrin-releasing peptide Neuromedin B	BB_1 BB_2 BB_3	pEQRLGNQWAVGHLM-CONH2 MYPRGNHWAVGHLM-CONH2 GNLWATGHFM-CONH2	-CONH2	C-end	(Mervic, et al., 1991; Lin, J. T., et al., 1995; Jensen, et al., 2008)
Neuromedin-U	NMU1-2	H2N-FRVDEEFQSPFASQSRGYFLFRPRN-CONH2	-CONH2	C-end	(Brighton, et al., 2004; Kawai, et al., 2014)
Substance-P /K Neurokinin, Tachykinin	NK_1, NK_2, NK_3	H2N-RPKPQQFFGLM-CONH2	-CONH2	C-end	(Couture, et al., 1979; Ganjiwale, et al., 2011)
Thyrotropin-releasing hormone	TRH1,	pEHP-CONH2	-CONH2	all	(Chang, et al., 1971; Engel and Gershengorn, 2007)

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Opioid peptides endorphines	DOR, KOR, MOR, NOP	H2N-YGGFL-COOH H2N-GGFM-COOH	H2N-	N-end	(Morley, 1983; Koehl, et al., 2018)
Neuropeptides B/W	NPBW1-2	H2N-WYKPAAGHSSYSVGRAAGLLSGL-COOH H2N-WYKHVASPRYHTVGRAAGLLMGL-COOH		N-end	(Kanesaka, et al., 2007)
Neuropeptide S	NPSR1	H2N-SFRNGVGTGMKKTSFQRAKS-COOH		N-end	(Roth, et al., 2006)
N-formyl peptide	FPR1-3	O=HC-NH-MLFK-COOH	O=HC-NH-	N-end	(Prossnitz and Ye, 1997)
Melanocyte-stimulating hormone /Melanocortins Adrenocorticotropic hormone	MC1-5	O=HC-NH-SYSMEHFRWGKPV-CONH2	O=HC-NH-	FRW	(Schioth, et al., 1997; Audinot, et al., 2001; Matsunaga, et al., 1989; Schwyzer, 1977; Haskell-Luevano, et al., 2001)
Motilin	MLNR	H2N-FVPIFTYGELQRMQEKERNKGQ-COOH		N-end	(Poitras, et al., 1992; Xu, L., et al., 2005)
Ghrelin	GHSR	H2N-GSS(octanoyl)-FLSPEHQRVQQRKESKKPPAKLQPR-COOH		N-end	(Charron, et al., 2017)
Gonadotropin-releasing hormone	GNRHR	CONHCHO-EHWSYGLRPG-CONH2	CONHCHO-	N-end	(Hoffmann, et al., 2000; Padula, 2005; Barran, et al., 2005)
Galanin	GAL1-3	H2N-GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS-COOH	H2N-	N-end	(Church, et al., 2002; Lang, R., et al., 2015)
Urotensin	UT	H2N-AGTAD**C**FWKY**C**V-COOH		Cyclic portion	(Labarrere, et al., 2003; Merlino, et al., 2013)
Melanin-concentrating hormone	MCH1-2	H2N-DFDMLR**C**MLGRVYRP**C**WQV-COOH		Cyclic portion	(Schioth, et al., 1997; Matsunaga, et al., 1989; Audinot, et al., 2001)
Somatostatin	SST1-5	H2N-AG**C**KNFFWKTFTS**C**-COOH		Cyclic portion	(Vale, et al., 1978; Martin-Gago, et al., 2013)
Fragments of a tethered ligand	PAR1-4	H2N-SFLLRN-COOH H2N-TFLLRN-COOH			(Gerszten, et al., 1994)

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3. GPCRs binding peptides with the C-terminal free carboxyl group interacting with the helical cavity.

The receptors binding angiotensin, apelin, complement C fragment, endothelin, neurotensin, bradykinin and relaxin peptides are grouped together (Figure 2A). From the alignment of the peptide binding site residues, we highlight positively charged residues at positions 4.64 (or placed within the EL2), 5.42 and 6.55 with a degree of conservation (67%, 46% and 41%, respectively).

AT_2, APLNR, ET_B, NTS_1 and $C5a_1$ have been crystallized in complex with a peptide, providing direct information about peptide-receptor interactions (Asada, et al., 2018; Ma, et al., 2017; Shihoya, et al., 2016; White, et al., 2012; Liu, et al., 2018). Arginine at position 4.64 forms polar interactions with the backbone of angiotensin, apelin and PMX53 in AT_2, APLNR and $C5a_1$, respectively (Figure 1). Lysine at position 5.42 forms a salt bridge with the free carboxyl group of angiotensin in AT_2. Arginine at this position in $C5a_1$ is 4.3 Å away from the backbone of PMX53. Lysine or arginine at position 6.55 forms a salt bridge with the C-terminal carboxyl group of apelin and endothelin in APLNR and ET_B, respectively (Figure 1). In the case of NTS_1, residue 6.55 is at a distance of 3.8 Å from the C-terminal carboxyl group of neurotensin and, instead, arginine 6.54 forms a salt bridge with the terminal group of the peptide. Mutation of the charged residues in these three conserved positions reduces the peptide activity at AT_2, APLNR, ET_B, NTS_1 and $C5a_1$ (Asada, et al., 2018; Ma, et al., 2017; Shihoya, et al., 2016; Higginbottom, et al., 2005; Labbe-Jullie, et al., 1998).

The two relaxin receptor subtypes, namely RXFP3 and RXFP4, have a positively charged residue in position 5.42, whereas the other two subtypes, RFXP1 and RFXP2, in position 6.55. Mutation of the positively charged residue in these positions in RXFP3 and RXFP1 decreases relaxin activity (Hu, X., et al., 2016; Wong, et al., 2018). Unlike other peptides of this group, relaxin is a cyclic peptide composed of two chains A and B (Table 2). The C-terminus of chain B carrying the peptide activity may interact with the positively charged residue of the relaxin receptors. In the bradykinin receptors (B_1 and B_2), arginine at position 4.64 is conserved, however, no information on its importance in peptide binding is available in the literature.

4. GPCRs binding peptides with the C-terminal amidated carboxyl group interacting with the helical cavity.

Amidation of the C-terminal carboxyl group in a large number of regulatory peptides is important for biological activity (Eipper, et al., 1992). This group of peptides includes cholecystokinin, gastrin, orexin, neuropeptide Y, prolactin-releasing peptide, pyroglutamylated RFamide peptide, neuropeptide FF, kisspeptin, vasopressin, oxytocin, bombesin, gastrin-releasing peptide, neuromedin B, neuromedin U, substance P, neurokinin and thyrotropin-releasing hormone. The receptors binding these peptides are grouped together and the alignment of binding site residues highlights three notably conserved asparagine or glutamine at positions 3.32, 4.60 and 6.55, with the conservation score of 66%, 44% and 52%, respectively (Figure 2B).

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Currently, there is no crystal structure of a GPCR bound to an amidated peptide demonstrating direct interactions between the peptide C-terminal amidated group and the receptor. However, the crystal structures of the neuropeptide Y1 (NPY_1), orexin (OX_1 and OX_2) and neurokinin (NK_1) receptors are available in complex with orthosteric non-peptide ligands (Figure 3), guiding thoughts on hydrophilic interactions between a peptide and the receptor helical bundle.

The NPY_1 crystal structure is obtained in complex with two synthetic antagonists, BMS-19885 and UR-MK299 (Yang, Z., et al., 2018). The carbonyl of BMS-19885 ester group forms H-bonding with glutamine and asparagine at positions 3.32 and 6.55 (Figure 3). The peptide bond-like moiety of UR-MK299 is engaged in two acceptor- and donor- hydrogen bonds with residue 6.55 (Figure 3). In mutagenesis studies, residues at positions 3.32 and 6.55 are predicted to be in contact with the peptide C-terminus and important for receptor activation (Sautel, et al., 1996; Kaiser, et al., 2015). Structurally, UR-MK299 imitates the C-terminal Arg and Tyr residues of the natural peptide and its binding helps to model the binding mode of the peptide in NPY_1 (Yang, Z., et al., 2018). Furthermore, in a very recent study, employing mutagenesis and synthesis of peptide analogues with the modified C-terminal amide group, it has been suggested that the C-terminal amide group forms interactions with asparagine 6.55 (Xu, B., et al., 2018).

The crystal structures of the orexin receptors are also available in complex with three synthetic antagonists, Suvorexant, SB-674042 and EMPA (Yin, et al., 2015; Yin, et al., 2016; Suno, et al., 2018). The tertiary amide carbonyl group of Suvorexant and SB-674042 forms a hydrogen bond with asparagine at position 6.55 in OX_1 and OX_2 (Figure 4, shown for Suvorexant only). This functional group of the antagonists mimics the peptide amide group, suggesting also a potential interaction with this residue either through the backbone or the amidated peptide C-terminus. Suvorexant and SB-674042 are in very close proximity to glutamine at position 3.32 (3.3 Å), whereas EMPA forms a water-mediated contact with this residue (Suno, et al., 2018) (Figure 3). Although the role of asparagine 6.55 has not yet been validated in mutagenesis, mutation of glutamine at position 3.32 reduces the functional activity of the orexin peptides (Malherbe, et al., 2010).

Residues 3.32 and 6.55 are also important in vasopressin binding to the vasopressin (V_{1A}) receptor (Mouillac, et al., 1995; Chini and Fanelli, 2000). In the case of the cholecystokinin receptors (CCK1 and CCK2), receptor mutagenesis and structure-affinity studies of modified CCK analogues suggest that asparagine 6.55 forms hydrogen bonds with the amidated carboxyl group of the peptide C-terminus (Figure 3) (Gigoux, et al., 1999; Gales, et al., 2003; Langer, et al., 2005).

The receptors binding the RF-amide peptides, including neuropeptide Y, prolactin-releasing peptide, pyroglutamylated peptide, neuropeptide FF and kisspeptin have conserved glutamine 3.32. However, apart from the NYP receptors, the role of this residue in the other RF-amide receptors has not been established yet by mutagenesis. Another conserved residue in the RF-amide receptors is aspartate 6.59, mutation of this residue significantly attenuates peptide activity (Findeisen, et al., 2011). From functional and modelling studies, it is suggested that aspartate 6.59 interacts with the arginine of the peptide RF-amide motif (Rathmann, et al., 2012).

Mutagenesis studies of the bombesin receptors (BB_2 and BB_3) indicate the importance of the asparagine or arginine 3.32 for bombesin binding (Akeson, et al., 1997; Nakamura, et al., 2016). Mutation of tyrosine at

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position 6.55 in BB_2 is critical for the activity of the peptide (Lin, Y., et al., 2000). Within the bombesin family, other three polar residues at positions 2.61, 6.51, and 7.39 are conserved and critical for peptide binding (Donohue, et al., 1999; Lin, Y., et al., 2000).

Glutamine 3.32 has not been mutated yet in the thyrotropin-releasing hormone receptor (TRH1). Mutations of other polar residues at positions 3.37, 6.52 and 7.39 significantly reduce the binding of thyrotropin-releasing hormone, suggesting direct interactions with the backbone of the peptide (Engel and Gershengorn, 2007).

In the neuromedin U receptors (NMU1 and NMU2), mutation of arginine 6.55 reduces peptide binding (Kawai, et al., 2014). In addition, the importance of glutamates at positions 2.61 and 3.33 for the interaction with the peptide C-terminus has been shown in mutagenesis (Kawai, et al., 2014).

The neurokinin receptors (NK_1, NK_2 and NK_3) do not have polar residues at positions 3.32 and 6.55. However, the amide-containing residue at position 4.60, which is conserved within the subfamily forms a hydrogen bond with the antagonists aprepitant (Figure 2), CP-99,994 and netupitant in the NK_1 crystal structure complexes (Yin, et al., 2018; Schoppe, et al., 2019) and is predicted to bind to the neurokinin C-terminus according to mutagenesis and modelling studies (Lundstrom, et al., 1997).

5. GPCRs binding peptides with the N-terminal end pointing to the helical bundle.

Peptides having an N-terminal end important for activity include opioid peptides, neuropeptides B/W, neuropeptide S, N-formyl peptide, melanocyte-stimulating hormone, motilin, ghrelin, gonadotropin-releasing hormone and galanin (Table 2). The sequence alignment of the binding site residues (Figure 2C) identifies negatively charged residues in helix 2 at position 2.61 or in helix 3 at positions either 3.29, 3.32 or 3.33 in a large number of the receptors binding these peptides, with the conservation score of 43%, 30%, 30% and 19%, respectively.

The crystal structures of the DOR and MOR receptors bound to a bifunctional peptide, DIPP-NH2 and the peptide agonist DAMGO, respectively, have been determined (Fenalti, et al., 2015; Koehl, et al., 2018). The N-terminus of both peptides binds in a similar way, with the ammonium group forming a salt bridge with the aspartate at position 3.32 (Figure 1). This interaction is conserved with opioid-like compounds as observed in their crystal complexes with the opioid receptors (Wu, F., et al., 2017). Mutation of this residue is critical for the activity of endorphins and opioid-like compounds (Surratt, et al., 1994; Li, et al., 1999).

The importance of negatively charged residue at position 3.32 for the receptors binding neuropeptides B/W (NPBW1 and NPBW2) is unknown. Mutagenesis of aspartate or glutamate at position 3.33 in the FPR, MLNR and GHSR receptors shows the significance of this residue for binding of the N-formyl peptide, motilin and ghrelin, respectively (Mills, et al., 2000; Xu, L., et al., 2005; Feighner, et al., 1998). Binding studies of various analogues of N-formyl peptide and motilin suggest that the N-terminal formyl-amide or the ammonium group interact with the negatively charged aspartate or glutamate (Mills, et al., 2000; Xu, L., et al., 2005).

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The importance of a negative charge at position 3.29 in the melanocortin receptors (MC1 and MC4) for the binding and potency of melanocortin peptides is demonstrated in mutagenesis studies (Yang, Y., et al., 1997; Haskell-Luevano, et al., 2001). However, it is not clear whether the N-terminal group or arginine of the critical FRW motif bind to the residue. Within the five melanocortin receptor subtypes, there are two other negatively charged residues at positions 2.61 and 4.64. While residue 4.64 does not change substantially the potency of the peptide, residue 2.61 has a profound effect on peptide binding (Yang, Y., et al., 1997; Haskell-Luevano, et al., 2001).

Receptors binding gonadotropin-releasing hormone, galanin and neuropeptide S do not have a negatively charged residue in helices 2 and 3. However, there are negatively charged residues in other helices that are known to be important for the peptide activity. Thus, aspartate at position 2.61 is critical for peptide binding and signalling in the gonadotropin-releasing hormone receptor (GNRHR) (Flanagan, et al., 2000; Hoffmann, et al., 2000). In the galanin receptor (GAL1), mutagenesis and galanin SAR studies suggest that the ligand ammonium group binds to glutamate 6.59 (Kask, et al., 1996; Church, et al., 2002). The role of other negatively charged residues in position 5.39 in GAL2 and GAL3 and; 6.55 and 6.58 in the neuropeptide S receptor (NPSR1) is unknown.

Over twenty GPCRs are known to bind chemokines. Because chemokines are classified as small proteins (8–10kDa), these receptors belong to the protein-bound GPCRs. As mentioned previously, several crystal structures of the chemokine receptors are available bound to short peptides, where the N-terminus points inside the helical bundle. Following the analysis of the receptor binding site residues we perform here, the negatively charged residues at positions 2.63, 6.58, 7.32 and 7.39 have a level of conservation among the chemokine receptors. The direct interactions with these residues are observed in the crystal structures of the chemokine receptors in complex with peptide and non-peptide ligands (Tan, et al., 2013; Wu, F., et al., 2017). Thus, in the crystal structure of the CXCR4 receptor with the viral chemokine antagonist, the peptide N-terminal ammonium group forms a salt bridge with residue 2.63 (Qin, et al., 2015). For a large peptide, like chemokine, it is especially true that many binding factors, including steric and electrostatic interactions of various peptide residues, contribute together to peptide activity, thus the binding of the terminal ammonium group to a counterpart residue in the binding site might not be crucial along.

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6. GPCRs binding cyclic peptides with a cyclic part important for activity and the PAR receptors.

The receptors binding urotensin, melanin-concentrating hormone and somatostatin form a group with a conserved negatively charged residue at position 3.32 and a conserved amide-based residue at position 6.55, having 100% of conservation in orthologs (Figure 2D). Residue 3.32 is known to be important for peptide binding (Sainsily, et al., 2013; Strnad and Hadcock, 1995; Macdonald, et al., 2000). From mutated peptide analogue studies, it appears that the positively charged residue within the cyclic part of the peptide forms polar interactions with aspartate 3.32 (Martin-Gago, et al., 2013; Labarrere, et al., 2003; Audinot, et al., 2001). The importance of residue 6.55 is demonstrated in the urotensin receptor (Holleran, et al., 2009). Unlike other peptides with the C-terminal free carboxyl group, this group is not important for urotensin binding, as its

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amidation does not affect the activity (Labarrere, et al., 2003; Merlino, et al., 2013). In contrast, the cyclic part of these peptides conveys the activity (Martin-Gago, et al., 2013; Labarrere, et al., 2003; Audinot, et al., 2001).

In the case of the PAR receptors, there are conserved charged residues at positions 2.61 and 4.64 and asparagine at position 6.52 (Figure 2E). Position 4.64 is similar to the first group. While the lysine at position 2.61 forms direct interactions with the synthetic ligand, AZ8838 in the crystal structure of PAR2 (Cheng, et al., 2017), positions 4.64 and 6.52 have not been yet explored. The importance of the extracellular loops in peptide binding has been examined in mutagenesis studies (Gerszten, et al., 1994), however, the role of residues in the helical cavity needs further investigation.

Concluding Remarks

In this review, using the earlier peptide SAR studies combined with the alignment of binding site residues derived from the recent GPCR-peptide complex crystal structures, we divided the peptide GPCRs of Class A into three major groups. In particular, we used the 25 residues of the GPCR helical bundle to characterize the hydrophilic properties of the receptor binding cavity.

The first group consists of the receptors that bind the free C-terminal carboxyl group terminus of peptides inside the helical bundle. These receptors have several relatively conserved positively charged residues at positions 4.64, 5.42 and 6.55. From the available crystal structures, these residues form polar interactions either with the C-terminal free carboxyl group or the peptide backbone. A recognition residue in a receptor for the peptide C-terminal carboxylate is not fully preserved and a positively charged residue at other non-conserved positions could contribute to peptide binding. Thus, the interaction with the terminal carboxylate involves arginine at position 6.54 in NTS_1 and lysine at positions 3.33 and 5.38 in ET_B.

The second large group involves the receptors binding amidated peptides. Interestingly, these receptors have relatively conserved asparagine or glutamine at positions 3.32, 4.60 and 6.55. The analysis of the peptide SAR studies combined with receptor mutagenesis and the available crystal structures of the receptors in complex with synthetic antagonists suggests that the peptide terminal amide forms interactions with a counterpart amide group of glutamine or asparagine at one of these positions. In contrast to the group of the receptors binding peptides with the terminal free carboxyl group, this group of the receptors does not have conserved polar residues at positions 4.64 and 5.42 but has conserved asparagine or glutamine at position 6.55, instead of arginine or lysine. Residue 6.55 appears to be critical in peptide binding and could contribute to the differentiation of the C-terminus property for several receptors of these two groups.

The third group includes the receptors binding peptides with the N-terminus interacting with the helical bundle. Our analysis identifies a regular presence of a negatively charged residue at positions 2.61, 3.29, 3.32 and 3.33. The crystal structures of the opioid receptors bound to peptides show the ionic interaction between the peptide N-terminal ammonium group and residue 3.32. Analysis of available SAR and mutagenesis data for several receptors of this group suggests the presence of ionic interaction between the peptide terminal ammonium group and a negatively charged residue of the receptor. In comparison with the first two groups,

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these receptors have a few positively charged and amide-containing residues in the binding cavity. Unlike the receptors binding amidated peptides, several receptors have aspartate at position 3.32 instead of glutamine.

The receptors binding the cyclic peptides with the cyclic part being important for the activity have conserved aspartate and glutamine/asparagine at positions 3.32 and 6.55, respectively. The importance of these residues is shown in mutagenesis for several receptors of this group. The PAR receptors form a separate group with conserved polar residues at positions 2.61, 4.64 and 6.52.

Comparison of the conservation score for hydrophilic residues in the alignment of the binding site residues in the three major receptor groups shows a clear pattern in the conserved residue preference in the receptor group. In particular, the highest conservation score in the first group is for the positively charged residues, in the second group is for the amide-containing residues and in the third one is for the negatively charged residues. Thus, the proposed grouping of the receptors based on the peptide SAR data is justified from the sequence analysis of the receptor orthologs.

The recent reporting of crystal structures of GPCRs bound to peptides allows structural diversity of peptide binding to be assessed for the first time. Furthermore, the structural data facilitates interpretation of peptide SAR studies and allows extrapolation of findings to related GPCRs. Thus, our polar residue analysis enables to group the peptide receptors highlighting conserved residues important for peptide binding. Despite a large heterogeneity in possible binding modes of peptides within GPCRs, some level of generalization of peptide binding can be established through the analysis of the peptide terminal end binding to the transmembrane helical cavity of GPCRs.

Further understanding of peptide-GPCR recognition has important implication for the design of peptides and peptide-like molecules as new pharmacological tools and medicines. From the conservation of a specific type of hydrophilic residues in a receptor, the binding orientation of peptides in the helical bundle could be identified and therefore, the important part of peptides for activity is determined, this, in turn, provides a rationale for peptide modification. This knowledge can also facilitate peptide receptor deorphanization. Although 32 mammalian GPCRs were only analysed here, studies could be expanded to neuropeptide GPCRs of parasitic nematodes and insects to facilitate the understanding of peptide binding sites and design of GPCR-targeting agrochemicals.

Acknowledgment. We thank Prof Karl Malcom and Dr Antonella Ciancetta for critical review and editing the manuscript.

Authorship Contributions

Participated in research design: I.G.T. and D.F. Performed data analysis: I.G.T., D.F., V.G. Wrote the manuscript: I.G.T.

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Footnotes

*Work described herein is supported in part by grants from NEXT-LPCNO-CNRS (to IGT, VG and DF) and the Biotechnology and Biosciences Research Council, [BB/R007101/1] (to IGT).

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Figure legends

Figure 1. Receptor-peptide terminal group interactions in the X-ray peptide-GPCR complexes. The overview of the peptide binding is on the left side and the zoom view of the peptide terminal group interactions is on the right side. **A**: Endothelin with the free C-terminal carboxylic group and ET_B; **B:** Neurotensin with the free C-terminal carboxylic group and NTS_1; **C**: Apelin with the free C-terminal carboxylic group and APLNR; **D:** [Sar1, Ile8] Angiotensin II with the free C-terminal carboxylic group and AT_2. **E**: DAMGO peptide with the N-terminal ammonium and MOR; **F:** CVX15 with the N-terminal ammonium and CXCR4. Peptide and receptor ribbons are in red and grey, respectively. Peptide carbon atoms are in green, only receptor residues forming interactions are shown. Salt bridges and hydrogen bonds are shown in pink and black dashed lines.

Figure 2. **The alignment of binding site residues for human Class A peptide GPCRs.**
A: GPCRs binding peptides with the C-terminal free carboxyl group pointing to the helical cavity. **B**: GPCRs binding peptides with the C-terminal amidated group pointing to the helical cavity. **C:** GPCRs binding peptides with the N-terminus pointing to the helical cavity. **D:** GPCRs binding the cyclic peptides; and **E:** PAR receptors. Positively charged, negatively charged and amide-containing residues are in cyan, purple and yellow, respectively. Receptors co-crystalized with a peptide and synthetic ligands are in grey. Residues forming direct interactions with peptides are in bold. Abbreviation: AT_1, AT_2, angiotensin receptors; APLNR, apelin receptor; C3a, $C5a_1$, $C5a_2$, complement peptide receptors; ET_A, ET_B, endothelin receptors; NTS1, NTS2, neurotensin receptors; RXFP1, RXFP2, RXFP3, RXFP4, relaxin receptors; B_1, B_2, bradykinin receptors; NPY_1, NPY_2, NPY_4, NPY_5, neuropeptide Y receptors; OX_1, OX_2, orexin receptors; V_{1A}, V_{1B}, V_2, vasopressin receptors; OT, oxytocin receptor; CCK1, CCK2, cholecystokinin receptors; QRFP, pyroglutamylated RFamide peptide receptor; PrRP, prolactin-releasing peptide receptor; NPFF1, NPFF2, neuropeptide FF receptor; KISS1R, kisspeptin receptor; BB_1, BB_2, BB_3, bombesin receptors; TRH1, thyrotropin-releasing hormone receptor; NMU1, NMU2, neuromedin-U receptors; NK1, NK2, NK3, neurokinin/tachykinin receptors; DOR, KOR, MOR, NOP, opioid receptors; NPBW1, NPBW2, neuropeptide B/W receptors; FPR1, FPR2, FPR3, N-formyl peptide receptors; MLNR, motilin receptor; GHSR, ghrelin receptor; MC1, MC2, MC3, MC4, MC5, melanocyte-stimulating hormone receptors; GNRHR, gonadotropin-releasing hormone receptor; GAL1, GAL2, GAL3, galanin receptors; NPSR1, Neuropeptide S receptor; UT, urotensin receptor; MCH1, MCH2, melanin-concentrating hormone; SST1, SST2, SST3, SST4, SST5, somatostatin receptors; PAR1, PAR2, PAR3, PAR4, PAR5, proteinase-activated receptors. Conservation score (%) for each residue position calculated from the analysis of receptor orthologs (bovine, chimpanzee, guinea pig, human, mouse, rabbit and rat) is shown at the bottom of each binding site residue alignment. The conservation score and the corresponded bar are shown for the most conserved positively charged (cyan), negatively charged (purple) or amide-containing residues (yellow).

Figure 3. Ligand binding sites of peptide GPCRs. Non-peptide antagonist interactions with residues at positions 3.32. 4.60 and/or 6.55 in the crystal structure of NPY_1, OX_1, OX_2 and NK_1. Validated homology model of the CCK2 receptor complexed with the CCK4 peptide (Langer, et al., 2005). The antagonists, the peptide CCK4 and the receptors are labelled and only the amide-containing residue is shown. Hydrogen bonding is in black-dashed lines.

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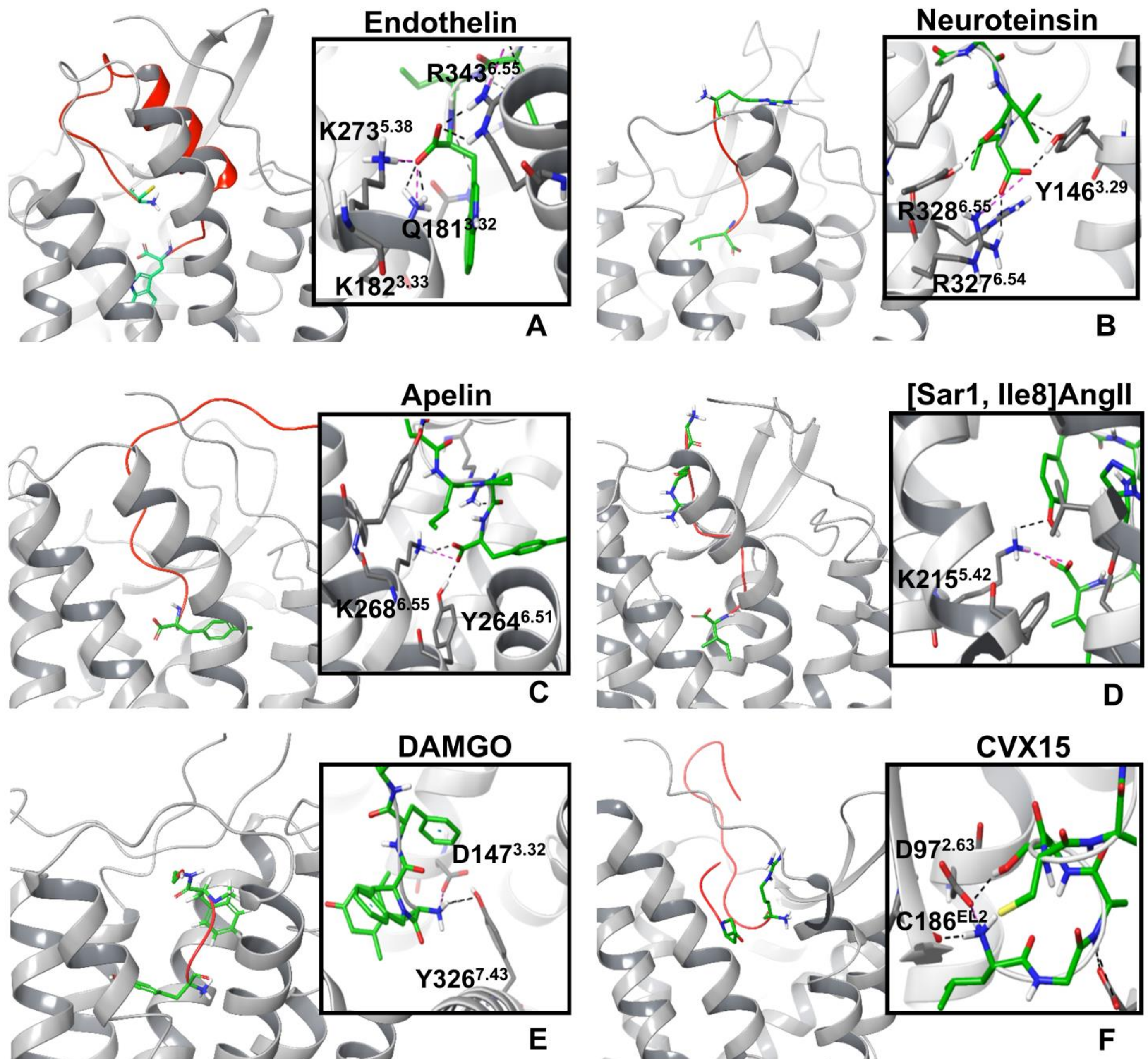

Figure 1

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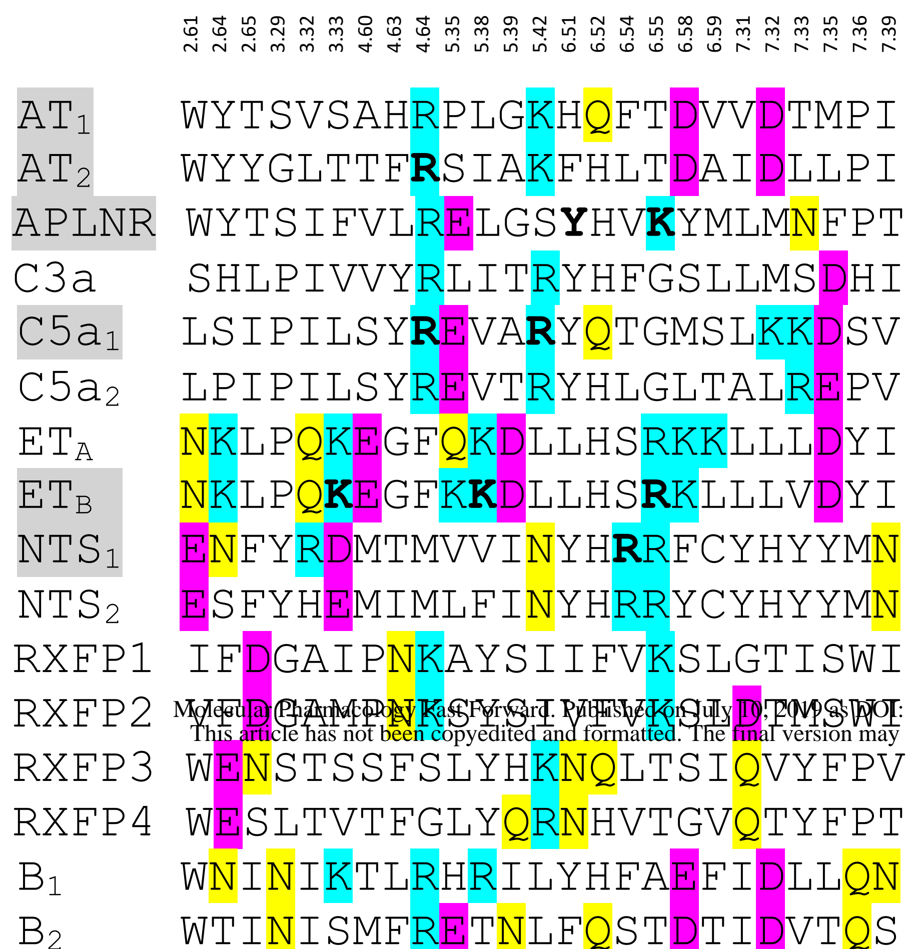
Figure 2

A



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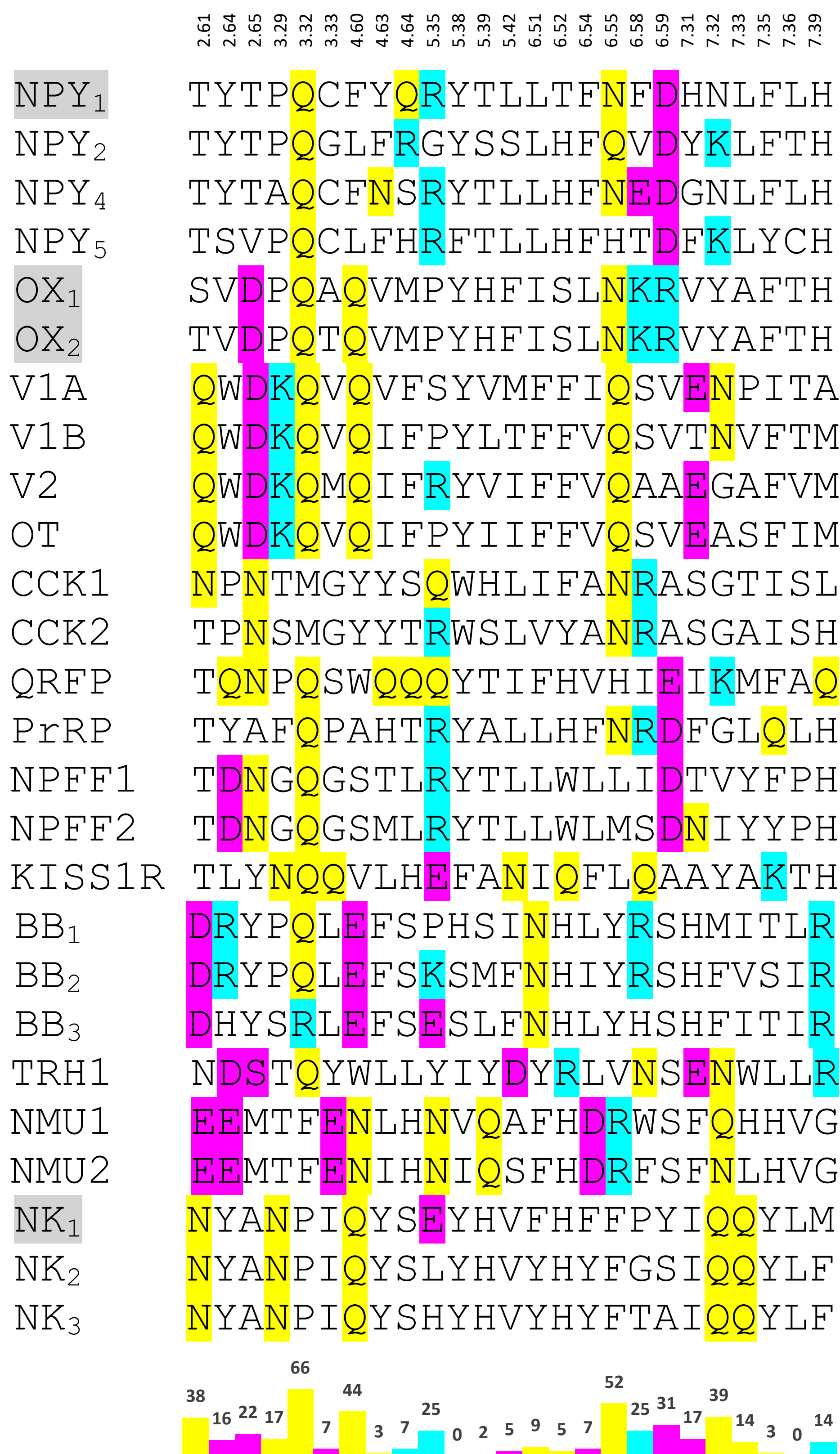
B



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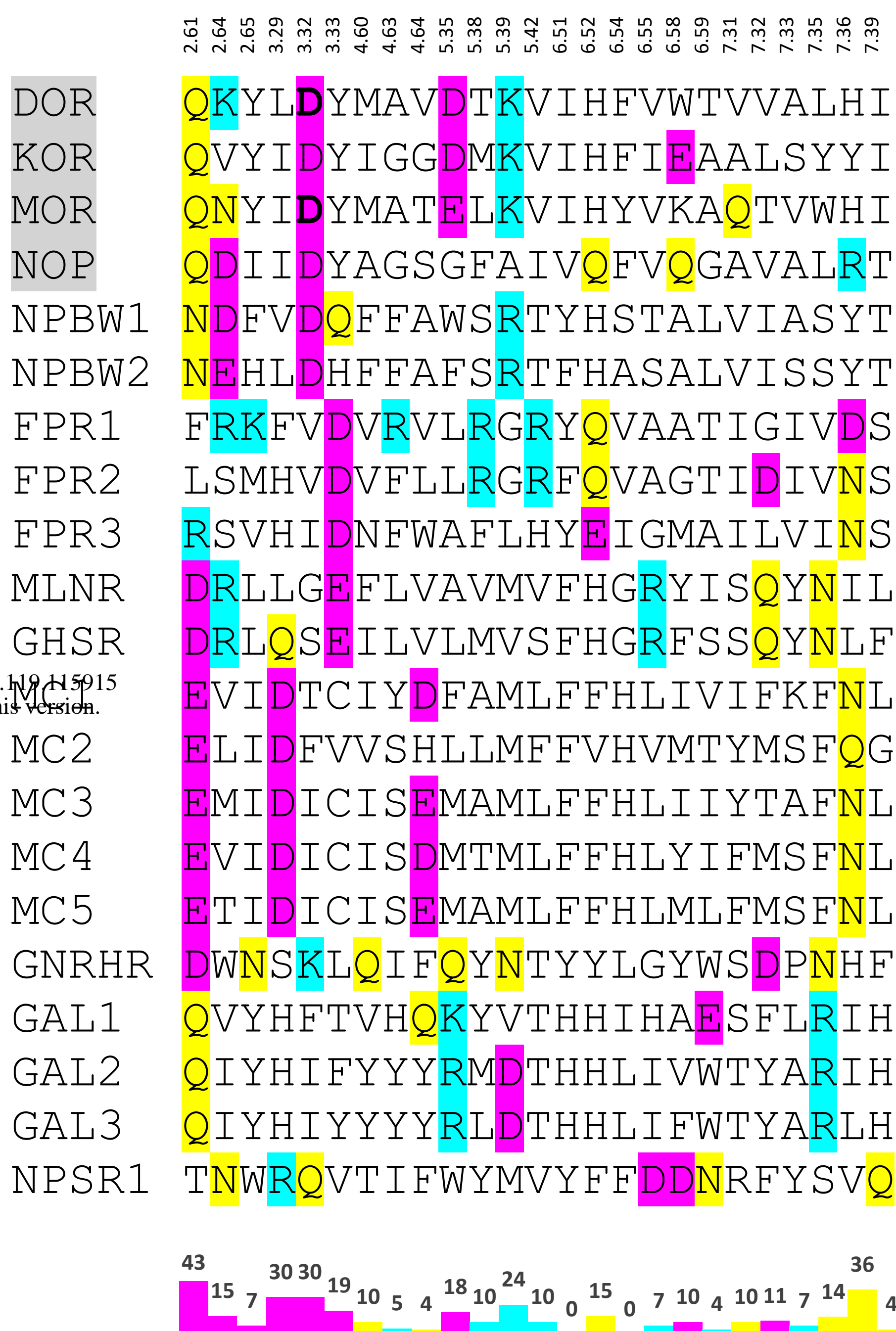

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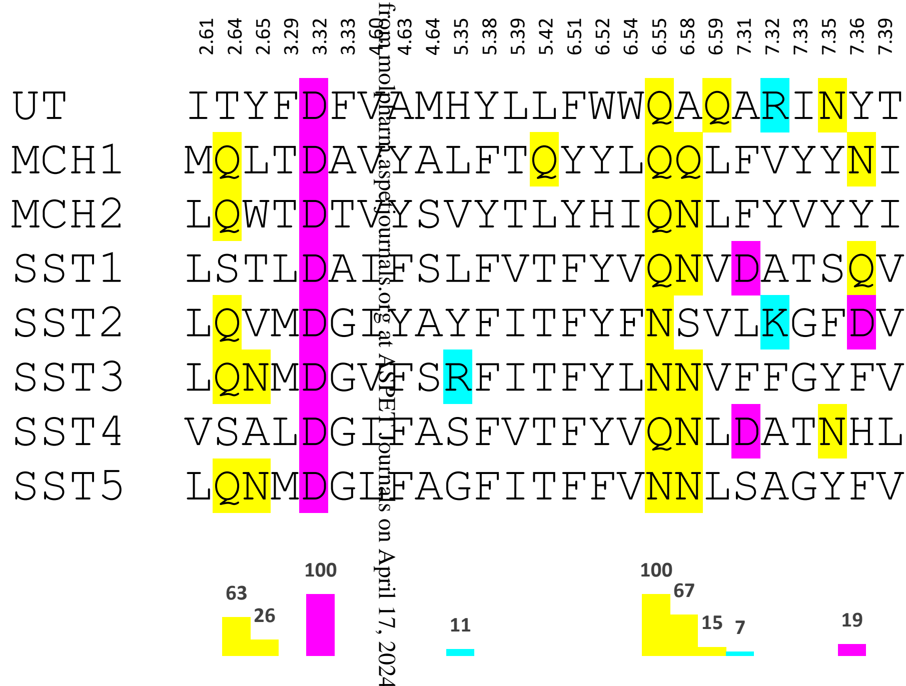
D



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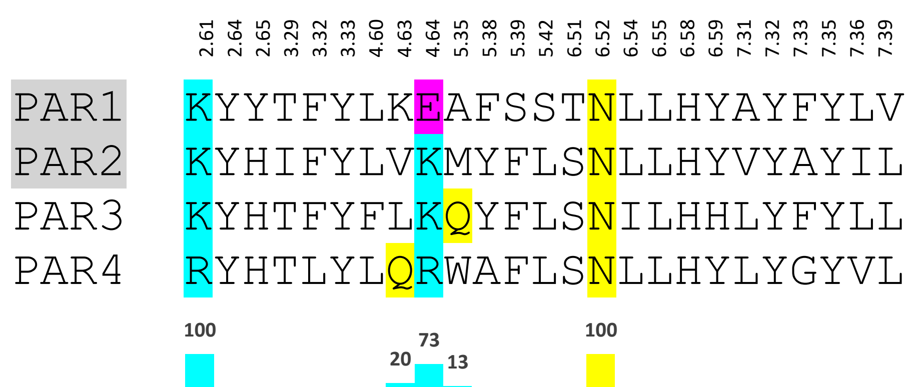

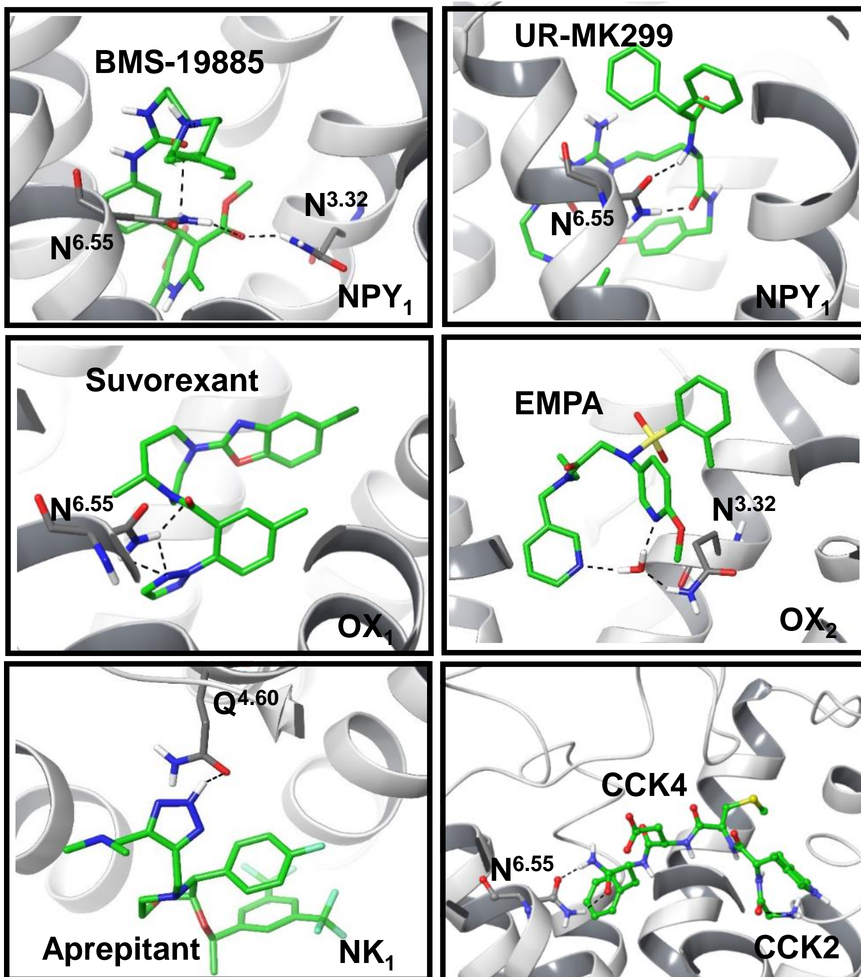

Figure 3

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