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Receptor Identification: Advances in Ligands and Transmitters Discovery

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Abstract: Receptor identification is an integral part of drug discovery and development. By the beginning of the next millennium, the search for the natural ligands of the orphan G-protein-coupled receptors will lead to the discovery of so many new peptides that it may well double their present number. It has recently become evident that all types of chemical messengers, hormones and transmitters act through membrane receptors which constitute our largest superfamily of proteins, i.e. the G protein-coupled receptors. The development of targeted therapies has revolutionized the treatment of various chronic diseases. Receptors have well-conserved regions that are recognized and activated by hormones and neurotransmitters. These ligands are peptides, lipids or biogenic amines, and act as transmitter molecules. Identification of orphan receptors include screening, binding and reverse engineering that help to find out cysteinyl leukotriene CysLT1 and Cys T2, hepatointestinal leukotriene B4, motilin, Ghrelin, Growth hormone-releasing peptide and growth hormone secretagogue receptor and many more. Techniques involved in screening of receptors include low stringency hybridization followed by PCR-derived approaches helps to discover various orphan g protein couple recptors (oGPCR). The discovery of the oGPCR represents a hallmark in neuroscience research, and the exploitation of its numerous physiological and pathophysiological functions is a promising avenue for therapeutic applications

Keywords: Orphan G-protein-coupled receptors, Hybridisation, PCR, cysteinyl leukotriene CysLT1 and Cys T2, hepatointestinal leukotriene B4, motilin, Ghrelin, Growth hormone-releasing peptide

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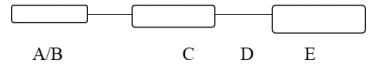
1. INTRODUCTION

1.0 Receptor identification is an integral part of drug discovery and development process. Most of receptors are initially marked as orphan receptors, with the transmitter or ligand to which they structurally bind for their pharmacological

and physiological activity not known. Most receptors show following gross structure



A/B: Amino terminal (With free amino group) (ligand independent Transcription activator)

C: DNA binding domain

D: hinge Domain

E: Ligand binding domain

Stimuli that elicit a response from a receptor vary from neurotransmitters, hormones, light, which specifically activate intracellular signaling events mediated by various intracellular proteins. Receptors are a necessary interface in the plasma membrane for interaction with diverse extracellular mediators and thereby initiation of cellular responses. Thus development of many pivotal therapeutic agents involves interactive and structural studies on membrane receptors. A natural activating ligand being integral for activation and response of a receptor, ligand discovery for orphan receptors might give an idea to discovering new cellular substances with physiological and therapeutic potential. This process of 'ligand discovery for orphan receptors (ligand not defined) is key to drug discovery.

The complete identification process may be done by use of two methodologies:

I. Identification of receptors after screening, those bind to a particular transmitter/ligand structure.
II. Identification of Ligand that bind to a particular receptor (reverse engineering)

Sarau, H.M. *et al.* (1999) carried out identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. Lynch, K.R. *et al.* (1999) carried out characterization of the human cysteinyl leukotriene CysLT1 receptor. Heise, C.E. *et al.* (2000) carried out characterization of the human cysteinyl leukotriene 2 (CysLT2) receptor.

Wang, S. *et al.* (2000) identified a novel hepatointestinal leukotriene B4 receptor and determined its cloning and functional characterization.

Feighner, S.D. *et al.* (1999) identified receptor for motilin in the human gastrointestinal system.

Kojima, M. *et al.* (1999) confirmed that Ghrelin is a growthhormone-releasing acylated peptide from stomach. Bowers, C.Y. (1998) confirmed Growth hormone-releasing peptide (GHRP).

Patchett, A.A. *et al.* (1995), carried out design and biological activities of L-163,191 (MK-0677): a potent, orally active growth hormone secretagogue.

1.1 Techniques for discovery:

Bruce Blumberg and Ronald M. Evans (1998)

I. Those focusing on concept first as above, that of screening receptors against a particular transmitter:

These include:

A. PCR based homology screening approaches.
B. Low stringency Hybridisation studies followed by PCR-derived approaches

Most of the GPCRs started as orphans. Their discoveries stemmed from the concept that GPCRs would belong to a supergene family and thus would share sequence similarities. Homology screening techniques, low stringency hybridization (Bunzow *et al*., 1988, 1992) soon followed by PCR-derived approaches (Libert *et al*., 1989), paved the way for the discoveries of new GPCRs, thus that, by the end of the 1980s, it became clear that the number of GPCRs would be large. This was confirmed, at the turn of the century, with the sequence of the genome. Today, one estimates that the number of GPCRs is about 800, of which more than half are olfactory GPCRs (Vassilatis *et al*., 2003).

II. Those focusing on concept second as above, that of screening transmitters against a particular receptor (Reverse engineering):

These include:

A. Quantification of intracellular cAMP level changes.
B. Quantification of Adenyl cyclase activity (Binding as well as activation confirmation).
C. Changes in intracellular Calcium release.
D. Phospholipase A2 activity
E. Phospholipase C activity

The first deorphanized GPCRs, the 5HT-1A and the D2 dopamine receptors, were already reported in 1988 (Fargin *et al*., 1988; Bunzow *et al*., 1988). The strategies used were the same, that is, membranes of eukaryotic cells, with orphan GPCR expressed by DNA transfection, used as targets to determine the binding of potential transmitters. This strategy has come to be known as reverse pharmacology (Libert *et al*., 1991a, 1991b; Mills & Duggan, 1994).

The second successful attempt at discovering novel transmitter through orphan GPCRs was done by measuring intracellular calcium release when GPCRs wee subjected to peptidic extracts. By this way, two ligand peptides were identified named the orexins (Oxs) (Sakurai *et al*., 1998), and hypocretins (Hcrts) (de Lecea *et al*., 1998) (also identified through an RNA subtraction approach). Two more novel ligand peptides, prolactin-releasing peptide, and apelin were discovered as the natural ligands of the orphan GPCRs GPR10 and APJ, respectively (Hinuma *et al*., 1998; Tatemoto *et al*., 1998).

1.2 Transmitter/Ligand classification

Most of the receptors are activated by a plethora of ‘‘transmitters’’ (Civelli *et al*., 2001), or messengers that carry a message to the cell as an endocrine, paracrine, or exocrine activity to initiate a particular physiological change. Transmitters include nucleotides, amino acid and derivatives, biogenic amines, olfactory and gustatory molecules, neuropeptides, chemokines, lipid mediators, pheromones, polypeptide hormones, and some other naturally occurring chemicals such as calcium ions and protons.

Traditionally, the GPCRs are expected to exhibit specificity for the transmitters (Goldstein, 1974). This specificity results from evolutionary processes that aim at diversifying the intercellular interactions. A transmitter may be the natural ligand of more than one GPCR, but those then share a higher degree of homology that groups them into a subfamily. Also a GPCR may bind more than one transmitter, but then these transmitters share structural similarities and are often part of the same synthesis pathway, as in the case of the neuropeptides synthesized from the same precursor (Douglass *et al*., 1984).

TRANSMITTERS ARE PUT INTO FOLLOWING BROAD CLASSIFICATION

1 Biogenic amines
2 Neuropeptides
3 Chemokines
4 Lipid mediators
5 Nucleotides
6 Aminoacids
7 Neuropeptides
8 Pheromones
9 Polypeptide hormones
10 Ca^{2+} and H^{+}

1.3 Ligands discovery over the year’s

Detailed analysis of oGPCRs has led to several significant discoveries, some of which are presented. Of particular interest is the sheer number of recent successful orphan searches driven in part by improvements in assay methodology. Since 1988 these include identification of the receptors for:

1 5-HT (Andrew D Howard *et al*, 2001) adenosine (Lee, Y.K., and Moore, D.D. 2008). (Lee, J.M., *et al* 2011).

2 Nociceptin/orphanin FQ (Reinscheid, R.K. *et al.,* 1995), (Meunier, J.C. *et al.,*1995) anaphylatoxin C3a Ames, (R.S. *et al.* 1996)

3 GHS (Howard, A.D. *et al.,* 1996)

4 Prolactin-releasing peptide (Hinuma, S. *et al.,* 1998)

5 Calcitonin generelated peptide or adrenomedullin (Aiyar, N. *et al.* 1996), (McLatchie, L.M. *et al.*1998)

6 Hypocretins (orexins) (Sakurai, T. *et al.* 1998), (de Lecea, L. *et al.*1998)

7 Apelin (Tatemoto, K. *et al.* 1998)

8 Thyrotropin-releasing hormone (Itadani, H. *et al.*1998), Cao, J. *et al.* 1998)

9 Cysteinyl leukotrienes (Sarau, H.M. *et al.* 1999), (Lynch, K.R. *et al.* 1999), (Heise, C.E. *et al.* 2000), (Wang, S. *et al.* 2000)

10 Motilin (Feighner, S.D. *et al.* 1999)

11 Melanin-concentrating hormone (Saito, Y. *et al.* 1999), (Chambers, J. *et al.* 1999), (Shimomura, Y. *et al.* 1999), (Bachner, D. *et al.* 1999), (Lembo, P.M. *et al.* 1999)

12 Urotensin II (Liu, Q. *et al.* 1999) (Mori, M. *et al.* 1999) (Ames, R.S. *et al.* 1999) (Nothacker, H.P. *et al.* 1999)

13 S1P (Okamoto, H. *et al.* 1999) (An, S. *et al.* 2000) (Pyne, S. and Pyne, N.J. 2000) (Lee, M.J. *et al.* 1998) (Im, D.S. *et al.* 2000)

14 Lysophosphatidic acid (Im, D.S. *et al.* 2000) (Fukushima, N. *et al.* 1998)

15 Leukotriene B4 (Kamohara, M. *et al.* 2000) (Yokomizo, T. *et al.* 1997)

16 Allostatin-like Birgul, N. *et al.* 1999) (Lenz, C. *et al.* 2000)

17 Histamine (Lovenberg, T.W. *et al.* 1999)

18 Eskine (Jarmin, D.I. *et al.* 2000)

19 UDPglucose (Chambers, J.K. *et al.* 2000)

20 Sphingosylphosphorylcholine (Xu, Y. *et al.* 2000)

21 Neuromedin U, neuropeptide FF (Hedrick, J.A. *et al.* 2000) (Elshourbagy, N.A. *et al.* 2000) (Bonini, J.A. *et al.* 2000)

MANY LIGANDS HAVE BEEN DISCOVERED OVER THE YEARS BY THE ABOVE TECHNIQUES. THESE INCLUDE:

1 Orphanin FQ (Polypeptide) from Brain tissue.
2 Nociceptin
3 Hypocretin (Hcrts)
4 Orexins (Oxs)
5 Prolactin releasing peptide
6 Apelin
7 PrPP ligands
8 Urotensin Ii transmitters
9 Neuropeptide S
10 Leptin
11 Neuropeptide Y
12 OFQ/N
13 RFamide dipeptide
14 Bam 22
15 Cortistatin
16 Prokinetin 2
17 NPS
18 NPFF
19 NPAF
20 MCH
21 Succinic acid
22 Citric Acid
23 Motilin
24 Anaphylatoxin
25 TRH
26 Cysteinyl Leukotriene
27 Lysophosphatidic acid

Thompson *et al*., (2012) studied and elucidated the structure of the nociceptin/ orphanin FQ receptor in complex with a peptide mimetic (Thompson *et al*., 2012)

Mogil and Pasternak, (2001) studied the molecular and behavioral Pharmacology of the Orphanin FQ/Nociceptin Peptide and Receptor Family. This heptadecapeptide binds to the NOP_1 (previously termed ORL1) receptor

with exceedingly high affinity, but does not interact directly with classical opioid receptors. Functionally, the actions of OFQ/N are diverse and intriguing. Most work has focused upon pain mechanisms, where OFQ/N has potent anti-analgesic actions supraspinally and analgesic actions spinally (Mogil and Pasternak, 2001).

Chandrasekaran *et al*., (2008) studied the role of apelin in cardiovascular function and heart failure. Apelin is a novel peptide, widely distributed in a number of tissues, mainly restricted to vascular endothelium, and is a ligand for APJ receptor similar to the angiotensin II–angiotensin II type 1 receptor pathway. It is a peripheral vasodilator, powerful inotrope and may affect central fluid homeostasis and is involved in the pathogenesis of heart failure by modulating the harmful effects of angiotensin II. Apelin is reduced in patients with heart failure and up regulated following favourable left ventricular remodelling.

Sato *et al* (2013) confirmed that **A**pelin is a positive regulator of ACE2 in failing hearts. Apelin, RAS and ACE2 together form an axis which helps increase the contractility of heart and provides a therapeutic target for cardiological disorders.

Smith *et al*., (2001) studied various growth hormone secretagogue receptor (GHS-R) family members and its ligands such as L-692,429 and MK-0677. Adenosine is a partial agonist of the GHS-R and that motilin is the endogenous ligand for GPR38.

Wurtman RJ (2006) studied relationship between narcolepsy and the hypocretins.

In a narcoleptic patient, "automatic behavior," refers to daytime sleep episode in which conventional functions are done but forgotten once awake. Genetic factors alone can not be singled out as a single major cause, as a mutation in chromosome 6 controlling the HLA antigen immune complex, is seen in narcoleptics as well as nonnarcoleptics. Narcoleptic patients show drastic decrease in Cerebrospinal fluid (CSF) hypocretin levels, and a specific reduction in hypocretin-containing neurons which may be due to mutation on chromosome 12 which disrupts the processing of Hypocretin, whereas HLA subtype resulting from the mutation on chromosome 6 may also increase the susceptibility of hypocretin-containing brain neurons to immune attack. This loss of neurons with Hypocretin causes REM sleep at inappropriate times.

The hypocretins and orexins are neuropeptides with same precursor expressed mainly in lateral hypothalamus neurons. Hypocretins act as ligands for two G-protein coupled receptors distributed within the central nervous system in Hypocretin neuron fibers in several areas implicated in regulation of the sleep/wakefulness cycle (De Lecea and Sutcliffe, 2005). Synthetic hypocretin-1 affects when administered, affects blood pressure, hormone secretion and locomotor

activity, causes wakefulness, suppressing REM sleep, by integrating homeostatic, metabolic and limbic information and providing a coherent output.

Brighton *et al*., (2004) elaborated neuromedin U and its receptors: structure, function, and physiological roles. Neuromedin U (NmU) a neuropeptide is variably distributed, mainly in the GIT and pituitary with two G-protein-coupled receptors for NmU being recently cloned and the receptors being widespread throughout the body but with differential distributions. It shows contraction of smooth-muscle (hence the suffix "U"), regulation of BP and local blood flow, feeding behavior, stress responses, gastric acid secretion, pronociception.

Expression of cortistatin was observed in peptidergic nociceptors of the peripheral nociceptive system, and endogenous cortistatin is involved in modifying pain sensitization, in inflammatory diseases, by acting both peripherally and centrally & modifying tactile allodynia and heat hyperalgesia due to arthritis and peripheral tissue inflammation in mice, via mechanisms different than antiinflammatory action, involving nociceptive neurons and central sensitization, which may be due to impairment of ERK signaling, activation of the G protein subunit Gαi, binding of the neuropeptide to somatostatin and ghrelin receptors, and decreased production of calcitonin gene-related peptide in primary nociceptors (Morell *et al*., 2013).

1.4 Pharmacological Targeting of receptors with Ligands

1.4.1 Retinoic Acid receptors

Discovered as RORα, RORβ, RORγ in liver, skeletal muscles, lungs, thymus tissues etc. RORγ is more expressed in immune tissues, thymus, liver, kidneys etc. RORα and RORγ play a key role in immune response and generation and differentiation of CD^{4+} T cells into TH1, TH2, TH17 etc. Many ligands have been found to configurate with RORα and include Cholesterol, Cholesterol Sulphate, Hydroxycholesterol, 0901317, T1317, SR10001 and Ursolic acid (Mangelsdorf & Evanst, 1995**).** The RXR Heterodimers and Orphan Receptors Crystallographic studies of RORα suggested that a sterol such as cholesterol or cholesterol sulfate may function as a natural ligand of this receptor (Kallen *et al*., 2004). It was also found that hydroxycholesterols were high affinity ligands for both RORa and RORg and that some of these such as 7-oxygenated sterols functioned as inverse agonists suppressing the constitutive activity of both of these receptors (Wang *et al*., 2010a, 2010b). Huh *et al*. (2011) also identified a small molecule inhibitor of RORg and this compound was the well-known cardiac glycoside, digoxin. Ursolic acid has also been recently shown to inhibit TH17 cell differentiation via targeting RORg (Xu *et al*.,

2011). Ursolic acid also delayed the onset and decreased the severity of EAE in mice (Xu *et al*., 2011).

1.4.2 PPARγ receptors and their ligands and Type 2 Diabetes

Type II Diabetes is caused due to development of insulin resistance on cell surface, sometimes by adipose tissue increase and obesity, and by the adipokines secreted by adipose tissues including TNFα, IL-1, resistin, which prevent proper movement of plasma glucose and its redistribution. is one of the prime reasons as which decrease insulin sensitivity. Adiponectin and compounds like Thiazolidine diones and SR 1664 act in a similar fashion as Adiponectin (Forman *et al*. 1995c; Kliewer *et al*. 1995; Chawla *et al*. 1994; Tontonoz *et al*. 1994; Ricote *et al*. 1998; Jiang *et al*. 1998) increase the insulin sensitization (Kershaw & Flier, 2004) (Hotamisligil *et al*., 1993; Lagathu *et al*., 2006; Steppan *et al*., 2001). Conversely, adipose tissues from lean individuals secrete higher levels of adiponectin, a circulating protein that has insulin-sensitizing effects on liver and other tissues (Berg *et al*., 2001; Hu *et al*., 1996; Yamauchi *et al*., 2006).

1.4.3 Liver receptor homolog-1 (LRH-1) and associated ligands

Many ligands have been screened against this receptor expressed in the intestine liver, pancreas and ovary and playing important role in embryonic development, and the prominent transmitters discovered include Dilaurylphosphatidyl Choline (DLPC) and Diundecanoyl PC and Dipalmitoyl PC. LRH-1 (Lee and Moore, 2008).It also controls critical enzymes for cholesterol homeostasis and bile-acid biosynthesis and expression of aromatase in the breast and ovaries (Lee *et al*., 2008; Santen *et al*., 2009).

1.4.4 CARβ and ligands

These have also been explored vigorously for possible ligands and the prominent transmitters found include Androstanol and Androstenol.

1.4.5. BγR receptors and Benzoate ligands

These receptors, identified as an obligate RXR heterodimeric partner, both in vitro and in vivo are mainly found with nervous tissues associated with embryonic fluid and show prominent affinity to benzoate derivatives as Ligands e.g. Paraaminobenzoic acid (PABA) (Smith *et al*. 1994a). Bioactivity guided HPLC fractionation of embryonic extracts, for stimulation of BXR target genes, revealed several components and scale-up purification followed by mass spectrometry and ^{1}H-NMR analysis identified alkyl esters of amino and

hydroxy benzoic acids as potent, stereo selective activators verified as ligands by in vitro (Blumberg *et al*. 1998a).

1.4.6. PαR Pregnane activated receptors:

These receptors show prominent binding and activation by synthetic steroids, steroid antagonists, spironolactones, Cyproterones, PLN and other catatonic compounds including Natural sterols.

Exogenous steroids and pharmacologic substances and such catatoxic compounds like synthetic glucocorticoid antagonist, pregnenolone-16-carbonitrile (PCN) may modify the expression of enzymes and induce the proliferation of hepatic endoplasmic reticulum and the expression of cytochrome P450 genes that would protect against subsequent exposure to toxic xenobiotic substances, endogenous steroids, hormones and drugs (Selye 1971) giving nonspecific protection or immunity against numerous drugs and xenobiotic compounds, by increased catabolism by acting through SXR 5 receptor systems (Burger *et al*. 1992; Gonzalez *et al*. 1986). The important transmitters include various in use drugs, Rifampicin, Nifedipine, Estrogen, Tamoxifen, Phytoestrogens etc. Many of the most potent catatoxic compounds (e.g., cyproterone acetate, PCN, spironolactone) are steroid receptor antagonists, others (e.g., dexamethasone) are receptor agonists (Burger *et al*. 1992).

1.4.7. PDARγ and ligands

Many compounds have been screened and the potent transmitters or ligands discovered include oxidized low density lipoproteins OX-LDL and 9 and 13 Hydroxy Octadecadienoic acid.

1.5 Concluding Remarks

The drug discovery process has benefitted well from the two way process of identification of ligands such as biologically active peptides and their counterpart receptors, thus giving a proper picture of various ligand–receptor systems, which are an integral part involved in the therapeutical action of various drugs used in various disease conditions. This drug discovery process is now utilizing three basic tools for discovery: genomics, high-throughput receptor and ligand interaction assays, and large library of biologically active molecules**. G**enomics has provided considerable opportunities in receptor identification and discovery and more of such orphan receptors can be confirmed after identification of their natural ligands. The future opportunities involve identification of more such new receptor–ligand system of therapeutic use in human physiology and disease.

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