

Review

Why Search for Alternative GPCR Agonists?

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Abstract: Intuitively, it is easy to understand why we search for G protein-coupled receptor (GPCR) antagonists. It is obviously to block a functionality of a specific receptor potentially linked to some aspects of disease. Whether by focused research or by serendipity, many drugs were discovered in the last century that function as antagonist at a precise receptor. A current idea is that at least half of the drugs on the market are antagonist ligands of GPCRs. Then, why are we searching for alternative receptor agonists while the endogenous activating molecule is known? In the present commentary we try to rationalize these fields of research, since they proved to be very successful over the years, with receptor pharmacology populated with dozens of alternative agonists, particularly to bioaminergic receptors, and to a lesser extent to peptidergic ones. However, the action of such compounds is not well-characterized: are they surrogates to the endogenous agonist, and if yes in which context and for which purpose? The present essay is a reflection on this subject that leads to fundamental interrogations of our understanding of GPCR roles and functions.

Keywords: peptidergic ligands; aminergic ligands; receptology

1. Introduction

Whenever one is attracted by G protein-coupled receptors (GPCRs), they stumble rapidly into a confounding complexity that one was maybe not prepared to cope with. During our younger times, our vision was that receptors worked in a simple way: they were activated by an agonist, most often a single one was available, and blocked by antagonists, most often several ones accessible. It was thus well accepted that agonists were natural endogenous compounds: biogenic amines, peptides, fatty acids, leukotrienes and so on, whereas antagonists were rarely unique and most often synthetic. Hence, it was tempting to generalize these observations: agonists were natural and antagonists were synthetic and without defined structural limits. Therefore, antagonists were considered the only drug candidates (or tools for pharmacological and physiological investigations) until synthetic agonists and natural endogenous antagonists were discovered (*vide infra*). However, we could consider those as exceptions since the endogenous agonists and synthetic antagonists largely outnumber them.

The present article is a reflection on different concepts, more or less recent, in the pharmacology of GPCRs based on some expertise we have either on melatonin receptors and on some peptidergic receptors. As we recently ask why pursuing the cloning of GPCRs, we thought it might be useful to reflect on the following questions:

1. Why search for endogenous agonists?
2. Do alternative natural agonists exist?
3. Why search for synthetic agonists?

Before tackling these questions, it seemed important to us to introduce few terms herein: we categorized three types of agonists: (i) the endogenous agonist is the reference agonist for a family of receptors. For instance, dopamine for dopamine receptors or



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bradykinin for bradykinin receptors; (ii) a natural agonist is a compound coming from natural living sources, such as plants, bacteria, fungi, or animal venoms. Take for example morphine, an alkaloid extracted from *Papaver somniferum*, or the venom peptide exendin-4, isolated from the Gila monster *Heloderma suspectum*, as agonists for opioid receptors and glucagon-like peptide 1 receptors (GLP1R), respectively; (iii) a synthetic agonist is a compound that is bench-synthesized such as Agomelatin[®] for melatonin receptors or GTPL2010, a peptide-based somatostatin type 1 receptor agonist. By the term alternative agonist, we mean all the compounds that are agonists except the endogenous compound, i.e., naturally occurring and chemically synthesized agonists. As touched upon above, these notions can be obviously transposed to antagonists as well.

2. Why Search for Endogenous Agonists?

Let us first ask the question of what is an endogenous agonist of a GPCR? The answer is simple and unambiguous, it is not one, but the compound synthesized by a living organism (thereafter we will only consider the case of mammalian organisms) able to activate a GPCR present in the same organism, at the whole capacities of the latter, at the lowest effective concentration. It is then considered as the reference agonist, a full agonist. In this context, this definition in no way excludes the uniqueness of this principle. Indeed, the possibility that the same endogenous agonist can recognize one or more other receptors, in the best possible manner, remains valid. Apart from these considerations, the notions of target selectivity/specificity and potency/efficacy take over (see *vide infra* or elsewhere) and lead to the concept of side effects or off-target effects, which are notable, insignificant, or irrelevant.

At this stage, let us also answer the question: do the terms endogenous and agonist always go hand in hand? It is definitively not unreasonable to say yes in the face of the outrageous number of cases where the endogenous ligand activates its receptor. This is the basis of physiology: the agonist triggers a physiological response which disappears with the dissociation of the agonist/receptor pair, often consecutive to internalization of the membrane complex. There are obviously negative feedback processes themselves under the guidance of another agonist. However, everything is not so dichotomous. Indeed, agouti-related protein (AgRP), a neuropeptide synthesized in the arcuate nucleus of the hypothalamus [1] is a competitive endogenous antagonist of the anorexigenic effects of α -melanocyte-stimulating hormone (α -MSH) mediated by melanocortin receptors [2,3]. More recently, liver enriched antimicrobial peptide-2 (LEAP2) has been identified as an endogenous high-affinity antagonist of the growth hormone secretagogue type 1 receptor (GHSR1) [4] which reverses the effects of ghrelin such as its insulinostatic effect [5]. To the best of our knowledge, AgRP and LEAP2 are the only two cases of endogenous GPCR antagonists [6]. Endogenous modulators that act on allosteric sites [7], such as gallamine on the M₂ muscarinic receptor [8] or JR64a on the cannabinoid receptor CB₂ [9], are out of the scope of the present commentary. Ions could bind to multiple GPCRs, such as Na⁺ for adenosine A_{2A} receptor [10] or Zn²⁺ for β_2 -adrenergic receptor [11] for which they could be endogenous modulators as reviewed by van der Westhuizen et al. [7].

This being stated, it is now relatively simple to answer the initial question. Knowledge of the endogenous agonist is mandatory in understanding the molecular mechanisms that orchestrate the physiology but that can also malfunction in pathophysiological conditions. Indeed, the gradual decrease in insulin secretion by deficient pancreatic β -cells leads to diabetes, the absence of circulating leptin due to missense mutations or to a shift in the reading frame of the *ob* gene results in scarce but severe obesity, a deficiency in aromatic L-amino acid decarboxylase, the final enzyme in the biosynthesis of monoaminergic neurotransmitters, leads to serious pathologies linked to the combined lack of mature serotonin and dopamine. This question is still relevant if we are interested in orphan receptors, without current identified endogenous ligand, or conversely in endogenous bioactive molecules whose target remains unknown.

3. Do Alternative Natural Agonists Exist?

We were naïve enough to believe that the endogenous ligand, which has served physiology during millions of years, is certainly the “best” optimized compound at a given receptor. In other words, melatonin is the best agonist for melatonin receptors, and neuropeptide Y (NPY) for NPY receptors. Biogenic amines have identical chemical structure throughout species suggesting that evolution did not find a better molecule. The situation might be a little bit more complex from the receptor point of view, although we showed that melatonin type-1 receptor (MT₁) exhibits a similar molecular pharmacology from hen to human [12], suggesting that evolutive pressure was minimal for these receptors. Indeed, a BLAST analysis shows that human and chicken MT₁ (UniProt access P48039 and P49288, respectively) shares 80.4% sequence similarity. For peptide-liganded GPCRs, the situation is quite different because the sequence of a peptide can diverge among species. However, global analyses have shown that neuropeptides and their cognate receptors have co-evolved over large evolutionary distances to maintain the full functionality of the homologous systems [13,14].

Before going further, let us go back to the truism: melatonin is the best ligand for melatonin receptors. It seems that this concept is almost completely wrong, at least for nonpeptide-liganded GPCRs. Indeed, as displayed in Table 1, some bioaminergic receptors have an extremely weak affinity for their endogenous ligand. For instance, dopamine has a pK_i < 7 at all dopamine receptors, while the affinity of melatonin for its two receptors MT₁ and MT₂ are within the sub-nanomolar range (pK_i between 9 and 10). To complete the picture, serotonin (5-HT) has a pK_i of 8 and 9 at 5-HT₆ and 5-HT_{1A} receptors, respectively, while its affinity is in the 10 micromolar range for 5-HT₄ or 5-HT_{5A} receptors (pK_i ~4–6) [15,16]. Furthermore, histamine has a weak apparent K_i of 3.8 for H₃ receptors [17]. As it seems amazing to have receptors with extremely low affinity for biogenic amines, in the micro to ten millimolar range, one can question the pairing of these molecules with these receptors. Indeed, while one might think that the affinity for its cognate receptor of a blood-borne endogenous agonist reflects its circulating concentration, they might be surprised to learn that the affinities (pK_i) of dopamine for its five receptors range from 4.3 to 7.6 [18] and can reach sub-millimolar concentrations that could never be achieved *in vivo*, excepted in the synaptic space. As a matter of fact, it has been reported that brain concentrations of dopamine in the ventral tegmental area is 4.8 ± 1.5 nM and 0.5 ± 1.5 nM in the red nucleus [19] whereas the basal level of dopamine in the blood is around 30 pg/mL (0.2 nM). In other words, the available quantity of circulating dopamine never reaches a level at which it could activate its extra-synaptic/ectopic receptors. However, it is fair to mention that in the synaptic cleft, the local concentration of dopamine can transiently reach millimolar values [20], after the exocytosis of a single densely packed presynaptic vesicle [21], more consistent with its affinity for its postsynaptic receptors. Noteworthy, receptor subtypes for a same ligand are usually defined on a high degree of sequence homology and to a lesser extent, if never, on pharmacological criteria, even though both often depend on each other. However, we concede that 5-HT has almost no affinity for a receptor outside its family, except that 5-HT has an affinity equivalent to that of dopamine at both D₁ and D₅ receptors [22,23]. The discovery of an endogenous agonist exhibiting a better affinity than that of 5-HT for its less well recognized receptors should lead to revisiting this statement. However, we do not know if this is a current active area of research. Nevertheless, this interplay between these monoaminergic neurotransmitters and the receptors raises many questions, including on the cross-regulation due to heterodimer formations, such as those described for MT₂ and 5-HT_{2C} [24], between MT₁ and the orphan receptor GPR50 [25] or μ-opioid receptor and 5-HT_{1A} [26].

Table 1. Bioaminergic receptors and their agonists.

GPCR	Reference Ligand and pKi	Number of Agonists with Higher Affinities than the Reference Ligand	pKi Max	Number of Agonists with Lower Affinities than the Reference Ligand	pKi Min
5-HT _{1A}	5-HT (pKi 9.5)	10	10.1	62	5.6
5-HT _{1B}	5-HT (pKi 8.1)	18	9.7	42	5.3
5-HT _{1D}	5-HT (pKi 8.5)	28	9.6	28	5.1
5-HT _{1e}	5-HT (pKi 8.1)	2	8.7	32	5.3
5-HT _{1F}	5-HT (pKi 7.2–8.0)	8	9.4	28	5.5
5-HT _{2A}	5-HT (pKi 8.9)	9	9.4	57	5.3
5-HT _{2B}	5-HT (pKi 9.0)	14	9.3	42	5.4
5-HT _{2C}	5-HT (pKi 7.5)	28	9.1	23	5.3
5-HT ₄	5-HT (pKi 7.7–8.6)	13	9.8	17	5.6
5-HT _{5A}	5-HT (pKi 6.8)	2	9.7	8	5.0
5-HT ₆	5-HT (pKi 7.9)	9	8.7	33	5.5
5-HT ₇	5-HT (pKi 9.1)	7	9.9	32	5.3
A ₁	Adenosine (pKi 7.1)	22	10.0	19	4.3
A _{2A}	Adenosine (pKi 6.8)	10	9.3	31	5.0
A _{2B}	Adenosine (pKi 5.3)	3	8.2	11	3.4
A ₃	Adenosine (pKi 6.5)	33	9.7	10	5.2
D ₁	Dopamine (pKi 5.0) *	20	9.1	1	4.3
D ₂	Dopamine (pKi 4.7–7.2)	38	10.2	3	4.7
D ₃	Dopamine (pKi 6.4)	28	10.1	1	6.1
D ₄	Dopamine (pKi 7.6)	8	9.4	7	5.4
D ₅	Dopamine (pKi 6.6) *	5	8.5	12	4.9
H ₁	Histamine (pKi 5.3)	10	6.5	5	3.7
H ₂	Histamine (pKi 3.8)	7	7.2	0	3.8
H ₃	Histamine (pKi 8.0)	25	10.0	6	6.1
H ₄	Histamine (pKi 7.4)	9	8.2	9	5.2
MT ₁	Melatonin (pKi 9.5)	13	10.9	13	5.0
MT ₂	Melatonin (pKi 9.6)	20	12.0	10	5.5

*: 5-HT has an equivalent pKi for these dopamine receptors [22]. This table was built based on the IUPHAR GPCR database (<https://www.guidetopharmacology.org>, accessed on 15 November 2022).

The last-mentioned considerations suggest that it is certainly possible, in some cases, to have better than, or at least as good as, the endogenous agonist. Let us see what else Mother Nature does. At the end of the 19th century, when scientists discovered drugs, it was on the basis of observations on whole animals. The administration of a compound into animals resulted in a change in some spontaneous or induced physiological parameters. The reverse is also true if we consider the effects, in humans, of an insect bite or a snakebite, or the simple contact of a stinging substance caused by a nettle sting. It was the genesis of pharmacology which rapidly appropriated the concept of receptor with the theories, at the time very controversial, of Ehrlich and Langley [27] and subsequently largely complexified with the advent of other concepts which have benefited from the development of cutting-edge techniques of biochemistry, molecular and cellular biology, in order to materialize them.

It is therefore obvious and indisputable that Mother Nature produces substances that activate different types of targets in humans, including GPCRs [28] and that our current knowledge (in ethnopharmacology for instance) is a minute fraction of what one could conceive even considering only the scientific discourses on the depletion or disappearance of biodiversity [29]. We have recently reviewed the molecular diversity in plants [30] like other authors [31,32] and all pharmacologists know that morphine, an alkaloid from poppy, is an agonist of opioid receptors or that caffeine and theophylline are adenosine receptor agonists. The vast majority of natural products acting as GPCR agonists are small molecules, but in recent years, nature-derived peptides have gained significant momentum in this landscape. The animal kingdom is the main supplier of bioactive peptides and, in this context, venomics has fully contributed to this [33,34]. One of the latest resounding examples is that of exendin-4, a 39-amino-acid-long peptide

isolated from the venom of the Gila monster *H. suspectum* [35], which acts as a more potent GLP1R agonist than the endogenous compound, and is long-lasting in the bloodstream [36]. Similarly, peptide P17, derived from the venom of the ant *Tetramorium bicarinatum*, is a potent agonist of the MRGPRX2 receptor [37]. To complete, this is salmon calcitonin, used as a calcitonin receptor agonist, which is prescribed in the treatment of osteoporosis and not human calcitonin. Other peptides, under their fish sequence, may find interest in the near future [38]. David Craik's work also shows that plants provide not only small GPCR agonists such as alkaloids or terpenoids but also complex peptides such as the cyclotide Kalata B7, a vasopressin/oxytocin receptor agonist [39,40]. Let us stop here without mentioning bacteria and *fungi* as sources of GPCR agonists.

Many studies therefore answer the question "does Mother Nature produce GPCR agonists?" but now let us come back to the pending question: what are they used for once discovered? As mentioned above, their main interest lies in their action of supplementation of the endogenous agonist based on possibly more potent, prolonged, more targeted effects (*vide infra*). Indeed, due to their often-complex core chemical structure, the natural products active on GPCRs present a greater chemical diversity and occupy larger regions of the chemical space [31,41], difficult to access by synthetic methods [30], which is often at the origin of a higher selectivity or biased biological outcomes [42,43]. Clearly, the positioning of natural agonists falls somewhere between endogenous agonists and therapeutic agonists with marked drug-likeness profiles [44].

4. Why Search for Synthetic Agonists?

IUPHAR receptor data base is extremely rich in synthetic agonists exhibiting pKi, for some of them, better than that of the corresponding endogenous agonist, suggesting that synthetic agonists were designed on purpose. We document below some of the reasons why those compounds have been designed.

The most obvious is undoubtedly to suggest that chemists designed them to fill a void, the absence of selective agonists. Take the case of the pituitary adenylate cyclase-activating peptide (PACAP) and its three receptors PAC₁, VPAC₁ and VPAC₂ [45]. Since PACAP has a sizable affinity on these three receptors [45], how can one specifically activate a subtype without a dedicated agonist? The alternative agonists find here all their interest. Maxadilan, a potent vasodilator peptide of 61 amino acids isolated from the blood-feeding sand fly *Lutzomyia longipalpis*, is currently considered as a specific and potent natural agonist of PAC₁ receptor [46]. For the VPAC₁ and VPAC₂ receptors, medicinal chemists have been able to develop, through structure–activity relationship studies, specific synthetic agonists based on the vasoactive intestinal peptide (VIP) [Ala^{11,22,28}]VIP [47] and Ro 25-1392 [48], respectively. Conversely, the absence of a specific agonist at MT₁ and MT₂ receptors (most of the disclosed compounds displaying balanced recognitions) leads to the uncomfortable situation in which no molecular tools are available to distinguish in a cellular natural context the action of one of them compared to the other's [49].

The void is not always created by the absence of specific agonists but sometimes by the incompatibility of the available molecules with a preclinical or clinical approach due, for example, to deplorable pharmacodynamic characteristics, more generally grouped under the term weak drug-likeness or low bioavailability. Indeed, a pharmacological tool used *in vitro*, as powerful, and selective as it is, does not make the drug. Medicinal chemists are all familiar with the Lipinski's rule of five which discriminates a bioactive compound from a drug [50]. Furthermore, these rules—apparently against the wishes of the initiator [51]—were frequently invoked to describe the projects from hit to drug. The design of new entities complying with this rule from the structure of natural agonists as leader compounds or *ex nihilo* by structure-based drug design [52] approaches must then be considered. For example, the druggability of GHSR1, physiologically activated by ghrelin, a 23-amino-acid *O*-octanoylated peptide [53], has led to the synthesis of numerous compounds including JMV 1843 [54]. This orally active pseudopeptide stimulates growth hormone secretion by activating GHSR1. It has been approved by the FDA for the diagnosis

of growth hormone deficiency in adults as MacrilenTM [55]. Alternatively, the initial goal may be to find more stable molecule(s) for a sustained action at a given receptor. This is typically the case for melatonin receptor, as melatonin has an alternate profile of expression (at night) and non-expression (at day) [56]. Wanting to activate those receptors in a timely controlled fashion needs selective alternative agonists, as melatonin itself is reported to be responsible of a plethora of actions [57].

Finally, the accessibility of certain natural compounds of major interest for activating a GPCR may be limited due to the protection of certain species, their rarefaction or even their disappearance. The complexity of these molecules, which often contain several asymmetric centers, makes their total chemical synthesis still today unaffordable by the methods available and therefore requires the search for other natural molecules that are easier to synthesize on a large scale or the design and the synthesis of equally potent compounds or antagonists.

Historically, it seems to us that the search for alternative agonists has been essentially pragmatic, and in some scarce occasions, a desired goal. Indeed, we have the feeling—to have experienced those types of situations—that once the screening process is launched, it results, depending on the size of the compound library, in the discovery of ligands at the targeted receptor. Their pharmacological characterization, with ad hoc functional assays, revealed that actually few compounds were full, partial, or inverse agonists while most of them were antagonists. This was a nice case of serendipitous discovery.

In the following sections, some technical approaches are documented to show the tools used to find those agonists, and to exemplify the profound differences between such a task addressing peptidergic or bioaminergic receptors.

5. Finding Synthetic Agonists for Peptidergic Receptors: The Case of Melanin-Concentrating Hormone

Most of endogenous agonist peptides at their cognate receptors are in a molecular mass range above 1500 Da, while most of small molecules are rather in the 300 Da vicinity. This fact bears an important and maybe underestimated feature: it means that the number of structural spots within the ligand is greater for peptides than for smaller ligands. Thus, the number of possible variations in the sequence of the peptide agonist at a given receptor is far larger than for small molecules. Intuitively, this could lead to a better specificity of the agonist peptide than for small molecules which, mechanically, have less contact points within their binding site. It could also suggest that a reasonable modification of the sequence could have negligible impact on the agonist activity and that therefore, designing a peptide antagonist from a peptide agonist is a complicated task. Many examples support this remark; let us mention, among others, the more than 300 analogues of 26RFa that we have synthesized without finding the slightest antagonist [58–62].

The specificity issue is, as always in therapeutics, a key caveat for a drug candidate. Indeed, except in some historical cases of multitarget drugs overly complicated to rationalize (see Section 6.1), a disease is simplified by the default of a given pathway along which the pharmacologist chooses one target to hit (activate, inhibit or silence). This single pathway, impaired along a single protein, would exclude as much as possible the risk of touching another (off-target) protein(s) and would lead to a safe drug that could fix the default if the default is due to a single monogenic failure.

Because of the possibility of altering the original sequence of the endogenous peptide agonist with natural and non-proteinogenic amino acids, literally thousands of analogues can be generated from a single sequence. We have widely used this approach with the ODN [63,64] and the urotensin II [64–68] peptidergic systems and outside the GPCR field with a hundred peptide analogues of the plasma membrane calcium ATPase channel inhibitors named caloxins [69]. This approach illustrates the process: one can modify the natural sequence of the endogenous agonist to generate alternative peptides that are, to a certain extent, more stable than the parent peptide, in particular because they encompass

unnatural residues. It would be vain and pretentious to generalize this observation to all the peptidergic systems.

Melanin-concentrating hormone (MCH) was found as a control of the darkening of fish skin [70], and was discovered in rat pituitary gland [71] and evolved in mammals as a possible control of energetic metabolism [72–74]. A first [75,76], and then a second [77] receptor for this peptide (MCHR₁ and MCHR₂, respectively) were discovered. As such, several dozens of analogues were reported in the literature, including about two hundred from our side [78]. We aimed at (i) understanding the structure–activity relationships through the preparation of a large series of MCH analogues and (ii) trying to derive antagonist(s) from the agonist using a limited number of modifications, believing as a general rule that the specificity of peptide ligands at their respective receptors were more restricted than that of small molecules. Unexpectedly, exploiting NMR data of MCH, we described a first super-agonist for MCHR₁ [79] from which the peptide antagonist prototype S38151 was serendipitously designed. Incidentally, this antagonist turned out to be stable for a short period of time while remaining active on many models of obesity [80]. Finally, a more stable version of S38151, GPS18169, comprising unnatural amino acids, was designed. This later was stable and active in vivo in reference rodent models of obesity [81]. A nice transition from bench to preclinical experiments has been developed in this program over a period of nearly 25 years in which a series of peptides designed on the binding and functionality of an agonist of a cloned receptor was transformed into a promising anti-obesity drug candidate.

6. Finding Synthetic Agonists for Aminergic Receptors: The Case of Bioamines

IUPHAR lists the 5-HT_{1A} agonists (<https://www.guidetopharmacology.org>, accessed on 15 November 2022) by beginning with the tritium-labelled molecules that served to study the binding profiles at this particular receptor: [³H]NLX-112, [³H]8-OH-DPAT and [³H]S15535. Then, other ligands are ranked from the most (S14671, pK_i = 10.5) to the least potent (SEP-363856, pIC₅₀ = 5.6) including S14671, LY293284, 5-CT, lisuride, U92016A, vilazodone, S-14506, roxindole, 5-HT, flesinoxan, L-694,247, S15535, lysergide, RU 24969, flibanserin, 8-OH-DPAT, LY 165,163, spiroxatrine, ipsapirone, FG-5893, pergolide, 7-methoxy-1-naphthylpiperazine, (R)-UH 301, NLX-101, terguride, ziprasidone, S16924, aripiprazole, tandospirone, lurasidone, asenapine, zalospirone, 1-naphthylpiperazine, ocaperidone, bromocriptine, buspirone, vortioxetine, LY334370, BRL-15572, cabergoline, donitriptan, eletriptan, naratriptan, nafadotride, LP-44, frovatriptan, LP-12, BMY-14802, xanomeline, avitriptan, GR 127935, apomorphine, clozapine, EMDT, fluparoxan, LP-211, zolmitriptan, quetiapine, piribedil, rizatriptan, LY344864, SB 216641, CP 93129, capeserod, sumatriptan, quinpirole, olanzapine, vilazodone, BMY-7378, L-772,405, and SEP-363856 [82]. Not less than 76 agonists were inventoried. Most of them present a pK_i for 5-HT_{1A} better than 100 nM, while pK_i of 5-HT itself is in the 9.5 range (1–5 nM).

A close examination of this Prévert-style inventory (i.e., long motley list of items, poetically rendered) leads to a couple of lines of questioning that are worth pointing out.

6.1. The Notion of Specificity

Indeed, some of those agonist compounds have, beside their affinity for the 5-HT_{1A} receptor, a non-negligible affinity for several other receptors. For instance, NLX-112 recognizes 5-HT₄, D₂, D₃, H₁ and the 5-HT reuptake transporter (SERT), lisuride binds to α_{2A} -adrenoceptor, α_{2B} -adrenoceptor, α_{2C} -adrenoceptor, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₆, 5-HT₇, D₂, D₃, D₄, and D₅ whereas bromocriptine has affinity for α_{2A} -adrenoceptor, α_{2B} -adrenoceptor, α_{2C} -adrenoceptor, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₆, 5-HT₇, D₂, D₃, and D₄. Obviously, the last-ranked compounds of the list, those that have a weak affinity for the 5-HT_{1A} receptor, recognize many other targets. These examples illustrate, once again, the lack of selectivity of small molecules compared to that of peptides.

This would have consequences in the use of these compounds in more complex paradigms including “non-cloned”/pure receptors of integrated biological systems. Indeed,

in *in vivo* pharmacological experiments, most of those compounds cannot be used because they target many different receptors, leading to bended results where the consequences of the pairing of the given agonist at its receptor, 5-HT_{1A} herein, cannot be decrypted, bearing in mind that this concerns only the receptors and the targets we know. As a matter of fact, according to a relatively trustable hypothesis, more than 3000 proteins of the human genome are proteins of which we do not know the function(s) of [83]. The fact that GPCRs have a unique sequence signature does not really help since about less than one hundred of the 400 physiological receptors have no known ligands [84], beside the orphan receptors, still waiting for adoption. Of particular note, nothing guarantees that a given agonist at its “cognate” receptor is not a ligand at another one from a different family where it can function as an antagonist. Another aspect of the lack of specificity is that historically, before the rise of cloning, some of those molecules were described as having a given impact onto an altered physiological system. Once the scientists realized that this system was made of several targets, they claimed that the *in vivo* effects of these agonists were due to the exquisite balance between those receptor binding properties; such typical reasoning forms the basis of more recent research, for example the pharmacology of roxindole [85]. The rationalization of those observations remains difficult to obtain.

Specificity, as discussed elsewhere (Leprince and Boutin, *in preparation*), comprises also the notion of “we do not know what we did not test.” Indeed, among the roughly four hundred GPCRs at disposition, only one to two hundred are actually included in those experiments, rendering the notion of specificity/selectivity even more difficult to rationalize. As long as all the targets in the human genome will not be cloned, expressed, evaluated individually or in association with one or more partners and confronted with any compound, no specificity can truly be concluded.

6.2. *The Chemical Lesson of Those Structures*

Even non-chemist scientists would see the chemical diversity of the serotonergic agonists displayed in Figure 1: the bicyclic compounds derived from the 5-HT indole moiety (5-CT, 8-OH-DPAT, 1-naphthylpiperazine or (R)-UH 301), the linear succession of two, three or four aromatic cycles (vilazodone, NLX-112, L-694,247 or flesinoxan), or the compact-fused polycyclic compounds (lysergide, U92016A or LY293284). While the search for alternative melatonin receptor agonists has led to a multi-thousand collection of bicyclic compounds, all closely derived from the original indole moiety [49], the series of 5-HT_{1A} agonists presented above shows a great diversity of chemical structures accelerating the molecular design of new chemical entities, through the study of structure–activity relationships, facilitated since the report of the crystal structure of the human receptor [86]. As a consequence, it is possible with those compounds to accurately map the receptor binding site.

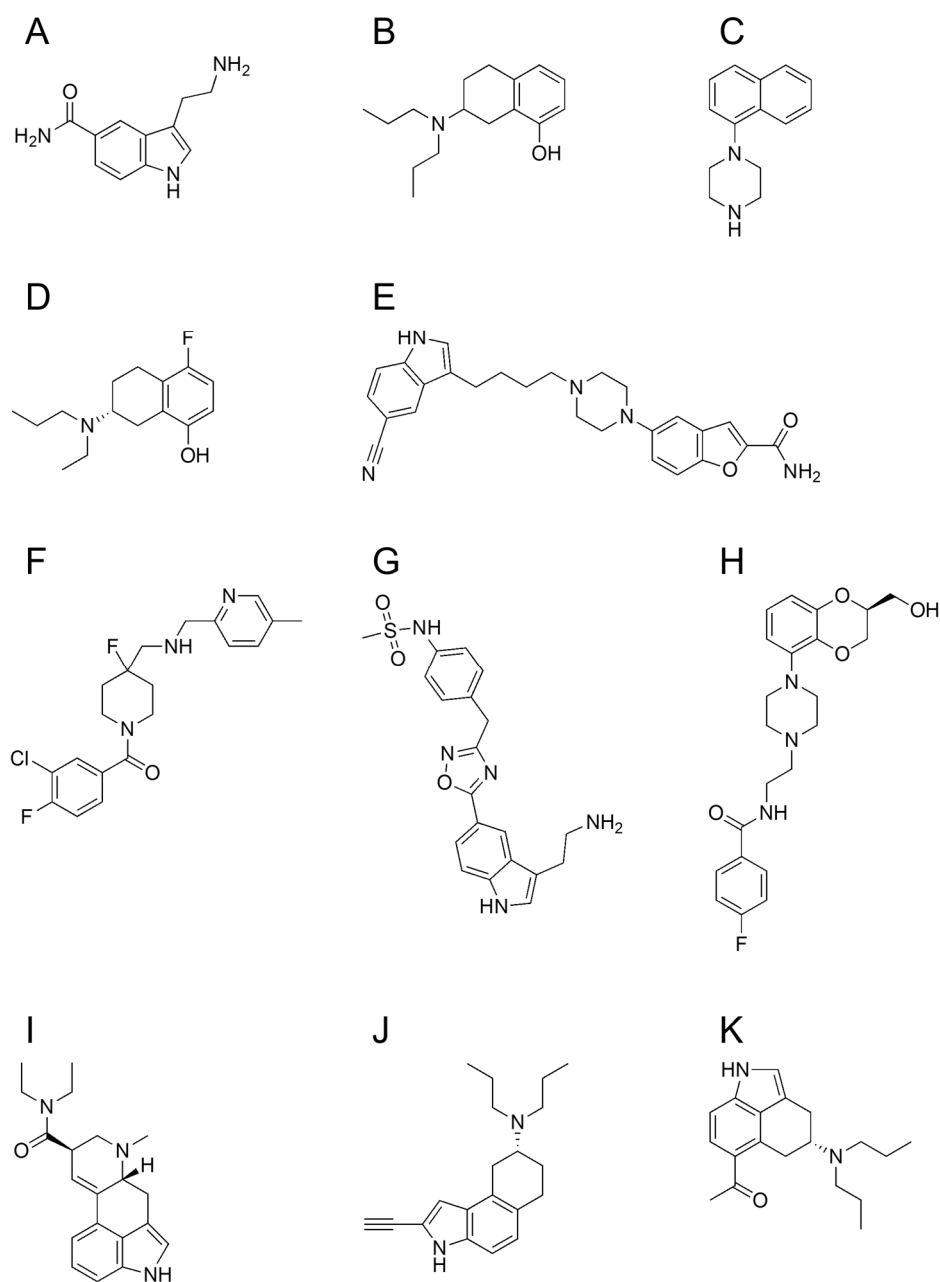


Figure 1. Chemical structures of various serotonergic agonists. (A), 5-CT; (B), 8-OH-DPAT; (C), 1-naphthylpiperazine; (D), (R)-UH 301; (E), vilazodone; (F), NLX-112; (G), L-694,247; (H), flesinoxan; (I), lysergide; (J), U92016A; (K), LY293284.

7. Practical Approaches

It is worth, at this point, refreshing the memory of the reader with the way those compounds are discovered at the bench level, even though some new approaches are rising, based on the *in silico* docking of millions of compounds on the crystallographic structure of a given GPCR. Today, the way to discover or design molecules able to interfere with the membrane-associated proteins called receptors, particularly GPCRs, is very rationalized.

Two technological revolutions have fostered the discovery of new ligands: the high-throughput screening and the miniaturization of assays. The former because it permits to screen literally thousands—if not millions—of compounds on a given assay, including functional and binding assays, and the later because it renders the former financially reachable. As examples, we screened more than a million compounds on the melatonin receptors [87] and others on GPR23 [88] or on μ - and δ -opioid receptors [89] while, thanks

to the availability of some structural features of crystal receptor [83], virtual screening was performed with millions compounds at the same melatonin receptors [90] or at other ones [91]. Of course, the screening and the further discovery for receptor agonists require, by definition, a functional assay compatible with miniature formats [88,89].

A special case of screening, less popular today, is the deorphanization of orphan GPCRs by reverse pharmacology [92–96]. The goal is to find the endogenous agonist of the orphan receptor. Thus, tissue or cell extracts are tested in a functional assay using the cloned candidate orphan receptor in a host co-expressing a “universal” chimeric G-protein (G α 16) which couples most receptors to calcium mobilization [77,97,98] until the total deconvolution of the extract and the characterization of the endogenous ligand [33,99,100]. By extension, this process has been used for the screening of hundreds of endogenous or natural compounds through all kinds of animal extracts (amphibians, insects, venoms, and so on) or more broadly chemical libraries of endogenous or natural compounds. This approach has been very popular in the 1990s, with some significant published successes, but a lot of unpublished (and thus undetermined) complete failures [101].

It is clear to us that one of the end points of the large screening campaigns, including those on melatonin receptors we conducted, was to find new ligands. Firstly, to extend our knowledge on the topology of the binding site of the ligands, and secondly to nourish the ideas of alternative ligands—mostly antagonists—away from the canonical structures of the natural ligand. We already discussed the “*melatonin-like syndrome*” of the 3000ish molecules reported in the melatonergic ligand data base. Indeed, most of those molecules were bicyclic analogues of melatonin, in which the indole scaffold was replaced by almost any of the possible cycles organic chemistry can offer: naphthalene, chromane, quinoline, furopyridine, coumarine, benzothiophene, or more complex moieties such as phenalene, acenaphthene, and so on. The goal was in that case to find alternative agonist ligands [49]. Other receptors suffered the same distorted approach. For instance, a large proportion of adenosine receptor ligands, particularly A_{2A} ligands, are adenosine-based compounds with various decorations, and only a minority of compounds (mainly antagonists) have a structure distanced from the purine core than the majority of them [102]. The situation for peptide liganded receptors is slightly different, as many antagonists derive from the natural ligand such as for angiotensin receptors [103,104], gonadotropin-releasing hormone receptors [105] or MCH receptors even if this passage is not so straightforward (*vide supra*).

8. Characterization of Agonism

Thus, one of the main purposes of screening campaigns is to confront the largest molecular diversity possible with the same target in order to disclose original alternative compounds potentially patentable. Expanding the chemical space also means diversifying biological activity [106]. Indeed, multiple recent discoveries rendered the domain increasingly complex. First the agonist/antagonist duality description was magnified by the discovery of partial agonists or partial antagonists [107] or even, in some cases, inverse agonists [108,109]. Inverse agonists are orthosteric ligands exhibiting the opposite effect of the agonist. For instance, while the binding affinity of ZEL-H16 is in the same range as histamine for H₃ receptor, this non-imidazole compound induces a dose dependent reduction of H₃ activation on HEK293T cells as measured by a CRE-driven luciferase reporter gene assay [110]. Other examples can be found in the literature, like inverse agonists of melatonin receptors [111], or SR48692 for neurotensin receptor 1 [112] among many others [113]. Furthermore, the rise of bias concept [114,115] rendered the receptor picture even more complex. The conformational flexibility of GPCRs suggests that these transmembrane proteins exist in either multiple distinct conformations or as a continuum of states. This also implies that different ligands may stabilize receptor conformations that interact selectively with different G proteins and, by so doing, initiate different signaling cascades. In brief, different agonists—different by their chemical structures—can activate different signaling pathways from the same receptor [116]. Finally, a super-agonist is a ligand that elicits a maximal effect greater than that of the endogenous ligand [117]. Super-

agonists at dopamine receptors have been produced with affinities in the nM range such as A77636 (pKi = 8.7) or SKF-81297 (pKi = 8.7). Interestingly, unlike other super-agonists for other receptors such as iodo-melatonin [116], the structures of these two compounds are extremely loosely related to the dopamine's one (Figure 2). Peptide analogs with potency and/or efficacy superior to the reference peptide have also been designed [118]. How do endogenous agonists fit in this picture [119]?

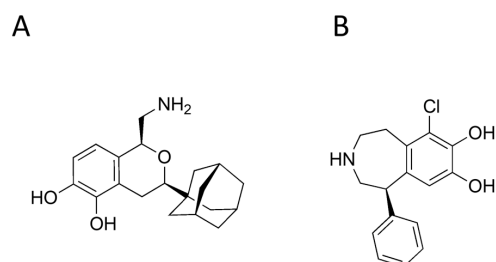


Figure 2. Chemical structures of two dopaminergic super-agonists. (A), A77636; (B), SKF-81297.

The concept of partial agonist applies perfectly to endogenous agonists. Indeed, in addition to NPY, mammalian NPY receptors recognize two other endogenous peptides with strong sequence identity to NPY, peptide YY (PYY) and pancreatic polypeptide (PP) [120]. However, the different receptor subtypes have certain affinity preferences for these three peptides (Table 2) which also affect their activation capacity. As a matter of fact, while PYY is as effective as NPY in mobilizing intracellular calcium in human erythroleukemia cells, PP evokes a weak Ca^{2+} increase and can be classified as a partial agonist of Y_1 -like receptor subtype [121]. The development of specific ligands for each member of the NPY receptor family is a very intensive area of research [122]. Moreover, the concept of bias is also valid for endogenous ligands. GPR120 recognizes several endogenous lipids such as palmitic acid-hydroxy stearic acid (PAHSA) [123]. In pancreatic δ cells, its activity is regulated by two long-chain unsaturated fatty acids, oleic acid (OA) and linoleic acid (LA), which both stimulate paracrine insulin secretion by inhibiting the release of somatostatin [124]. At the molecular level, the Gq, Gi and β -arrestin pathways are known to couple with GPR120, thus generating signaling diversity. OA and LA show similar abilities to PAHSA to trigger the Gi pathway [123] but much weaker on β -arrestin signaling [125]. In particular, LA exhibits a significantly stronger activity and β -arrestin2 than OA which echoes greater potency in promoting β -arrestin2-dependent insulin secretion [124]. The concept of bias is also documented for endogenous peptide ligands. Although calcitonin gene-related peptide, adrenomedullin and adrenomedullin-2 behave as full agonists on cAMP signaling irrespective to the calcitonin-like receptor/RAMP complex expressed in HEK cells, only the cognate agonists elicit full β -arrestin recruitment on their receptors [126]. Similarly, AgRP, the endogenous antagonist of α -MSH-evoked cAMP signaling at MC_3R and MC_4R , exhibits MAPK/ERK signaling agonist activity for the same melanocortin receptors [127]. In contrast, urotensin II and urotensin II-related peptide do not show biased activity on the UT receptor [128]. Characterization or design of biased GPCR agonists is now a promising approach to develop selective drugs with increased efficacy and reduced side effects [129–131]. Interestingly, it has been recently reported that the 26RFa peptide receptor GPR103 [132] is a $\text{G}\alpha_q$ and $\text{G}\alpha_i$ /o-dually coupled receptor and that two naturally occurring mutations Y68H and R371W exhibit distinct but opposite signaling bias, $\text{G}\alpha_q/\text{PLC}/\text{Ca}^{2+}$ and $\text{G}\beta\gamma/\text{PKC}\zeta/\text{ERK1}/2$, respectively [133]. For the obvious reasons given in the Introduction section, the concept of super-agonist cannot be applied to endogenous molecules.

Table 2. Endogenous ligand preference for NPY receptors.

Receptor Subtype	NPY ₁	NPY ₂	NPY ₄	NPY ₅	NPY ₆ *
Endogenous ligand preference	NPY = PYY >> PP	NPY = PYY >> PP	PP >> NPY = PYY	NPY > PYY > PP	NPY = PYY > PP

*: npy6 is a pseudogene. Adapted from Beck-Sickinger et al. [120]

9. Conclusions

In conclusion, the search for endogenous or alternative agonists of GPCRs must be dictated by the finality of the need. Knowledge on the endogenous ligand allows for the study of the physiology of the considered system and potentially explains its pathophysiology. Based on about 350–400 non-olfactory GPCRs including the orphan receptors [134,135], one could theoretically expect as many endogenous ligands. However, a recent study shows that there are more than 520 endogenous ligands in human with only 34 unequivocal pairings, i.e., one receptor for one ligand, and amazing combinations of 17 endogenous ligands for the same receptor and up to 8 different receptors for the same ligand [134]. The discovery of selective natural agonists certainly helps to clarify this situation by increasing the number of pairs with a ligand/receptor ratio of 1:1. These agonists are then important tools to serve the system to finely detail the functioning of these receptors and determine their physiological and pathophysiological involvements. It is obvious that agonists could be useful tools to study phenomena where the receptor(s) are clearly involved or are target for a mechanism which could confer a potential improvement in therapy. Finally, the rational design of highly selective and specific agonists, but also antagonists or more subtle molecules such as biased ligands with therapeutic merit of their own, expands the palette for a complete instrumentalization of these systems. Let us specify, if necessary, that there is no need to know the endogenous ligand to discover an alternative agonist. It therefore seems tempting to us to rule that an endogenous, natural, and synthetic agonist can be qualified as physiological, pharmacological, and therapeutic, respectively.

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Abbreviations

5-HT: serotonin; A₁, adenosine type-1 receptor; AgRP, agouti related peptide; D₁, dopamine type-1 receptor; GHSR₁, growth hormone secretagogue type 1 receptor; GLP1R, glucagon-like peptide 1 receptor; GPCR, G protein-coupled receptor; H₃, histamine type-3 receptor; LA, linoleic acid; LEAP2, liver enriched antimicrobial peptide-2; MC₃R, melanocortin type-3 receptor; MCH, melanin-concentrating hormone; MCHR, melanin-concentrating hormone receptor; MT₁, melatonin type-1 receptor; MT₂, melatonin type-2 receptor; NPY, neuropeptide Y; OA, oleic acid; PACAP, pituitary adenylate cyclase-activating peptide; PAHSA, palmitic acid-hydroxy stearic acid; PP, pancreatic polypeptide; PYY, peptide YY; SERT, serotonin reuptake transporter; VIP, vasoactive intestinal peptide; α -MSH, α -melanocyte-stimulating hormone.

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