The History of N/OFQ and the NOP Receptor

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Abstract

The discovery of nociceptin/orphanin FQ (N/OFQ) marks the genuine start of the reverse pharmacology era, when systematic hunting for ligands of orphan receptors began. The choice of this particular target was no coincidence as the orphan receptor ORL-1 displayed high similarity to known opioid receptors,

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and thus its elusive ligand held promise to find more than a ligand but a missing opioid peptide. N/OFQ indeed turned out to belong to the opioid peptide family, but with significant pharmacological and functional distinctions. The quest for understanding N/OFQ's physiological functions has produced some novel insights into stress regulation and many other body functions but is still ongoing almost 25 years after its discovery. This chapter highlights the early steps of orphan receptor research and some of the protagonists who helped to advance the field.

Keywords
Analgesia · Bioassay · G protein-coupled receptor · Nociceptin/orphanin FQ · Opioid peptide · Orphan receptor · Stress

1 Introduction

In the beginning stood a pivotal question for neuroscience: Do we know all the transmitters in the brain, or are the numerous sequences of orphan receptors suggesting that we are still missing many? When on January 31, 1995, we saw the sequence of orphanin FQ/nociceptin, we had an answer. Preceding this moment, the quest had all the ingredients of science: doubts, rejection, competition, and of course hard work.

The notion that the G protein-coupled receptor (GPCR) family had many more members than expected arose, for us, in 1987 when we used a known GPCR probe to identify related but novel gene sequences by low stringency screening (Bunzow et al. 1988). This observation was greatly supported by the landmark study of Libert et al. (1989), who used degenerate primers for conserved regions of known GPCRs to amplify a series of novel receptor sequences. These "homology cloning" approaches were rapidly applied to identify novel but sequentially closely related GPCRs and led to the discovery of most of the receptors in specific families such as the adrenergic, dopamine, and serotonin receptors. Some had been predicted by pharmacology; many were not. The obvious limitation of this approach was, of course, that only GPCRs for known ligands could be discovered.

However there were also a number of putative GPCRs which did not belong to the known GPCR families. These receptors had obviously not been conserved in the genome without matching ligands, thus immediately suggesting that many more ligands remained to be identified. Since these novel GPCRs stayed "alone" until identification of their cognate ligands, they were termed "orphan receptors."

Until 1995, no truly novel ligand had been identified for any of the growing number of orphan receptors. Using an orphan GPCR as bait to identify its natural ligand from tissue extracts was later termed "the orphan receptor strategy" or "reverse pharmacology," promising the towering prize of discovering an entirely new ligand-receptor-physiology system that might even offer novel therapeutic targets. Few believed it was possible; even fewer tried.

Half way to this goal came the discovery of the first cannabinoid receptor using the plant-derived ligand Δ^9-tetrahydrocannabinol and related cannabinoid compounds as tools (Matsuda et al. 1990). The researchers noticed that certain cell lines and particular brain regions – both previously reported to express cannabinoid binding sites – showed overlapping expression of the novel GPCR. Similar to the homology cloning approaches, this study represents a good example of the so-called matching strategy, using libraries of known ligands together with anatomical information to identify ligands for orphan GPCRs. Most importantly, the discovery of the first cannabinoid receptor opened the door to finding its natural mammalian ligands anandamide and 2-arachidonoylglycerol a few years later (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995), using in essence the orphan receptor strategy but with a synthetic ligand as a critical aide.

2 The "Orphan Receptor Strategy" Launches the Era of "Reverse Pharmacology"

The main steps of the orphan receptor strategy can be summarized as follows:

1. An unknown GPCR sequence with variable homology (high-moderate-none at all) to known GPCRs, including anatomical information about sites of expression.
2. By definition, not even synthetic ligands are available to test expression or functionality. Thus no binding assays are available, and second messenger coupling is unknown or can only be postulated by homology to closely related GPCRs.
3. Heterologous expression of the orphan GPCR produces a cell-based assay tool.
4. Second messenger responses can sometimes be guided to a common readout by co-expression of promiscuous or engineered G proteins, such as $G\alpha_{16}$ or $G\alpha q_{i3}$.
5. Fractionated tissue extracts suspected to contain the natural ligand(s) are tested for specific activity at orphan GPCR-expressing cells vs. non-transfected cells.
6. Purification of activity to (near) homogeneity and determination of its structure by physicochemical methods.

It is easily conceivable that this strategy contains many unknowns. For example, functional expression of the orphan GPCR cannot be verified in the absence of any ligand. Tagging of receptor proteins at either the N- or C-terminus carries the risk of accidental interference with functionality. The presence of a natural ligand in a given tissue cannot always be inferred by anatomical vicinity, especially for GPCRs mainly expressed in peripheral tissues. And finally, the chemical nature of the sought-after ligand can only be predicted for orphan GPCRs with closely related family members. In addition, tissue content of highly potent ligands that naturally act in the nanomolar range can be incredibly low, challenging the detection limits of even the most advanced analytical methods. Considering this long list of uncertainties, "deorphanizing" an orphan receptor was an extremely high-risk

project which needed to be carried out in a scientific environment that was not risk-averse. Consequently, most of the pioneering breakthroughs on orphan receptors were made in the pharmaceutical industry as well as the European and Japanese university systems, which are less dependent on short-term funding cycles.

3 The Quest for the Endogenous Ligand of ORL1 (and Other Orphan GPCRs)

3.1 Uncertainties Setting the Stage

Out of all the uncertainties that we faced in 1993, the one we were most concerned with was the issue of predicting the second messenger response of a GPCR. There were no generally applicable rules, as it is still now. There were no automated activity measurement tools. There was, however, an instrument that monitored pH changes (lactic acid, bicarbonate) around cultured cells, called the "microphysiometer," which in principle should be able to monitor any second messenger response, since GPCR activation "consumes" energy leading to increased cellular metabolism. Using this "general" assay tool, we embarked on searching for the ligands of several orphan GPCRs, which included a novel opioid receptor, GPR7 and 8, and a number of GPCRs with poor homology to any known family members.

The stage for the first successful isolation of a natural ligand for an orphan GPCR was set in 1994 when numerous groups reported the cloning of a fourth member of the opioid receptor family that did not bind any natural or synthetic opioid ligands at reasonable concentrations (Mollereau et al. 1994; Bunzow et al. 1994; Chen et al. 1994; Fukuda et al. 1994; Wang et al. 1994; Lachowicz et al. 1995). The three main subtypes of opioid receptors (mu, delta, and kappa) had just been cloned 2 years earlier (Kieffer et al. 1992; Evans et al. 1992; Yasuda et al. 1993; Chen et al. 1993). Given the inherent fascination and long history of opioid research (starting with Sertürner 1806) together with the untypically large research community in this field, it was even more surprising that a fourth opioid receptor had eluded discovery for so long. The many simultaneous reports of this unexpected opioid receptor immediately produced a Babylonian multiplicity in nomenclature. For simplicity reasons, the term ORL1 (for "opioid receptor-like") proposed in the first report by Mollereau and colleagues should serve as a synonym. Efforts to match ORL1 to previously postulated opioid receptor subtypes, such as an enigmatic kappa3 subtype, contained little convincing evidence (Pan et al. 1995), so that ORL1 remained a scientific and intellectual challenge.

3.2 In the Eye of the Storm: It Is Back to cAMP

During the annual meeting of the Society for Neuroscience in Miami in the fall of 1994, a perplexed opioid research community presented more than ten posters on ORL1 without an answer about its natural ligand. On a memorable evening in the

midst of a tropical storm, the first author of this article who had attempted to deorphanize ORL1 as well GPR7 and 8 using the microphysiometer (with little success and major technical obstacles) came to the conclusion that it should be possible to find the ligand of ORL1 by monitoring inhibition of adenylyl cyclase in analogy to all the other opioid receptors. That launched the project back. Fortunately we were at the time in the CNS Department of Hoffmann-La Roche in Basel, Switzerland, which would not resist at providing the funds necessary to carry out such a screening project using numerous and expensive cAMP assays.

In the case of ORL1, its high similarity to the three known opioid receptors held a few advantages that increased the likelihood of success for finding its natural ligand. First of all, the ligand should be a peptide in analogy to all other endogenous opioids. Second, as presented above, the receptor was likely coupling to G_i-type G proteins, thus predicting an inhibition of adenylate cyclase and consequently inhibition of cAMP accumulation. Third, the endogenous ligand was most likely synthesized in the brain, in particular the hypothalamus, as this brain region showed highest levels of ORL1 expression. We could therefore devise a purification strategy that was based on traditional protocols for peptide isolation, which had been developed in the 1970s and 1980s. Nevertheless, peptides are known to occur at notoriously low quantities, even in enriched preparations.

3.3 The Isolation

We started with collecting a large amount (close to 10 kg) of porcine hypothalamic tissue at the local Basel slaughterhouse. Special thanks for this effort goes to Robert A. Henningsen, who overcame more than one natural inhibition during that long morning and the ensuing isolation. A batch of 4.5 kg porcine hypothalamic tissue was frozen and then homogenized in acetic acid using a kitchen blender. The combined supernatants were supposed to contain all soluble material, including peptides, and we further enriched peptides by batch adsorption on C_{18} reversed-phase silica. This step also depleted most small and highly water-soluble molecules while irreversibly trapping lipids on the reversed-phase matrix. The concentrated peptide extract then underwent the first fractionation using preparative cation-exchange chromatography. Since almost all natural peptides carry at least one positive charge under mildly acidic conditions, we employed a gradient of increasing salt for separating differently charged molecules. Due to the inherent chemical complexity of the crude homogenates or even the enriched peptide concentrate, it was not possible to test any of the previous steps for biological activity that would indicate an ORL1-activating molecule. Only at the stage of well-separated cation-exchange fractions the first and most critical proof-of-concept could be obtained in a functional ORL1 assay. Using small aliquots, we monitored inhibition of cAMP accumulation in cells stably expressing ORL1 and wildtype cells as controls. Positive controls for the presence of endogenous opioid peptides were kappa opioid receptor (KOR)-expressing cells. After a few pilot experiments, we noticed that ORL1-specific activity was found only in fractions eluting at high

salt concentrations, indicating a peptide carrying multiple positive charges. These fractions also contained dynorphin-like material as they robustly activated KOR-expressing cells.

ORL1-specific activity "survived" when we further purified the cation-exchange fractions by reversed-phase HPLC and remained intact during a reluctant Christmas break. Using a total of five reversed-phase purification steps, a single peak was finally isolated that contained the only biological activity from porcine brain to produce profound inhibition of cAMP accumulation in ORL1-expressing cells. Fortunately, the isolated amount was more than sufficient for Sanger peptide sequencing, as we later calculated that we had purified 200 pmol of active peptide. When we saw the sequence on January 31, 1995, we immediately knew that we had not only found a ligand for ORL1 but also the missing fourth member of the opioid peptide family.

4 The Novel Opioid Peptide from Basel...

All natural opioid peptides start with the canonical sequence YGGF (Tyr-Gly-Gly-Phe), and this motif is considered to be critically required for opioid receptor activity, with highest stringency for the amino terminal tyrosine residue (Fig. 1). Instead, the new peptide sequence started with FGGF (Phe-Gly-Gly-Phe), or in other words, one single oxygen as the difference between phenylalanine and tyrosine. The evolutionary relatedness to the known opioid peptides is obvious, while the subtle deviation from the conserved opioid motif immediately offers an intuitive explanation for the pharmacological separation. Structure-activity studies later confirmed our early hypotheses: This ligand still looks like an opioid peptide but is pharmacologically distinct, founded in its structure. Included in this thought is another important postulate: There must be a biological reason for the separation from classical opioids.

Because of its ancestry and structural features, we termed this peptide "orphanin FQ," marking its relation to a former orphan receptor and its first and last amino acids as unique identifiers. The naming was a courageous guess, since we did not know at the time that the first and last amino acids of this peptide are indeed conserved across all vertebrate animals (Sundström et al. 2010). Later

F**GGF**TGAR**KS**AR**KL**A**NQ**	Nociceptin/Orphanin FQ
Y**GGF**LRRIRPKL**K**WD**NQ**	Dynorphin A
Y**GGF**L	Leu-Enkephalin
Y**GGF**MTSE**KS**QTP**L**VTLFKNAIIKNAYKKGE	β-Endorphin

Fig. 1 Sequence alignment of natural opioid peptides (human). Identical amino acids between N/OFQ and classical opioid peptides are highlighted in bold

structure-activity relationship studies identified the structural components that provide and ensure functional separation between the classical opioids and this fourth member of the ligand family (Reinscheid et al. 1996, 1998; Shimohigashi et al. 1996; Mollereau et al. 1999). In one of the first experiments following our discovery, we observed that changing the N-terminal phenylalanine to tyrosine was not sufficient to render orphanin FQ into a functional opioid ligand, as Tyr^1-orphanin FQ was unable to activate classical opioid receptors while remaining a full agonist at ORL1.

5 ... Is Simultaneously Discovered in Toulouse

As is often the case in science, you are never alone with a good idea for long. In June 1995, we learned about an upcoming presentation at the International Narcotics Research Conference (INRC) that announced the identification of an endogenous ligand for ORL1. At the meeting, a team consisting of the group of Jean-Claude Meunier from the University of Toulouse, France, and the group of Gilbert Vassart from the University of Brussels, Belgium, presented data showing that they had isolated a peptide ligand for ORL1 from rat brain. They named their peptide "nociceptin" since they had early evidence that the novel transmitter was producing hyperalgesia-like behaviors in vivo. Although they did not show the sequence (since their manuscript was still under review), one of their graphs showed that a Tyr^1-nociceptin analogue had equal potency as the native peptide. This detail told us that we had found the same sequence.

6 Race to the Finish Line

What followed was a frantic race to the finish line by both teams: As an example, the first complete version of our later paper in Science was written in a single night in June 1995. Since our discovery in January, we had accumulated data about tissue distribution, initiated extensive structure-activity studies, launched a project to clone the orphanin FQ precursor protein (which took until September, after submission and acceptance of our manuscript), and, importantly, collected the first in vivo data about behavioral responses. We found that central administration of orphanin FQ profoundly reduced locomotor activity in rats. More importantly, we also saw an apparent increase in pain responsiveness after central orphanin FQ administration, similar to the data reported at INRC. However, we opted against naming the new peptide after a physiological effect since we could not exclude that later investigations might discover a more dominant or entirely different function (there are some examples in the orphan receptor field where a first-glance functional effect of a newly discovered ligand was used for naming but later turned out to be less important). The multiplicity of names, however, has remained to this date, as both reports appeared almost simultaneously in October and November of 1995. Meunier's paper in Nature beat ours in Science by 3 weeks (Meunier et al. 1995; Reinscheid et al. 1995). Since then, the novel peptide has been alternatingly referred

to as nociceptin/orphanin FQ (N/OFQ) or orphanin FQ/nociceptin (OFQ/N). For the remainder of this text, we will refer to the natural ligand of ORL1 as N/OFQ, giving credit to the earlier publication date of the paper by Jean-Claude Meunier's team. It is also important to mention that a third team around Seiji Itoh at Kansai Medical University in Japan successfully isolated the endogenous ligand of ORL1 from bovine brain at the time of the first two publications (Okuda-Ashitaka et al. 1996).

7 Early Steps to Uncover the Physiological Functions of N/OFQ

Surprisingly, and although both teams came from a background of opioid research, both original publications lacked an important control experiment in their studies on nociceptive effects of N/OFQ: There were no uninjected control animals correcting for the effects of intracerebroventricular (ICV) injections on basal pain perception. If we and Meunier's team had included such animals, we both would have noticed that ICV injections alone produce profound stress-induced analgesia, an effect well-known in the field. Instead of causing pronociceptive effects, N/OFQ merely reversed this procedure-induced analgesia, as later studies demonstrated (Mogil et al. 1996). Rather than modulating pain sensitivity on its own, central N/OFQ reversed a number of stress-related behavioral effects, including most notably anxiety and fear responses (Jenck et al. 1997; Köster et al. 1999). Since stress is a natural trigger for release of endogenous opioid peptides, N/OFQ can indeed be viewed as a functional anti-opioid peptide as it reverses the initial protective analgesic effects of classical opioid peptides. At the same time, N/OFQ produces profound anxiolysis that may be required to initiate defensive behaviors in situations of severe stress. In fact, the reversal of some opioid effects may constitute the physiological reason for the pharmacological separation of classical opioids from the N/OFQ system. But they all serve the same goal: to preserve the individual's ability to respond to a potentially life-threatening challenge.

8 Hopes for Clinical Applications

It is probably the dream of every neuroscientist to discover a new transmitter in the brain. To discover an endogenous opioid peptide has essentially happened only four times in history, and we feel honored to have been part of this scientific milestone. But part of our dreams was also the hope to see new therapeutic drugs being developed based on our discovery. Since our work occurred in the midst of a large pharmaceutical company, it was probably the first time in history that a drug discovery program was launched even before publication of the target. Synthetic ORL1 agonists with potent anxiolytic and anti-stress profiles were indeed identified in preclinical research efforts (Wichmann et al. 1999; Jenck et al. 2000; Ciccocioppo et al. 2002), but unfortunately never went into clinical trials, despite their lack of reinforcing effects in contrast to the prototypical benzodiazepine anxiolytics. In the

meantime, potential applications have also emerged for ORL1 antagonists as possible adjuvants during chronic morphine therapy in order to prevent or attenuate development of analgesic tolerance (Ueda et al. 1997, 2000; Lutfy et al. 2001; Chung et al. 2006). However, none of these promising targets has been pursued in clinical trials thus far. More progress has been made on the somewhat unexpected finding that ORL1 antagonists can produce antidepressant-like effects. Early studies in animal models (Gavioli et al. 2003, 2004; Gavioli and Calo 2013) were recently followed up by the first human clinical trials with promising results (Post et al. 2016). More recently, renewed interest in the N/OFQ system has been resurrected by identification of bifunctional compounds such as cebranopadol that target both mu-opioid receptors and ORL1 (recently renamed by IUPHAR into "NOP receptor," standing for "nociceptin/orphanin FQ peptide receptor") to produce analgesia in chronic pain conditions but with limited abuse liabilities (Linz et al. 2014; Günther et al. 2018). Results from phase II clinical trials with cebranopadol appear promising (Scholz et al. 2018), and we hope that one not too-distant day real patients will ultimately benefit from our work.

9 Reverse Pharmacology Success Stories

In the end, it was possible to find the natural ligand of an orphan GPCR, against all the odds and doubts. Since 1995, numerous ligands for other orphan GPCRs have been discovered, using the orphan receptor strategy. Most productive and successful proved to be a team around Shuji Hinuma and Masahiko Fujino at Takeda Pharmaceuticals in Tsukuba, Japan, who discovered more than a dozen of new ligands for orphan receptors (Hinuma et al. 1998, 2000; Tatemoto et al. 1998; Shimomura et al. 1999; Mori et al. 1999; Fujii et al. 2000, 2002; Ohtaki et al. 2001; Masuda et al. 2002; Kawamata et al. 2003; Itoh et al. 2003; Fukusumi et al. 2003; Sugo et al. 2003; Shinohara et al. 2004). Other big successes were the isolation of ghrelin (Kojima et al. 1999) as a major regulator of food intake and the discovery of the orexins/hypocretins (de Lecea et al. 1998; Sakurai et al. 1998) together with their genetic link to narcolepsy (Chemelli et al. 1999; Lin et al. 1999). The orexin/hypocretin system is currently the first and only example of a former orphan GPCR with a drug on the market. Since 2015, the nonselective orexin/hypocretin receptor 1/2 antagonist suvorexant is marketed as a treatment for insomnia under the name of Belsomra®. More examples are certainly going to follow, as drug development speed is lagging notoriously far behind basic science.

10 Conclusion

This should serve as a final remark: Risk taking and tropical storms can have benefits, some even long lasting.

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