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Mei-Chuan Ko
Girolamo Caló *Editors*

The Nociceptin/ Orphanin FQ Peptide Receptor

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In memory of Professor Domenico Regoli,
an outstanding scientist and a great friend

Preface

Since 1994, when the nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor (aka, opioid receptor like 1 (ORL1) receptor) was discovered, there has been tremendous effort to study the N/OFQ-NOP receptor system. The current volume in Springer's renowned series of *Handbook of Experimental Pharmacology* highlights ongoing and exciting research in the NOP receptor field and compiles all pivotal findings from key investigators in the past 25 years.

This volume includes 19 chapters, which are divided into three parts. Part I introduces readers with the discovery of N/OFQ and the NOP receptor, fascinating pharmacological tools of peptide and nonpeptide nature, to ligands with mixed NOP/opioid receptor agonist activities and a vast range of assays for studying the NOP receptor pharmacological profiles. In addition, the electrophysiological actions of N/OFQ, the NOP receptor signaling cascades, and the state-dependent changes in the gene expression of this ligand-receptor system are elaborated. Part II provides readers with pleiotropic effects of NOP-related ligands in animal models. Targeting NOP receptors can modulate pain, substance abuse, Parkinson's disease, anxiety, mood disorders, memory, food intake, immune and respiratory functions. Translational aspects of NOP-related ligands are discussed in nonhuman primate models in terms of their therapeutic potential for pain and substance use disorders. Part III advances exciting preclinical findings of NOP-related ligands into clinical contexts. In particular, Rec 0438 (aka, UFP-112) for treating overactive bladder, cebranopadol as a potent analgesic with an improved side effect profile, and BTRX-246040 (aka, LY2940094) as a treatment for major depressive disorder and alcohol use disorder are encouraging.

We would like to thank Dr. James Barrett, Editor in Chief of the *Handbook of Experimental Pharmacology*, for contacting us to prepare this volume. We thank Susanne Dathe, Anand Venkatachalam, and M. Rajasekar from Springer for overseeing the production of this volume. Finally, we would like to thank all NOP receptor researchers who have persevered and collectively contributed to this volume which substantiates the biological functions and pharmacology of the NOP receptor, and their therapeutic applications. It is our hope that this

timely volume with a comprehensive scope will stimulate more research and development of NOP-related ligands in the next decade and eventually advance human medicine.

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The History of N/OFQ and the NOP Receptor

Rainer K. Reinscheid and Olivier Civelli

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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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NOP-Targeted Peptide Ligands

Delia Preti, Girolamo Caló, and Remo Guerrini

Contents

Abstract

The nociceptin/orphanin FQ (N/OFQ)-N/OFQ peptide (NOP) receptor system is widely distributed at both the peripheral and central level where it modulates important biological functions with increasing therapeutic implications. This chapter wants to provide a comprehensive and updated overview focused on the available structure–activity relationship studies on NOP receptor peptide ligands developed through different rational approaches. Punctual modifications and cyclizations of the N/OFQ sequence have been properly combined furnishing potent NOP selective ligands with different pharmacological activities (full and partial agonists, pure antagonists) and enhanced metabolic stability in vivo. The screening of peptide libraries provided a second family of NOP ligands that have been successfully optimized. Moreover, recent findings suggest the possibility to

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apply different multimerization strategies for the realization of multi-target NOP/opioid receptor ligands or tetrabranched N/OFQ derivatives with extraordinarily prolonged duration of action in vivo. The diverse approaches led to the identification of important pharmacological tools along with drug candidates currently in clinical development such as Rec 0438 (aka UFP-112) for the treatment of overactive bladder and SER 100 (aka ZP120) for the clinical management of systolic hypertension.

Keywords
Nociceptin/orphanin FQ · NOP · Peptide ligands · SAR studies

1 Introduction

Nociceptin/orphanin FQ (N/OFQ; FGGFTGARKSARKLANQ, see Fig. 1) shows significant similarities in the primary sequence with other endogenous peptides of the opioid family of which dynorphin A can be considered its closer analogue (Calo' and Guerrini 2013). Nevertheless, due to its unique structure, N/OFQ is unable to interact with classical opioid receptors (MOP, DOP, and KOP), as well as opioid peptides are unable to bind the N/OFQ peptide (NOP) receptor. Typically, the first four N-terminal residues of the heptadecapeptide sequence of N/OFQ (FGGF) represent the "message" domain responsible for NOP activation, while the "address" fragment is composed of the last C-terminal residues (7–17) and promotes NOP binding affinity and receptor selectivity (Calo' et al. 2013; Mustazza et al. 2018). The central dipeptide Thr^5Gly^6 constitutes a hinge region between message and address sequences. The message tetrapeptide of N/OFQ is clearly superimposable to the canonical YGGF N-terminus of mammalial opioid peptides. Thus, the simple Tyr^1/Phe^1 replacement is fundamental for N/OFQ selectivity being able to preclude NOP/opioid receptors cross activation (Calo' et al. 2013). A series of crystallographic studies performed with NOP (Thompson et al. 2012) and classical opioid receptors (Wu et al. 2012; Manglik et al. 2012; Granier et al. 2012) in complex with specific antagonists suggested that the Phe^1 phenyl ring of N/OFQ would face a hydrophobic region of the receptor binding pocket while the phenolic function of Tyr^1 in opioid peptides would be involved in a hydrogen bond network with the conserved His residue present in position 52 of TM VI of opioid receptors.

Truncation studies on N/OFQ indicated the exclusive possibility to shorten the C-terminal portion up to the identification of N/OFQ(1–13) as the minimal active sequence (Calo' et al. 1996; Dooley and Houghten 1996). Moreover, C-terminal amidation proved to reduce the susceptibility to carboxypeptidases of both N/OFQ and N/OFQ(1–13) (Guerrini et al. 1997). It has to be remarked that, according to NMR studies, the C-terminal "address" portion of N/OFQ would assume a typical alpha helix conformation in physiological conditions (Orsini et al. 2005; Tancredi et al. 2005). This region is characterized by the presence of two couples of Arg-Lys dipeptide units at 8–9 and 12–13 positions, which are important for the affinity/selectivity of the peptide because of the capability to promote the α-helix bioactive

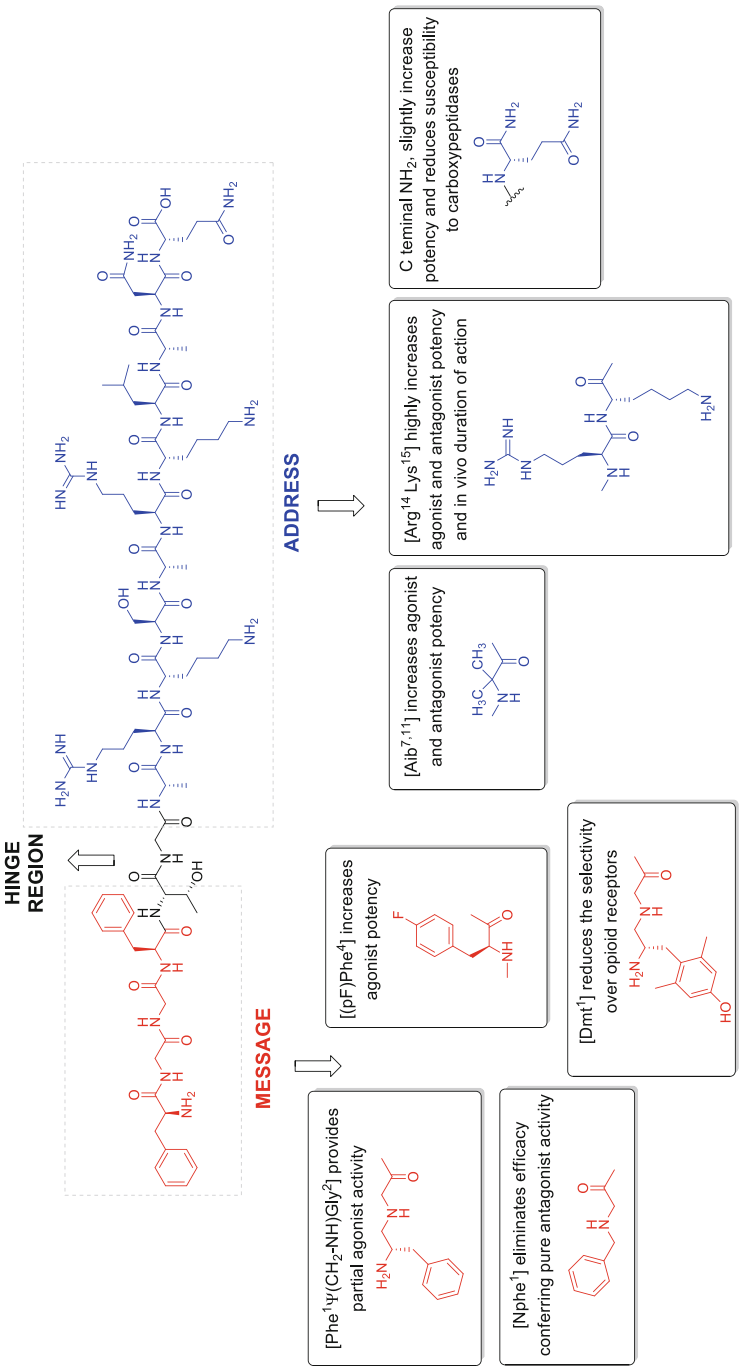


Fig. 1 Structure–activity relationship studies on the native sequence of N/OFQ

motif as well as to interact with negative residues in the second extracellular loop of the NOP receptor (Daga and Zaveri 2012).

Advances in the pharmacology and medicinal chemistry of the N/OFQ-NOP system have been recently reviewed by different authors (Toll et al. 2016; Mustazza et al. 2018; Zaveri 2016). This chapter wants to provide a comprehensive and updated overview focused on SAR studies on NOP receptor peptide ligands that have been thus far developed following different rational approaches.

2 Structure–Activity Relationship Studies on N/OFQ-Related Peptides

2.1 Linear N/OFQ Derivatives

The key structural modifications applied to N/OFQ sequence for generating useful NOP peptide ligands have been summarized in Fig. 1. Phe^1 is one of the most essential residues for NOP binding/activation/selectivity (Calo' et al. 2013). The saturation of the Phe^1 benzyl moiety (Cha^1) and the replacement of Phe^1 with a Leu^1 are highly tolerated while Ala^1 derivatives are essentially inactive. In addition, the inversion of the configuration of Phe^1 (D-Phe^1) abolished the activity. This suggests that a bulky and lipophilic side chain at the first residue is required while aromaticity is not mandatory for receptor binding. The N-terminal residue seems to be of key relevance for ligand efficacy if considering that the reduction of the peptide bond between Phe^1 and Gly^2 [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$] or the shift of the benzyl side chain to the N-terminal nitrogen ($Nphe^1$) led to partial agonism (i.e., [F/G]N/OFQ(1–13)NH_2) or antagonism (i.e., [$Nphe^1$]N/OFQ(1–13)-NH_2), respectively (see Table 1) (Calo' et al. 2013; Toll et al. 2016). Of note, a significant loss of selectivity over opioid receptors was observed when Phe^1 has been replaced with Tyr^1 that was even more evident with the introduction of a 2,6-dimethyltyrosine (Dmt) residue at the same position (Molinari et al. 2013). This led to the identification of [Dmt^1]N/OFQ (1–13)-NH_2 and [Dmt^1]N/OFQ-NH_2 as mixed NOP/opioid agonists with an interesting potential as innovative spinal analgesics. In contrast, the replacement of Phe^1 with an amino phosphonate moiety in N/OFQ(1–13)NH_2 resulted in low potency though NOP selective agonists (Todorov et al. 2012).

Unlike other opioid ligands, N/OFQ is particularly sensitive to substitutions at the Gly^2-Gly^3 dipeptide spacer. X-ray analysis (Thompson et al. 2012) and docking studies (Daga and Zaveri 2012) confirmed the importance of both the composition and length of the Gly^2-Gly^3 unit that imposes the right distance between Phe^1 and Phe^4 and confers high conformation flexibility. This allows the N-terminal nitrogen atom of the message tetrapeptide to establish an ionic interaction with the Asp^{130} of the NOP receptor (Toll et al. 2016). Different substitutions have been also performed at the Phe^4 residue that is critical for NOP binding/activation (Guerrini et al. 2001). Noteworthy, the potency of the endogenous peptide was significantly enhanced with the introduction of electron withdrawing moieties (especially a fluorine atom) at the para-position of the Phe^4 phenyl ring (Guerrini et al. 2001; McDonald et al. 2002).

Table 1 In vitro biological activity of N/OFQ-related peptide ligands

	Human NOP			Mouse NOP	
		Functional potency		Functional potency	
	Binding affinity	[^{35}S] GTPγS	Ca^{2+}	mVD	Reference
NOP agonists	pK_i	pEC_{50}	pEC_{50}	pEC_{50}	
N/OFQ	9.91	8.75	9.54	7.47	McDonald et al. (2003), Camarda et al. (2009), and Toll et al. (2016)
N/OFQ(1–13)-NH_2	10.24	9.28	9.30	7.40	McDonald et al. (2003), Camarda et al. (2009), and Toll et al. (2016)
[Dmt^1]N/OFQ(1–13)-NH_2	10.59	9.46	8.94	ND	Molinari et al. (2013) and Cerlesi et al. (2017)
[(pF)Phe^4]N/OFQ (1–13)-NH_2	9.40	9.55	nd	8.19	Guerrini et al. (2001) and McDonald et al. (2002)
[Arg^{14}Lys^{15}]N/OFQ	9.49	9.85	9.56	8.93	Okada et al. (2000), Rizzi et al. (2002a), and Camarda et al. (2009)
UFP-112	10.55	10.55	9.05	9.24	Arduin et al. (2007) and Camarda et al. (2009)
NOP partial agonists	pK_i	pEC_{50}	pEC_{50}	pEC_{50}	
[F/G]N/OFQ(1–13)-NH_2	9.27	8.05	8.03	Slight transient effect	Wright et al. (2003) and Camarda et al. (2009)
UFP-113	10.26	9.73	7.97	Variable effects	Arduin et al. (2007), Camarda et al. (2009)
NOP antagonists	pK_i	pA_2	pA_2	pA_2	
[Nphe^1]N/OFQ(1–13)-NH_2	8.39	7.33	6.29	6.04	McDonald et al. (2003), Camarda et al. (2009), and Calo' et al. (2000)
UFP-101	10.24	8.85	7.66	7.29	McDonald et al. (2003), Camarda et al. (2009), and Calo' et al. (2002)

[^{35}S]GTPγS: [^{35}S]GTPγS binding in membranes from CHO cells expressing the human NOP; Ca^{2+}: calcium mobilization in CHO cells coexpressing the human NOP and the $G\alpha_{qi5}$ chimeric protein; mVD electrically stimulated mouse vas deferens; pEC_{50}: agonist potency; pA_2/pK_B: antagonist potency; UFP-112: [(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2; [F/G]N/OFQ(1–13)-NH_2: [Phe$^1\Psi$(CH_2-NH)Gly^2]N/OFQ(1–13)-NH_2; UFP-113: [Phe$^1\Psi$(CH_2-NH)Gly^2(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2; UFP-101: [Nphe^{1}Arg^{14}Lys^{15}]N/OFQ-NH_2

A series of N/OFQ(1–13)-NH_2 analogues in which the Thr^5 residue was substituted with both natural and nonnatural amino acids has been recently investigated (Guerrini et al. 2015). These analogues behaved as NOP full agonists with highly variable potency thus suggesting that Thr^5 would contribute to the binding to the receptor more than to its activation. Yet, neither the size of X^5 side chain nor its lipo/hydrophilic nature and hydrogen bond capability seemed of significant relevance for receptor binding. In particular, the simple removal of the side chain hydroxyl function of Thr^5 ([Abu^5]N/OFQ(1–13)-NH_2) determined a substantial preservation of NOP activity.

The effect of various modifications of the C-terminal motif of N/OFQ and N/OFQ (1–13)-NH_2 has been also investigated (Calo' et al. 2013). Notably, a highly potent NOP agonist was obtained when a third Arg-Lys couple was introduced at the 14–15 positions of the native peptide sequence (Okada et al. 2000). [$Arg^{14}Lys^{15}$]N/OFQ displayed indeed higher binding affinity (threefold) and potency (17-fold) compared to N/OFQ. Similar results have been achieved with the introduction at the same positions of different combinations of positively charged residues such as Lys-Arg, Lys-Lys, and Arg-Arg (Okada et al. 2008). On the contrary, when a single positive residue was introduced in either 14 or 15 position, a moderate enhancement of binding and biological activity was observed. In addition, with the aim to investigate the importance of the C-terminal secondary structure, both alpha helix inducers (Aib, alpha-aminoisobutyric acid) and breakers (Pro) have been alternatively introduced at key positions of the address domain (Zhang et al. 2012; Tancredi et al. 2005). Aib^7, Aib^{11}, and Aib^{15} peptide derivatives were significantly more potent than the native ligand. On the other hand, Pro^5, Pro^6, Pro^7, and Pro^{11} substitutions severely compromised the activity of the endogenous peptide.

Finally, Thr^5 or Ser^{10} have been scrutinized as possible glycosylation sites of N/OFQ-related peptides (Biondi et al. 2006; Arsequell et al. 2011). These investigations agree in the identification of Ser^{10} as preferred anchoring point for a monosaccharide unit. Of the reported compounds, [Ser^{10}-O-α-D-GalNAc]-N/OFQ exhibited a pK_i value of 8.42 in competition binding experiments with similar affinity as N/OFQ (Arsequell et al. 2011). A NMR analysis performed in membrane mimicking environments indicated that, unlike for Thr^5 derivatives, Ser^{10} glycosylated analogues exist prevalently as linear α-helix motifs that are supposed to interact in a more favorable binding pose with the NOP compared to folded structures.

Some of the chemical modifications of the N/OFQ sequence described above have been profitably combined in the search for potent NOP peptide ligands with different pharmacological activities spanning from full agonists (N/OFQ(1–13)-NH_2, [(pF)Phe^4]N/OFQ(1–13)-NH_2, [$Arg^{14}Lys^{15}$]N/OFQ, UFP-112) to partial agonists ([F/G]N/OFQ(1–13)-NH_2, UFP-113) and antagonists ([$Nphe^1$]N/OFQ (1–13)-NH_2, UFP-101). The structures and the in vitro pharmacological profile of these molecules at the human and murine NOP receptor are reported in Table 1.

2.2 Cyclic N/OFQ Derivatives

As mentioned above, different approaches including circular dichroism (CD) and NMR spectroscopy have been applied with the aim to elucidate the bioactive conformation of N/OFQ(1–17)-NH_2, N/OFQ(1–13)-NH_2, and related peptides (Lohman et al. 2015). These studies consistently suggest that the 7–17 address domain of N/OFQ would rather assume an amphipathic α-helical conformation in the binding pocket of NOP receptor thanks to a motif in which the positive Arg-Lys couples are regularly spaced by Ala residues. However, the α-helicity of linear N/OFQ analogues is irrelevant in aqueous medium making these compounds particularly subjected to the action of serum peptidases (Lohman et al. 2015). For these reasons, different efforts have been made in the design of proper C-terminal cyclization strategies that could promote water-stable α-helix motifs thus improving peptidase resistance and in vivo potency and duration of action of N/OFQ analogues.

As largely known, the addition of cysteine residues to peptides makes them prone to cyclization via the formation of intramolecular disulfide bridges. Following this approach, a small series of cyclic analogues of N/OFQ(1–13)-NH_2 has been reported in 2001 (Ambo et al. 2001). These compounds came from the cyclization of either the N-terminal or the C terminal region of N/OFQ thanks to the addition/replacement of Cys residues at key positions of the parent peptide sequence. Any cyclization involving the N-terminal part led to a complete loss of activity. On the other hand, the cyclization of the C-terminal portion led to potent derivatives especially when the disulfide linkage was introduced between the positions 10–14 where a serine and a leucine residue were replaced by two cysteines, respectively. Cyclo[Cys^{10},Cys^{14}]N/OFQ(1–14)-NH_2 (compound **1**, Fig. 2) can be considered the first example of constrained N/OFQ-related peptide whose receptor affinity ($pIC_{50} = 9.91$), potency ($pEC_{50} = 8.36$), and efficacy were comparable to those of the native peptide (Ambo et al. 2001). In an attempt to identify a cyclic antagonist, Kitayama et al. reported in 2003 the synthesis of the $Nphe^1$ derivative of compound **1** (Kitayama et al. 2003) that however suffered from a severe reduction of NOP affinity.

As an alternative to the disulfide bridge strategy, a few N/OFQ cyclic analogues with side chain to side chain lactam linkage have been firstly reported by Charoenchai et al. (2008). Of this series, the cyclo-peptide **2** (cyclo[D-Asp^7,Lys^{10}]N/OFQ(1–13)NH_2, Fig. 2) showed subnanomolar binding affinity and high potency for the NOP receptor ($pK_i = 9.57$, $pEC_{50} = 8.80$). The usefulness of the

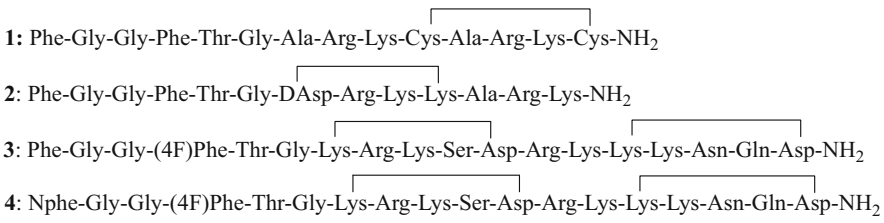


Fig. 2 Structures of selected cyclic analogues of N/OFQ

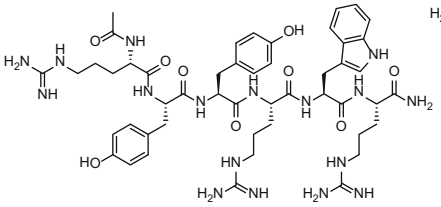
lactamization strategy was later confirmed by Harrison et al. who performed multiple cyclizations of the address domain of N/OFQ(1–17)-NH_2 (Harrison et al. 2010) each involving the minimum number of residues that span a canonical α-helix (five amino acids). In the latter case, cyclizations were combined with strategical substitution of the message sequence intended to enhance ligand potency (i.e., (pF)Phe^4) or induce antagonist activity (i.e., $Nphe^1$). The study resulted in the identification of the highly potent NOP agonist **3** (Fig. 2) with picomolar potency ($pEC_{50} = 10.40$) in vitro and significantly higher and longer lasting antinociceptive activity in vivo in comparison with unconstrained peptides (Harrison et al. 2010). With the same approach, the potent NOP antagonist **4** ($pIC_{50} = 8.12$) was discovered (Fig. 2). CD spectroscopy highlighted that constrained analogues of this series adopted α-helical conformation even in aqueous phosphate buffer where all the investigated unconstrained analogues exhibited minimal helicity. In addition, lactame derivatives showed longer half-life in human serum (Lohman et al. 2015).

3 Structure–Activity Relationship Studies on N/OFQ-Unrelated Peptides

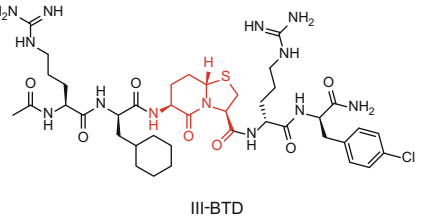
Dooley et al. have reported in 1997 the first examples of peptide ligands of the NOP receptor featuring an amino acid sequence totally unrelated to that of N/OFQ (Dooley et al. 1997). This study originated from the screening of a large peptide combinatorial library and resulted in the identification of a small series of positively charged hexapeptides reflecting the general structure Ac-RYY(R/K)(I/W)(R/K)-NH_2. The functional profile of such compounds evaluated in three different assays (stimulated [^{35}S]GTPγS binding and inhibition of forskolin-stimulated cAMP in cells expressing the recombinant human NOP, and the electrically stimulated mouse vas deferens bioassay) revealed a partial agonist behavior. One of the most representative member of this class namely Ac-RYYRWR-NH_2 (see Table 2) was later employed in photoaffinity labeling studies with the aim to determine its binding domain on the NOP receptor (Bes and Meunier 2003). Interestingly, these studies indicated that N/OFQ and hexapeptides interact with the NOP receptor in distinct, although partially overlapped, regions and this could be the reason of their diverse pharmacological activities. Of this series, Ac-RYYRWR-NH_2 showed the highest efficacy in inhibiting cAMP accumulation (75% versus 84% for N/OFQ), while the analogue Ac-RYYRWK-NH_2 (Table 2) exhibited lower maximal effect (58%). This suggests that the higher basicity of Arg compared to Lys at the 6-position could contribute to promote NOP activation. Of note, it was found that the side chain of Arg/Lys^6 can be replaced with shorter moieties (such as in Orn, Dab, or Dap) without significant loss of activity in vitro (Kasakov et al. 2010).

The negligible in vivo activity of Ac-RYYRWR-NH_2 prompted further efforts aimed at improving proteolytic resistance. The resulting SAR profile indicated the importance of each Arg residue to maintain high binding affinity while Tyr^2 and Tyr^3 seem to be less essential residues, although at least one of these must maintain its

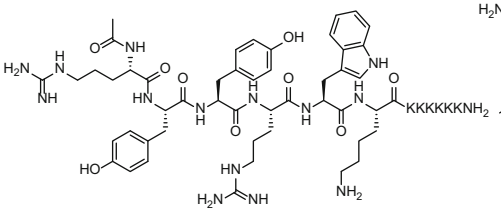
Table 2 Structures and in vitro biological activity of N/OFQ-unrelated NOP peptide ligands



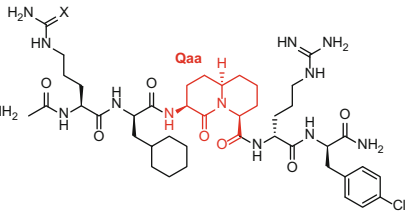
Ac-RYYRWR-NH$_2$



III-BTD



ZP120 (SER 100)



5: Ac-Arg-D-Cha-Qaa-D-Arg-D-pClPhe-NH$_2$ (X = NH)
6: Ac-Cit-D-Cha-Qaa-D-Arg-D-pClPhe-NH$_2$ (X = O)

	Human NOP		Mouse NOP	
	Binding affinity	Functional potency	Functional potency[a]	Reference
NOP partial agonists	pK_i	pEC$_{50}$	pEC$_{50}$	
Ac-RYYRWR-NH$_2$	9.22	8.66[b] 9.28[c]	nd	Dooley et al. (1997)
Ac-RYYRWK-NH$_2$	9.15	8.68[b] 9.28[c]	8.07	Dooley et al. (1997) and Rizzi et al. (2002b)
Ac-R-(3Cl)Y-YRWR-NH$_2$	10.5	9.30[b]	nd	Judd et al. (2004)
ZP120 (SER100)	9.6	9.30[c] 7.15[d]	8.88	Rizzi et al. (2002b), Kapusta et al. (2005), and Camarda et al. (2009)
NOP antagonists	pK_i	pA$_2$/pK$_B$	pA$_2$/pK$_B$	
Isovaleryl-RYYRIK-NH$_2$	8.13	Inactive[b]	9.7	Li et al. (2008)
III-BTD	7.62	7.89[b] 7.49[c]	6.57	Becker et al. (1999), Hashiba et al. (2001), McDonald et al. (2002), and Bigoni et al. (2000)
5	7.46	6.52[b]	nd	Halab et al. (2002)
6	7.14	6.87[b]	nd	Van Cauwenberghe et al. (2004)

pEC$_{50}$: agonist potency; pA$_2$/pK$_B$: antagonist potency
[a]mVD
[b][^{35}S]GTPγS
[c]cAMP
[d]Ca^{2+}

phenol group to sustain the agonist efficacy of the peptide (Kawano et al. 2002; Judd et al. 2004). Indeed, Ac-R-Phe(4-F)-Phe(4-F)-RWR-NH_2 ($pK_i = 8.82$) behaved as NOP antagonist while compound Ac-R-Tyr(3-Cl)-YRWR-NH_2 (Table 2) exhibited picomolar affinity for the NOP receptor with a functional profile of low efficacy partial agonist in different assays (Judd et al. 2004). Moreover, Trp^5 was substituted with a series of nonnatural aromatic amino acids and the following biological investigation suggested that the indole moiety of the side chain at this position is not mandatory for biological activity (Carra' et al. 2005). Nevertheless, it was found that the fifth amino acid residue plays a crucial role in the modulation of agonist/antagonist activity. Indeed an L-aliphatic/hydrophobic amino acid seems to favor antagonist activity, while a D-residue (especially D-Trp or a D-Arg) resulted in potent agonist activity (Ambo et al. 2007). More recently, Zamfirova et al. reported the synthesis and biological evaluation of a new series of congeners of Ac-RYYRWK-NH_2, modified at the 5-position with nonnatural tryptophan analogues (Zamfirova et al. 2013). This study highlighted the contribution of the 5-position to the modulation of selectivity over opioid receptors. Indeed, when a 5-methoxy β^2-tryptophan residue was incorporated at the 5-position of Ac-RYYRWK-NH_2 a compound with higher affinity for opioid receptors than for NOP was obtained.

Other modifications of the original hexapeptide scaffold include the substitution of the N-terminal acetyl group that led in some cases to reduced or abolished intrinsic activity. In particular, the peptide pentanoyl-RYYRWR-NH_2 displays high NOP affinity ($pK_i = 9.89$) with barely measurable agonist activity (Judd et al. 2003). Furthermore, the compounds Isovaleryl-RYYRIK-NH_2 (Table 2) (Li et al. 2008) and Ac-Diaminobutyl-YYRWR-NH_2 (Judd et al. 2004) are examples of pure NOP antagonists obtained with modifications of the N-terminal residue. Arg^1 was also substituted with aminophosphonate moieties with a severe loss of NOP affinity and selectivity (Naydenova et al. 2010). The effect of modifications of the C-terminal portion of the hexapeptide scaffold has been explored as well. A reduction of ligand efficacy has been observed when the C-terminal amide moiety has been replaced by a primary hydroxyl function like in the NOP antagonist Ac-RYYRIK-ol (Kocsis et al. 2004; Gündüz et al. 2006). Noteworthy, the parent hexapeptide structure has been extended at the C-terminal position with an oligo-lysine frame in compound ZP120 (Ac-RYYRWKKKKKKK-NH_2, Table 2) developed by Zealand Pharma (Rizzi et al. 2002b). The $(Lys)_6$ sequence of this derivative would likely induce an α-helix conformation with consequent reduction of enzymatic vulnerability (Larsen 1999). The pharmacological profile of ZP120 (more recently known as SER100) has been extensively investigated both in vitro and in vivo consistently demonstrating its action as a potent and selective NOP partial agonist (Toll et al. 2016). This compound is of particular interest since SER100 is now in clinical development as innovative treatment for systolic hypertension (Kantola et al. 2017).

A screening of a synthetic combinatorial library of β-turn-constrained peptides resulted in the identification of the pseudohexapeptide III-BTD (Ac-Arg-DCha-BTD-DArg-D(pCl)Phe-NH_2, Table 2) as NOP antagonist with low binding selectivity over opioid receptors (Becker et al. 1999). Interestingly, the compound behaves

as weak agonist toward all the three classical opioid receptors. The thiazolidinone bicycle inserted within the sequence of III-BTD as turn inducer has been later replaced with different azabicycloalkane amino acids in the search for more selective ligands (Halab et al. 2002). The introduction of a 6,6-bicyclic moiety (i.e., quinolizidine) in compound **5** determined the maintenance of NOP affinity with a significant improvement of selectivity especially over DOP receptors. However, at high concentration, **5** behaved as weak partial agonist at MOP and KOP receptors. Further SAR studies on this molecule focused on the importance of the Arg residues whose position, structure, and charge were modified (Van Cauwenberghe et al. 2004). This investigation resulted in the identification of the citrulline derivative **6** with slightly lower NOP affinity but improved potency and selectivity.

4 Bivalent NOP Peptide Ligands

With the aim to investigate the possible existence of NOP receptor homodimers, we recently reported a series of dimeric NOP ligands obtained by the linkage of two peptide or non-peptide pharmacophores with spacers of different length and chemical composition (Pacifico et al. 2017). A subset of homobivalent ligands have been obtained connecting the C-terminal portions of two N/OFQ(1–13) fragments chosen as pharmacophore units. The spacers varied from 18 to 32 atoms and were composed of Gly, Ala, β-Ala, Gaba, and Cys residues, in variable combinations. Neither ligand dimerization nor spacer length/composition seemed to affect agonist potency or efficacy. However, when low potency agonists, e.g., N/OFQ(1–12) and N/OFQ (1–11), were chosen as pharmacophores, dimerization resulted in total recovery of ligand potency. This effect of dimerization depends on the doubling of the C-terminal address sequence rather than the presence of an additional N-terminal message sequence or modifications of peptide conformation (Pacifico et al. 2017).

Together with homobivalent ligands, a few examples of peptide-based bifunctional MOP/NOP agonists have been recently examined. This research area is of particular interest since molecules that are able to modulate multiple opioid receptors may result into novel opioid analgesics possibly with reduced side effects (Günther et al. 2018). In particular several recent studies demonstrated that the mixed NOP/opioid receptor agonist cebranopadol displays analgesic effects similar to morphine associated with reduced side effects (reviewed in Calo' and Lambert 2018). Moreover, the small molecule AT-121, with a bifunctional NOP/MOP agonist profile, has been shown to promote nonaddictive analgesia in nonhuman primates with potential as treatment for opioid abuse disorders (Ding et al. 2018).

As far as heterobivalent peptides are concerned, Kawano et al. obtained a first hybrid template linking dermorphin, as mu receptor agonist, to the NOP peptide ligand Ac-RYYRIK-NH_2 (Kawano et al. 2006). A synergistic effect was observed on both MOP and NOP binding potency when a relatively long spacer −Gly-Gly-Gly-Lys(Gly-Gly-)-NH_2 was incorporated between the pharmacophores. In fact, compound **7** (Fig. 3) exhibited picomolar affinities for the investigated targets (pK_i NOP = 10.33; pK_i MOP = 11.63); despite this, the chimeric compound did not show

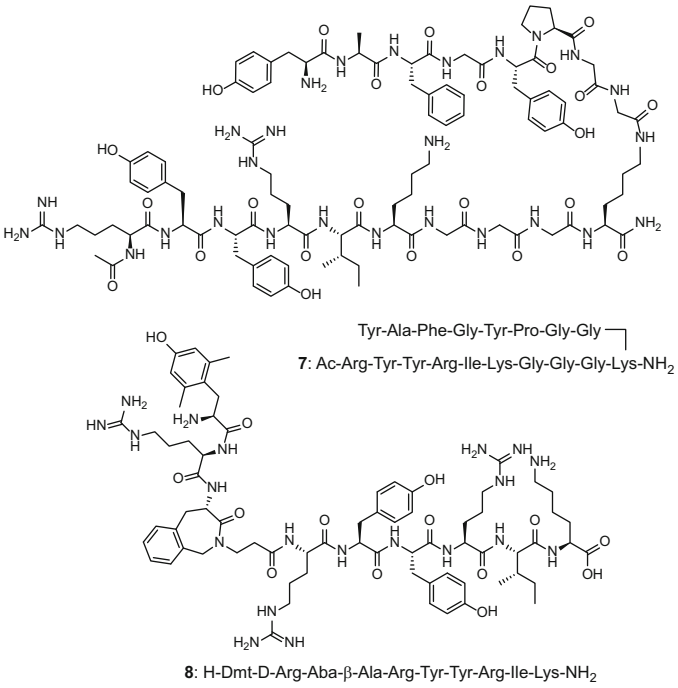


Fig. 3 Structures of bifunctional NOP/opioid receptor peptide ligands

improved antinociceptive activity in vivo compared to the single peptides (Kawano et al. 2007). Dermorphin was also hetero-dimerized with the endogenous NOP agonist N/OFQ (Bird et al. 2016). The resulting compound showed in vitro the expected profile (i.e., the sum of that of the single monomeric components) but exhibited in vivo only weak antinociceptive properties.

More recently, Guillemyn et al. described chimeric compounds obtained through the linear combination of the N-terminal fragment H-Dmt-D-Arg-Aba-β-Ala-NH_2, as opioid pharmacophore, with different NOP peptide ligands at the C-terminal portion (Guillemyn et al. 2016; Lagard et al. 2017). Such hybrids were able to simultaneously activate opioid receptors and block NOP when evaluated in vitro. In this case, the merging strategy led to a slight loss of affinity toward both opioid and NOP receptors. Among the investigated compounds, the bifunctional peptide **8** (pK_i NOP = 7.38; pK_i MOP = 8.30, Fig. 3) elicited high and long lasting antinociceptive efficacy in vivo upon i.v. administration in mice. Of note, this molecule demonstrated higher analgesic efficacy in neuropathic pain models compared to morphine with limited effects on the respiratory function and reduced tolerance liability (Starnowska et al. 2017).

5 Tetrabranched NOP Peptide Ligands

Bracci et al. provided in 2003 the first evidences of the positive effect of N/OFQ multimerization on its stability in plasma and serum (Bracci et al. 2003). A high efficacy chemical strategy for the synthesis of multi-branched peptides, the peptide welding technology (PWT), has been developed more recently (Calo' et al. 2018). This approach showed to significantly extend the typical short half-life of a series of peptides of therapeutic interest possibly because of a reduced proteolytic metabolism. The methodology is based on the thiol-Michael conjugation of three different tetra-maleimide functionalized cores (PWT1, PWT2, and PWT3; see Fig. 4) with four linear peptide monomers strategically functionalized with a cysteine residue. N/OFQ was employed in early studies to produce the first examples of PTW homotetravalent peptides (Guerrini et al. 2014). PWT-N/OFQ derivatives displayed higher binding affinity (threefold) for NOP than N/OFQ and comparable selectivity over opioid receptors (Rizzi et al. 2014). Moreover, PWT-derivatization of N/OFQ preserved its behavior as NOP full agonist in vitro with even improved potency. Interestingly, N/OFQ clustering impacts also on the capability to discriminate between NOP/G protein and NOP/β-arrestin 2 interaction. In particular, PWT2-N/OFQ displayed a significant bias toward G protein (Malfacini et al. 2015). Remarkably, PWT-N/OFQ derivatives showed enhanced potency (40-fold) compared to

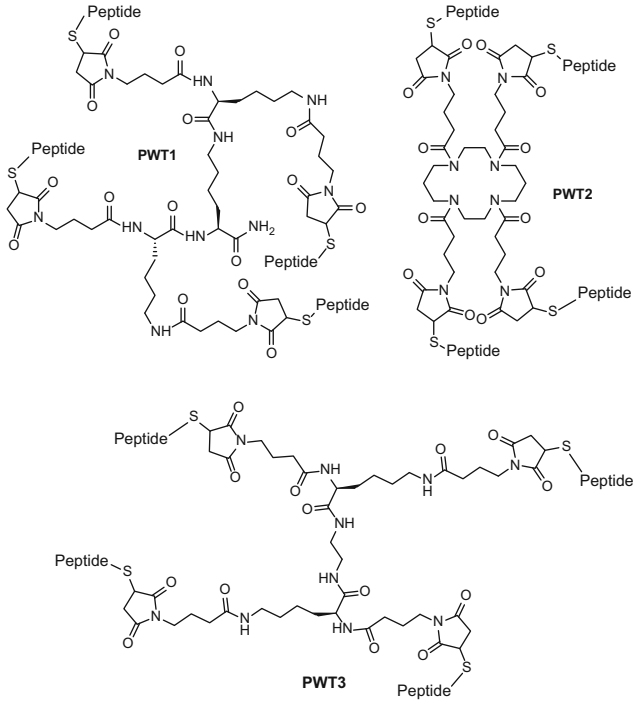


Fig. 4 General structures of tetrabranched peptide ligands of the NOP receptor (PWT technology)

N/OFQ when evaluated in vivo for its inhibitory effects on mouse locomotor activity after supraspinal administration (Rizzi et al. 2014). Similar results, i.e., 40-fold higher potency than N/OFQ, were obtained investigating the spinal antinociceptive effects of PWT2-N/OFQ in neuropathic pain models in mice and in nonhuman primates (Rizzi et al. 2015).

[Dmt^1]N/OFQ(1–13)-NH_2 has been clustered into PWT cores with the aim to provide a potential example of a tetrameric ligand with mixed NOP and opioid receptor agonist properties (Cerlesi et al. 2017). PWT2-[Dmt^1]N/OFQ(1–13) displayed reduced NOP affinity if compared to the linear peptide monomer, with a similar selectivity profile (NOP = MOP = KOP > DOP). In functional assays, this compound behaved as a G protein biased NOP/MOP dual agonist. In addition, the compound prompted antinociceptive effects following spinal administration in monkeys, with tenfold higher potency than [Dmt^1]N/OFQ(1–13)-NH_2 and longer lasting effects (Cerlesi et al. 2017).

PWT2 technology was also employed to synthetize a tetrabranched derivative of the NOP antagonist UFP-101 (see Table 1). PWT2-UFP-101 preserved the NOP antagonist pharmacological activity displaying a value of potency ($pA_2 = 8.58$) comparable to that of UFP-101 ($pA_2 = 8.32$). Of note, tetramerization of UFP-101 determined a significant reduction of selectivity over opioid receptors, particularly for the DOP. When evaluated in vivo in the mouse forced swimming test, PWT2-UFP-101 exhibited antidepressant properties with higher potency (nearly tenfold) compared with UFP-101. In line with the PWT derivatives described above, the onset of the in vivo effects of PWT2-UFP-101 was significantly delayed. Nevertheless, unlike other tetrabranched derivatives, the duration of action of PWT2-UFP-101 was similar to that of UFP-101. It has been speculated that the latter finding may be due to the presence of the unnatural amino acid residue Nphe at N-terminal portion of UFP-101 resulting in an intrinsic proteolytic resistance that cannot be further improved through the PWT approach (Calo' et al. 2018).

6 Concluding Remarks

Collectively the diverse approaches to the obtainment of NOP peptide ligands resulted in the identification of important tools with pharmacological profiles spanning from full and partial agonists to pure antagonist. Some of these ligands largely contributed to the elucidation of the physiopathology of the N/OFQ-NOP system and its translational potential to the pharmacological treatment of different diseases. Of note, some of the design and synthetic efforts described above also contributed to reduce significantly the known pharmacokinetic weaknesses of peptide molecules paving the way to the concrete option of employing NOP-targeted peptide ligands for clinical purposes. In this regard, the extensive SAR studies performed on the endogenous sequence of N/OFQ drove to the identification of the NOP full agonist UFP-112 that is now under clinical development by Recordati under the name Rec 0438. This compound is currently in Phase II studies as innovative treatment for overactive

bladder patients (ClinicalTrials.gov Identifier: NCT03482037). Furthermore, SER 100 (alias ZP120) distinguishes as the most advanced N/OFQ-unrelated peptide that reached phase II clinical studies aimed at assessing its safety and efficacy after subcutaneous administration in patients with systolic hypertension (Kantola et al. 2017). Finally, preclinical studies performed in rodents and nonhuman primates suggested that the spinal administration of NOP selective as well as of mixed NOP/opioid peptides could be of value for the treatment of chronic pain (Toll et al. 2016). A relevant contribution in this area might come from N/OFQ-related PWT derivatives whose spinal analgesic action last for more than one day from injection (Cerlesi et al. 2017; Rizzi et al. 2015). These kind of compounds together with technological advances of targeted intrathecal drug delivery systems may hopefully provide in the near future interesting options for the management of chronic pain patients.

Author Contributions DP, GC, and RG wrote the chapter and approved its final version.

Declaration of Interests DP has nothing to declare. GC and RG are inventors of the patent applications WO2006/087340 and U.S. Serial No. 14/782,578 covering UFP-112 and PWT derivatives of N/OFQ, respectively, and are among the founders of the University of Ferrara spin-off company UFPeptides s.r.l., the assignee of these patents.

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NOP-Targeted Nonpeptide Ligands

Nurulain T. Zaveri and Michael E. Meyer

Contents

Abstract

The development of nonpeptide systemically active small-molecule NOP-targeted ligands has contributed tremendously to validating the NOP receptor as a promising target for therapeutics. Although a NOP-targeted compound is not yet approved for clinical use, a few NOP ligands are in clinical trials for various indications. Both successful and failed human clinical trials with NOP ligands provide opportunities for rational development of new and improved NOP-targeted compounds. A few years after the discovery of the NOP receptor in 1994, and its de-orphanization upon discovery of the endogenous peptide nociceptin/orphanin FQ (N/OFQ) in 1995, there was a significant effort in the pharmaceutical industry to discover nonpeptide NOP ligands from hits obtained from high-throughput screening campaigns of compound libraries. Depending on the therapeutic indication to be pursued, NOP agonists and antagonists were discovered, and some were optimized as clinical candidates. Advances such as G protein-coupled receptor (GPCR) structure elucidation,

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functional selectivity in ligand-driven GPCR activation, and multi-targeted ligands provide new scope for the rational design of novel NOP ligands fine-tuned for successful clinical translation. This article reviews the field of nonpeptide NOP ligand drug design in the context of these exciting developments and highlights new optimized nonpeptide NOP ligands possessing interesting functional profiles, which are particularly attractive for several unmet clinical applications involving NOP receptor pharmacomodulation.

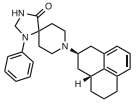
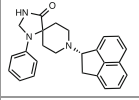
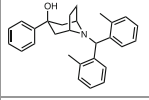
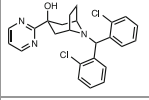
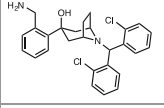
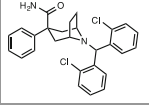
Keywords
Nociceptin ligands · NOP agonists · NOP antagonists · NOP ligands · Small-molecule NOP ligands

1 Nonpeptide NOP Ligands As Tools and Candidate Drugs in Development

The endogenous natural ligand for the nociceptin opioid peptide receptor (NOP) is a 17-residue peptide, nociceptin/orphanin FQ (N/OFQ), which is very similar to the endogenous kappa peptide ligand, dynorphin, also a heptadecapeptide. All endogenous opioid peptides contain Tyr as the N-terminal residue, with the exception of N/OFQ, which contains Phe at the N-terminus. Although there is significant similarity in the primary sequence of N/OFQ and the other endogenous opioid peptides, there is an exquisite selectivity of N/OFQ, which does not bind to the classical opioid receptors despite the 65% homology between NOP and the classical opioid receptors (Meunier et al. 2000). A reciprocal selectivity extends to the "nonpeptide" opium alkaloids and most semisynthetic opioid ligands, which have high affinity for the three classical opioid receptors, but not the NOP receptor (Hawkinson et al. 2000; Zaveri et al. 2001). Soon after this characterization, there was a major effort in several pharmaceutical companies to discover high affinity, nonpeptide ligands that were selective for the NOP receptor. As discussed in this review, several such nonpeptide, small-molecule NOP ligands have facilitated the evaluation and validation of the N/OFQ-NOP system as a pharmacological target for therapeutics and have emerged as drug candidates in recent clinical development for a variety of conditions such as major depressive disorder, alcohol dependence, Parkinson's disease motor symptoms (NOP antagonist LY-2940094, now BTRX-246040) (NCT03608371 2018; Post et al. 2016a, b) and as analgesics for neuropathic and postoperative pain (e.g., NOP/MOP bifunctional agonist cebranopadol) (Christoph et al. 2017; Scholz et al. 2018).

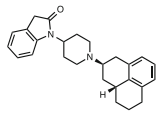
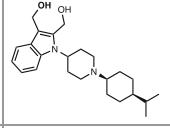
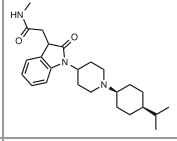
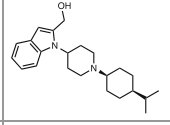
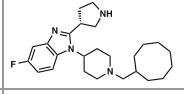
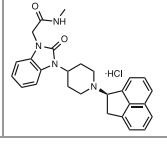
Given the prevailing technologies at the time during the 1990s, most nonpeptide NOP ligands were discovered from high-throughput screening of corporate compound libraries and extensive chemical optimization of hits, to enhance binding affinity and selectivity for the NOP receptor. In 1999, Banyu reported the discovery and structure-activity relationships (SAR) of the first nonpeptide NOP ligand, the NOP antagonist J-113397 (see Table 3) (Kawamoto et al. 1999). Soon thereafter, Hoffmann La-Roche reported the first high affinity nonpeptide NOP agonist Ro 64-6198 (see Table 1), which showed anxiolytic efficacy in rodent models of anxiety (Jenck et al. 2000). Both

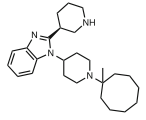
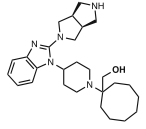
Table 1 Receptor binding affinities and functional activity of NOP agonists

	Structure	Receptor binding K_i (nM)[a]				NOP [^{35}S]GTPγS		Reference[a]
		NOP	MOP	KOP	DOP	EC_{50} (nM)	% Stim	
Ro 64-6198		0.39 ± 0.06	46.8 ± 9.7	89.1 ± 13.2	138 ± 128	38.9	100	Wichmann et al. (2000)
Ro 65-6570		0.52	5.90	26.0	250	40.0	100	Rover et al. (2000)
SCH221510		0.30 ± 0.05	65.0 ± 10.0	131 ± 33	2,854	12.0 ± 3.0	100	Varty et al. (2008)
SCH486757		4.60 ± 0.61	972 ± 40	590 ± 40	>10 K	79.0 ± 12.0	100	McLeod et al. (2010)
SCH225288		0.38 ± 0.02	21 ± 0.57	39 ± 0.28	773 ± 8	1.35 ± 0.14	100	McLeod et al. (2009)
SCH655842		1.70	38.0	268	233	6.00	100	Ho et al. (2009)

(continued)

Table 1 (continued)

	Structure	Receptor binding K_i (nM)[a]				NOP [^{35}S]GTPγS		Reference[a]
		NOP	MOP	KOP	DOP	EC_{50} (nM)	% Stim	
AT-202 (SR16835)		11.4 ± 0.9	79.9 ± 3.9	681 ± 62	368 ± 1,108	46.0 ± 20.5	107 ± 7	Toll et al. (2009)
AT-390		0.9 ± 0.3	53.1 ± 16.5	85.3 ± 19.8	113 ± 10	15.2 ± 0.4	110 ± 11	Arcuri et al. (2018)
AT-403		1.1 ± 0.1	97.9 ± 15.0	156 ± 204	407 ± 17	6.3 ± 1.4	104 ± 1	Arcuri et al. (2018)
AT-312		0.34 ± 0.13	5.99 ± 0.97	73.5 ± 28.3	128.7 ± 57.4	29.9 ± 1.4	102.3 ± 0.75	Zaveri et al. (2018b)
SR-8993		Not reported	Not reported	Not reported	Not reported	8.8 ± 1.4 (cAMP assay)	100	Andero et al. (2013)
MT-7716 (W-212393)		0.50 ± 0.1	76.0 ± 27.8	>1,000	>1,000	13.9	≥100	Teshima et al. (2005)

MCOPPB		0.09	1.1	23.1	>667	0.39	140	Hayashi et al. (2009)
HPCOM		1.4	201	>577	>5,000	12	144	Hayashi et al. (2010)

[a]The binding affinities and functional activities for the various compounds are from different laboratories and may use different experimental protocols to determine K_i and functional activity. These data are included for discussion purposes only and cannot be directly compared

these first reported nonpeptide NOP ligands remain, to this day, two of the most widely used NOP ligand tool compounds (Zaveri 2016). In the nearly two decades after Ro 64-6198 and J-113397 were reported, there have been >200 patents claiming nonpeptide NOP ligands. More recent advances such as the X-ray crystallographic resolution of the structure of the NOP receptor bound to an antagonist (Thompson et al. 2012) and the use of structure-based drug design approaches (Daga et al. 2014; Daga and Zaveri 2012) provide new opportunities for the discovery of novel NOP ligands. As discussed below, the concepts of functional selectivity (biased agonism) of GPCR ligands and the multifunctional targeting of opioid receptors for pharmacological manipulations of efficacy versus side effects provide further opportunities to refine nonpeptide NOP ligands that can be advanced into therapeutic development for several disorders.

2 Structure-Activity, Structure-Selectivity, and Structure-Function Relationships of Nonpeptide NOP Ligands

Nonpeptide NOP ligands that were identified from refining high-throughput screening hits from various companies show strikingly similar pharmacophoric features, with very few notable exceptions. These early lead compounds were also non-morphinans by structural class and bore close resemblance to neuroleptics and serotonergic drugs. For example, NOP antagonist J-113397 was structurally similar to neuroleptic pimozide, whereas NOP agonist Ro 64-6198 was similar to the 5-HT partial agonist spiroxatrine, each differing only in the substituent on the piperidine nitrogen. A pharmacophore and SAR analysis of early reported NOP ligands showed that most nonpeptide NOP ligands contain three main pharmacophoric features that determine binding affinity, selectivity versus the classical opioid receptors, and intrinsic activity. These were (1) an alicyclic core containing a protonatable nitrogen (most commonly a piperidine ring), (2) an aromatic or heterocyclic moiety distal to the protonatable nitrogen (at the 4-piperidine position), and (3) a lipophilic substituent on the protonatable nitrogen (e.g., see Ro 64-6198 and J-113397 in Tables 1 and 3) (Zaveri et al. 2005). SAR analysis of various nonpeptide NOP ligands shows that the heterocyclic pharmacophore and the lipophilic nitrogen substituent are important determinants of high binding affinity and selectivity versus the other opioid receptors, particularly the MOP receptor. The lipophilic nitrogen substituent also plays an important role in the intrinsic activity of the NOP ligands, as we have shown that subtle one-carbon differences in the C-moiety substituents can convert a NOP agonist into an antagonist, without affecting binding affinity (Zaveri et al. 2005).

The protonatable nitrogen is an essential pharmacophoric feature in all nonpeptide NOP ligands and makes an anchoring interaction with the Asp130 in the NOP binding pocket. This mimics the interaction of the N-terminus Phe of N/OFQ with Asp130 (see Fig. 1 for N/OFQ docked into the NOP active-state homology model (Daga and Zaveri 2012)). The importance of this interaction was further confirmed with the resolution of the NOP receptor crystal structure bound to the potent NOP antagonist C-24 (see (Thompson et al. 2012)) (Fig. 2a) and SB-612111 (see Table 3)

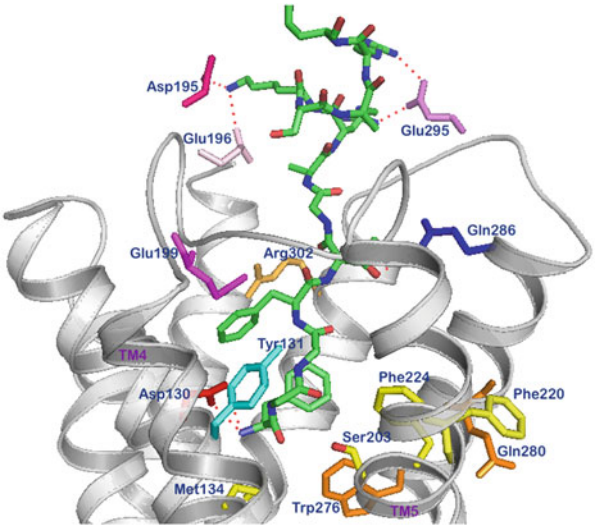


Fig. 1 Molecular model of the N/OFQ (1–13) peptide (depicted as green sticks) bound to the active-state homology model of the NOP receptor (Daga and Zaveri 2012). The TM helices are depicted in gray. The side chains of amino acids interacting with the peptide are labeled. The Asp130 interacts with the N-terminus Phe-1 of N/OFQ. The acidic residues of the ECL2 loop (D195, E196) interact with the basic residues (8–13) of N/OFQ

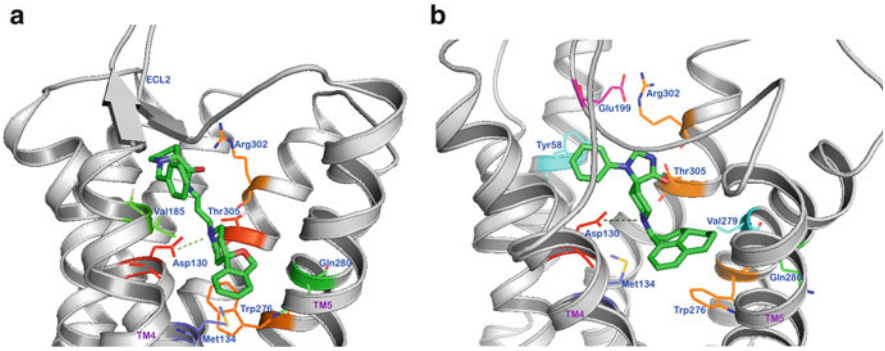


Fig. 2 (**a**) Structure of the NOP receptor bound to NOP antagonist C-24 (green) (PDB ID: 4EA3). The TM helices are colored in gray and labeled. Side chains of amino acids interacting with the antagonist are shown as sticks and labeled. The spiro-substituent on the 4-piperidinyl position is oriented toward the intracellular end of the binding pocket. (**b**) NOP agonist Ro 64-6198 (green sticks) bound to the active-state NOP receptor model developed by (Daga and Zaveri 2012). The NOP agonist interacts with the Thr305 (orange sticks) and Y309 (blue sticks). The phenalenyl group of the NOP agonist is in close proximity to V279 (cyan sticks, labeled). This residue is isoleucine in the classical opioid receptors, which is likely responsible for the lower affinity of Ro 64-6198 for the classical opioid receptors

(Miller et al. 2015), in which the piperidine nitrogen of the NOP antagonist makes an ionic interaction with Asp130. Although the agonist-bound NOP crystal structure has not yet been solved, the active-state NOP receptor structure was obtained by homology modeling and used for docking NOP agonist ligands such as Ro 64-6198 (Daga and Zaveri 2012), which also showed the ionic interaction of the piperidine nitrogen with Asp130. Interestingly, the most-favored binding orientation of the NOP agonist Ro 64-6198 placed the N-substituent of the piperidine nitrogen toward the intracellular end of the ligand binding pocket and the heterocyclic imidazolone ring oriented toward the extracellular end, making a hydrogen-bonding interaction with Thr305, located at the extracellular end of TM7 (transmembrane helix 7) (See Fig. 2b) (Daga and Zaveri 2012). This binding orientation was also consistent with a previously reported docking of Ro 64-6198 conducted by Broer et al. using a NOP homology model (Broer et al. 2003), as well as the docking of other NOP agonists (Daga et al. 2014). However, the binding orientations of the NOP antagonists in the antagonist-bound NOP co-crystal structure were flipped 180° to what is observed with NOP agonists, such that the N-substituent on the piperidine nitrogen is oriented toward the "extracellular end" of the binding pocket and the heteroaromatic moiety (benzofuran in NOP antagonist C-24 and dichlorophenyl in SB-612111) is oriented toward the intracellular end of the binding pocket (Miller et al. 2015). Notably, however, docking of the NOP antagonist J-113397 showed that it bound in the same orientation as the NOP agonists, with its benzimidazolone heteroaromatic moiety positioned at the extracellular end and the lipophilic N-substituent on the piperidine nitrogen oriented toward the intracellular end of the ligand-binding pocket (Miller et al. 2015). These observations suggest that the nature of the piperidine N-substituent and the heteroaromatic moiety affects the binding mode of NOP ligands. It appears that large substituents on the piperidine nitrogen and a relatively nonpolar heteroaromatic moiety favor the "antagonist" orientation of the NOP ligand in the receptor, as seen with C-24 and SB-612111, whereas small nonpolar lipophilic groups on the piperidine nitrogen and polar heteroaromatic moieties around the central alicyclic ring favor the "agonist" orientation seen with Ro 64-6198 and also with some antagonists like benzimidazolone J-113397 and indolinone AT-207 (previously SR14148) (Table 3) (Zaveri et al. 2005).

We have shown that modifying NOP-selective agonist ligands on the heteroaromatic moiety, as well as on the lipophilic substituent attached to the piperidine nitrogen, leads to increased binding affinity to the MOP receptor, and provides NOP/MOP bifunctional ligands. This structure-based design of multifunctional NOP-opioid ligands from NOP-selective ligands takes into account the differences in several key residues between the NOP and opioid receptors that typically preclude the binding of N/OFQ to opioid receptors and opioid ligands to the NOP receptor (Ding et al. 2018; Journigan et al. 2014; Zaveri et al. 2013a, b).

3 Nonpeptide NOP Agonist Ligands

Several nonpeptide NOP agonists continue to be investigated for their pharmacological efficacy in various therapeutic indications. Table 1 shows the structures and in vitro pharmacological profile of some well-characterized NOP agonists. While the

in vitro binding affinities given in Table 1 are from different laboratories and cannot be directly compared, a few trends are evident among the various NOP agonists. There are several nano-to-subnanomolar affinity NOP agonists with >100-fold selectivity versus the opioid receptors (particularly the MOP receptor), such as Ro 64-6198, SCH221510, SCH486757, AT-403, MT-7716, and HPCOM, with some possessing high agonist potency (AT-403 and MT-7716), whereas Ro 64-6198, SCH221510, and SCH486757 showing modest potency compared to their subnanomolar binding affinity. Other NOP full agonists have high binding affinity but modest selectivity (10–50-fold) versus the MOP receptor (Ro 65-6570, SCH225288, SCH655842, AT-202, AT-390, AT-312, MCOPPB), although some of these modestly selective NOP agonists have high agonist potency and are full agonists (SCH655842, MCOPPB). Ro 64-6198, the first reported nonpeptide NOP agonist is also the most widely employed NOP tool compound. It is interesting that the agonist potency (EC_{50} nM) of Ro 64-6198 in the GTPγS functional assay is nearly 100-fold lower than its binding affinity (K_i nM) at the human NOP receptor. The reasons for such a significant difference between the binding affinity and functional potency of some NOP full agonists are not clearly understood (Adapa and Toll 1997). However, several other compounds in Table 1 show higher agonist potency (AT-403, MT-7716, MCOPPB, SCH221510, SCH225288, and SCH655842), similar to the natural peptide agonist N/OFQ, and have been recently characterized as tool compounds in several in vivo pharmacological assays involving NOP function, e.g., AT-403 (Arcuri et al. 2018; Ferrari et al. 2017; Rekik et al. 2017), MT-7716 (Ciccocioppo et al. 2014; de Guglielmo et al. 2015), and SCH221510 (Fichna et al. 2014; Sukhtankar et al. 2014a).

Nonpeptide NOP agonists have been investigated for efficacy in vivo in several pharmacological models predicting therapeutic utility, as discussed below. Ro 64-6198 has been the most widely employed tool compound to investigate NOP pharmacology in vivo (Shoblock 2007); however, more recently, other NOP agonists (shown in Table 1), such as Ro 65-6570, SCH221510, and AT-403, have also been used.

Anxiolytics One of the earliest therapeutic indications pursued for NOP agonists was as anxiolytics, with a profile differentiated from benzodiazepines. Indeed, Jenck et al. first reported the anxiolytic-like effects of N/OFQ at low nonsedating doses (given intracerebroventricularly, i.c.v.) in several behavioral paradigms of anxiety in rodents (Jenck et al. 1997). Soon after, the same group demonstrated the anxiolytic efficacy of Ro 64-6198 in several rat models of spontaneous and conditioned anxiety, but observed no dose separation between anxiolytic activity and general disruption in behavior in the mouse (Jenck et al. 2000). These observations were further confirmed by Varty et al. in their extensive characterization of Ro 64-6198 (Varty et al. 2005). Several other NOP agonists have shown anxiolytic efficacy in both rat and mouse models with a better dose separation from motor-disrupting behavioral effects, as shown for SCH221510 (Varty et al. 2008), SCH655842 (Lu et al. 2011), and MCOPPB (Hirao et al. 2008).

Among related studies, NOP agonist SR-8993 showed efficacy in impairing fear memory consolidation in a post-traumatic stress disorder (PTSD)-like rodent model,

when administered prior to or immediately after a cued-fear event (Andero et al. 2013). PTSD is an anxiety disorder that develops after exposure to a highly traumatic event and involves altered fear learning and fear memory consolidation. Rekik et al. recently showed that N/OFQ and systemically administered NOP agonists Ro 65-6570 and AT-403 impair reconsolidation of contextual fear memory in mice, a pharmacological correlate of suppressing maladaptive contextual memories, for example, those associated with PTSD (Rekik et al. 2017).

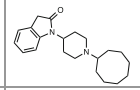
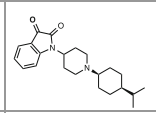
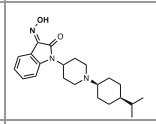
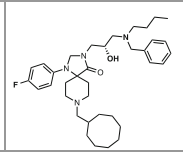
Chronic and Neuropathic Pain Selective NOP agonists, upon systemic administration, show significant antinociceptive efficacy in several animal models of chronic, neuropathic, and inflammatory pain, but not acute pain. Both Ro 64-6198 and Ro 65-6570 showed anti-allodynic and antihyperalgesic activity in rat models of neuropathic pain only after local (intraplantar) or spinal (intrathecal, i.t.) administration but not systemic administration (Obara et al. 2005; Schiene et al. 2015). Even SCH221510 was shown to have anti-allodynic efficacy only after spinal (i.t.) but not systemic administration in the chronic constriction injury (CCI) model and the carrageenan-induced inflammatory pain model in mice (Sukhtankar et al. 2013) and in rat (Wu and Liu 2018). On the other hand, NOP agonist AT-202 (Table 1) was shown to have significant anti-allodynic and antihyperalgesic efficacy after systemic (subcutaneous, s.c.) administration in a mouse spinal nerve ligation model (Khroyan et al. 2011b), whereas agonist HPCOM (Table 1)-administered s.c. and i.t. showed anti-allodynic activity without producing motor-suppressing effects in the rat CCI model of neuropathic pain.

Unlike in rodents, NOP agonists Ro 64-6198 and SCH221510 show significant antinociceptive and antihyperalgesic efficacy in nonhuman primates after systemic and intrathecal administration (Ko et al. 2009; Podlesnik et al. 2011; Sukhtankar et al. 2014b). The antinociceptive efficacy of NOP agonists was comparable to that of morphine and observed at doses at which there was no suppression of motor activity or opioid-like effects of itch and dependence formation, suggesting that NOP agonists may have a more tolerable and safer profile than opioid-based analgesics with comparable analgesic efficacies.

A further demonstration that NOP agonists may have superior efficacy than classical opioids in chronic and neuropathic pain conditions comes from the study by Vang et al., which demonstrated that selective NOP agonist AT-200 (Table 2) showed significantly higher antinociceptive, antihyperalgesic, and anti-allodynic efficacy than morphine in a spontaneously hyperalgesic transgenic mouse model of sickle cell disease. This analgesic efficacy was reversed by a NOP antagonist but not by naloxone and did not develop tolerance, unlike morphine, in the same animal model (Vang et al. 2015).

Overall, several preclinical studies suggest that nonpeptide NOP agonists that can be systemically administered may have a better profile as nonaddicting and potent analgesics for chronic and neuropathic pain conditions, compared to classical opioids (Schröder et al. 2014).

Table 2 Receptor binding affinities and functional activity of NOP partial agonists

	Structure	Receptor binding K_i (nM)				NOP [^{35}S]GTPγS		MOP [^{35}S]GTPγS		Reference
		NOP	MOP	KOP	DOP	EC_{50} (nM)	% Stim	EC_{50} (nM)	% Stim	
AT-200 (SR14150)		1.39 ± 0.4	29.9 ± 2.1	42.7 ± 1.0	424 ± 212	20.8 ± 3.1	54.2 ± 10.9	99 ± 12	23.4 ± 3.2	Zaveri et al. (2004)
AT-090		5.6 ± 1.7	95.4 ± 3.5	233 ± 18.1	244 ± 7.0	50.1 ± 6.4	21.0 ± 6.5	>10,000	–	Ferrari et al. (2016)
AT-127		1.18 ± 0.2	71.7 ± 27.8	149 ± 18.3	47.2 ± 10.2	15.5 ± 3.1	61.0 ± 2.0	59.2 ± 3.0	37.0 ± 2.0	Ferrari et al. (2016)
1		34.0 (IC_{50} nM)	119 (IC_{50} nM)	240 (IC_{50} nM)	Not reported	Not reported	Not reported	Not reported	Not reported	Battista et al. (2009) and Ross et al. (2015)

Substance Abuse Therapy Several NOP agonists show efficacy in decreasing the rewarding effects of various abused drugs like morphine, alcohol, and cocaine. Ro 64-6198 decreased rewarding effects of morphine (Shoblock et al. 2005) and alcohol (Kuzmin et al. 2003) in the mouse conditioned place preference (CPP) paradigm and decreased alcohol self-administration and relapse-like alcohol drinking in rats (Kuzmin et al. 2007). A more recently reported NOP agonist AT-312 (Table 1) was shown to decrease rewarding effects of ethanol, morphine, and cocaine, in the CPP paradigm, when administered systemically (Zaveri et al. 2018a, b). The potent NOP agonist MT-7716 was shown to have significant efficacy after systemic administration in decreasing ethanol intake in rats dependent on ethanol (Ciccocioppo et al. 2014; de Guglielmo et al. 2015). NOP agonist SR-8993 was also reported to reduce alcohol intake and alcohol seeking in naïve rats. Together, efficacies of chemically distinct NOP agonists in various models of alcohol addiction behaviors strongly suggest the potential therapeutic utility of NOP agonists for treating alcohol use disorders.

Parkinson's Disease Dyskinesia In elegant detailed studies, Morari and colleagues have shown that N/OFQ-NOP-system is differentially dysregulated in different brain regions affected in Parkinson's disease and levodopa treatment-induced dyskinesias (LID). Exogenously administered N/OFQ and NOP agonist Ro 65-6570 were shown to inhibit LID expression in dyskinetic rats and macaques without attenuating the antiparkinsonian effect of L-DOPA (Marti et al. 2012). Recently, two different NOP agonists AT-390 and AT-403 (Table 1) were also shown to have a significant but mild anti-dyskinetic effect in an animal model of LID (Arcuri et al. 2018). However, there appeared to a differential dose separation and narrow therapeutic window between the two agonists, where AT-403 attenuated dyskinesia expression without causing sedation within a narrow lower dose range, whereas AT-390 delayed the expression of LID at doses that also caused sedation.

Inflammatory Bowel Disease NOP agonists have been proposed as a new pharmacological approach for the treatment of intestinal pathologies such as inflammatory bowel syndromes (IBS) (Agostini and Petrella 2014). Indeed, NOP agonist SCH 221510 demonstrated a potent inhibitory effect on GI contractility and an antitransit and analgesic action after i.p. and oral administration, in mouse models of intestinal bowel syndrome (Fichna et al. 2014; Sobczak et al. 2014). Whether these effects can be separated from the central motor-suppressing effects of NOP agonists or by modulating the degree of brain permeability of NOP agonists remains to be investigated and validated with other chemically distinct NOP agonists; nevertheless, these studies provide a potentially new therapeutic utility for NOP agonists.

Antitussives Several NOP agonists discovered by Schering Plough such as SCH225288 and SCH486757 were shown to have significant cough-suppressing efficacy in several preclinical models of cough (McLeod et al. 2009, 2010). Ro 64-6198 was also shown to have cough-suppressing activity in a guinea pig model of cough (McLeod et al. 2004). SCH486757 was advanced to Phase 1b human clinical

trials but failed to show antitussive efficacy at any dose without producing a somnolence effect in patients and was not further developed (McLeod et al. 2011; Woodcock et al. 2010).

4 Nonpeptide NOP Partial Agonist Ligands

Among the earliest selective nonpeptide NOP partial agonists reported, AT-200 (previously called SR14150) has moderate binding selectivity (20-fold) for NOP over the MOP receptor and a fivefold higher potency as a NOP agonist than as a MOP agonist (see Table 2). AT-200 has an interesting profile in pain models in vivo, which highlights the complexity of NOP agonist efficacy in pain as being dependent on the type of pain assay (acute versus chronic), route of administration, and species. In the mouse tail-flick acute pain assay, AT-200 increased tail-flick latency, reversible by naloxone, showing that it was a MOP-mediated antinociceptive effect (Spagnolo et al. 2008). However, in the mouse spinal nerve ligation chronic pain model, AT-200 showed anti-allodynic activity reversible by a NOP antagonist but not by naloxone, indicating that the anti-allodynic effect was due to its NOP agonist efficacy (Khroyan et al. 2011b). AT-200 also shows potent antihyperalgesic and anti-allodynic activity in the transgenic sickle cell pain mouse model reversible by a NOP antagonist but not naloxone (Vang et al. 2015). Even though AT-200 shows some MOP-mediated acute antinociceptive efficacy, it shows no rewarding effects in the mouse conditioned place preference paradigm (Toll et al. 2009). Together, these studies with AT-200 suggest that NOP partial agonist efficacy is sufficient for NOP-mediated antihyperalgesic efficacy in chronic pain models.

Other well-characterized recently reported NOP partial agonists are AT-090 and AT-127, which show high binding affinity and selectivity for NOP over the other opioid receptors (Table 2). As discussed in further detail later in this article, both these two NOP partial agonists show arrestin recruitment in vitro as well as G protein-mediated functional efficacy, resulting in an unbiased or modestly arrestin-biased profile of functional selectivity (Ferrari et al. 2016). In vivo, AT-090 showed anxiolytic-like activity in the elevated plus maze (EPM), but not in NOP (−/−) mice, mimicking the action of NOP full agonists (Asth et al. 2016). Furthermore, AT-090 showed no suppression of motor activity at anxiolytic doses, suggesting that NOP partial agonists may have a better dose separation between anxiolytic efficacy and locomotor suppression unlike NOP full agonists like Ro 64-6198.

Ross et al. also reported the anxiolytic efficacy in the EPM assay of a triazaspirodecanone, compound 1, (Table 2), which they labeled as a NOP partial agonist (Ross et al. 2015). However, there was no functional efficacy data in this paper or in their cited patent showing that compound 1 is indeed a NOP partial agonist (Battista et al. 2009).

5 Nonpeptide NOP Antagonist Ligands

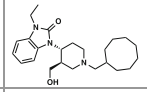
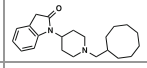
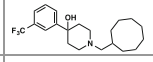
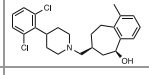
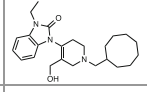
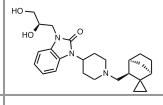
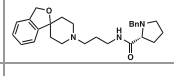
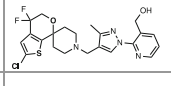
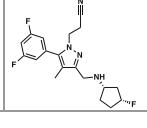
Nonpeptide NOP antagonists have been invaluable in investigating NOP pharmacology, particularly after systemic administration of ligands for therapeutic benefit. One of the very first nonpeptide NOP ligands reported was indeed a NOP antagonist, J-113397 (see chemical structure in Table 3) (Kawamoto et al. 1999). J-113397 is a benzimidazolone-derived NOP antagonist, with nanomolar affinity for NOP but modest selectivity versus the MOP receptor compared to the other widely used NOP antagonist tool compound SB-612111, reported by GSK (Zaratin et al. 2004). SB-612111 is a phenylpiperidine class of NOP ligand and shows subnanomolar affinity for NOP and excellent selectivity versus the classical opioid receptors. Both these NOP antagonists are systemically active and brain-penetrant and are very useful as tool compounds. Banyu Pharmaceuticals also developed another potent, orally active NOP antagonist, MK-5757 (Table 1) from the benzimidazolone series of NOP ligands, which was advanced into clinical trials (Satoh et al. 2009). Other benzimidazolone-based NOP antagonists reported include Trap-101 (Table 3), closely related to J-113397, reported by Trapella et al. (2006).

Almost all NOP antagonists contain the three pharmacophoric elements important for high NOP affinity (discussed in Sect. 2) and possess a piperidine ring as the central pharmacophoric motif with a basic nitrogen important for binding to NOP. The cyclooctylmethyl moiety on the piperidine nitrogen appears to be a common pharmacophore that affords a NOP antagonist profile, as seen on the benzimidazolone-based J-113397 (Table 3). Other chemical classes of NOP antagonists such as the dihydroindolinone-based SR14148 (Table 3) and the phenylpiperidine-based SR16430 (Table 3) were also reported as selective NOP antagonists that were systemically active and reversed the pharmacological effects of N/OFQ or NOP agonists in vivo (Khroyan et al. 2007, 2009; Spagnolo et al. 2008).

While the early reported NOP antagonists (J-113397, SB-612111, SR14148, SR16430) contained smaller lipophilic groups on the piperidine nitrogen (such as c-octyl methyl), Banyu scientists also reported new NOP antagonists with significantly larger and novel substituents on the piperidine nitrogen, such as C-24 (see Table 3) (Goto et al. 2006), a spiropiperidine-based compound, which is a potent and selective NOP antagonist optimized from high-throughput screening hits. C-24 was subsequently co-crystallized with the NOP receptor protein for the first determination of the three-dimensional structure of NOP receptor by X-ray crystallography (Thompson et al. 2012).

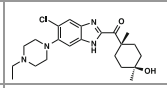
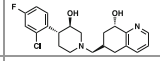
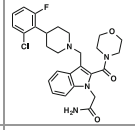
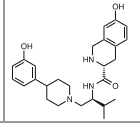
A novel series of potent NOP antagonists were also reported by Eli Lilly, from which LY2940094 (Table 3) was advanced into clinical development. LY2940094 and its analogs contain a novel dihydrospiropiperidine-thienopyran scaffold, with a bulky, aromatic 1-aryl-4-methylpyrazole substituent on the spiropiperidine nitrogen (Toledo et al. 2014). LY2940094 was optimized for oral bioavailability and shown to have high NOP receptor occupancy in vivo in rats and reversed NOP agonist Ro 64-6198-induced hypothermia in rats in a dose-dependent manner, confirming its antagonist profile in vivo (Toledo et al. 2014).

Table 3 Binding affinities and functional activity of NOP antagonists

	Structure	Receptor binding K_i (nM)				NOP [^{35}S]GTPγS	Reference
		NOP	MOP	KOP	DOP	Antagonist potency	
J-113397		2.0 ± 0.7	30.7 ± 7.6	58.3 ± 6.9	>2,500	IC_{50} = 5.3 ± 0.1	Kawamoto et al. (1999) and Ozaki et al. (2000)
SR14148 (AT-206)		6.0 ± 0.4	14.4 ± 1.1	229 ± 33.5	>10 K	K_e = 15.3 ± 1.6	Zaveri et al. (2004)
SR16430 (AT-207)		6.5 ± 1.4	61.0 ± 15.0	220 ± 18.0	2,350 ± 196	Not reported	Khroyan et al. (2007)
SB-612111		0.3 ± 0.1	57.6 ± 8.0	161 ± 24	2,100 ± 570	K_b = 5 (luciferase)	Zaratin et al. (2004)
Trap-101		2.2	251	676	>10 K	pA_2 = 8.55	Trapella et al. (2006)
MK-5757		2.3 ± 0.2	4,500 ± 200	10 K ± 900	>10 K	IC_{50} = 16 ± 7	Satoh et al. (2009)
C-24		0.27 ± 0.04	6,700 ± 4,300	2,500 ± 200	>10 K	IC_{50} = 0.15 ± 0.01	Goto et al. (2006)
LY2940094		0.11	>375	>375	>375	K_b = 0.17	Toledo et al. (2014)
MK-1925		8.2 IC_{50}	Not reported	Not reported	Not reported	IC_{50} = 4.6	Kobayashi et al. (2009b)

(continued)

Table 3 (continued)

	Structure	Receptor binding K_i (nM)				NOP [^{35}S]GTPγS	Reference
		NOP	MOP	KOP	DOP	Antagonist potency	
7c		1.4 IC_{50}	Not reported	Not reported	Not reported	$IC_{50} = 1.3$	Kobayashi et al. (2009c)
10 1		11 ± 2	Not reported	Not reported	Not reported	$IC_{50} = 12 \pm 2$	Yoshizumi et al. (2008b)
Nik-21,273		Not reported	Not reported	Not reported	Not reported	$K_b = 41.7$ (Ca^{2+} mobilization assay)	Marti et al. (2013)
AT-076		1.8 ± 0.7	1.1 ± 0.6	1.7 ± 0.6	19.6 ± 1.3	$K_e = 30.1 \pm 21.9$	Zaveri et al. (2015)

Several other novel chemical series of NOP antagonists were discovered by Banyu Pharmaceuticals and optimized for oral activity, CNS permeability and hERG selectivity for advancement as clinical candidates. Lead compounds identified from each series were confirmed as NOP antagonists by reversal of NOP agonist-induced hypolocomotion. Some of these NOP antagonists are shown in Table 3. Compound 7c from a series of 6-piperazinyl-substituted benzimidazoles (Kobayashi et al. 2009c) was a single-digit nanomolar potent NOP antagonist, obtained by extensive optimization to reduce P-glycoprotein efflux and hERG channel affinity (Kobayashi et al. 2009a). Compound 7c was also shown to inhibit carrageenan-induced hyperalgesia in rats after oral administration. Banyu also reported an optimized series of 3-hydroxy-4-arylpiperidines, structurally similar to arylpiperidine SB-612111, from which compound 10l (Table 3) (Yoshizumi et al. 2008b) was shown to reverse NOP agonist-induced hypolocomotion in mice after oral dosing. A chemically novel and distinct series of NOP antagonists based on bis-arylpyrazoles were also reported by Banyu, from which MK-1925 (Table 3) was identified after optimization, and advanced as a clinical candidate (Kobayashi et al. 2009b; Yoshizumi et al. 2008a). MK-1925 has different pharmacophoric features than most NOP antagonists (and most NOP ligands) shown in Table 3. Nevertheless, it is likely that the 2-substituted-3-aryl-4-methylpyrazole moiety functions as the lipophilic substituent on the exocyclic secondary amine nitrogen and is notably similar to the piperidine nitrogen substituent in the LY2940094 series of NOP antagonists.

While considerable effort has been expended into developing highly selective NOP antagonists as tools and for clinical development, nonselective NOP antagonist-opioid antagonists have also been reported. AT-076 was reported as a potent nonselective pan antagonist at NOP and all three classical opioid receptors (Zaveri et al. 2015). AT-076 is structurally similar to the kappa antagonist JDTic but has significantly higher affinity for the NOP receptor than JDTic itself, resulting in a ligand that has high affinity at all four opioid receptors (Zaveri et al. 2015). SAR studies suggest that AT-076 represents a new "universal opioid ligand" motif, which could be a useful tool and chemical scaffold for structure-based design and discovery of selective- or multifunctional opioid ligands (Journigan et al. 2017).

NOP antagonists have been investigated in various preclinical models of major depressive disorder, chronic pain, alcohol use disorders, and Parkinson's disease motor symptoms. At least two NOP antagonists (LY2940094 and MK-5757) have been advanced into human clinical trials, as discussed below.

Major Depressive Disorder (MDD) There is a significant rationale for the role of the NOP receptor in anxiety and mood disorders (Gavioli and Calo 2013; Mallimo and Kusnecov 2013; Reinscheid 2006; Witkin et al. 2014). In fact, early studies demonstrated an antidepressant phenotype of the NOP(−/−) mice (Gavioli et al. 2003). Furthermore, NOP antagonists J-113397 and SB-612111 show antidepressant-like activity in vivo in the mouse forced swim and tail suspension tests (Gavioli and Calo 2006; Rizzi et al. 2007). Indeed, NOP antagonist drug candidate LY2940094 shows excellent efficacy in several preclinical models of depression (Witkin et al. 2016) and in phase II clinical trials (Post et al. 2016a). The progress and success of the

clinical development of LY2940094 (now called BTRX246040) will be important in validating NOP antagonism as an approach for psychiatric disorders.

Alcohol Use Disorders It is well-known (and discussed earlier in this article) that activation of the NOP receptor with N/OFQ or NOP agonists blunts the motivational and reinforcing effects of alcohol in a range of behavioral measures, such as conditioned place preference, self-administration, and relapse to alcohol seeking (Ciccocioppo et al. 2009; Martin-Fardon et al. 2010; Ubaldi et al. 2013). However, the dysregulation of the N/OFQ-NOP system in rats genetically modified for alcohol preference (Ciccocioppo et al. 2006; Economidou et al. 2008) and recent evidence that genetic deletion of NOP receptors in rats confers resilience to drug abuse (Kallupi et al. 2017), including lower alcohol intake, appears to support the concept that NOP antagonists may have promising efficacy in alcohol addiction. Indeed, the orally active NOP antagonist LY2940094 was demonstrated to attenuate ethanol drinking, seeking, and relapse in alcohol-preferring rats (Rorick-Kehn et al. 2016). LY2940094 was advanced to a Phase 2 proof-of-concept trial in alcohol-dependent subjects and showed a decrease in heavy drinking and increased abstinence days, but did not appear to reduce alcohol intake per se (Post et al. 2016a). Clearly, more translational studies are needed to determine whether NOP agonists or NOP antagonists are clinically useful for alcohol addiction disorders (Litten 2016).

NOP Antagonists in Pain Models The pharmacology of the NOP system in pain is complex, and therefore characterization of NOP ligands in preclinical models of acute, chronic, neuropathic, or inflammatory pain is highly dependent on species, the model, the site of action, and measurement of efficacy. NOP antagonist tool compounds J-113397 and SB-612111, as expected, inhibit hyperalgesia elicited by i.c.v. N/OFQ in the mouse tail-flick or hot-plate assay, but have no effect on latency per se (Ozaki et al. 2000; Rizzi et al. 2007; Zaratin et al. 2004). However, NOP antagonists SB-612111 and Banyu antagonist 7c have been shown to be effective in reversing thermal hyperalgesia in the rat carrageenan inflammatory pain model (Kobayashi et al. 2009c; Zaratin et al. 2004). No other NOP antagonists have been investigated for efficacy in pain models.

Parkinson's Disease Motor Symptoms Perhaps the most consistent demonstration of the in vivo activity of chemically distinct NOP antagonists for therapeutic benefit has been their efficacy in relieving parkinsonian motor deficits in preclinical rodent and nonhuman primate models of Parkinson's disease (PD). Seminal work conducted by Morari and colleagues provide evidence that the N/OFQ-NOP system undergoes changes in basal ganglia following dopamine depletion and that upregulation of N/OFQ transmission in the substantia nigra contributes to motor symptoms in PD (Marti et al. 2004). NOP receptor blockade provides symptomatic benefit in normalizing the motor deficits in animal models of PD (Marti et al. 2005). Systemic administration of various NOP antagonists consistently attenuates parkinsonian-like akinesia/hypokinesia in 6-hydroxydopamine hemilesioned or haloperidol-treated rat model of PD and MPTP-treated nonhuman primates. This efficacy was demonstrated

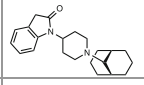
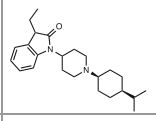
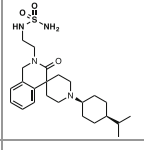
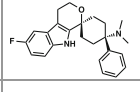
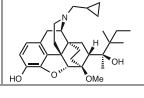
for J-113397 (Marti et al. 2004, 2007; Viaro et al. 2008), Trap-101 (Marti et al. 2008), SB-612111 (Marti et al. 2013) and Nik-21,273 (Table 3) (Marti et al. 2013). NOP antagonist LY2940094 is currently in Phase 2 clinical trials for motor symptoms in PD patients (NCT03608371 2018).

6 Nonpeptide NOP/MOP-Targeted Bifunctional Agonists

Selective NOP agonists can attenuate opioid agonist-induced rewarding effects in rodents and nonhuman primates (Podlesnik et al. 2011; Shoblock et al. 2005; Sukhtankar et al. 2014a; Zaveri et al. 2018a) and also show significant antinociceptive and antihyperalgesic efficacy in chronic or neuropathic pain in rodent models (Khroyan et al. 2011b) and in acute and inflammatory pain in nonhuman primates (Podlesnik et al. 2011; Sukhtankar et al. 2014b). Furthermore, NOP agonists have synergistic antinociceptive efficacy with MOP agonists in nonhuman primates after spinal (Hu et al. 2010) and systemic administration (Cremeans et al. 2012). Given that the abuse liabilities as well as other side effects of MOP analgesics can be modulated by NOP agonists, there is a compelling hypothesis that dual-targeted NOP/MOP ligands with bifunctional NOP and MOP agonist activity may have a nonaddicting analgesic profile and be devoid of opioid liabilities such as tolerance and dependence.

One of the first nonpeptide NOP/MOP bifunctional agonists to be characterized was SR16435 (now named as AT-201) (Table 4) (Khroyan et al. 2007; Zaveri et al. 2004), which has high affinity for the NOP and MOP receptors and partial agonist efficacy at both receptors, as measured in the GTPγS functional assay. AT-201 has acute antinociceptive activity in the mouse tail-flick assay, reversible by naloxone, but produced a place preference in the CPP assay, indicative of rewarding effects, after systemic administration (Khroyan et al. 2007). To explore whether full agonist activity at NOP would better attenuate the MOP-mediated rewarding effect in the bifunctional compound, a NOP full agonist and MOP partial agonist SR16507 (now called AT-212, Table 4) was developed using medicinal chemistry (Zaveri et al. 2013a) and characterized in vivo. AT-212 showed potent, naloxone-reversible antinociceptive efficacy in the mouse tail-flick assay but also induced a modest CPP response even though it suppressed morphine CPP (Toll et al. 2009). However, Zaveri and colleagues also reported that AT-200 (SR14150) (Table 2), a NOP partial agonist with moderate binding selectivity for NOP over MOP and partial agonist activity at MOP, showed naloxone-reversible analgesia in the mouse tail-flick assay and did not produce a CPP response, suggesting lack of rewarding effects (Toll et al. 2009). Together, the in vivo profile of these three NOP/MOP bifunctional compounds suggests that a nonaddicting, but effective, analgesic profile in NOP/MOP bifunctional agonists may be obtained by modulating the selectivity between the NOP and MOP receptor and that even modest selectivity in favor of NOP over MOP, both in binding affinity and in agonist potency, may be important for overcoming MOP-mediated rewarding effects in the bifunctional ligand.

Table 4 Binding affinities and functional activity of NOP/MOP bifunctional agonists

		Receptor binding K_i (nM)				NOP [^{35}S]GTPγS		MOP [^{35}S]GTPγS		
	Structure	NOP	MOP	KOP	DOP	EC_{50} (nM)	% Stim	EC_{50} (nM)	% Stim	Reference
AT-201 (SR16435)		7.5 ± 0.8	2.70 ± 0.0	31.7 ± 4.8	2066 ± 130	28.7 ± 0.6	45.0 ± 5.0	29.5 ± 10.0	30 ± 0.0	Zaveri et al. (2004)
AT-212 (SR16507)		5.2 ± 0.7	1.1 ± 0.2	82.4 ± 16.4	574 ± 87	8.5 ± 0.8	95.0 ± 12.0	5.2 ± 1.6	47.0 ± 1.5	Zaveri et al. (2013a)
AT-121		3.7 ± 1.1	16.5 ± 2.1	301 ± 35.4	146 ± 25.5	34.7 ± 6.3	41.1 ± 0.3	19.6 ± 6.9	14.2 ± 0.40	Ding et al. (2018)
Cebranopadol		0.9 ± 0.2	0.7 ± 0.3	2.6 ± 1.4	18.0 ± 20.0	13.0 ± 2.0	88.9 ± 3.9	1.2 ± 0.4	103.5 ± 4.7	Schunk et al. (2014)
BU08028		8.5 ± 1.3	2.1 ± 0.8	5.6 ± 1.3	1.6 ± 0.3	78.6 ± 49.0	48 ± 13	6.0 ± 2.1	21.1 ± 8.7	Khroyan et al. (2011a)

This hypothesis was further confirmed by the recent report of a NOP/MOP bifunctional partial agonist AT-121, from a different chemical series than AT-201 or AT-212 (Table 4), which was optimized to produce the profile of modest NOP binding selectivity over MOP and partial agonist efficacy at both NOP and MOP receptors (Ding et al. 2018). AT-121 was shown to have morphine-comparable antinociceptive and antihyperalgesic efficacy in nonhuman primates after systemic administration and did not show reinforcing effects or other opioid liabilities such as respiratory depression, tolerance, itch, or dependence after chronic dosing. It is further notable that not only does AT-121 lack innate reinforcing activity, it also attenuates the reinforcing effects of oxycodone, an abused prescription opioid, in nonhuman primates (Ding et al. 2018). Thus, NOP/MOP bifunctional agonists with an appropriate profile discussed above may be developed as "nonaddicting analgesics" and have potential as treatments for opioid use disorders for the current opioid crisis.

A chemically distinct NOP-/opioid-targeted agonist cebranopadol was developed by Grunenthal (Germany) and is currently in phase III clinical development for chronic pain indications (Christoph et al. 2017; Scholz et al. 2018). Cebranopadol (Table 4) has high binding affinity for the NOP, MOP, and KOP receptors and slightly lower affinity for the DOP receptor. It shows high potency, full agonist efficacy at the MOP receptor, and slightly lower potency and efficacy at the NOP receptor (Linz et al. 2014; Schunk et al. 2014). It also has partial agonist efficacy at the KOP receptor (Linz et al. 2014). However, in line with the hypothesis posited above, cebranopadol, having higher selectivity in favor of MOP in both binding and functional potency (similar to AT-212) (Table 4), appears to have reward-like effects recently demonstrated in clinical trials (Gohler et al. 2019) and also produces a morphine-like discriminative stimulus in preclinical animal models, suggestive of opioid-like tendency for abuse liability (Tzschentke and Rutten 2018).

While the attenuation of rewarding effects of NOP/MOP bifunctional agonists seem to be dependent on the selectivity for NOP over MOP agonist potency, bifunctional NOP/MOP efficacy does attenuate other opioid liabilities regardless of the selectivity between NOP and MOP potencies. Indeed, cebranopadol produces limited physical dependence compared to MOP analgesics (Tzschentke et al. 2017) and has a wider therapeutic window for respiratory depression than classical opioids, as demonstrated in clinical trials (Dahan et al. 2017). Further, bifunctional NOP/MOP efficacy, regardless of the balance between NOP and MOP efficacy, also produces potent antinociception and antihyperalgesic efficacy in chronic, neuropathic, and inflammatory pain models in rodents, as shown with AT-201 (Khroyan et al. 2011b; Sukhtankar et al. 2013), AT-200 (Khroyan et al. 2011b; Vang et al. 2015), cebranopadol (Christoph et al. 2018; Linz et al. 2014; Rizzi et al. 2017; Salat et al. 2018; Schiene et al. 2015, 2018), and in human clinical trials with cebranopadol (Christoph et al. 2017; Eerdekens et al. 2018; Scholz et al. 2018).

While the abovementioned bifunctional NOP/MOP ligands (AT-201, AT-212, AT-121, and cebranopadol) were all based on non-morphinan scaffolds (see Table 4), efforts to increase NOP activity in morphinan-type scaffolds, such as buprenorphine, resulted in the first universal multifunctional opioid agonist

BU08028 (Table 4), which had single-digit nanomolar affinity at all four opioid receptors (Cami-Kobeci et al. 2011). BU08028 showed potent antinociceptive activity in the mouse tail-flick assay but produced a significant CPP response (Khroyan et al. 2011a). While BU08028 has partial agonist activity at NOP, it has higher affinity and agonist potency at MOP (Table 4). Consistent with the hypothesis above, BU08028 shows significant rewarding effects in rodents, given that its selectivity is in favor of its MOP activity (Table 4). Nevertheless, BU08028 shows lower reinforcing effects than remifentanil or cocaine in a progressive ratio schedule of reinforcement in nonhuman primates and no physical dependence or respiratory suppression at antinociceptive doses (Ding et al. 2016).

Taken together, these data on bifunctional NOP/MOP agonists suggest that the balance of NOP versus MOP efficacy in favor of NOP selectivity affords a promising profile for nonaddicting analgesia and for opioid use disorders.

7 NOP Ligands and Functional Selectivity

Biased agonism (functional selectivity or biased signaling) of GPCR ligands is the ability of agonists to selectively activate one or more intracellular signaling pathways resulting in differential and selective functional responses. For the MOP receptor agonists, biased agonism was developed as an approach that progressed from concept to clinical trials, as a means to improve their therapeutic profiles and reduce side effects such as constipation, respiratory depression, and abuse liability (Kingwell 2015). Indeed, TRV-130 (oliceridine), a nonpeptide MOP ligand which possesses functional selectivity for G protein signaling over arrestin signaling (DeWire et al. 2013), showed effective analgesia in acute pain with a wider therapeutic window for side effects such as constipation and respiratory events, as shown in clinical trials (Soergel et al. 2014). The concept of biased signaling has been investigated for all three classical opioid receptor GPCRs as an approach to dissociate analgesic effects from unwanted side effects such as dysphoria (for KOP agonists) (Dunn et al. 2018) or analgesic tolerance (for DOP agonists) (Pradhan et al. 2016) or respiratory depression (for MOP agonists) (Schmid et al. 2017). Being an opioid GPCR, the NOP receptor also has multiple intracellular signaling cascades that may be linked to differential functional responses (Toll et al. 2016).

The investigation of functional selectivity of nonpeptide NOP ligands is still in nascent stages; however, several NOP agonists have been characterized for their "bias" for activating G protein signaling or arrestin signaling in cellular functional assays in vitro. Malfacini et al. conducted a systematic analysis of the functional selectivity of a large panel of peptide and nonpeptide NOP ligands to promote or block NOP/G protein and NOP/arrestin interactions and found that most known NOP agonists they tested (shown in Table 1) show a bias for the G protein-mediated signaling interactions (Malfacini et al. 2015). Further analysis of several other nonpeptide NOP agonists such as MCOPPB, AT-403, Ro 65-6570, SCH-221510, AT-202, and SCH-486757 and NOP partial agonists AT-090 and AT-127 conducted by Ferrari and colleagues showed that while most NOP agonists showed a bias

toward G protein recruitment, AT-403, AT-090, and AT-127 showed significant arrestin recruitment similar to N/OFQ, such that they had an "unbiased" functional profile in vitro (Ferrari et al. 2016, 2017). Although the correlations of such functional selectivity with in vivo pharmacological properties of NOP ligands remain to be investigated, the differential signaling profiles of some structurally unrelated NOP ligands open up possibilities for dissociating the undesired effects of NOP agonists (such as locomotor suppression) from the beneficial effects such as analgesic efficacy in chronic pain and suppression of drug reward. Preliminary investigations along these lines have already been reported (Asth et al. 2016).

8 Conclusion

Nonpeptide NOP agonists and antagonists appear to have promising, differentiated pharmacological profiles for several therapeutic applications such as nonaddicting analgesia and substance abuse treatment for NOP agonists and Parkinson's disease and depression treatment for NOP antagonists. Successful translation of preclinical findings to human clinical trials will be important for validating the NOP receptor as a therapeutic approach in these indications. Future research should address investigations into NOP pharmacology that will overcome barriers to such translation. For instance, further studies of NOP-selective partial agonists are warranted to determine if they have a better profile of (or lack of) neurological side effects (such as sedation) compared to NOP full agonists. Future studies of the role of biased agonism in NOP pharmacology may also lead to innovative NOP-targeted therapies.

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Pharmacological Assays for Investigating the NOP Receptor

Davide Malfacini and Girolamo Caló

Contents

Abstract

The nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) is a G protein-coupled receptor involved in the regulation of several physiological functions and pathological conditions. Thus, researchers from academia and industry are pursuing NOP to discover and study novel pharmacological entities. In a multi-disciplinary effort of pharmacologists, medicinal chemists, and molecular and structural biologists the mechanisms of NOP activation and inhibition have been, at least partially, disentangled. Here, we review the in vitro methodologies employed, which have contributed to our understanding of this target. We hope this chapter guides the reader through the mostly established assay platforms to investigate NOP pharmacology, and gives some hints taking advantage from what has already illuminated the function of other GPCRs. We analyzed the

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pharmacological results obtained with a large panel of NOP ligands investigated in several assays including receptor binding, stimulation of GTPγS binding, decrease of cAMP levels, calcium flux stimulation via chimeric G proteins, NOP/G protein and NOP/β-arrestin interaction, label-free assays such as dynamic mass redistribution, and bioassays such as the electrically stimulated mouse vas deferens.

Keywords
N/OFQ · Pharmacological assays · Recombinant and native NOP receptors · Signal transduction

1 Introduction

The nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) is endogenously activated upon interaction with N/OFQ, a 17 amino acid peptide sharing high degree of sequence similarity with opioid peptides particularly dynorphin A (Reinscheid et al. 1995; Meunier et al. 1995). The NOP receptor is the last member of the opioid receptor family cloned and N/OFQ is the first successful example of reverse pharmacology (Civelli et al. 2013). Insights in N/OFQ–NOP interaction and drug development attempts have been taking advantage of the application of receptor binding assay (Dooley and Houghten 1996). The activity of NOP has been disentangled by mutational and structure–activity relationship (SAR) approaches. For instance, NOP mutants prompted the delineation of its selectivity (Mollereau et al. 1996), constitutive activity (Kam et al. 2002), signaling (Miyakawa et al. 2007; Mouledous et al. 2000), and phosphorylation patterns (Zhang et al. 2012; Wang et al. 2006). On the other hand, N/OFQ SAR studies contributed to clarify its minimum active sequence, chemical requirement crucial for affinity, selectivity, and efficacy (Calo' and Guerrini 2013). More recently, efforts in understanding structural features of NOP in complex with various antagonists determined the NOP binding pocket in great detail (Miller et al. 2015; Thompson et al. 2012). This class A GPCR shares with the other opioid receptors not only structural (Filizola and Devi 2013) but also signaling features (Corder et al. 2018). NOP perpetrates its intracellular effects mainly by activating inhibitory heterotrimeric G proteins. Following G protein dissociation, alpha subunits of $G_{i/o}$ lead in turn to the inhibition of adenylate cyclase (AC) with corresponding decrease of cAMP levels. Beta gamma subunits instead open inwardly rectifying potassium channels leading to cell hyperpolarization. In addition, activated NOP inhibits voltage-dependent calcium channels (Lambert 2008). G protein activation is mainly assayed by applying stimulation of [^{35}S]-GTPγS binding (McDonald and Lambert 2010; Sim et al. 1996), chimeric G proteins forcing a $G_{i/o}$-coupled receptor to trigger calcium release (Camarda et al. 2009; Coward et al. 1999), and more recently with bioluminescence resonance energy transfer (BRET) methods (Malfacini et al. 2015; Wang et al. 2004). cAMP levels alteration when linked to GPCR activation is mainly albeit not exclusively caused by Gα; this phenomenon is assayed with radioactively

labeled cAMP (Wu et al. 1997; Brown et al. 1971), and also with luminescent/fluorescent-based biosensors (Zhang et al. 2012; Liao et al. 2012). Following NOP activation, the C-tail of this GPCR undergoes GRKs-mediated phosphorylation with consequent β-arrestin recruitment (Zhang et al. 2012), an event occurring also in the absence of functional $G_{i/o}$ proteins. These phosphorylation events control the internalization of NOP, whose trafficking has been mainly evaluated by measuring the binding of radiolabeled N/OFQ (Spampinato et al. 2001, 2002, 2007; Spampinato and Baiula 2006) or by confocal microscopy (Corbani et al. 2004). Due to the lack of selective NOP antibodies, the generation of knock-in mice with fluorescently labeled NOP receptor importantly contributed to define NOP cellular and regional localization in vivo (Ozawa et al. 2015). Downstream of G protein coupling, NOP activation is involved in a whole plethora of events, including phosphorylation of kinases such as mitogen-activated protein kinase (MAPK) (Fukuda et al. 1997), protein kinase C (Lou et al. 1997), modulation of gene transcription/transduction (Wnendt et al. 1999), cytoskeleton rearrangement (Lowry et al. 2002), and chemotaxis (Trombella et al. 2005). Integrated cellular responses caused by NOP activation can be monitored using label-free assays including the dynamic mass redistribution (DMR) assay (Malfacini et al. 2018). This is a useful approach that together with primary cell cultures, cells obtained from animal models of pathology, and eventually from normal subjects and patients will be of great value for clarifying the NOP role in physiology and pathology. In Fig. 1 we schematically summarized the cascade of events leading from ligand binding to cellular response.

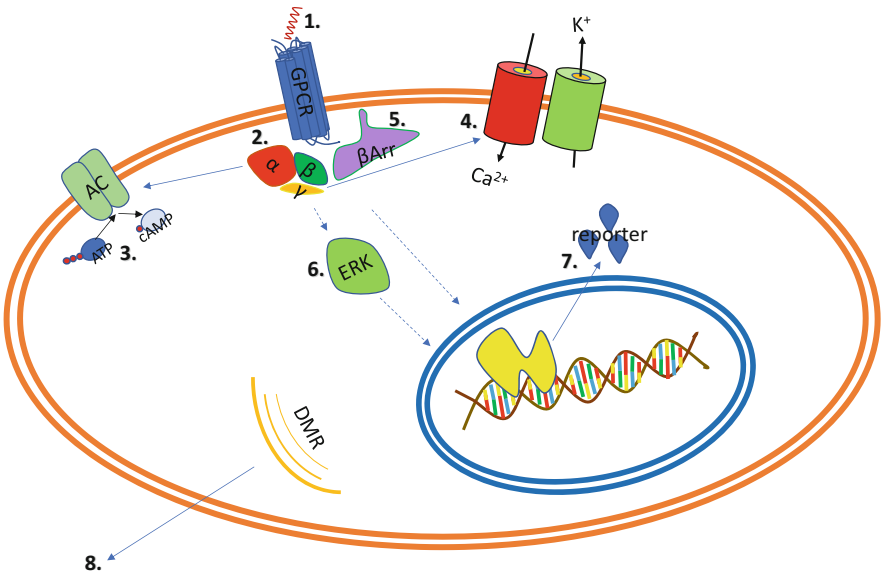

Fig. 1 Pharmacological investigations at NOP receptor. 1. Ligand–receptor interaction. 2. Receptor–G protein coupling. 3. cAMP levels 4. βγ-mediated channel modulation. 5. β-arrestin recruitment/internalization. 6. ERK phosphorylation. 7. Gene reporter assay. 8. Holistic response (dynamic mass redistribution)

Finally, as we consider still valid the typical statement of our mentor Prof. D. Regoli "Classical pharmacology performed on isolated organ preparations is an essential tool for receptor characterization and classification" (Pheng and Regoli 1998), the last section of this chapter is dedicated to N/OFQ-sensitive isolated tissues such as the electrically stimulated mouse vas deferens (mVD) (Calo et al. 1996; Berzetei-Gurske et al. 1996).

In the next sections we will review the pharmacological assays used to investigate the N/OFQ–NOP receptor system and briefly mention novel approaches and techniques that can be used in future studies for deepening our understanding of this peptidergic system.

2 Receptor Binding

Quantitative pharmacology is grounded on assumptions originated by the Langmuir adsorption isotherm model. In particular, receptor binding approaches take great advantage of such a theoretical background (Kenakin 2014a). Radioactive labeled ligands are used in receptor binding studies as useful instruments to get information concerning ligand affinity (see Fig. 2a), kinetics of association and dissociation, density of membrane receptors, and receptor trafficking. N/OFQ radiolabeled derivatives employing ^{125}I or ^{3}H isotopes (Dooley and Houghten 1996; Reinscheid et al. 1995) aided the evaluation of N/OFQ affinity determinants (Reinscheid et al. 1996). Of note, the study of N/OFQ binding might be nontrivial, for example as reviewed in (Dooley and Houghten 2000), NOP affinity reported from different laboratories ranges between 2 pM and 5 nM. Complicating the picture, this peptide binds to harvesters' filters even in the presence of polyethyleneimine that represents a common treatment for diminishing undesired binding; the use of bovine serum albumin is recommended for minimizing artifacts due to N/OFQ displaceable nonspecific binding (Dooley and Houghten 2000).

Recently, a fluorescent derivative of N/OFQ (i.e., N/OFQ$_{ATTO594}$) has been proposed as novel NOP ligand for studying ligand–receptor interaction in living cells. The application of such tool will be important not only to study NOP binding in radioactive-free conditions, but also to measure receptor trafficking (Bird et al. 2018). We envisage N/OFQ$_{ATTO594}$ together with fluorescently labeled NOP receptors will be instrumental for fluorescence correlation spectroscopy (FCS) (Briddon et al. 2018), single-molecule imaging (Calebiro and Sungkaworn 2018), and fluorescence anisotropy (Rinken et al. 2018) studies. Furthermore, given the renewed importance of binding kinetics in drug discovery (Strasser et al. 2017), the availability of novel radioactive and particularly fluorescence labeled NOP ligands will certainly empower the field of innovative perspectives.

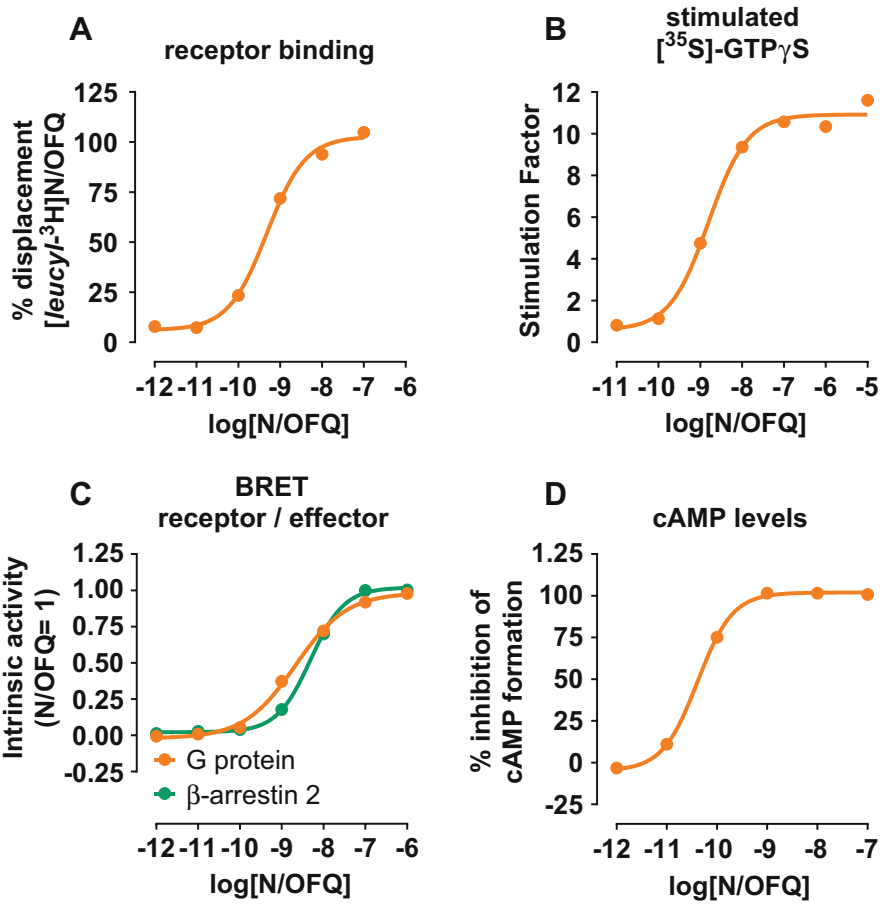

Fig. 2 Concentration response curves to N/OFQ in equilibrium assays: panel (**a**) receptor binding, panel (**b**) stimulated [35S]-GTPγS, panel (**c**) BRET receptor/effector, and panel (**d**) cAMP levels

3 G Proteins

NOP activation fosters GDP/GTP exchange in the Gα subunits of the $G_{i/o}$ subfamily; moreover, NOP coupling to G_{16}, G_{12}, and G_{14} has also been reported (Yung et al. 1999; Chan et al. 1998). Stimulation of [^{35}S]-GTPγS binding (McDonald and Lambert 2010; Sim et al. 1996) shed light onto functional pharmacological properties of NOP ligands (McDonald and Lambert 2010; McDonald et al. 2003a) (see Fig. 2b). Interestingly, NOP activation and its blockage were also characterized in coronal sections of mouse brain (Berger et al. 2000), contributing to explain how high levels of receptor expression together with G proteins reserve and GDP/GTP levels promote agonism of low-efficacy ligands.

The NOP-dependent triggering of G proteins has also been studied through chimeric G proteins (Camarda et al. 2009; Coward et al. 1999) (see Fig. 3a) and BRET methods (Malfacini et al. 2015) (see Fig. 2c). By chimeric G proteins we refer here only to those engineered entities created by stepwise exchanging of $G\alpha_q$

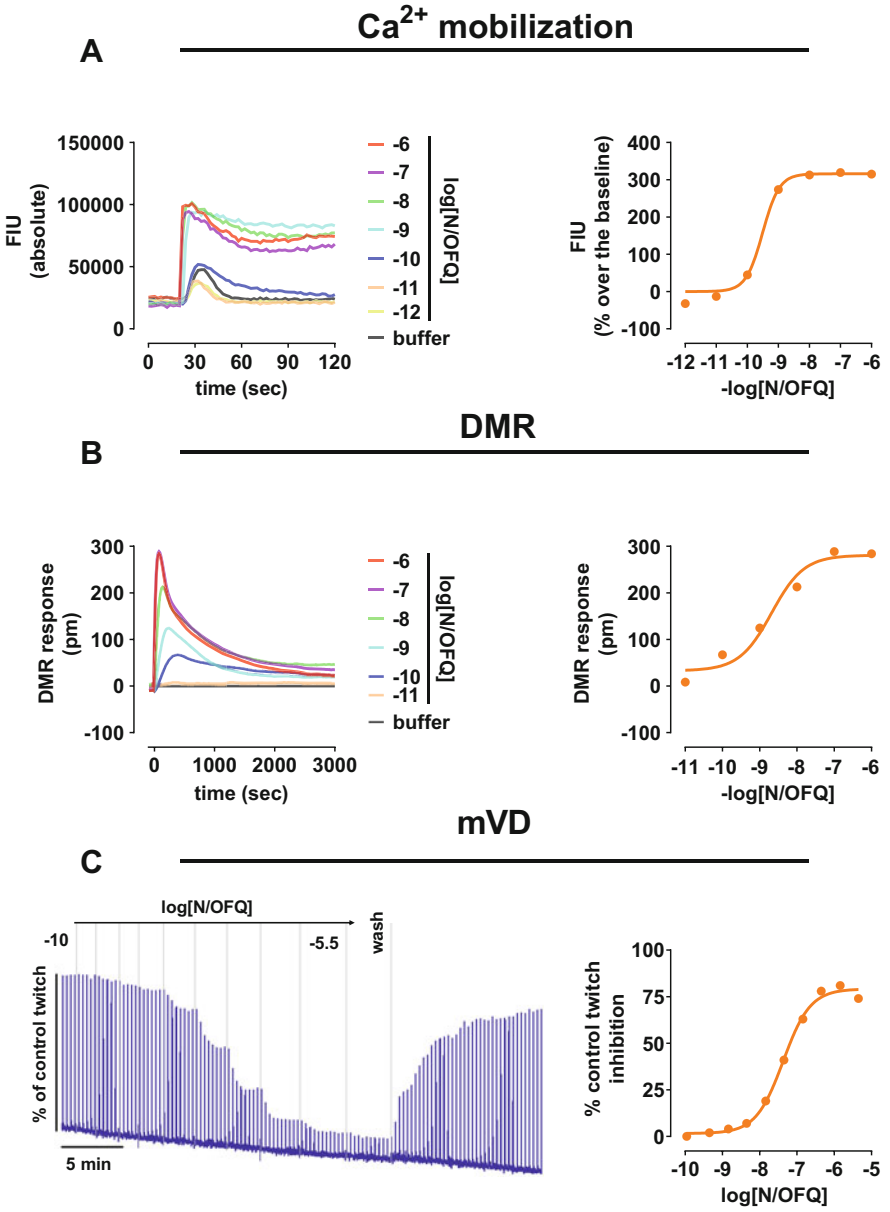

Fig. 3 Concentration response curves to N/OFQ in real-time assays: panel (**a**) calcium mobilization, panel (**b**) dynamic mass redistribution, and panel (**c**) electrically stimulated mouse vas deferens

C-terminal with that of $G\alpha_i$. Conklin et al. (1993) demonstrated that the substitution of only three amino acids switches the $G\alpha_q$–GPCR coupling specificity towards GPCRs normally coupled to Gαi. Given their increased GPCRs promiscuity, these chimeric G proteins have become common tools to study calcium fluxes upon stimulation of G_i-coupled receptors including NOP. The calcium mobilization assay has been successfully applied to study the pharmacological properties of the NOP receptor and of novel ligands. The pharmacological profile of NOP receptors coupled with chimeric G proteins did not show major deviations from that obtained in native systems (Camarda et al. 2009). However, disturbing phenomena should be considered both when studying agonists and antagonists with this assay. On one hand, amplification phenomena (overexpression of receptors and chimeric G proteins) may leftward shift agonists concentration response curves thus overestimating agonist potency and increase their maximal effects thus overestimating agonist efficacy (Camarda et al. 2009). On the other hand, kinetic artifacts due to hemi equilibrium conditions caused by the rapid and transient nature of calcium peaks (discussed in detail in (Charlton and Vauquelin 2010)) might lead to underestimation of the potency of slow interacting agonists (Rizzi et al. 2014) and to an apparent unsurmountable behavior of competitive antagonists (Fischetti et al. 2009). Nevertheless, this assay is a very useful tool that accelerated the identification and characterization of numerous NOP ligands.

BRET and fluorescence/Förster resonance energy transfer (FRET) systems are widely adopted in basic and molecular pharmacology. In BRET experiments the donor is an enzyme capable of generating chemiluminescent light, while in FRET studies the donor is a fluorescent molecule (i.e., protein or dye). In both BRET and FRET techniques the acceptor is a fluorescent entity (i.e., protein or dye) that emits light at a different wavelength. BRET- and FRET-based assays can be roughly divided into two groups, i.e., proximity-based vs intramolecular assays: in the former, donor and acceptor are linked to different proteins (e.g., GPCR and a GPCR interacting protein), see for example (Gales et al. 2005); in the latter, donor and acceptor are linked to different parts of the same protein and can therefore sense the rearrangement occurring upon interaction with another protein, see for example (Charest et al. 2005). NOP receptor–G protein interaction has been measured with a classical Renilla luciferase (Rluc)–Renilla green fluorescent protein (RGFP) BRET system where the intracellular C-terminal region of NOP is linked with RLuc and the Gβ1 N-terminal tagged with RGFP. In this system, NOP binding to endogenous Gα subunits results in a reduced distance between the receptor C-terminal region and the N-terminal region of the Gβ1 subunit. This NOP/G protein interaction BRET assay leads to very similar results as the stimulation of [^{35}S]-GTPγS binding that is also performed on cell membranes (Malfacini et al. 2015).

GPCR's constitutive activity can also be easily assayed by employing such BRET approaches as elegantly demonstrated by Vezzi et al. (2013). Interestingly, NOP receptor basal G protein recruitment is the least affected by GDP concentrations in comparison with mu and delta opioid receptors. This suggests low liability of the NOP protein to adopt constitutively active conformations under physiological conditions. In fact, evidence for NOP constitutive activity has been

obtained only under extreme conditions such as sympathetic neurons microinjected with NOP coding cDNA (Mahmoud et al. 2010). Of note, NOP constitutive activity was negatively regulated by Na^+. Intriguingly, a single mutation of residue N3.35 (N133W) of NOP sequence is sufficient for increasing the receptor ligand-independent signaling (Kam et al. 2002), and this residue is involved in the sodium binding pocket that allosterically regulates receptor binding and functions in several class A GPCR (Katritch et al. 2014).

In terms of native second messenger assaying, cAMP level measurements were employed alone or in combination with other methods since the very beginning of the N/OFQ-NOP system discovery (see Fig. 2d). In fact, N/OFQ identification as endogenous NOP agonist has been carried out by testing the effects of fractions of tissue extracts in wild-type cells and in cells expressing NOP with a forskolin (FSK)-stimulated cAMP inhibition assay (Reinscheid et al. 1995; Meunier et al. 1995). This assay was also useful for the investigation of the relationship between the level of receptor expression and signal amplitude using prolonged agonist exposure (Hashimoto et al. 2002) or receptor inducible systems (Barnes et al. 2007; McDonald et al. 2003a). Although most of the existing investigations were conducted with radioactive methods, nowadays fluorescent or luminescent cAMP-biosensors are often adopted as useful and cheaper alternatives (Wang et al. 2004; Zhang et al. 2012; Liao et al. 2012). Other approaches employed were based on cAMP-sensitive reporter gene assays; for example, Wnendt et al. (1999) utilized a cAMP reporter plasmid pSE66 that transcripts luciferase in a cAMP concentration-dependent manner to compare activities of N/OFQ, [F/G]N/OFQ(1–13)-NH_2, and buprenorphine. In terms of future perspectives, the findings recently obtained with Gs-coupled GPCRs in terms of cellular nanodomain cAMP levels are certainly exciting (Musheshe et al. 2018) since such discoveries shed light on how a cell discriminates the impulse coming from different receptors. We envisage that the development of better cAMP sensors will allow to disentangle how inhibitory GPCRs, including NOP, determine their specific effects.

G proteins are complex entities that upon interaction with guanine nucleotide exchange factors undergo a GDP/GTP exchange followed by Gα dissociation from Gβγ subunits; GPCRs are de facto the mostly studied guanine nucleotide exchange factors. Studying G protein-specific effects on second messenger modulation and kinases phosphorylation cascades is facilitated by the application of inhibitors such as PTX for Gi/o (Bokoch et al. 1984) and FR900359 for Gq/11/14 (Schrage et al. 2015), and of novel genetic tools based on CRISPR-Cas9 technologies (Grundmann et al. 2018). Gβγ subunits remain a difficult target for pharmacological interdiction despite the availability of molecules such as gallein and its derivatives (Bonacci et al. 2006), and of a recently developed nanobody capable of blocking multiple Gβγ subunits functions (Gulati et al. 2018). Even with these limitations, a large body of evidence indicates that after NOP activation Gβγ is involved in calcium channel modulation (Mahmoud et al. 2012, 2016). These results have been measured employing patch-clamp techniques and validated by applying small interference RNA directed against Gβ or Gγ. Moreover, patch-clamp approaches showed how NOP plays an important role in controlling G protein-activated K^+ channels and thus increasing potassium conductance (Connor et al. 1996; Ikeda et al. 1997).

4 GRK, β-Arrestin, and Internalization

GPCR kinases (GRKs)-mediated phosphorylation is a key regulatory mechanism for receptor desensitization, internalization, and trafficking. It has been proposed that phosphorylation patterns are directly involved in regulating receptor–arrestin interaction (Yang et al. 2017). An elegant study carried out by Zhang et al. (2012) demonstrated that the NOP receptor S363A substitution, making the receptor unable to undergo GRK-mediated phosphorylation, dramatically decreases receptor internalization and desensitization. The latter event was investigated in terms of calcium conductance alteration through whole-cell patch clamp and as reduction of cAMP levels. Small interference RNA-dependent knocking down of GRK2, GRK3, β-arrestin 1, and β-arrestin 2 demonstrated that GRK3 and β-arrestin 2 are primarily involved in NOP internalization processes. Furthermore, through western blot evaluations NOP S363A displayed superimposable extracellular signal-related kinases (ERK) phosphorylation patterns as the wild-type receptor, while JUN phosphorylation kinetics were significantly altered when wild-type and mutated receptor activities were compared. The Zhang study represents the first attempt to investigate G protein and β-arrestin contribution in NOP-mediated signaling. The role of β-arrestins in GPCRs signaling is nowadays object of intense debate (Grundmann et al. 2018; Shenoy et al. 2006); however, also for the NOP receptor, ligands capable of producing different degrees of receptor/G protein vs receptor/β-arrestin 2 interaction (biased agonists) have been described (Ferrari et al. 2016, 2017; Rizzi et al. 2016; Chang et al. 2015a, b; Malfacini et al. 2015) (see Fig. 2c for the effect of N/OFQ). These results, mainly based on BRET methods, indicate that several NOP ligands behave as G protein biased agonists while none are biased toward β-arrestins. The reasons for the lack of NOP (and more in general opioid) biased agonists toward β-arrestins are still obscure. Nevertheless, the generation of strongly NOP-β-arrestins biased agonists will be instrumental for answering important questions regarding the role of β-arrestins in NOP signaling and in the biological functions controlled by the NOP receptor, and ultimately regarding the therapeutic potential of this kind of NOP ligands. For the NOP as for other receptor systems also the calculation of the amplitude of bias (bias factor) has been approached differently (Ferrari et al. 2017; Malfacini et al. 2015; Chang et al. 2015b). For detailed discussion of this topic please refer to Rajagopal et al. (2011) and Kenakin (2014b). In addition to a rigorous comparison of such methods, independent semi-quantitative calculation techniques for determining bias have been recently proposed by Onaran et al. (2017).

As far as receptor internalization is concerned, NOP binding on intact cells or confocal approaches were rigorously employed to determine the proportion of NOP internalized following addition of diverse compounds (Corbani et al. 2004; Spampinato et al. 2001). Interestingly enough, ligand's effects in internalization studies resemble that obtained in arrestin recruitment; in other words, studies employing arrestin recruitment or receptor internalization will likely generate similar patterns of bias.

5 Downstream Effectors

Signal transduction cascades vary between receptor and receptor. In addition, stimulation of the same receptor with different ligands triggers to evoke diverse physiological outcomes. This might be based on the proportion and amplitude of effectors recruited. Therefore, the investigation of signals downstream of the NOP cascade is needed to forecast/explain the effects of a ligand in vivo. A radioactive-based protein kinase C (PKC) was applied to membrane fractions of CHO cells stably expressing the NOP receptor by Lou et al. (1997). The authors demonstrated a robust NOP-dependent activation of PKC via a PTX-sensitive phospholipase C (PLC)/calcium-triggered manner. Nevertheless, a systematic characterization of PKC activation with a panel of NOP ligands characterized by diverse pharmacological properties is still lacking. ERK phosphorylation is linked to several cellular outcomes including proliferation, differentiation, migration, senescence, and apoptosis, but it has also been reported a role for ERK1/2 in learning and memory (Johnson and Lapadat 2002). Fukuda and colleagues measured the incorporation of [^{32}P] from [^{32}P]ATP into a synthetic substrate peptide of ERK upon N/OFQ stimulation of the NOP receptor (Fukuda et al. 1997). Importantly, it has been demonstrated that ERK activity is completely abolished in the presence of the Gi/o blocker PTX and that the phosphorylation occurs in a PKC-dependent and -independent manner as clarified by the use of enzymatic inhibitors (Hawes et al. 1998). ERK phosphorylation can be assessed by employing western blot evaluation using phospho-specific antibodies (Zhang et al. 2012) or with other commercially available approaches, such as that based on the Perkin Elmer's AlphaScreen technology (Garbison et al. 2004), on enzyme-linked immunosorbent assays (ELISA) or adaptations of that method (e.g., Cisbio's homogenous time-resolved fluorescence (HTRF) phospho-ERK assay).

Other easy approaches to measure NOP activity are those based on the modulation of transcripts unveiled through reporter gene assays. In Bevan et al. (1998), for example, N/OFQ-mediated regulation of Elk-1 and Sap1a was measured by using chimeric transcription factors (i.e., Ga14/Elk-1 and Ga14/Sap1a) and a firefly luciferase reporter gene under the transcriptional control of a Ga14 responsive promoter. The results obtained demonstrated that NOP stimulation triggers Elk-1 and Sap1a transcription via ERK-dependent modulation.

Very recently, Liu and coworkers applied an intriguing approach to investigate the kappa receptor function; by combining bioinformatic analysis and advanced mass spectrometry of samples obtained from different brain region, they quantified how 50,000 different phosphosites were modified upon selective kappa stimulation, thus offering a fascinating picture of how the kappa receptor works in vivo (Liu et al. 2018). We wish that such innovative methods will contribute to better clarify the mechanism of action also of the other members of the opioid receptor family, including the NOP receptor.

6 Label-Free

Label-free methods provide integrated measures of receptor biology in whole cells and in real-time without the need of radioactive or fluorescent dyes. These assays are generally very sensitive and allow to detect pleiotropic signaling elicited by GPCRs stimulation. Mostly known approaches are impedance- and optical-based assays. Among those that are measuring variations of cellular impedance we mention CellKey System (Molecular Devices) (Grundmann 2017; Peters et al. 2007) and xCELLigence (Lundstrom 2017; Scott and Peters 2010), while Epic is known as an optical method employing a nano-grating waveguide biosensor for diffracting an incident light that is then perturbed by the cell in a stimulus-dependent manner (Fang 2011). Detailed dissertation on the use of Epic and its application to perform dynamic mass redistribution (DMR) measurements with different GPCRs is reported by Schröder and colleagues (Schroder et al. 2010, 2011). Recently, we used the DMR technique to investigate the NOP receptor pharmacology in stably transfected cells (Malfacini et al. 2018) (see Fig. 3b). We found the effects of a large panel of NOP ligands of different pharmacological activities overall in line with previous reports, including the absence of inverse agonism that is easily detected with this technique (Lee et al. 2014). Quite surprisingly, most of the NOP agonists displayed a PTX-insensitive amount of signal even with very high toxin concentrations, and this was somehow more evident for those compounds with higher efficacy. However, preliminary data indicate that in cells deprived of all relevant G proteins functionality the NOP-dependent DMR signal is completely ablated. Therefore, in line with previous findings obtained with different GPCR (Grundmann et al. 2018), in the absence of functional G proteins the NOP receptor is no longer able to promote the DMR response. In perspective label-free studies performed in cells expressing the native NOP receptor, particularly in cells obtained from animal models of pathology, and also in human primary samples (Hillger et al. 2017) will increase our knowledge on NOP signaling and functions under physiological and pathological conditions, thus contributing to identify the therapeutic potential of NOP ligands.

7 Bioassays

Since the advent of molecular biology and subsequent receptor cloning, most of the current in vitro pharmacology is performed with cell- or membrane-based assays. Nevertheless, bioassay studies substantially contributed to define the pharmacology of the NOP receptor and its ligands. As reviewed by Giuliani et al. (2000), several tissues/preparations have been described as N/OFQ sensitive and used for pharmacological studies. In most of the cases NOP receptor activation inhibited the release of neurotransmitter evoked by electrical field stimulation with examples for the sympathetic (e.g., mouse vas deferens (Calo et al. 1996; Berzetei-Gurske et al. 1996)) (see Fig. 3c), parasympathetic (e.g., guinea pig ileum (Zhang et al. 1997; Calo et al. 1997)), and non-adrenergic-non-cholinergic (e.g., guinea pig renal pelvis (Giuliani and Maggi 1996)) systems. N/OFQ-sensitive isolated tissues were used

for identifying and characterizing novel NOP receptor ligands. These bioassay studies allowed to estimate the basic pharmacological parameters of NOP ligands (receptor selectivity, efficacy and potency of agonists, apparent affinity, and surmountable/insurmountable behavior of antagonists, see Tables 2 and 3 in Toll et al. 2016) as well as to get information about interesting aspects of ligand behavior including kinetics of action (Rizzi et al. 2001, 2002), sensitivity to washing (Spagnolo et al. 2007; Rizzi et al. 2001), and susceptibility to peptidases (Bigoni et al. 2001; Calo et al. 2000a). The electrically stimulated mouse vas deferens has been probably demonstrated, among the different N/OFQ-sensitive preparations, the most useful for pharmacological studies. This preparation generates very consistent results regarding NOP agonists and antagonists of peptide and non-peptide nature (Toll et al. 2016). Moreover, the availability of NOP knockout mice allows the use of this preparation to investigate ligand selectivity in "real life" as recently showed by comparing the action of the most used non-peptide NOP agonists in vas deferens tissues taken from wild-type and NOP knockout animals (Ferrari et al. 2017); the results of this study suggest that the NOP selectivity evaluated in recombinant systems is overestimated compared to what obtained in the knockout tissues which should be considered the acid test for ligand selectivity.

8 Conclusions

This chapter is addressed to both academic and industrial researchers who aim to begin the investigation of the NOP receptor and/or to establish drug development attempts at this intriguing member of the opioid receptor family. The reader will take advantage of the comparison of NOP ligands in terms of pharmacological effects in the different assays (Table 1).

NOP seems structurally very similar to the other members of the opioid receptor family, yet we know its pharmacology and biology are rather distinct. It should be underlined that even small divergence produced in terms of signaling fingerprint may translate into important different biological outputs, and thus the parallel examination of the ligand properties in different assays is recommended. It is reasonable to assume that the cellular background, the local composition of the membrane, the proximity of other GPCRs, the presence of diverse effectors, and most probably other still unknown factors are influencing the activity of NOP. Can we simply define a signaling fingerprint for each ligand available in physiologically relevant cellular context? Can we link a signaling fingerprint to a specific biological output? We envisage that whole-cell label-free and broad phosphoproteomic studies, together the identification of NOP biased agonists (toward G protein and toward arrestin) eventually obtained via structure-based rational drug design will help answering these questions. Hopefully, this will allow to precociously discriminate between molecules and select drug candidate with substantially higher rate of success.

Table 1 Pharmacological parameters of standard NOP ligands obtained in the assays described in this chapter

	Receptor binding	Stimulated [^{35}S]-GTPγS		BRET G protein		cAMP levels		Calcium $G\alpha_{qi5}$ cells		BRET β-arrestin 2		DMR		mVD	
	CR	CR	α	CR	α	CR	α	CR	α	CR	α	CR	α	CR	α
N/OFQ	1	1	1.00	1	1.00	1	1.00	1	1.00	1	1.00	1	1.00	1	1.00
UFP-112	0.2[a]	0.4[a]	1.03	0.1[b]	0.98	ND		3[c]	1.04	0.4[b]	0.89	0.5[d]	1.21	0.02[e]	0.97
PWT2-N/OFQ	0.1[f]	0.1[f]	1.14	0.2[b]	1.10	ND		4[f]	1.00	3[b]	1.3	2[d]	0.97	0.4[f]	1.06
Ro 65-6570	35[g]	12[h]	1.00	5[b]	0.96	8[i]	1.00	10[c]	0.95	45[b]	0.84	11[d]	1.02	5[j]	>1
AT-403	ND	1[h]	0.91	4[h]	0.94	ND		1[h]	0.96	5[h]	0.79	ND		0.4[h]	>1
MCOPPB	0.4[k]	0.3[h]	1.00	0.5[h]	1.04	~0.1[l]	1.00	0.5[h]	1.03	1[h]	0.99	ND		0.7[h]	>1
[F/G]	0.3[m]	14[n]	0.67	4[b]	0.72	603[m]	0	32[c]	0.54	3[b]	0	2[d]	0.98	7[o]	0
UFP-113	0.2[a]	0.2[a]	0.79	0.1[b]	0.45	ND		37[c]	0.62	0.04[b]	0	ND		0.02[a]	0
AT-090	47[j]	14[j]	0.21	21[h]	0.49	ND		8[j]	1.00	11[j]	0.57	1[d]	1.23	3[j]	>1
[Nphe^1]	53[p]	26[q]	0	39[b]	0.55	3030[r]	0	1778[c]	0	10[b]	0	28[d]	0.88	26[o]	0
UFP-101	2[s]	1[q]	0	13[b]	0	302[t]	0	76[c]	0	5[b]	0	5[d]	0	3[s]	0
J-113397	5[u]	0.8[v]	0	3[b]	0	182[w]	0	46[c]	0	6[b]	0	5[d]	0	0.6[w]	0
SB-612111	3[v]	0.3[v]	0	0.3[b]	0	14[x]	0	24[c]	0	1[b]	0	1[d]	0	0.1[v]	0
C-24	1[y]	0.1[y]	0	0.2[b]	0	ND		3[h]	0	0.1[b]	0	1[d]	0	0.1[y]	0

CR concentration ratio (EC_{50} or K_B ligand/EC_{50} N/OFQ), *α* intrinsic activity (Emax ligand/Emax N/OFQ), *ND* not determined, *mVD* mouse vas deference
To be noted: the rank order of potency of agonists is similar in the different assays with AT-403 and MCOPPB consistently behaving as the most potent non-peptide NOP full agonists; the efficacy of NOP partial agonists is variable with some assays overestimating (e.g., DMR) and other assays underestimating (mVD) this parameter; the apparent higher efficacy of non-peptide agonists compared to N/OFQ in the mVD is actually due to off-target actions as demonstrated using tissues taken from NOP(−/−) animals; the rank order of potency of NOP antagonists, i.e., C-24 > SB-612111 > J-113397 > UFP-101 is consistently demonstrated in all the assays. All compounds with α equal to 0 behaved as antagonists and CR were computed employing K_B values
References: [a] (Arduin et al. 2007), [b] (Malfacini et al. 2015), [c] (Camarda et al. 2009), [d] (Malfacini et al. 2018), [e] (Rizzi et al. 2007), [f] (Rizzi et al. 2014), [g] (Hashiba et al. 2001), [h] (Ferrari et al. 2017), [i] (Hashiba et al. 2001), [j] (Ferrari et al. 2016), [k] (Hirao et al. 2008), [l] (Chang et al. 2015b), [m] (Okawa et al. 1999), [n] (Wright et al. 2003), [o] (Guerrini et al. 1998), [p] (Calo et al. 2000b), [q] (McDonald et al. 2003b), [r] (Hashimoto et al. 2000), [s] (Calo et al. 2002), [t] (Calo et al. 2002), [u] (Trapella et al. 2006), [v] (Spagnolo et al. 2007), [w] (Bigoni et al. 2000), [x] (Spagnolo et al. 2007), [y] (Fischetti et al. 2009)

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Declaration of Interests DM and GC have nothing to declare.

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Electrophysiological Actions of N/OFQ

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Contents

Abstract

Whilst the nociceptin/orphanin FQ (N/OFQ) receptor (NOP) has similar intracellular coupling mechanisms to opioid receptors, it has distinct modulatory effects on physiological functions such as pain. These actions range from agonistic to antagonistic interactions with classical opioids within the spinal cord and brain, respectively. Understanding the electrophysiological actions of N/OFQ has been

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crucial in ascertaining the mechanisms by which these agonistic and antagonistic interactions occur. These similarities and differences between N/OFQ and opioids are due to the relative location of NOP versus opioid receptors on specific neuronal elements within these CNS regions. These mechanisms result in varied cellular actions including postsynaptic modulation of ion channels and presynaptic regulation of neurotransmitter release.

Keywords
Electrophysiology · G-protein coupled receptors · Nociceptin/orphanin FQ · Opioids · Pain

1 Introduction

The nociceptin/orphanin FQ (N/OFQ) receptor (NOP) was the fourth opioid receptor to be cloned on the basis of its high sequence homology to the 'classic' opioid receptors: mu (μ-OR/MOP), delta (δ-OR/DOP) and kappa (κ-OR/KOP) (Toll et al. 2016). Its endogenous ligand, N/OFQ, was later identified as a 17-amino acid peptide that structurally resembled the κ-OR ligand dynorphin A (Meunier et al. 1995; Reinscheid et al. 1995). Despite similar sequence identity between receptors and endogenous ligands, NOP and N/OFQ are pharmacologically and functionally distinct from their classic opioid ligand/receptor counterparts. Pharmacologically, NOP has very low affinity for most opioid ligands, and N/OFQ displays poor affinity for μ-OR, δ-OR and κ-OR. Both classic opioids and N/OFQ have been shown to modulate many of the same physiological responses/systems including anxiety, learning and memory, locomotor activity and pain (nociception). This broad influence in function reflects the wide distribution of both classic opioids/opioid receptors and N/OFQ-NOP, which are expressed in many of the same regions throughout the central and peripheral nervous system. This includes the spinal cord and brain regions involved in pain and analgesia, such as the amygdala, midbrain periaqueductal grey (PAG), dorsal raphe nucleus (DRN), locus coeruleus (LC), rostral ventral medulla (RVM), spinal cord and primary sensory neurons. Functionally, however, the actions of N/OFQ often seem to oppose those of opioids. For example, classic opioids can be administered to all levels within pain-related circuitry and are generally antinociceptive. In contrast, NOP agonists affect nociceptive transmission in a site-specific manner, and this can often vary depending on the agonist administered, the species tested and the pain state of the animal (i.e. acute, chronic: inflammatory/neuropathic). Further, activation of NOP has been shown to drastically alter the antinociceptive properties of μ-OR agonists such as morphine, and this too is in a site-specific manner (Schroder et al. 2014; Tian et al. 1997).

What is remarkable is that despite these functional differences, at a cellular level, the actions of N/OFQ through NOP signalling are comparable to those of classic opioids. Like classic opioid receptors, NOP is a G-protein-coupled receptor (GPCR) coupled preferentially (although not exclusively) to the pertussis toxin (PTX)-sensitive $G_{i/o}$ protein (Connor and Christie 1999; Margas et al. 2008) (see also Chan et al. (1998) and Yung et al. (1999) who report NOP coupling to

PTX-insensitive G_z, G_{12}, G_{14} and G_{16}). Activation of NOP alters cellular activity through typical $G_{i/o}$ signalling, which involves the dissociation and independent signalling of the $G\alpha_{i/o}$ and $G\beta\gamma$ subunits. $G\alpha_{i/o}$ downregulates adenylyl cyclase activity, decreases cyclic AMP (cAMP) accumulation and attenuates protein kinase A (PKA)-dependent signalling. Meanwhile, $G\beta\gamma$ directly interacts and activates G-protein-activated inwardly rectifying potassium channels ($K_{ir}3$, also known as GIRKs) and inhibits voltage-gated calcium channels ($Ca_V2.x$). Through $G\beta\gamma$ signalling, activation of NOP can also upregulate cell signalling cascades such as the MAPK (mitogen-activated protein kinase) pathways. Further, $G\beta\gamma$ may alter the activity of exocytotic machinery involved in neurotransmitter release, although this has not been directly linked to NOP signalling (Blackmer et al. 2005; Gerachshenko et al. 2005).

The overall effect of activation of all opioid receptors, including NOP, is a decrease in neuronal excitability and synaptic transmission due to increased $K_{ir}3$ conductance, reduced Ca_V conductance and suppression of neurotransmitter release (Toll et al. 2016). These neurophysiological changes can be detected using electrophysiological methods such as whole-cell patch-clamp and sharp electrode intracellular recordings. The advantage of these methods is they provide direct measures of neuronal activity, and by applying different recording conditions, it is possible to distinguish whether a bioactive compound acts via a pre- or postsynaptic mechanism. By using these methods, classic opioids and N/OFQ have been shown to reduce neuronal activity at both pre- and postsynaptic sites throughout the CNS.

Extensive literature indicates the cellular localisation of opioid receptors, and NOP can dictate the functional consequence of their activation. Although the direct consequence of activation maybe inhibition, this does not necessarily reflect the overall output of the target region. For example, opioid receptors are well-established disinhibitors of excitatory transmission. This involves the selective inhibition of GABAergic interneurons, which disinhibits excitatory neurons and thus increases neuronal output (see Lau and Vaughan (2014) for a comprehensive review of opioid disinhibition). Opioid disinhibition has been demonstrated most extensively in nociceptive (pain)-related pathways. In particular, μ-opioids have been shown to disinhibit and activate several supraspinal regions that constitute descending 'analgesic' pathways. This includes the amygdala, the periaqueductal grey, the dorsal raphe nucleus and the rostral ventral medulla, which, when activated, inhibit transmission of nociceptive signals within the dorsal horn of the spinal cord.

Whilst NOP signalling mirrors that of classic opioid receptors, there are marked differences both in where they act and how this affects overall cellular activity. These differences are likely to underlie the functional discrepancies between N/OFQ and classic opioid receptor signalling. In this review, we will discuss the neurophysiological actions of N/OFQ and other NOP ligands on the basis of their electrophysiological properties and compare this with what is known on classic opioid receptor signalling. We will focus primarily on regions involved in pain sensation, and, where possible, we will attempt to resolve the direct cellular activity of N/OFQ with overall functional outcome.

2 Postsynaptic Actions of N/OFQ

2.1 G-Protein-Activated Inwardly Rectifying K^+ Channels ($K_{ir}3$)

Numerous whole-cell or intracellular electrophysiological recordings have shown activation of NOP or classic opioid receptors cause an outward hyperpolarising current, which reduces cell excitability (see below for details). This current is associated with a decrease in membrane input resistance, indicating opening of membrane channel pores. It is also inwardly rectifying, it reverses at E_k for K^+ ions (calculated with the Nernst equation), and it is dependent on G-protein activity. Each of these characteristics, together with reports that compounds such as tertiapin-Q (a partial antagonist of $K_{ir}3$) inhibit these outward currents, indicates NOP and opioid receptors activate $K_{ir}3$s. Henceforth we shall refer to these currents as $K_{ir}3$-mediated.

$K_{ir}3$ channels belong to a larger family of inwardly rectifying K^+ channels ($K_{ir}1$-$K_{ir}7$; Hibino et al. 2010). They are tetrameric channels, and in the CNS, they are formed from the subunits $K_{ir}3.1$, $K_{ir}3.2$ and $K_{ir}3.3$. Another subunit $K_{ir}3.4$ exists, but it is expressed at low levels within the CNS (Karschin et al. 1996). Whilst $K_{ir}3.2$ subunits are able to form homomers, $K_{ir}3.1$ and $K_{ir}3.3$ are unable to form functional channels independently and exist as heteromers ($K_{ir}3.1/K_{ir}3.2$, $K_{ir}3.1/K_{ir}3.3$ or $K_{ir}3.2/K_{ir}3.3$). $K_{ir}3$ channels typically have relatively low conductance at resting membrane potentials and are activated by Gβγ subunits, which dissociate from $G\alpha_{i/o}$ upon GPCR activation (Reuveny et al. 1994; Wickman et al. 1994). Direct binding of Gβγ to the N- and C-termini of $K_{ir}3$ induces a conformational change, opening the channel to allow selective conductance of K^+ ions (Lei et al. 2000; Luscher and Slesinger 2010). $K_{ir}3$ channels are termed 'inwardly rectifying', as they allow large inward K^+ conductance at potentials negative to E_k (equilibrium potential for K^+) but permit less outward current flow at potentials more positive to E_k. This is due to a voltage-dependent cation (Mg^{2+}/polyamine) block which restricts ion conductance at more positive potentials (Yamada et al. 1998). Thus at resting membrane potentials, which are usually more positive to E_k, $K_{ir}3$ activation induces a small outward K^+ current. This current directly hyperpolarises neurons, driving their membrane potential away from threshold, which reduces neuronal excitability and action potential firing. The increased membrane conductance also shunts excitatory currents from dendrites and the cell body, thus rendering neurons less responsive to excitatory inputs. Importantly, it should be noted that $K_{ir}3$, opioid receptors and NOP can be distributed in various subcellular compartments, including their cell bodies, dendrites, spines and synaptic contacts (Cheng et al. 1997; Lujan and Aguado 2015; Ozawa et al. 2015; Reyes et al. 2009). Receptor activation of $K_{ir}3$ will therefore depend on proximity to the receptor, and the functional consequence of opioid- or N/OFQ-dependent $K_{ir}3$ activation will depend on the subcellular localisation of the receptor-effector signalling complex.

$K_{ir}3$-mediated currents, induced by the activation of NOP and classic opioid receptors, have been demonstrated in a multitude of cells including heterologous systems (e.g. Ikeda et al. 1997) and native neurons throughout the CNS. This

includes several cortical regions, the hippocampus, hypothalamus and ventral tegmental area (Chee et al. 2011; Parsons and Hirasawa 2011; Xie et al. 2008; Zheng et al. 2002), plus numerous regions directly involved in the modulation of pain such as the amygdala, periaqueductal grey, dorsal raphe nucleus, locus coeruleus, rostral ventral medulla and spinal cord (see below). Although N/OFQ and classic opioids appear to activate $K_{ir}3$s in many of the same brain regions, distinct subpopulations of cells within these regions display selective sensitivity to these signalling peptides. Here we will discuss the differences in N/OFQ and classic opioid sensitivity within nociception-related pathways (see also Table 1).

2.1.1 The Periaqueductal Grey

The periaqueductal grey (PAG) is a midbrain structure which has a crucial role in descending modulation of pain where it sends strong excitatory glutamatergic projections to the rostral ventral medulla (RVM), which in turn projects to the spinal cord where it inhibits nociceptive signalling (Basbaum and Fields 1984; Millan 2002) (Fig. 1). Microinjection of classic opioids into the ventrolateral PAG activates this pathway and produces robust analgesia by disinhibiting PAG to RVM projection neurons (Basbaum and Fields 1984). By contrast, intra-PAG microinjection of N/OFQ produces hyperalgesia and impairs μ-opioid-induced analgesia (Lu et al. 2010; Morgan et al. 1997), although N/OFQ-induced analgesia has also been reported (Shane et al. 2003).

At the cellular level, N/OFQ induces $K_{ir}3$ currents in all PAG neurons irrespective of location along the dorsoventral axis (Chiou 2001; Vaughan et al. 1997, 2003) (Fig. 1). This is similar to the $GABA_B$ receptor agonist baclofen, which also induces a $K_{ir}3$ current in all PAG neurons (Chieng and Christie 1995). In contrast, agonists of classic opioid receptors induce $K_{ir}3$ currents in a subpopulation of PAG neurons, and this depends on the dorsoventral location and output target of the recorded cell. In general, μ-opioids produce $K_{ir}3$ currents in 50–78% of all PAG neurons, whilst δ- and κ-opioids produce an outward current in ~24% and ~32% of cells, respectively (Behbehani et al. 1990; Chiou 2001; Vaughan et al. 2003). These numbers drastically change when more selective sampling methods are employed. Indeed, only 44% of PAG neurons that project to the RVM (identified with a retrograde tracer) displayed μ-OR-induced inwardly rectifying K^+ conductance (Osborne et al. 1996) (Fig. 1). Further, these opioid-responding projection neurons were not uniformly distributed throughout the PAG. Rather, responding neurons were restricted to specific dorsoventral locations. The majority of responding projection neurons (~56%) were located within the lateral PAG, whilst few projection neurons located in the ventrolateral PAG (~14%) responded to μ-opioids (Osborne et al. 1996). A similar distribution of μ-opioid responsive neurons was described using sharp electrode intracellular recordings that favoured sampling from larger diameter PAG neurons, which were presumed non-GABAergic (Chieng and Christie 1994a). Interestingly these neurons were not affected by δ- or κ-opioids. Thus whilst N/OFQ likely dampens excitability of all neurons within the PAG, μ-opioids selectively hyperpolarise PAG neurons depending on their dorsoventral location and

Table 1 Summary of N/OFQ and classic opioid effects

N/OFQ	References	Classic opioids	References
Periaqueductal grey (PAG)			
Kir3			
Kir3-mediated currents detected in all PAG neurons. No distinction in sensitivity between dorsoventral locations	Chiou (2001) and Vaughan et al. (1997, 2003)	μ-Opioids activate Kir3 currents in subpopulations of PAG neurons depending on their dorsoventral location and output target. Few vlPAG neurons that project to the RVM responded to μ-opioids. Only a small population of PAG neurons respond to δ- and κ-opioids. None of these projected to the RVM	Behbehani et al. (1990), Chieng and Christie (1994a), Chiou (2001), Chiou et al. (2004), Liao et al. (2011), Osborne et al. (1996), and Vaughan et al. (2003)
CaV			
Inhibited I_{Ca} in 113 out of 114 PAG cells. Predominantly inhibited N- and P-/Q-type currents but had little effect on L- or R-currents	Connor and Christie (1998)	μ-Opioids inhibited I_{Ca} in a subpopulation of PAG neurons (30–40% all cells), but efficacy was markedly lower than N/OFQ. μ-Opioids preferentially inhibit N-type currents. δ- and κ-opioids had no effect on I_{Ca} in any PAG neuron	Cho et al. (2001), Connor and Christie (1998), and Kim et al. (1997)
Presynaptic			
Inhibited IPSCs and EPSCs in subpopulations of neurons	Vaughan et al. (1997)	Inhibited IPSCs and EPSCs in all neurons	Chieng and Christie (1994b) and Vaughan et al. (2003)
Rostral ventral medulla (RVM)			
Kir3			
N/OFQ-induced Kir3 currents detected in all RVM neurons	Pan et al. (1990) and Vaughan et al. (2001)	Primary neurons insensitive to μ-opioids but κ-opioids induced Kir3 currents. Secondary neurons insensitive to κ-opioids, but μ-opioids induce Kir3 currents	Pan et al. (1990) and Vaughan et al. (2001)

(continued)

Table 1 (continued)

N/OFQ	References	Classic opioids	References
CaV			
Inhibits HVA currents in all RVM neurons	Vaughan et al. (2001)	κ- and μ-opioids inhibit I_{Ca} in a subset of neurons that were nearly mutually exclusive	Vaughan et al. (2001)
Presynaptic			
Inhibited IPSCs, but not EPSCs in subpopulations of neurons	Vaughan et al. (2001)	Inhibited IPSCs and EPSCs in all neurons	Pan et al. (1990) and Vaughan et al. (2001)
Amygdala			
Kir3			
Lateral amygdala (LA) Kir3 currents induced in most 'type 1' pyramidal projection neurons	Meis and Pape (1998)	*Lateral amygdala* μ-Opioids had no effect on 'type 1' pyramidal neurons but induced Kir3 currents in all 'type 2' non-pyramidal (presumed GABAergic) interneurons. δ- and κ-opioids have no effect on any LA neuron	Sugita and North (1993) and Sugita et al. (1993)
Central amygdala (CeA) Kir3 currents only detected in a subpopulation of neurons. Most were low-threshold spiking neurons. Higher proportion of responsive neurons located in medial central amygdala (CeM), the putative output nucleus. PAG projecting neurons were N/OFQ-sensitive	Chen et al. (2009), Chieng and Christie (2010), and Meis and Pape (1998)	*Central amygdala* μ-Opioids activate Kir3 in ~2/3 all CeA neurons. δ-Opioids only induce Kir3 currents in subset of CeM neurons, all of which are μ-opioid sensitive. κ-Opioids activate Kir3 in different subsets of CeM neurons that are μ-opioid insensitive. All CeM projection neurons are μ-opioid sensitive	Chieng and Christie (2009) and Chieng et al. (2006)
Intercalated cells (ITCs) Not determined	N/A	*Intercalated cells* μ-Opioids induced Kir3 currents in all ITCs	Blaesse et al. (2015) and Winters et al. (2017)

(continued)

Table 1 (continued)

N/OFQ	References	Classic opioids	References
CaV (not determined for N/OFQ or classic opioids)			
Presynaptic			
Inhibited IPSCs and EPSCs in LA neurons	Meis and Pape (2001)		
Locus coeruleus (LC)			
Kir3			
Kir3 currents induced in all LC neurons	Connor et al. (1996a, 1999)	μ-Opioids induce Kir3 currents in all LC neurons	Ingram et al. (1997), North and Williams (1985), Torrecilla et al. (2002), and Williams et al. (1982)
CaV			
Inhibits HVA I_{Ca} in all LC neurons	Connor et al. (1999)	μ-Opioids inhibit HVA I_{Ca} in all LC neurons	Ingram et al. (1997)
Presynaptic			
Not determined		Inhibited IPSCs but not EPSCs	Pan et al. (2002b)
Dorsal raphe nucleus (DRN)			
Kir3			
Kir3 currents induced in all DRN neurons with sevenfold higher potency than in the LC	Vaughan and Christie (1996)	κ-Opioids induce Kir3 currents in all serotonergic neurons, whilst μ-opioids induce currents primarily in non-serotonergic neurons and a subpopulation of serotoninergic neurons	Jolas and Aghajanian (1997) and Lemos et al. (2012)
CaV (not determined for N/OFQ or classic opioids)			
Presynaptic			
Not determined			
Spinal cord			
Kir3			
Kir3 currents induced in all LII dorsal horn neurons from young adolescent and adult rats	Jennings (2001), Lai et al. (1997), and Luo et al. (2001)	Complex distribution of classic opioid-sensitive cells in LI and LII neurons of the dorsal horn of the spinal cord which varies depending on the phenotype used to classify subpopulations of neurons (see main text for details)	Eckert et al. (2001, 2003), Grudt and Williams (1994), Jeftinija (1988), Marker et al. (2006), Randic et al. (1995), Santos et al. (2004), Schneider et al. (1998), Smith et al. (2016), and Wang et al. (2018)

(continued)

Table 1 (continued)

N/OFQ	References	Classic opioids	References
CaV			
Not determined	N/A	μ-Opioids inhibited I_{Ca} in a subpopulation (~36%) of dorsal horn neurons. N-, P-/Q-, R- and L-currents all inhibited, but μ-opioids had highest efficacy at L-currents which constituted the highest proportion of overall I_{Ca}	Lee et al. (2004)
Presynaptic			
Inhibited primary afferent evoked EPSCs, but not local evoked IPSCs	Ahmadi et al. (2001a, b) and Liebel et al. (1997)	Inhibited EPSCs and IPSCs	
Primary sensory neurons			
CaV			
Trigeminal ganglion neurons (TGs) N/OFQ inhibited N- and P-/Q-currents in ~82% small diameter neurons that did not contain T-type currents ('type 1'), were assumed $IB4^+$ and capsaicin responsive. N/OFQ had no effect on T-type currents and only affected a small proportion of HVA currents in cells that contained T-type currents ('type 2')	Borgland et al. (2001)	*Trigeminal ganglion neurons (TGs)* μ-Opioids had no effect on 'type 2' cells and inhibited I_{Ca} in the same population of type 1 cells that responded to N/OFQ. μ-Opioids also inhibited I_{Ca} in 77% of large diameter type 1 cells. μ-Opioid DAMGO had higher efficacy inhibiting I_{Ca} than N/OFQ. δ- and κ-opioids had no effect on I_{Ca} in any TG neuron	Borgland et al. (2001)
Dorsal root ganglion neurons (DRGs) Mainly inhibits N- and P-/Q-type currents in small diameter neurons (< 20 μm) that are $IB4^-$ and presumed peptidergic. Has little effect on L- and R-type currents. Conflicting reports on N/OFQ effect on	Abdulla and Smith (1997), Beedle et al. (2004), and Murali et al. (2012)	*Dorsal root ganglion neurons (DRGs)* μ- and κ-opioids preferentially inhibit N- and P-/Q-type currents in small diameter DRGs and have little effect on L-, R- or T-type currents. δ-Opioids have no effect in any DRG. A larger population of	Abdulla and Smith (1998), Andrade et al. (2010), Ingram et al. (1997), Moises et al. (1994), Murali et al. (2012), Schroeder and McCleskey (1993), Taddese et al. (1995), and Wu et al. (2004)

(continued)

Table 1 (continued)

N/OFQ	References	Classic opioids	References
T-type currents with majority reporting no effect. N/OFQ had a higher efficacy inhibiting I_{Ca} than μ-opioids in DRGs that responded to both agonists		DRGs respond to μ-opioids compared with N/OFQ, but the degree of I_{Ca} inhibition is variable. μ-Opioids have higher efficacy in $IB4^-$ neurons but inhibit I_{Ca} in all $IB4^+$ neurons albeit to a lesser degree	

afferent target, with PAG-RVM projection neurons located in more ventral regions being more resistant to classic opioid agonist control (Fig. 1).

Whilst N/OFQ has been shown to induce $K_{ir}3$-mediated hyperpolarising currents in almost all PAG neurons irrespective of location in the dorsoventral axis, there is some evidence to suggest there maybe heterogeneity in NOP-subtype expression within this region. Two small molecule NOP agonists, (+)5a-Compound and Ro-64-6198, were shown to induce a $K_{ir}3$ current in the same subpopulations of vlPAG neurons, which were distinct from N/OFQ-responsive neurons (Chiou et al. 2004; Liao et al. 2011). Although the electrophysiological characteristics of responsive and unresponsive cells did not differ, (+)5a-Compound responsive cells were shown to have more complex dendritic arbours and were predominantly GABAergic neurons (Liao et al. 2011). Functional heterogeneity of NOPs has also been reported in the vas deferens and ileum of different species (Rizzi et al. 2001), behaviourally in spontaneous locomotor activity (Kuzmin et al. 2004) and in radioligand binding assays in the mouse brain (Mathis et al. 1997). Although the basis of this heterogeneity is not fully understood, alternative NOP splice variants (Peluso et al. 1998; Xie et al. 1999), high-/low-affinity binding sites (Mathis et al. 1997, 1999) and heterologous receptor dimerization (Pan et al. 2002a) have all been implicated. However, it should be noted that in approximately half of (+)5a-Compound/Ro-64-6198 responsive cells, neither effect could be reversed by putative NOP antagonists (Chiou et al. 2004; Liao et al. 2011). Thus the possibility of these compounds acting via another unidentified receptor cannot be ruled out. Nevertheless, given the potential for selectively targeting NOP-mediated signals in subpopulations of neurons, heterogeneity of NOP warrants further investigation.

2.1.2 The Rostral Ventral Medial Medulla

The RVM consists of several nuclei including the serotonergic nucleus raphe magnus (NRM), the nucleus reticularis gigantocellularis-pars alpha and the nucleus paragigantocellularis lateralis. The RVM is thought to act as a final relay in controlling descending pain modulation (Fig. 1). Indeed, depending on the RVM cell type and target within the spinal cord, activation of the RVM can either facilitate or inhibit ascending pain signals (Ossipov et al. 2014). Like the PAG, a similar

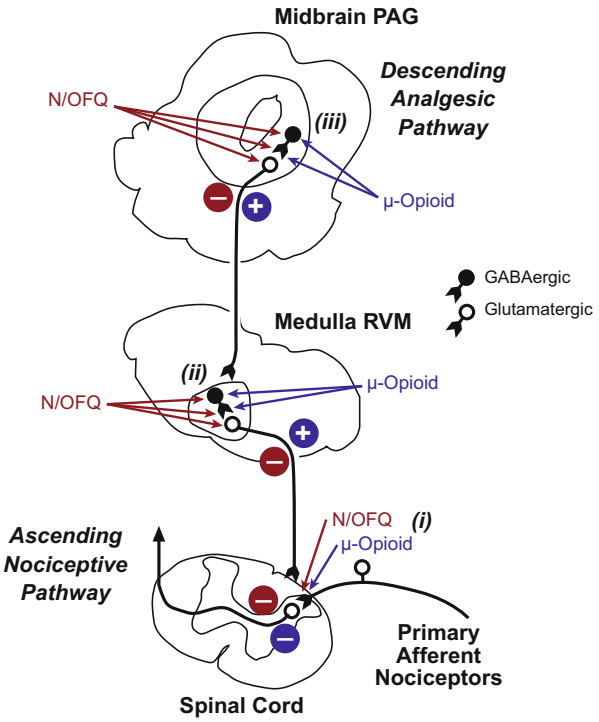

Fig. 1 Simplified schematic comparing the actions of N/OFQ (red) and μ-opioids (blue) on descending antinociceptive and ascending nociceptive pathways. On the left is an ascending pathway – primary nociceptive afferents synapse directly and indirectly (not shown) onto dorsal horn projection neurons. On the right is one major descending antinociceptive pathway – projects via the midbrain PAG and RVM (in the medulla) to the dorsal horn of the spinal cord; within both the PAG and RVM there are putative inhibitory interneurons. (i) In the ascending system, both N/OFQ and μ-opioids inhibit Ca_Vs in primary afferent neurons. This leads to an inhibition of excitatory nociceptive transmission into the dorsal horn. (ii) and (iii) Within the descending pathway, μ-opioid inhibition is largely restricted to non-projection neurons – direct postsynaptic inhibition and presynaptic inhibition of transmitter release from their terminals. This leads to activation of the descending outputs via GABAergic disinhibition within the RVM and PAG. By contrast, N/OFQ also directly inhibits the projection neurons. This leads to a reversal of μ-opioid-induced disinhibition

segregation in N/OFQ and classic opioid sensitivity has been reported in the rostral ventral medulla (RVM). Neurons within the RVM have been classified into distinct neuronal classes on the basis of their in vitro opioid sensitivity (Pan et al. 1990) and in vivo activity during nociceptive signalling (Basbaum and Fields 1984). In vivo electrophysiological studies have defined three classes of neurons, including on-, off- and neutral-cells. On-cells display increased activity in response to noxious stimuli and are inhibited by μ-opioids, whilst off-cells cease firing immediately prior to a nocifensive reflex (e.g. tail-flick) and are activated by μ-opioids and inhibited by κ-opioids (Fields et al. 1983a, b; Meng et al. 2005).

In vitro electrophysiological studies have identified primary neurons, which are presumed on-cells, that are insensitive to μ-opioids, but κ-opioids induce $K_{ir}3$ currents. On the other hand, secondary neurons, which are presumed on-cells (although see Cleary et al. 2008), are insensitive to κ-opioids, but μ-opioids induce a K^+ conductance (Pan et al. 1990; Vaughan et al. 2001). In contrast, N/OFQ was found to activate $K_{ir}3$-mediated currents in all RVM neurons irrespective of their μ-/κ-opioid sensitivity (Pan et al. 2000; Vaughan et al. 2001). Thus, like its action within the PAG, N/OFQ would be expected to dampen overall RVM activity, whilst classic μ-opioids selectively inhibit a subpopulation of neurons which results in activation of its descending outputs to the spinal cord (Fig. 1).

2.1.3 The Amygdala

The amygdala is a medial temporal lobe structure that plays an integral role in fear associative learning; it provides emotional valence to painful stimuli and constitutes part of the descending analgesic pathway upstream of the PAG (Duvarci and Pare 2014; Pape and Pare 2010; Thompson and Neugebauer 2018; Veinante et al. 2013). The amygdala consists of several distinct nuclei including the lateral amygdala (LA), the basal amygdala (BA), the lateral central amygdala (CeL), the medial central amygdala (CeM), the latero-capsular region (CeLC) and the intercalated cells (ITC). The LA and BA (together known as the basolateral amygdala, BLA) constitute the putative input nucleus and relay incoming signals from other brain regions to the putative output nucleus, the CeM (see Duvarci and Pare (2014) for an in-depth review on amygdala circuitry). Whilst there is distinct segregation between amygdala neurons that are either N/OFQ- or μ-/δ-/κ-opioid-sensitive, unlike the PAG and RVM, N/OFQ does not appear to affect all amygdala nuclei equally. In the LA, N/OFQ has been shown to induce a $K_{ir}3$ current in the majority of pyramidal neurons, which are presumed projection neurons due to their pyramidal, spiny morphology (Meis and Pape 1998). In contrast, μ-opioids had no effect on these pyramidal neurons but instead induced a $K_{ir}3$ current in LA 'type 2' non-pyramidal interneurons (Sugita et al. 1993). Neither δ- nor κ-opioids had any effect on the excitability of LA neurons (Sugita and North 1993).

The central amygdala (CeA), which is made up of the CeL and CeM, differs to the LA in that it is less sensitive to N/OFQ, with approximately a third of neurons displaying a $K_{ir}3$-mediated current, which was smaller in amplitude than in LA neurons (Meis and Pape 1998). Interestingly, if the sampled neurons are taken primarily from the CeM subdivision, the proportion of N/OFQ-responsive cells increases dramatically, indicating these output neurons are preferentially controlled by N/OFQ (Chieng and Christie 2010). In particular, neurons that displayed a low-threshold spiking firing phenotype were consistently N/OFQ-sensitive, and this included neurons located outside the CeM (Chieng and Christie 2010). The CeM sends projections to numerous regions throughout the brain, including the lateral and ventrolateral PAG, which form part of the descending antinociceptive circuitry (Chieng and Christie 2010; Li and Sheets 2018; Veinante et al. 2013). N/OFQ was shown to induce hyperpolarising, inwardly rectifying K^+ currents in CeM to PAG projecting cells (Chen et al. 2009; Chieng and Christie 2010).

However, this varied depending on the recording conditions and retrograde tracer used since either 100% (Chieng and Christie 2010) or 30% (Chen et al. 2009) of projection cells were reportedly N/OFQ-sensitive. The distribution of classic opioid-sensitive neurons within the CeA is quite different to N/OFQ. Whilst N/OFQ hyperpolarised neurons predominantly located in the CeM, μ-opioids were shown to activate $K_{ir}3$-mediated currents in approximately two thirds of all CeA neurons, irrespective of lateral/medial subdivision (Chieng et al. 2006). In contrast, δ-opioids activated a $K_{ir}3$ conductance in a subset of neurons located solely within the CeM, all of which were μ-opioid sensitive, whilst κ-opioids produced an outward current in a small subpopulation of CeA neurons that were largely insensitive to μ-opioids (Chieng et al. 2006). Interestingly, all CeM projection neurons, which include those that project to the PAG, parabrachial nucleus and bed nucleus of the stria terminalis, were unequivocally sensitive to μ-opioids (Chieng and Christie 2009; Chieng et al. 2006). Thus, μ-opioids would be expected to reduce the excitability of all CeM output neurons. Since N/OFQ appears to affect a higher proportion of CeM neurons more selectively than μ-opioids (82% vs 61%, respectively) and CeM to PAG projecting neurons are sensitive to N/OFQ, it is possible, at least at a cellular level, N/OFQ and μ-opioids perform a similar function and reduce the excitability of all amygdala output neurons.

The ITCs are small spiny GABAergic neurons arranged in heterogeneous clusters that encapsulate the BLA and putatively act as an inhibitory interface between the BLA and CeA. Several ITC nuclei have been classified; the lateral and medial paracapsular ITCs are located within the external and intermediate capsules, respectively, whilst the main island (Im) are located ventromedial to the BLA. Of these, μ-opioids have been shown to induce $K_{ir}3$ currents in all ITCs located within the Im, which is consistent with their high expression of μ-OR (Blaesse et al. 2015; Poulin et al. 2006; Winters et al. 2017). In contrast, δ-ORs are primarily expressed in the BLA and regulate presynaptic inputs from the BLA to ITC neurons (Poulin et al. 2006; Winters et al. 2017). The effect of N/OFQ on ITCs remains to be determined; however, given the high expression of NOP within the amygdala, which includes the ITC clusters (Neal et al. 1999, 2001), it is possible N/OFQ may also control ITC excitability through activation of $K_{ir}3$.

2.1.4 The Locus Coeruleus

The LC is a pontine region containing a major group of tonically active noradrenergic neurons. The LC plays a complex role in the pain experience; it constitutes part of a descending analgesic pathway (Llorca-Torralba et al. 2016) but also provides an affective component to pain (Hirschberg et al. 2017), plays a role in physical opioid dependence/withdrawal (Christie et al. 1997) and facilitates pain sensation under chronic pain contexts (Taylor and Westlund 2017). The LC projects noradrenergic afferents to the superficial dorsal horn of the spinal cord and to numerous other higher brain regions (Howorth et al. 2009). When the LC is electrically or chemically stimulated, this inhibits nociception in acute and inflammatory pain states, which is dependent on noradrenaline release and activation of α2-adrenoceptors in the spinal cord (Jones and Gebhart 1986; West et al. 1993). Paradoxically, microinjection of

μ-opioids into the LC, which inhibits tonic firing, is also analgesic (Jongeling et al. 2009). It is not fully understood how both activation and apparent inhibition of the same nucleus produce analgesia. However, recent evidence indicates functional dichotomy within the LC with ventral regions that project to the spinal cord being antinociceptive, whilst dorsal regions that likely project to higher brain regions are pronociceptive (Hickey et al. 2014; Hirschberg et al. 2017).

LC neurons express high levels of NOP (Anton et al. 1996) and μ-OR (Mansour et al. 1994), whilst κ-OR and δ-OR (Pan et al. 2002b; van Bockstaele et al. 1997) are expressed primarily in presynaptic axon terminals. Consistent with this expression profile, N/OFQ (Connor et al. 1996a, 1999) and μ-opioids (Ingram et al. 1997; North and Williams 1985; Williams et al. 1982) induce postsynaptic $K_{ir}3$-mediated currents in all LC neurons, with no reported distinctions between dorsal and ventral regions. In the case of μ-opioids, this reduced spontaneous discharge and dampened overall activity of this noradrenergic nucleus. Since N/OFQ and μ-opioids produced comparable hyperpolarisation (Connor et al. 1996a), N/OFQ would be expected to produce similar inhibition to overall LC activity. $K_{ir}3.2$ and $K_{ir}3.3$ channels have been implicated in underlying the majority of opioid-induced hyperpolarisation in the LC since the K^+ conductance was nearly abolished in $K_{ir}3.2/K_{ir}3.3$ double knockout mice (Torrecilla et al. 2002). Interestingly, LC neurons from these transgenic mice were markedly more depolarised suggesting these Kir3 channels also contribute to resting membrane potential.

2.1.5 The Dorsal Raphe Nucleus

The DRN is a midbrain structure located below the aqueduct, ventral to the PAG, and contains the largest population of serotonergic neurons and a subpopulation of GABAergic interneurons (Weissbourd et al. 2014). Like the LC, the DRN's role in nociception is complex. It participates in both ascending and descending nociceptive pathways (Wang and Nakai 1994), and direct stimulation or microinjection of μ-opioids into the DRN is antinociceptive (Campion et al. 2016). It is likely this μ-opioid effect is via disinhibition of serotonergic projection neurons since μ-OR agonists have been shown to increase serotonin efflux in vivo (Tao and Auerbach 2005). At a cellular level, N/OFQ induced $K_{ir}3$-mediated currents and reduced the firing rate in all DRN neurons (Nazzaro et al. 2010; Vaughan and Christie 1996). This is similar to κ-opioids, which induce $K_{ir}3$ currents in all serotonergic DRN neurons (Lemos et al. 2012). It was also noted that N/OFQ had a sevenfold higher potency in DRN neurons than in the LC, possibly indicating DRN neurons are more strongly coupled to $K_{ir}3$ channels, perhaps due to a larger receptor reserve and thus N/OFQ would be expected to provide greater control of DRN neuron excitability (Connor et al. 1996a; Vaughan and Christie 1996). In contrast, μ-opioids induce a $K_{ir}3$ current primarily in non-serotonergic (presumed GABAergic) neurons and in a subpopulation of serotonergic neurons (Jolas and Aghajanian 1997). Although δ-ORs are expressed within the DRN, they're primarily restricted to presynaptic sites (Arvidsson et al. 1995), and no reports have indicated δ-opioids induce a postsynaptic $K_{ir}3$ current. Therefore like in the PAG, N/OFQ would be expected to decrease all DRN activity which could explain its anti-analgesic effect, where it

has been shown to inhibit antinociception of morphine/μ-agonists (see Ge et al. 2007).

2.1.6 The Spinal Cord

The dorsal horn of the spinal cord receives sensory information from peripheral primary afferents that respond to specific noxious or non-noxious stimuli. Depending on the sensory modality and location of the input, these afferents terminate within different regions of the dorsal horn, which can determine the sensory experience. In general, noxious stimuli are detected by Aβ and C fibres which transmit this information to superficial laminae I-II_o of the dorsal horn (Fig. 1). This signal is then processed by complex circuits within the spinal cord before being transmitted for relay supraspinally via ascending pain pathways (see Todd (2010) for an in-depth review on nociceptive transmission in the dorsal horn). In addition to primary afferents, the dorsal horn receives descending inputs from brain regions such as the RVM, which can either facilitate or inhibit pain signals (Millan 2002).

Opioids play a major role in descending inhibition of nociceptive signals where they act at both supraspinal regions and within the spinal cord. Indeed, μ-OR, κ-OR, δ-OR and NOP are highly expressed throughout the dorsal horn of the spinal cord both at pre- and postsynaptic sites. Consistent with this, early reports indicate μ- and κ-opioids hyperpolarise the majority of lamina II neurons within the dorsal horn or trigeminal nucleus caudalis (an extension of the spinal cord located in the brainstem), whilst δ-opioids had no effect (Grudt and Williams 1994; Jeftinija 1988; Randic et al. 1995). However, the majority of these initial reports were performed using sharp intracellular recordings which favour neurons with larger cell bodies. Using whole-cell recordings of a more representative sample of lamina II dorsal horn neurons, it emerged these classic opioids differentially affect sub-populations of neurons within this region (e.g. see Eckert et al. 2003; Schneider et al. 1998). In general μ-opioids activate $K_{ir}3$ currents in 40–60% of all lamina II dorsal horn neurons, whilst the population of δ- and κ-opioid-sensitive neurons is much smaller (Eckert et al. 2001; Marker et al. 2006; Schneider et al. 1998). Distinct subpopulations emerged when these neurons were segregated on the basis of their electrophysiological characteristics, neurotransmitter content, morphology or molecular identity. For example, calretinin-positive excitatory interneurons were insensitive to μ- and δ-opioids agonists, whilst both induced $K_{ir}3$ currents in calretinin-positive inhibitory interneurons (Smith et al. 2016). Others have shown μ-opioids preferentially induced $K_{ir}3$ currents in tonic firing neurons or neurons with larger whole-cell capacitances but not in adapting or delayed firing neurons or neurons with smaller whole-cell capacitances (Marker et al. 2006; Santos et al. 2004). δ-opioids, however, induced $K_{ir}3$ currents in a subpopulation of neurons that were somatostatin-positive excitatory interneurons and could be distinguished by their adapting or delayed firing phenotype (Wang et al. 2018). Interestingly, within lamina II, μ- and δ-opioids affect completely separate subpopulations of neurons, whilst there was a high degree of overlap in lamina I projection neurons (Wang et al. 2018). Thus the overall effect of classic opioids mediated entirely

through $K_{ir}3$ activity is complex. In contrast to classic opioids, N/OFQ appears to have no selectivity and induced outward hyperpolarising $K_{ir}3$ currents in all lamina II dorsal horn neurons sampled from adult (Luo et al. 2001) or young to adolescent rats (P10–25) (Jennings 2001; Lai et al. 1997). Interestingly, one report indicated N/OFQ had no effect on membrane holding potential in young juvenile rats (P7–14), indicating there may be a developmental limitation on N/OFQ-dependent $K_{ir}3$ activation (Liebel et al. 1997). This is unlikely to be due to developmental changes in N/OFQ or NOP expression as both are highly expressed in the spinal cord from as early as embryonic day 13 (Neal et al. 2001). Whilst some evidence suggests $K_{ir}3$ channel expression and localisation to the cell membrane is developmentally regulated within the brain, it is not known whether the spinal cord displays similar developmental changes (Fernandez-Alacid et al. 2011; Lujan and Aguado 2015).

In summary, N/OFQ activates $K_{ir}3$-mediated currents in almost all neurons tested within nociceptive-related regions in the CNS, whilst classic opioids are more discerning and activate $K_{ir}3$ currents in distinct subpopulations of neurons, which varies with each region. One interesting characteristic noted in neurons sensitive to both N/OFQ and classic opioids is the $K_{ir}3$-mediated current appears to be homologous (Connor et al. 1996a). High concentrations of N/OFQ occlude the effect of simultaneous applications of classic opioid agonists, which indicates NOP and opioid receptors activated the same K^+ conductance. This homology in K^+ conductance also persists between NOP, somatostatin receptors, α2-adrenoceptors and $GABA_B$ receptors, all of which are coupled to $G_{i/o}$. This suggests there is a certain amount of redundancy in activating $K_{ir}3$ currents between the various $G_{i/o}$-coupled receptors that may be expressed in one neuron. In addition, it suggests prior activation of any one of these receptors may mask the effects of subsequent $G_{i/o}$ selective neuromodulators. Since N/OFQ tends to affect all cells within a region, its effect may be expected to dominate.

2.2 Voltage-Gated Calcium Channels (Ca_V)

Ca_V channels are heteromultimers consisting of the pore-forming α1 subunit and auxiliary subunits β and α2δ and in some cases γ subunits. In addition to contributing to neuronal excitability, by virtue of Ca^{2+} being an integral second messenger, Ca_Vs have the potential to upregulate numerous signalling cascades and regulate a range of neuronal functions. This includes (but is not limited to) neurotransmitter release, neurite outgrowth, synaptic plasticity, synaptogenesis and excitation-transcription coupling (Nanou and Catterall 2018; Zamponi et al. 2015). Therefore, any neuromodulator that alters Ca_V activity has the capacity to profoundly alter neuronal function.

To date, ten α1 subunits have been identified, and these are grouped into three subfamilies on the basis of their voltage sensitivity, deactivation kinetics and pharmacology (Zamponi et al. 2015). Of the Ca_V1 subfamily, $Ca_V1.2$ and $Ca_V1.3$ are primarily expressed in neurons and give rise to L-type currents which are high-voltage activated (HVA) and distinguished by their slow voltage-dependent

inactivation. They are also typically expressed on postsynaptic membranes (Hell et al. 1993); thus their primary function is to shape neuronal firing and activate Ca^{2+}-dependent signalling pathways. Ca_V2 subfamilies are expressed presynaptically (Westenbroek et al. 1995); they are high-voltage activated, have faster voltage-dependent inactivation kinetics and are regarded as drivers of evoked synaptic transmission. $Ca_V2.1$ give rise to P-/Q-type currents that are often defined by their sensitivity to ω-agatoxin IVA, whilst $Ca_V2.2$ underlie N-type currents, which are sensitive to ω-conotoxin GVIA. $Ca_V2.3$ trigger R-type currents that are poorly distinguished by their pharmacological profile and are often attributed as the remaining current when all other Ca_V channels have been inhibited (Zamponi et al. 2015). Finally, $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ give rise to T-type currents which are low voltage activated (LVA). They require brief membrane hyperpolarisation to recover from partial inactivation at resting membrane potentials, and they have fast inactivation kinetics (Cheong and Shin 2013). They also underlie the phenomenon of rebound bursting, which drives rhythmic generation of action potentials (Cheong and Shin 2013).

Changes in Ca_V activity can be recorded using whole-cell, cell-attached or nucleated patch techniques. It is important to note, however, due to 'space clamp' issues, which result from dendritic and axonal processes not reaching isopotential, it is difficult to accurately record key biophysical parameters of voltage-gated ion channels in whole-cell configuration in intact brain slices (Williams and Mitchell 2008). Electrophysiological measurements such as kinetics, voltage dependence and conductance can become severely distorted due to inadequate voltage clamp of distal membranes that are only a small distance away from the recording electrode (Williams and Mitchell 2008). Although this is a caveat for all whole-cell electrophysiological recordings, it is especially problematic for studying ion channels that depend on voltage changes for their activation. Thus, the majority of research into the effect of N/OFQ or classic opioids on Ca_V channels has been conducted in acutely isolated cell somas, which allow more accurate recordings of somatic ion conductance. For these reasons, Ca_V currents are regarded as postsynaptic although it should be recognised that these effects would also be expected to affect presynaptic neurotransmitter release (see Sect. 3).

The mechanisms by which GPCRs exert significant control over Ca_V function have been reviewed extensively elsewhere (Zamponi and Currie 2013). Briefly, voltage-dependent inhibition of Ca_Vs involves direct binding of Gβγ to the α1 subunit of Ca_Vs. This shifts the gating properties of Ca_V which renders the voltage dependence of channel activation to become less prominent (Zamponi and Currie 2013). Like the classic opioids, N/OFQ, through activation of NOP, has been shown to inhibit L- (Ca_V1), P-/Q- ($Ca_V2.1$), N- ($Ca_V2.2$), R- ($Ca_V2.3$) and T-type (Ca_V3) currents in heterologous cells (Connor et al. 1996b) and a wide range of native neurons throughout the central and peripheral nervous system. This includes the basal forebrain (Chin et al. 2002), LC (Connor et al. 1999), PAG (Connor and Christie 1998), hippocampus (Knoflach et al. 1996; Pu et al. 1999), hypothalamus (Parsons and Hirasawa 2011), nucleus tractus solitarius (Endoh 2006) and primary sensory neurons (see below). This inhibition is characterised by reduced current

density, increased rise time indicating slower activation kinetics and voltage dependence. Voltage dependence refers to both diminished Gβγ-induced inhibition of Ca_Vs at depolarised membrane potentials and block of Ca_V inhibition by a strong depolarising pulse which causes Gβγ to transiently dissociate from the Ca_V. These characteristics are typical of Gβγ-dependent inhibition of Ca_Vs (Zamponi and Currie 2013). In each case, there was often a voltage-independent component which could not be reversed by strong depolarising prepulses, which likely reflect alternative splicing of specific Ca_V subtypes (see below).

2.2.1 Primary Sensory Neurons

As is the case with $K_{ir}3$, N/OFQ and classic opioids do not always inhibit Ca_V-mediated currents (I_{Ca}) in the same subpopulation of neurons. Because the majority of reports have been conducted in acutely isolated neurons, it is more difficult to identify these subpopulations on the basis of their exact location or projection target. However, some distinction has been made between cell size and protein expression profile. This is most prominent in first-order primary sensory neurons. Dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons are pseudo-unipolar neurons that transduce sensory information from the periphery to the CNS. Whilst TGs primarily innervate the head, DRGs innervate the rest of the body. Both TGs and DRGs are exceptionally heterogeneous, and they contain a diverse population of neurons which can be loosely classified on the basis of their cell body size, protein expression profile or electrophysiological characteristics. This has been reviewed extensively elsewhere (Le Pichon and Chesler 2014). Briefly, primary sensory neurons can be classified as small (<20 μm), medium (20–30 μm) or large (>30 μm) depending on their cell body diameter or relative whole-cell capacitance. Typically large diameter cell bodies give rise to Aβ myelinated fibres, whilst medium/small diameter cells give rise to lightly myelinated Aδ or unmyelinated C nociceptive fibres. Small/medium cells are putative nociceptors and can be further divided into peptidergic or non-peptidergic subtypes. Peptidergic cells include those that express CGRP (calcitonin gene-related peptide) or substance P, whilst non-peptidergic cells possess cell surface glycol-conjugates and can be identified by the binding of isolectin B4 (IB4). Many other subdivisions have been described including those based on differing neurotrophic support (NGF/TrkA, BDNF/TrkB or GDNF/RET), capsaicin sensitivity (TRPV1) and T-type current (Le Pichon and Chesler 2014).

In acutely dissociated mouse TG neurons, Borgland et al. (2001) distinguished two classes of neurons based on the absence (type 1) or presence (type 2) of a prominent T-type current. They found N/OFQ inhibited N- and P-/Q-type currents in the majority of small diameter type 1 neurons (~82%). These neurons were also primarily $IB4^+$ (presumed non-peptidergic) and responded to the TRPV1 receptor agonist capsaicin. In type 2 cells, however, N/OFQ had no effect on the LVA T-type current and only marginally affected the HVA current in a small subset of cells (~23%). The μ-OR agonist DAMGO was shown to affect the same population of small diameter type 1 neurons as well as ~77% of large diameter type 1 neurons, but it had no effect on I_{Ca} in type 2 neurons (Borgland et al. 2001). In contrast κ-OR and

δ-OR agonists had no effect on I_{Ca} in any TG neuron. Interestingly, whilst N/OFQ and DAMGO appeared to primarily target the same population of TG neurons, DAMGO was substantially more efficacious and could inhibit I_{Ca} by up to 58%, whilst N/OFQ only inhibited I_{Ca} by up to ~37% (Borgland et al. 2001). This could indicate μ-ORs are more strongly coupled to Ca_Vs in these small diameter T-current lacking TG neurons.

Immunohistochemical, in situ hybridisation and radioligand binding studies indicate NOP is primarily expressed in large and medium diameter DRGs (Neal et al. 1999; Pettersson et al. 2002), whilst a recent NOP-eGFP knock-in mouse indicates NOP is distributed more heterogeneously throughout all DRGs (Ozawa et al. 2015). Electrophysiological studies, however, indicate NOP-dependent inhibition of Ca_V-mediated currents is largely restricted to a subpopulation of small/medium diameter DRGs (Abdulla and Smith 1998; Beedle et al. 2004; Borgland et al. 2001; Murali et al. 2012). Although it is not clear why there is this discrepancy, it likely represents preferential functional coupling of NOP to Ca_Vs in smaller cells, whilst NOP may couple to other effectors in larger cells. Further classification showed no distinction in N/OFQ sensitivity between capsaicin-responsive and capsaicin-unresponsive cells (Murali et al. 2012). In contrast, a subpopulation of small (<20 μm) $IB4^-$ (presumed peptidergic) DRGs acutely isolated from rats were shown to be highly N/OFQ-responsive with 85% of these neurons displaying over 50% I_{Ca} inhibition, whilst only 30% of $IB4^+$ cells responded to N/OFQ (Murali et al. 2012). This preference for $IB4^-$ neurons appears to conflict with N/OFQ sensitivity reported in TGs (Borgland et al. 2001). However, since concurrent IB4 binding and N/OFQ responsiveness were not directly specified in TGs, it is difficult to determine whether this region has a different population of N/OFQ-sensitive neurons. It may also reflect differences in NOP expression or NOP-Ca_v coupling between species.

A very similar profile of I_{Ca} inhibition has been reported for the classic opioids. Of these, only μ- and κ-opioids inhibit Ca_V currents in DRGs, whilst δ-opioids are without effect (Abdulla and Smith 1998; Moises et al. 1994). Although most reports concentrate on the effects of μ-opioids, those that investigated κ-opioids report the same population of neurons are affected by both opioids (Abdulla and Smith 1998; Moises et al. 1994). Further, inhibition was nonadditive, and each opioid could mutually occlude the action of the other, indicating μ-OR and κ-ORs are functionally coupled to the same Ca_V effectors (Moises et al. 1994). It is not known whether NOP also affect this same Ca_V pool. Of those that have studied μ-opioid and N/OFQ effects in the same cell, both similar and differing levels of inhibition have been reported (Abdulla and Smith 1998; Borgland et al. 2001; Murali et al. 2012). For example, Murali et al. (2012) report μ-opioids inhibited I_{Ca} to a much lesser degree than N/OFQ in small N/OFQ-sensitive DRG neurons, which is in contrast to what was reported in TG neurons (Borgland et al. 2001). Therefore, whilst the same Ca_V effector cannot be excluded, the strength of NOP and μ-OR coupling to Ca_Vs can differ substantially between cell subtype and region. In general, μ-opioids inhibit I_{Ca} in ~88% of all DRGs irrespective of size, IB4 binding or any other classification (Moises et al. 1994; Schroeder and McCleskey 1993; Wu et al. 2004), which is a much larger proportion of cells compared with those that are sensitive to N/OFQ.

However, the degree to which μ-opioids inhibit this Ca_V current varies dramatically, with ranges of 10–90% inhibition reported (Abdulla and Smith 1998; Moises et al. 1994; Schroeder and McCleskey 1993; Wu et al. 2004). Like N/OFQ, large diameter neurons appear to be least sensitive to μ-opioids, whilst small/medium neurons respond most frequently (Abdulla and Smith 1998; Andrade et al. 2010; Taddese et al. 1995). Interestingly, small/medium neurons that bind IB4 invariably respond to μ-opioids, but the degree of Ca_V-current inhibition is significantly less than in $IB4^-$ neurons (Schroeder and McCleskey 1993; Wu et al. 2004). This was attributed to higher levels of μ-OR expression in $IB4^-$ (presumed peptidergic) neurons (Wu et al. 2004). Thus like N/OFQ, μ-opioids preferentially reduce I_{Ca} in $IB4^-$ neurons. Since $IB4^-$ neurons predominantly synapse onto lamina I projection neurons of the superficial dorsal horn of the spinal cord (Saeed and Ribeiro-da-Silva 2012), N/OFQ and opioids would be expected to have greater control over nociceptive inputs to these neurons. By contrast, $IB4^+$ fibres, which primarily innervate lamina II_{inner} dorsal horn neurons (Saeed and Ribeiro-da-Silva 2012), may be expected to have limited μ-opioid or N/OFQ control. Indeed this was shown in spinal cord slices, where N/OFQ inhibited excitatory postsynaptic currents (EPSC), evoked by stimulation of primary sensory fibres, more strongly in lamina I neurons than in lamina II_{inner} neurons (Murali et al. 2012).

Interestingly, both N/OFQ and μ-opioids preferentially target N-type currents, which are inhibited to a greater degree than other Ca_V-mediated currents in DRGs and TGs. Neither L- nor R-currents are affected, and whilst P-/Q-currents are notably reduced by both neuromodulators, these currents only contribute ~17% of all I_{Ca} in DRGs (Beedle et al. 2004). One report indicated N/OFQ strongly supressed T-type currents in medium diameter DRGs, which reduced their propensity to undergo burst firing (Abdulla and Smith 1997). This was unique to N/OFQ, since the μ-opioid morphine had little effect. It was also G-protein independent since non-hydrolysable analogues, GTPγS and GDPβS, did not block inhibition (Abdulla and Smith 1997). However, this finding has not been replicated, and several other groups report LVA T-type currents are unaffected by both N/OFQ and classical opioids (Beedle et al. 2004; Borgland et al. 2001; Moises et al. 1994; Wu et al. 2004). The reason for this conflict is not altogether clear; however the EC_{50} for N/OFQ-dependent T-current inhibition was fivefold or tenfold higher than HVA current inhibition (Borgland et al. 2001; Murali et al. 2012). This may reflect weaker effector coupling of T-type channels to NOP; alternatively, N/OFQ maybe acting at an unknown off-target receptor at these higher concentrations. Nevertheless, since N-type currents predominate in small diameter primary sensory neurons (Beedle et al. 2004; Borgland et al. 2001) and $Ca_V2.2$ channels are more sensitive to N/OFQ or μ-opioids (Andrade et al. 2010; Borgland et al. 2001; Taddese et al. 1995; Wu et al. 2004), decreases in overall I_{Ca} would primarily reflect a decrease in $Ca_V2.2$ conductance. $Ca_V2.2$ channels are expressed presynaptically, and calcium entry through these channels triggers neurotransmitter release (Westenbroek et al. 1995; Zamponi et al. 2015). Consistent with this, both N/OFQ and μ-opioids reduce excitatory inputs from primary sensory fibres to superficial dorsal horn neurons (Kohno et al. 2005; Murali et al. 2012; see also Sect. 3.1).

It is possible NOP may mediate agonist-independent, tonic inhibition of $Ca_V2.2$ via a direct interaction between their respective C-termini. This was shown in the tsA-201 cell line (transformed from HEK293 cells), where a large depolarising prepulse revealed voltage-dependent, tonic inhibition of I_{Ca} in cells that were transfected with both Cav2.2 and NOP, but not in cells that lacked NOP (Beedle et al. 2004). This appeared to be unique to NOP since co-transfection of μ-OR with Cav2.2 had no effect on I_{Ca} (Beedle et al. 2004). Tonic inhibition was also observed in a subset of small diameter DRG neurons (<25 μm), which correlated with their responsiveness to N/OFQ (Beedle et al. 2004). It was later suggested NOP-$Ca_V2.2$ exist in a signalling complex and this interaction facilitates co-internalisation and lysosomal degradation of both NOP and $Ca_V2.2$, following prolonged exposure to N/OFQ (30 min) (Altier et al. 2006). Similarly, heterodimerisation of NOP and μ-OR facilitated μ-opioid-induced internalisation of $Ca_V2.2$, which was absent if $Ca_V2.2$ and μ-OR were co-expressed alone (Evans et al. 2010). Like classic opioid receptors, NOP undergoes β-arrestin-dependent endocytosis following prolonged agonist activation, which underlies receptor desensitisation (see Sect. 2.4). Thus, NOP could be acting as a molecular link between μ-OR and $Ca_V2.2$ as well as Cav2.2 and the endocytic machinery (Altier et al. 2006; Evans et al. 2010). However, Murali et al. (2012), who used electrophysiological methods in native DRGs, report a conflicting finding. Whilst prolonged N/OFQ induced rapid desensitisation of NOP, they observed no corresponding desensitisation of I_{Ca} or decrease in presynaptically evoked spinal cord response, thus indicating no functional loss of CaV2.2. The reason for this discrepancy is unclear. It is possible the electrophysiological studies sampled neurons from a different subpopulation, since only 10% of all acute DRGs displayed overlapping NOP/CaV2.2 internalisation (Altier et al. 2006). Differences in enzyme digestion protocols that favour acute isolation of distinct DRG populations may also play a role. Yet the majority of internalisation work was conducted in heterologous expression systems; therefore it is also possible the NOP-CaV2.2 interaction may be an artefact of overexpression. GST pull-down assays indicate the C-terminal of NOP could precipitate Cav2.2 from whole brain lysates (Beedle et al. 2004), thus indicating this interaction remains relevant in a physiological setting. Further, other GPCRs have been shown to facilitate Ca_V internalisation (Hermosilla et al. 2017; Kisilevsky et al. 2008; Macabuag and Dolphin 2015). Therefore, it is still an open question whether NOP-μ-OR heterodimerisation or NOP-facilitated CaV2.2 internalisation occurs in native neurons. Given the connotations this may have for therapeutic targeting of NOP to treat pain syndromes, further study is required to ascertain the physiological effects of NOP in primary sensory neurons.

An interesting property of CaV2.2 is the e37a/e37b splice variant site, which has been shown to affect μ-opioid sensitivity and pain sensation (Andrade et al. 2010; Raingo et al. 2007). In a series of elegant studies, Lipscombe and colleagues identified a pair of mutually exclusive exons, 37a and 37b, that encode the proximal C-terminal region of $Ca_V2.2$ (Andrade et al. 2010; Bell et al. 2004; Castiglioni et al. 2006; Marangoudakis et al. 2012; Raingo et al. 2007). Remarkably, they found expression of the $Ca_V2.2_{e37a}$ isoform is three times higher in a subset of small DRGs

that respond to capsaicin and contain the voltage-gated sodium channel $Na_V1.8$ (Bell et al. 2004; Castiglioni et al. 2006). This $Ca_V2.2_{e37a}$ splice variant activated at more hyperpolarised potentials and conducted larger current densities than the ubiquitously expressed $Ca_V2.2_{e37b}$ isoform. Further, $Ca_V2.2_{e37a}$ was more susceptible to G-protein inhibition, which was partially voltage-independent (Andrade et al. 2010; Raingo et al. 2007). This voltage-independent component was independent of $G\beta\gamma$ but dependent on $G\alpha_{i/o}$ and on Src-mediated phosphorylation of the tyrosine residue Y1747, contained within the region encoded by e37a (Raingo et al. 2007). Intriguingly, in both TGs and DRGs, strong depolarising pulses only partially blocked N/OFQ and μ-opioid inhibition of I_{Ca}, signifying a voltage-independent component that may be indicative of $Ca_V2.2_{e37a}$ expression (Beedle et al. 2004; Borgland et al. 2001). Indeed, by using an exon replacement strategy to create transgenic mice homozygous for either e37a* or e37b*, Andrade et al. (2010) showed e37a* genotype had no overall effect on DAMGO inhibition of I_{Ca} in DRGs, but it markedly increased the proportion of voltage-independent inhibition. This voltage-independent mechanism appears to be important for maximally effective morphine analgesia, since this was dramatically reduced in e37b* mice (Andrade et al. 2010). It is unclear whether the $Ca_V2.2_{e37a}$ similarly affects N/OFQ inhibition of I_{Ca}. However, given the characteristics that define $Ca_V2.2_{e37a}$-rich DRGs are very similar to those that identify N/OFQ-responsive neurons, together with a component of N/OFQ inhibition being voltage-independent, it is very likely N/OFQ affects this $Ca_V2.2$ isoform. Voltage-independent block of the main Ca_V conductance in a subset of DRGs has significant implications for their function under high periods of neuronal activity. Whilst the voltage-dependent component of μ-opioid or N/OFQ-induced I_{Ca} inhibition would be blocked under such conditions, voltage-independent inhibition would be preserved. Given under both acute and chronic pain conditions primary sensory neuron activity may be greatly enhanced (Basbaum et al. 2009; Li et al. 2017), this indicates μ-opioids and perhaps N/OFQ would retain a reasonable degree of control over $Ca_V2.2_{37a}$ expressing DRGs.

2.2.2 CNS Neurons

Although the majority of work on Ca_Vs has been conducted in primary sensory neurons, some reports have characterised N/OFQ and opioid effects in CNS neurons. This includes acutely dissociated cells of the hippocampus, PAG, LC, RVM, nucleus tractus solitarius and dorsal horn of the spinal cord (Table 1). Here we will briefly review what is known about N/OFQ and classic opioid actions on I_{Ca} in CNS regions concerned with nociception.

As with primary sensory neurons, N/OFQ and classic opioids inhibit Ca_V conductance which is pertussis toxin-sensitive and largely blocked with a strong depolarising pre-pulse. Yet in most cases a small voltage-independent component remained (Connor and Christie 1998; Connor et al. 1999; Vaughan et al. 2001). Although identifying cell subpopulations is more problematic, the distribution of N/OFQ and classic opioid-sensitive neurons is often very different, which is consistent across all reported actions of these neuropeptides. Indeed, in acutely isolated RVM neurons, N/OFQ dose-dependently inhibited I_{Ca} in all cells tested; however,

κ- and μ-opioids only inhibited the current in a subset of neurons which were nearly mutually exclusive (Vaughan et al. 2001). Curiously, it was noted that N/OFQ was more potent at inhibiting I_{Ca} than it was activating $K_{ir}3$s, which may reflect stronger effector coupling of NOP to Ca_Vs. This may be a phenomenon of all opioid receptors since the same has also been reported for μ-ORs in acutely isolated LC neurons (Ingram et al. 1997). Both N/OFQ and μ-opioids were found to strongly inhibit I_{Ca} in all LC neurons (Connor et al. 1999; Ingram et al. 1997), which is similar to their action on $K_{ir}3$ conductance (see Sect. 2.1.4). Although the specific subtype of Ca_V conductance was not identified in either RVM or LC studies, it was noted the voltage-dependent type was typical of HVA currents (Connor et al. 1999; Ingram et al. 1997; Vaughan et al. 2001).

In acutely dissociated PAG neurons, N/OFQ inhibited I_{Ca} in nearly all cells tested with a maximum effect of ~52% inhibition (Connor and Christie 1998). This was similar to the $GABA_B$ receptor agonist baclofen, which also inhibited I_{Ca} in nearly all PAG neurons albeit to a slightly lesser degree (Connor and Christie 1998). In contrast, μ-opioids inhibited I_{Ca} in a subpopulation of PAG neurons (30–40%), and the level of inhibition was substantially smaller than both N/OFQ and baclofen (Cho et al. 2001; Connor and Christie 1998; Kim et al. 1997). Neither κ- nor δ-opioids had an effect on I_{Ca} in any PAG neurons (Connor and Christie 1998). N/OFQ predominantly inhibited N- (~50% inhibition) and P-/Q-type (~33% inhibition) currents whilst having little effect on L- or R-type currents (Connor and Christie 1998). Interestingly, there was a gender difference in the contribution of N- and R-type currents to overall I_{Ca} measured in these PAG neurons. N-type currents were markedly higher in females, whilst males had greater R-type currents, but overall I_{Ca} did not differ between genders (Connor and Christie 1998). Since N/OFQ preferentially inhibited N-type currents, this could suggest I_{Ca} in the PAG of females may be more susceptible to N/OFQ control. The μ-opioid DAMGO was also shown to preferentially inhibit N-type currents in PAG neurons isolated from neonatal rats (Kim et al. 1997). Thus like N/OFQ, μ-opioids may also be more efficacious at inhibiting I_{Ca} in females. Interestingly, activation of protein kinase C (PKC) was shown to block the inhibition of I_{Ca} by μ-opioids (Cho et al. 2001), indicating potential regulation of this opioid effect by $G\alpha_q$ signalling (via canonical $PIP_2 \rightarrow DAG \rightarrow PKC$ pathways). A similar finding in acutely isolated spinal cord dorsal horn neurons was reported by the same group (Lee et al. 2004). DAMGO inhibited I_{Ca} in approximately 36% of all neurons tested, which was partially voltage-dependent. These neurons seemed to display distinct morphology, and they had medium-sized, oval-shaped somas, whilst DAMGO-insensitive neurons had either large or small somas that were round or pyramidal in shape (Lee et al. 2004). Like in the PAG, activation of PKC completely abolished this DAMGO inhibition. Interestingly, L-type currents constituted the majority (~58%) of I_{Ca} in these neurons, with N- (~29%), P-/Q- (~12%) and R- (~3.4%) currents contributing much less to the overall conductance. DAMGO appeared to inhibit each Ca_V component but was most efficacious on L-type currents (Lee et al. 2004). This is very different to what has been reported in TGs, DRGs and the PAG, where a combination of N- and P-/Q-type currents appears to dominate. Given Cav1.2 and

Cav1.3 (CaVs most likely to underlie L-type currents in the CNS) are expressed primarily in postsynaptic regions (Zamponi et al. 2015), this indicates μ-opioids may downregulate postsynaptic Ca^{2+} signalling. Thus, in addition to reducing inputs from a subpopulation of dorsal horn neurons, μ-opioids may also alter Ca^{2+}-dependent signalling cascades in these neurons, which could dramatically alter their function. To our knowledge, the effect of N/OFQ on I_{Ca} has not been studied in dorsal horn neurons and therefore remains an open question.

2.3 Other Postsynaptic N/OFQ Actions

Although NOP and classic opioid receptors are primarily coupled to $K_{ir}3$ and Ca_V, a few reports indicate these receptors also modulate other postsynaptic ion conductances, in particular those mediated by voltage-gated potassium channels (K_V). K_Vs are an extremely diverse class of ion channels, which when activated drive membrane potential away from threshold and influence action potential (AP) threshold, waveform and frequency. These channels give rise to currents that have been classified on the basis of their activation/inactivation kinetics, which has been reviewed extensively elsewhere (Yuan and Chen 2006). Briefly, I_A and I_D are fast activating at subthreshold potentials and can delay action potential firing. I_K is late rectifying, and it slowly activates at more depolarised potentials, contributes to repolarisation after AP firing and can determine AP duration. I_M slowly activates at low thresholds close to RMP and is non-inactivating. I_{BK} is mediated by Ca^{2+}-activated K^+ channels (BK channels) that are also voltage-dependent and account for the rapid repolarisation and after hyperpolarisation of a single AP.

There is limited evidence that N/OFQ and opioids may modulate I_A, I_K, I_M and I_{BK}. N/OFQ has been reported to inhibit I_K in acutely dissociated neurons from the parietal cortex (Qu et al. 2007; Wang et al. 2010) and the diagonal band of broca in the basal forebrain (Chin et al. 2002). N/OFQ has also been reported to inhibit I_A in broca neurons (Chin et al. 2002). N/OFQ has also been shown to potentiate I_M currents in CA1 and CA3 hippocampal neurons (Madamba et al. 1999; Tallent et al. 2001). In addition, N/OFQ has been reported to inhibit I_{BK} in the basal forebrain (Chin et al. 2002) and DRGs (Abdulla and Smith 1998), via inhibition of Ca_Vs which would decrease calcium influx. By contrast, N/OFQ has been shown to augment spontaneous transient outward currents that were mediated by BK channels (I_{BK}) in acutely isolated hippocampal dentate gyrus cells (Shirasaki et al. 2001). N/OFQ has also been shown to potentiate I_M currents in CA1 and CA3 hippocampal neurons and reduce epileptiform activity (Madamba et al. 1999; Tallent et al. 2001). The reason for these discrepancies is not clear and could be due to differences between different brain regions. Nevertheless, there is a paucity in the literature for opioid and N/OFQ effects on ion conductances that contribute to intrinsic membrane properties.

2.4 NOP Desensitisation

Receptor desensitisation has been attributed as one of the mechanisms underlying opioid tolerance (Williams et al. 2013). Like classic opioid receptors, NOP undergoes desensitisation following acute or chronic agonist exposure. The mechanisms underlying NOP desensitisation have been reviewed extensively elsewhere (Donica et al. 2013). Briefly, following activation, G-protein-coupled receptor kinases (GRK) are recruited to the membrane and phosphorylate agonist bound NOP. Phosphorylation, particularly at S363 within NOP C-terminus (Zhang et al. 2012), targets the receptor for β-arrestin binding and subsequent clathrin-mediated endocytosis. Following endocytosis, NOP may be targeted to either recycling endosomes for return to the cell surface or lysosomes/proteosomes for proteolytic degradation and downregulation (Donica et al. 2013).

Both homologous and heterologous NOP desensitisations have been demonstrated electrophysiologically. Homologous desensitisation describes a state where the receptor becomes less responsive to agonists following prolonged agonist exposure. This has been shown in both $K_{ir}3$ currents (Connor et al. 1996a, 1999; Vaughan et al. 2001) and Ca_V currents (Murali et al. 2012; Pu et al. 1999; Zhang et al. 2012). Interestingly, in the LC, it was shown that N/OFQ activates the same population of $K_{ir}3$ channels as μ-opioids, somatostatin and α2-adrenoceptors, all of which target $G\alpha_{i/o}$-coupled receptors. However, whilst prolonged (15 min) exposure to high concentrations of N/OFQ induced marked desensitisation in the measured $K_{ir}3$ current, μ-opioids were still able to induce $K_{ir}3$ currents that were close to their original amplitude during this desensitised period (Connor et al. 1996a). Thus in this case, desensitisation was specific to NOP and not to $K_{ir}3$ channels or μ-ORs, indicating under conditions of prolonged N/OFQ signalling, classic opioids would still be expected to evoked $K_{ir}3$-mediated responses and vice versa. Heterologous desensitisation describes the phenomenon whereby desensitisation occurs due to the activation of a second receptor system. This is often not consistent between different cell types, which likely reflects differences in signal transduction components native to each cell or cell line. For NOP, heterologous desensitisation is most well described in assays that measure cAMP accumulation or other downstream signal effectors, which is outside the scope of this review (see Donica et al. 2013). However NOP desensitisation has been demonstrated in the hippocampus using electrophysiological measures (Pu et al. 1999). Pretreatment (1 min) of acutely isolated hippocampal neurons with N/OFQ markedly reduced subsequent N/OFQ-induced inhibition of I_{Ca} (Pu et al. 1999). This desensitisation was acute, as the level of N/OFQ inhibition recovered to near control levels after 20 min washout. It was also shown that pretreatment of neurons with the $GABA_BR$ agonist baclofen could substantially decrease N/OFQ inhibition and vice versa (Pu et al. 1999). Thus in the hippocampus, NOP and $GABA_BR$ display cross desensitisation in their ability to inhibit I_{Ca}.

Homologous and heterologous desensitisation of classic opioid receptors has been extensively described (see Williams et al. (2013) for a review). The effector, cell identity, receptor subtype, concentration, duration and efficacy of the agonist can determine the mechanism and homo-/heterogeneity of desensitisation, which

varies widely with each circumstance. For example, in acutely isolated DRGs, long-term (24 h) exposure to high concentrations of high-efficacy μ-opioids (3 μM DAMGO) caused marked homologous desensitisation of μ-OR-induced inhibition of I_{Ca} (Samoriski and Gross 2000). Remarkably however, whilst long-term μ-opioid exposure induced homologous desensitisation, short-term exposure (7–12 min) induced heterologous desensitisation of $GABA_BR$-induced inhibition of I_{Ca}. Both short-term desensitisation of μ-OR and heterologous desensitisation of $GABA_BR$-mediated inhibition appeared to be specific to the voltage-dependent component of I_{Ca}, which was likely mediated by N-type ($Ca_V2.2$) channels (Samoriski and Gross 2000). Conversely, in the LC, the duration of μ-opioid exposure appears to differentially induce homologous and heterologous desensitisation, respectively, when measuring activation of $K_{ir}3$ conductance (Blanchet and Luscher 2002; Dang et al. 2009, 2012; Llorente et al. 2012). Indeed, 5 min exposure to the high-efficacy μ-/δ-opioid met-enkephalin (ME, 30 μM) induced primarily homologous desensitisation, whilst 10 min exposure dramatically reduced the $K_{ir}3$ current induced by an α2-AR agonist (Dang et al. 2012). Recovery from heterologous desensitisation was markedly slower than homologous desensitisation, which may reflect their differing underlying mechanisms (Dang et al. 2009, 2012). Similarly, heterologous desensitisation was also reported between μ-ORs, somatostatin and $GABA_B$ receptors (Llorente et al. 2012). Interestingly in this study, prolonged exposure (15 min) to supramaximal concentrations of μ-opioids (30 μM met-enkephalin) did not desensitise μ-opioid inhibition of presynaptic GABA release (see Sect. 3.1) (Llorente et al. 2012), whilst a similar exposure (10 min, 30 μM met-enkephalin) induced heterologous desensitisation of α2-AR control over presynaptic GABA release (Dang et al. 2012). Importantly, the age of the animal can drastically alter the contribution of heterologous or homologous desensitisation, with younger animals (<P20) displaying greater heterologous desensitisation between μ-ORs and α2-ARs, which appears to correlate with higher levels of GRK2 expression (Llorente et al. 2012). It is likely NOP desensitisation displays similar dichotomy depending on the cell type, agonist or effector studied. Given the implication desensitisation may have on drug tolerance, further work is warranted to fully characterise NOP desensitisation.

3 Presynaptic Actions of N/OFQ

3.1 Short-Term Modulation of Neurotransmitter Release

In addition to its postsynaptic effects, N/OFQ acts presynaptically in a number of regions throughout the CNS. Most of these studies have examined short-term plasticity, that is, the modulation of synaptic transmission which persists only during agonist activation of the NOP receptor. In general, N/OFQ reduces K+ evoked release of the major excitatory and inhibitory neurotransmitters, GABA and glutamate, within the brain (Nicol et al. 1996). In addition, N/OFQ has been shown to inhibit the release of transmitters such as noradrenaline and dopamine (Kawahara et al. 2004; Olianas et al. 2008; Werthwein et al. 1999).

The whole-cell patch-clamp technique has been used to study the underlying mechanisms in more detail. In these studies, it has been shown that N/OFQ inhibits electrically evoked $GABA_A$ and/or GlyR-mediated inhibitory postsynaptic currents (IPSCs) in the spinal cord and brain regions such as the midbrain periaqueductal grey, amygdala, rostroventral medial medulla and suprachiasmatic nucleus (Finnegan et al. 2006; Gompf et al. 2005; Roberto and Siggins 2006; Vaughan et al. 1997, 2001) (Table 1). In addition, N/OFQ inhibits non-NMDA-mediated evoked excitatory postsynaptic currents (EPSCs) and excitatory postsynaptic potentials (EPSPs) in the spinal cord and brain regions such as the midbrain periaqueductal grey, amygdala, nucleus ambiguous, hypothalamus, hippocampus, suprachiasmatic nucleus and ventral tegmental area (Brailoiu et al. 2002; Chieng and Christie 1994b; Emmerson and Miller 1999; Faber et al. 1996; Gompf et al. 2005; Liebel et al. 1997; Luo et al. 2002; Meis and Pape 2001; Vaughan et al. 1997; Venkatesan et al. 2003; Yu et al. 1997; Yu and Xie 1998; Zheng et al. 2002) (Table 1). It is interesting to note that unlike its postsynaptic actions, N/OFQ-induced presynaptic inhibition does not display desensitisation during prolonged application (Pennock et al. 2012).

The inhibition of synaptic transmission by N/OFQ is mediated by the NOP receptor because it is concentration dependent and is reduced/blocked by NOP antagonists and partial agonists (Ahmadi et al. 2001b; Faber et al. 1996; Kallupi et al. 2014; Liebel et al. 1997; Liu et al. 2001; Meis and Pape 2001; Nazzaro et al. 2007; Sulaiman et al. 1999; Zheng et al. 2002). It might also be noted that one study has shown that N/OFQ has dose-dependent inhibitory and excitatory effects on evoked excitatory field potentials in the spinal cord, although the mechanism underlying this biphasic activity is unclear (Ruscheweyh and Sandkuhler 2001). Finally, the presynaptic inhibition by N/OFQ is absent in NOP, but not μ-opioid receptor-deficient mice (Ahmadi et al. 2001a; Vaughan et al. 2003).

The synaptic actions of N/OFQ have subtle, regionally specific differences to those of opioids. For example, within the PAG, N/OFQ inhibits both evoked IPSCs and EPSCs in approximately 50% of neurons throughout the PAG, except for the ventrolateral PAG where it inhibits evoked IPSCs in all neurons (Vaughan et al. 1997) (Fig. 1). Within the RVM, N/OFQ inhibits evoked IPSCs, but not evoked EPSCs (Vaughan et al. 2001). In the spinal cord dorsal horn, N/OFQ inhibits excitatory evoked EPSCs, but not inhibitory evoked IPSCs (Ahmadi et al. 2001a, b; Liebel et al. 1997; see also Zeilhofer et al. 2000). This might be contrasted to μ-opioids which inhibit both evoked IPSCs and EPSCs in all neurons within these spinal cord and brain regions (Fig. 1). In addition, unlike μ-opioids, N/OFQ has no effect on evoked EPSCs in subthalamic neurons (Shen and Johnson 2002). It should also be noted that the differential presynaptic actions of N/OFQ differ to its less targeted postsynaptic actions in regions such as the midbrain PAG (see Sect. 2.1.1). Thus, N/OFQ differentially couples to presynaptic and postsynaptic effectors throughout the brain and spinal cord.

3.1.1 Locus of Action: Presynaptic

The locus of action of N/OFQ has been determined using a number of approaches. Firstly, N/OFQ has no effect on the currents induced by exogenously applied GABA or glutamate in regions where it inhibits evoked IPSCs and/or EPSCs, respectively (Luo et al. 2002; Yu and Xie 1998). This indicates that the effect of N/OFQ is not mediated by an effect on the postsynaptic ion channels mediating these forms of synaptic transmission. In other experiments on evoked synaptic transmission, it has been shown that N/OFQ produces an increase in the paired-pulse ratio (PPR) of closely spaced evoked IPSCs and/or EPSCs (Emmerson and Miller 1999; Roberto and Siggins 2006; Vaughan et al. 1997, 2001; Yu et al. 1997). Such changes in the paired-pulse ratio are traditionally thought to be mediated by altered presynaptic excitability (Katz and Miledi 1968).

In other experiments, it has been shown that N/OFQ reduces the frequency of spontaneous miniature EPSCs and/or IPSCs in the presence of tetrodotoxin but has no effect on their amplitude or rise and decay kinetics (Finnegan et al. 2006; Gompf et al. 2005; Kallupi et al. 2014; Liebel et al. 1997; Meis and Pape 2001; Roberto and Siggins 2006; Vaughan et al. 1997, 2001; Zheng et al. 2002). The reduction in miniature synaptic current frequency, without any concomitant change in amplitude, is indicative of a presynaptic site of action without an effect on membrane conductance or $GABA_A$/non-NMDA ligand-gated ion channels. Together, these experimental approaches indicate that N/OFQ acts to reduce the probability of neurotransmitter release from nerve terminals.

3.1.2 Mechanisms of Presynaptic Action

Few studies have examined the presynaptic mechanism by which N/OFQ modulates transmitter release. A clue to potential mechanisms arises from its differential effects on neuronal inputs to the PAG, RVM and spinal cord. Within the RVM, it has been shown that, unlike opioids, N/OFQ does not affect spontaneous miniature IPSC frequency under basal conditions (Vaughan et al. 2001). However, N/OFQ reduces spontaneous miniature IPSC frequency when external K^+ is elevated, and this inhibition is abolished by the voltage-gated calcium channel (VGCC) blocker Cd^{2+} and by removal of external Ca^{2+}. In addition, the inhibition of evoked synaptic currents by N/OFQ reduces N- and P-/Q-type VGCC blockers in the hypothalamus (Gompf et al. 2005). Together, these studies indicate that presynaptic VGCCs make a substantial contribution to the presynaptic actions of N/OFQ.

3.2 Long-Term Synaptic Plasticity

In addition to its immediate short-term effects on synaptic transmission, some studies have shown that exogenously applied N/OFQ can produce long-lasting adaptations, i.e. long-term synaptic plasticity. Within the hippocampal CA1 and dentate gyrus, N/OFQ inhibits glutamatergic synaptic transmission via a presynaptic mechanism, as observed in pain modulatory regions (Yu et al. 1997). In addition to this short-term inhibition, however, N/OFQ inhibits long-term potentiation (LTP)

and long-term depression within hippocampus (CA1 and dentate gyrus) (Wei and Xie 1999; Yu et al. 1997). Interestingly, endogenously released N/OFQ also appears to influence long-term plasticity. Thus, NOP knockout animals display a greater degree of LTP in the CA1 compared to wild-type mice (Manabe et al. 1998). This is observed with LTP induced by intense tetanic stimulation, but not by theta-burst stimulation (Bongsebandhu-phubhakdi and Manabe 2007).

The long-term plasticity produced by N/OFQ has been proposed to be mediated by a postsynaptic mechanism because it is not associated with a change in the paired-pulse ratio of evoked field excitatory postsynaptic potentials (fEPSPs) (Yu and Xie 1998). These studies have suggested that the effect of N/OFQ on long-term plasticity is NMDA receptor-dependent because, in addition to K_{ir}-mediated postsynaptic inhibition, it inhibits NMDA agonist-induced currents. Brain-derived neurotrophic factor (BDNF) has a key role in postsynaptically mediated LTP. Long-term application of BDNF to hippocampal cultures induces the expression of mRNAs preproN/OFQ, and N/OFQ has an inhibitory effect on dendritic growth (Alder et al. 2013; Ring et al. 2006). Conversely, knockout of ppN/OFQ leads to an increase and dendritic growth and spine density (Alder et al. 2013). These findings indicate that the N/OFQ system has a major impact on learning and memory (Mouledous 2018; Taverna et al. 2005).

Studies on long-term plasticity in pain pathways are largely restricted to the spinal and medullary dorsal horn where it is thought to have a role in the generation of chronic pain states (Ruscheweyh et al. 2011). It might be predicted that N/OFQ has long-term plastic effects in spinal pain pathways given its inhibitory influence on primary afferent transmission into the dorsal horn (see Sect. 3.1). To date, however, there has only been one study which has shown that N/OFQ inhibits synaptic fEPSPs in subpopulations of neurons within the nucleus of the solitary tract (Bantikyan et al. 2009). Thus, the role of N/OFQ on long-term plasticity in pain modulatory pathways remains to be explored.

4 Implications of the Electrophysiological Actions of N/OFQ in Nociception

Given that N/OFQ-NOP receptor coupling and MOP receptor coupling are similar in many ways, similarities and differences in their functional effects are likely to be related to receptor location (Table 1). This chapter has highlighted specific differences in the cellular actions of N/OFQ and opioids which are related to the distribution of receptors. Interestingly, N/OFQ appears to have a different effect than μ-opioids on nociception at the spinal and supraspinal levels in rodents. Within the spinal cord, endogenously administered N/OFQ has an antinociceptive action which is similar to that of opioids (Gunther et al. 2018; Kiguchi et al. 2016; Tian et al. 1997). Indeed, these findings are reflected in the partially similar postsynaptic actions of N/OFQ and μ-opioids on DRGs and spinal neurons, plus their presynaptic actions on primary afferent transmission into the dorsal horn.

Supraspinally, N/OFQ is generally described as having anti-opioid effects on nociception. Thus, intracerebroventricular injection of N/OFQ reduces the antinociceptive actions of μ-opioids, although some studies suggest that alone it can produce hyperalgesia (Calo et al. 1998; Meunier et al. 1995; Mogil et al. 1996a, b; Reinscheid et al. 1995). These findings are generally consistent with the electrophysiological actions of N/OFQ described in this chapter and can be understood in the context of their effects on descending analgesic pathways. Within components of the major descending analgesic pathways, such as the midbrain PAG and RVM, opioids tend to inhibit presumptive GABAergic interneurons but have no direct effect on the descending projection neurons which mediate antinociception. In addition, opioids inhibit GABAergic and glutamatergic inputs onto these neurons. The net effect of these opioid pre- and postsynaptic actions is disinhibition, or activation, of the descending projection neurons. By contrast, N/OFQ indiscriminately inhibits most neurons within these regions, including both opioid-sensitive and opioid-insensitive neurons. In addition, N/OFQ also inhibits synaptic transmission onto these neurons. Thus, the net effect of N/OFQ is inhibition of neuronal activity within these brain regions and the transmission of information between these brain regions. The net effect of N/OFQ is therefore a reversal of the opioid-induced activation of descending systems. It might be noted, however, that there are exceptions. For example, N/OFQ is antinociceptive when microinjected into the amygdala (Shane et al. 2003). This is consistent with the more opioid-like actions of N/OFQ within this brain region.

Nonetheless, the functional implications of the electrophysiological actions of N/OFQ are complex. It must be remembered that N/OFQ is an endogenous neurotransmitter and the above studies have all focused on the actions of exogenously applied agonists. Whilst agonist studies have implications for the development of analgesics, they are not necessarily related to the physiological role of this neurotransmitter system as this is determined by its endogenous release within specific circuits. Furthermore, it must be noted that the actions of N/OFQ are state dependent and will vary with pathological conditions such as chronic inflammatory and neuropathic pain (Schroder et al. 2014).

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NOP Receptor Signaling Cascades

Kyle E. Parker and Michael R. Bruchas

Contents

Abstract

The nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor is a G protein-coupled receptor with wide distribution throughout the peripheral and central nervous system. Similar to other opioid receptors, NOP receptors couple to intracellular second messengers and regulatory proteins to affect biological systems. In this chapter, we review the current literature for NOP signaling cascades including their role as classic GPCRs, the investigation of their kinase and arrestin signaling pathways, and the importance of examining biased signaling to critically evaluate the therapeutic potential of novel NOP agonists.

Keywords

Arrestin · Bias · G protein · N/OFQ · Nociceptin · NOP receptors

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1 Classic Gi-Signaling Pathways

Similar to all GPCRs, nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor couples to the inhibitory G protein $G\alpha_{i/o}$, and following agonist stimulation, the G protein exchanges GDP for GTP and permits Gα and Gβγ subunits to dissociate and act on the various intracellular pathways (Childers and Snyder 1978; Childers et al. 1979). Early research in opioid receptor pharmacology revealed that guanine nucleotides, such as GTP, control agonist binding to opioid receptors in membrane preparations from brain tissue (Blume et al. 1979). Later, Barchfeld and Medzihradsky (1984) determined that opioid agonists stimulate GTPase activity, while other groups determined that NOP receptor activation distinctly promotes guanine nucleotide exchange (Sim et al. 1996; Narita et al. 1999). Similar to the manner of other GPCRs, agonist stimulation of opioid receptors was also shown to reduce cyclic adenosine monophosphate (cAMP) production. Several studies confirmed that NOP receptor activation inhibits adenylyl cyclase activity, and it is broadly accepted that the NOP receptor couples to pertussis toxin-sensitive G proteins, including Gαi, to initiate inhibition of cAMP formation (Meunier et al. 1995; Butour et al. 1997; Zhang et al. 2012). Indeed, the initial identification of endogenous NOP ligand, N/OFQ, did so based on N/OFQ's ability to inhibit cAMP. Collectively, this inhibition reduces cell regulatory signaling through decreased activity of cAMP-dependent protein kinase, as well as cell proliferation and gene regulation.

It has also been suggested that NOP receptors can couple to other G proteins, G_Z and G_{16}. This noncanonical NOP G protein signaling has been less well characterized in physiologically relevant systems and has only been demonstrated in heterologous expression studies and SH-SY5Y cells (Chan et al. 1998). Similar to canonical opioid receptors, NOP receptors couple to Kir3 and Ca2+ channels via Gβγ pathways (Connor et al. 1996; Connor and Christie 1998). Channel deactivation for Kir3 interactions happens after GTP to GDP hydrolysis and Gβγ removal from interaction with the channel (Wickman and Clapham 1995). This opening of Kir channels causes cellular hyperpolarization and inhibits tonic neural activity. NOP receptors have also been shown to reduce Ca2+ currents sensitive to P/Q-type, N-type, and L-type channel blockers when activated (Connor et al. 1996; Zhang et al. 2012). Specifically, NOP receptor inhibition of N-type calcium conductance is likely mediated by binding of the dissociated Gβγ subunit directly to the channel and reduces voltage activation of channel pore opening (Zamponi and Snutch 1998, 2002; Beedle et al. 2004; Yeon et al. 2004; Ruiz-Velasco et al. 2005). Recently, it has also been shown that NOP receptors use Rho-associated coiled-coil-containing protein kinase (ROCK) and LIM domain kinase (LIMK) in the regulation of voltage-dependent Ca2+ channels (Mittal et al. 2013).

2 NOP Receptors and Kinase Signaling

As all GPCRs couple to various intracellular kinase cascades, opioid receptors have been shown to couple to protein kinase A (PKA) and protein kinase C (PKC) pathways as well as signaling through mitogen-activated protein kinase (MAPK) cassettes. In general, these pathways are key regulatory mechanisms within cellular signaling that control diverse physiological outcomes. Beginning in the 1990s, investigators learned that the phosphorylated arrestin-bound GPCR complex is not simply inactive but that it recruits alternate signal transduction cascades, including MAPKs (Fukuda et al. 1997; Hawes et al. 1998; Bruchas and Chavkin 2010; Whalen et al. 2011; Chang and Bruchas 2014). Similarly, signaling to MAPK cassettes in opioid receptors and NOP receptors can be mediated through this process (Zhang et al. 2012). NOP receptor activity can induce activation of PKC (Armstead 2002) as well as activation of phospholipase A2 and C (Fukuda et al. 1998; Yung et al. 1999). NOP receptor-dependent activation of all three MAPK cascades (ERK1/ERK2, p38, and JNK1/JNK2/JNK3) has also been demonstrated. These pathways are of significant importance as the ERK1/ERK2 pathway communicates signaling that facilitates cell proliferation, cell cycle progression, cell division, and differentiation. Further, these cascades regulate apoptosis, transcription factor regulation, ion channel regulation, neurotransporter regulation, and protein scaffolding (Raman et al. 2007). Both JNK and p38 signaling pathways are responsive to cellular stressors, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in adaptation to stress, apoptosis, or cell differentiation (Raman et al. 2007). Although NOP receptor-induced extracellular signal-regulated kinase (ERK) phosphorylation has not been extensively studied, endogenous agonist N/OFQ has been demonstrated to cause NOP receptor-mediated increases in ERK1/ERK2 phosphorylation levels in heterologous expression systems (COS7, CHO, and HEK293 cells) (Lou et al. 1998; Zhang et al. 2012). Recently, it was reported that ERK1/ERK2 signaling via NOP receptors was independent of receptor phosphorylation and GRK/arrestin signaling (Zhang et al. 2012). However, further examination within alternate model systems and with other ligands is warranted.

Recently, opioid receptor activation of p38 MAPK cassettes has gained attention due to the effects of kappa receptor-induced p38 phosphorylation and resulting aversion-like behaviors (Bruchas and Chavkin 2010; Bruchas et al. 2011). Similar to kappa receptors, NOP receptor activation has been linked to this phosphorylation of p38 MAPK in vitro as Zhang et al. (1999) demonstrated that NOP receptors activate p38 signaling via protein kinase A and PKC pathways in NG108-15 cells. However, further examination of NOP receptor-mediated p38 signaling in endogenous systems during pathologic conditions (as demonstrated in Armstead 2006) and in specific tissues will provide important insights into the coupling of NOP receptors to this MAPK cassette.

Additionally, the activation of c-Jun N-terminal kinase (JNK) signaling by opioid receptors has been recently examined for its interesting mu and kappa regulatory properties (Bruchas et al. 2007; Melief et al. 2010; Al-Hasani and Bruchas 2011). For the NOP receptor, important early studies in NG108 cells showed that N/OFQ

could induce phosphorylation of JNK in a time- and concentration-dependent manner (Chan and Wong 2000). This report suggested that JNK activation via NOP receptors could occur in a pertussis toxin (PTX)-sensitive and pertussis toxin (PTX)-insensitive manner. PTX-insensitive G proteins, G_Z, G12, G14, and G16, were all reported to potentially play a role (Chan and Wong 2000). Moreover, PTX-insensitive NOP-mediated JNK signaling was determined to be mediated through G protein-coupled receptor kinase 3 (GRK3) and arrestin-3 as late-phase JNK phosphorylation was absent following selective siRNA knockdown of GRK and arrestin (Zhang et al. 2012). Additionally, this report confirmed this GRK-/arrestin-mediated effect using cells expressing a C-terminal phosphorylation NOP receptor mutant (S363A) and also corroborated reports that NOP receptors couple to JNK in a PTX-sensitive fashion during the early phase of activity.

3 NOP Receptors and Arrestin Signaling

GPCR internalization is mediated through recruitment of arrestin and typically via either a clathrin-dependent or clathrin-independent process. Similar to other opioid receptor subtypes, phosphorylation by GRK2 or GRK3 of the NOP receptor leads to arrestin-2 or arrestin-3 recruitment (Zhang et al. 2012). This arrestin-2 and arrestin-3 binding modulates NOP receptor desensitization and ultimately assists in determining receptor status.

Several groups have examined the many stages of NOP receptor trafficking (for review, see Donica et al. 2013). Although initial study in the NOP receptor field had difficulty demonstrating agonist-induced internalization (Dautzenberg et al. 2001), Spampinato et al. (2001, 2002, 2007) clearly demonstrated that N/OFQ treatment induces NOP internalization. Similar to the kappa opioid receptor disparities in internalization conditions (Bruchas and Chavkin 2010), differences reported in the internalization of NOP receptors are likely due to expression variability and the model system implemented. Indeed, most studies implicating arrestin in this signaling have been conducted in heterologous expression systems using overexpressed arrestins and opioid receptor subtypes. Indeed, knockdown of arrestin-3, but not arrestin-2, blocks NOP receptor internalization after treatment with N/OFQ (Zhang et al. 2012). When Ser363, a putative GRK phosphorylation site on the NOP receptor, was mutated to an alanine, arrestin-3 was not recruited to the cell surface after N/OFQ treatment and the mutant S363A demonstrated significantly reduced NOP receptor internalization (Zhang et al. 2012). Similarly, the dominant positive arrestin-3-(R170E), which does not require receptor phosphorylation, was able to rescue a NOP receptor S363A mutant's internalization (Zhang et al. 2012). A recent study has also demonstrated that NOP receptors use arrestin-2 to regulate downstream signaling (Mittal et al. 2013). Here investigators demonstrated enhanced NOP function in dorsal root ganglia neurons that lacked arrestin-2. Using patch clamp, whole-cell recording, the authors found that nociceptin has greater inhibition of voltage-dependent Ca^{+2} channels in arrestin-2 KO mice compared to wild-type mice. Further, they demonstrated that NOP agonist Ro 65-6570 administration

produces a hypolocomotor response in arrestin-2 KO mice while having no effect in wild-type mice. Currently, investigators are examining how the NOP receptor engages these various arrestins and whether agonists with varying efficacies and potencies can induce different rates of internalization and divergent arrestin-2 and arrestin-3 recruitment (Chang et al. 2015; Malfacini et al. 2015). Recently, evidence suggests that compounds acting as partial agonists with respect to NOP/G protein signaling behave as antagonists with little to no activity in NOP/arrestin coupling (Chang et al. 2015; Malfacini et al. 2015; Asth et al. 2016). In fact, although NOP receptors functionally recruit both arrestin-2 and arrestin-3, arrestin-3 recruitment is likely more efficacious (Chang et al. 2015). Many NOP ligands also differ in the kinetics of arrestin recruitment as demonstrated using bioluminescence resonance energy transfer (BRET) techniques (Chang et al. 2015). Certainly, it is possible that agonist, cell type, and environment have a significant impact on NOP internalization and arrestin recruitment properties (Malfacini et al. 2015). Indeed, Asth et al. (2016) demonstrated that some NOP ligands can have similar G protein interaction yet have divergent arrestin recruitment (partial agonism vs antagonism) that drive behaviorally relevant outcomes for anxiety and depression. Based on these initial reports, it may be possible to further design arrestin-biased NOPR ligands in figure efforts (Fig. 1).

In most cases, NOP receptor internalization starts rapidly, within 5–10 min after agonist treatment, with very robust internalization at 1 h posttreatment in transfected cells (Spampinato et al. 2001; Corbani et al. 2004; Zhang et al. 2012). As with other

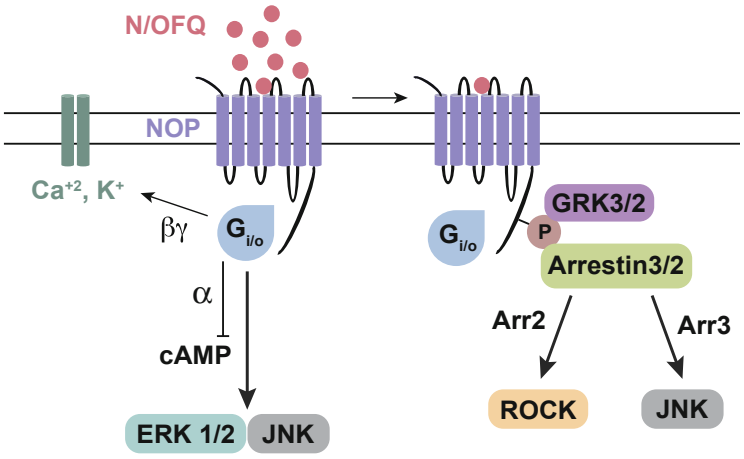

Fig. 1 Summary of NOP receptor signaling: Figure highlights basic NOP receptor signal transduction and trafficking pathways and features canonical NOP receptor coupling to inhibition of calcium channels and activation of inward rectifying potassium channels (Connor et al. 1996; Connor and Christie 1998). Figure additionally highlights reports of NOP receptor activation of MAPKs and desensitization mechanisms via GRK3 and GRK2 (Zhang et al. 2012). Figure also depicts findings that NOP receptor activation can initiate downstream signaling to JNK and ROCK pathways via arrestin signaling (Zhang et al. 2012; Mittal et al. 2013). Arrows refer to activation; T lines refer to block or inhibition of function. Figure adapted from Toll et al. (2016)

opioid receptor subtypes, the level of internalized receptor depends on the ligand. However, hexapeptide partial agonists do not induce receptor internalization or robust GRK translocation (Spampinato et al. 2001; Corbani et al. 2004). This could be due to their partial agonism as other NOP partial agonists such as [F/G] N/OFQ(1–13)-NH2 also lack receptor internalization or due to hexapeptides having a different NOP-binding site (Bes and Meunier 2003). It has been proposed that receptor regulation is dependent on the specific agonist examined and that peptides and small molecule agonists may impact the regulation of NOP receptors via different mechanisms (Donica et al. 2013; Chang et al. 2015; Malfacini et al. 2015; Asth et al. 2016).

N/OFQ via selective NOP receptor activation can control several biological functions; however the relative role of G protein and arrestin in mediating these actions is not completely understood. It is known that other ligands may act as biased agonists at the NOP receptor since they can have different efficacies in activating G protein versus arrestin pathways. Recent investigation has used BRET assays to assess multiple ligands to determine these distinct biases and have demonstrated the diverse impact NOP ligands have in these signaling pathways (Chang et al. 2015; Malfacini et al. 2015; Ferrari et al. 2016, 2017; Rizzi et al. 2016). This distinction is critical to dissecting the biological actions that follow the activation of this receptor as these biased ligands may act as more effective therapeutics. Critically, studies suggest that actions on arrestin signaling, rather than G protein efficacy, may be a better predictor of behavioral outcomes in vivo (Asth et al. 2016). Comparing in vitro and in vivo actions, it appears that NOP ligands able to promote NOP/arrestin-3 interaction (N/OFQ, Ro 65-6570, and AT-090) are also able to induce anxiolytic-like effects in an elevated plus maze test (Asth et al. 2016). However, compounds that inhibit NOP/arrestin-3 interaction (UFP-101, SB-612111, UFP-113, and [F/G]N/OFQ(1–13)-NH2) produced antidepressant-like effects in a forced swim test. Given this critical divergence, thorough in vitro and in vivo investigation of full and partial agonists and pure antagonists is required to elucidate their therapeutic potential.

New studies that reveal the signaling profiles of NOP receptor ligands previously only classified as agonists, antagonists, inverse agonists, or partial agonists offer the prospect to connect these biases to observed biological effects and better understand NOP receptor function. Further studies are also needed to identify new lead molecules that will help to understand the structural requirements underlying the difference in efficacy of NOP agonists for G proteins and arrestins and the potential therapeutic indications of G protein- or arrestin-biased NOP agonists.

4 Conclusion

This chapter intends to describe the extensive effort made to illuminate the NOP receptor's cellular signaling pathways. As the most recently unveiled opioid receptor, this avenue of research continues to be of significant importance for researchers who aim to utilize this receptor in the development of novel drug therapeutics for the

treatment of pain, substance use, and psychiatric disorders. The divergent activation of G protein-biased and arrestin-biased pathways is particularly informative as understanding distinct differences in agonists' propensity to activate these pathways will guide investigation to feasible therapeutic interventions with minimal side effects.

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Regulation of the Genes Encoding the ppN/OFQ and NOP Receptor

Francesca Felicia Caputi, Patrizia Romualdi, and Sanzio Candeletti

Contents

Abstract

Over the years, the ability of N/OFQ-NOP receptor system in modulating several physiological functions, including the release of neurotransmitters, anxiety-like behavior responses, modulation of the reward circuitry, inflammatory signaling, nociception, and motor function, has been examined in several brain regions and at spinal level. This chapter collects information related to the genes encoding the ppN/OFQ and NOP receptor, their regulation, and relative transcriptional control mechanisms. Furthermore, genetic manipulations, polymorphisms, and epigenetic alterations associated with different pathological conditions are discussed. The evidence here collected indicates that the study of ppN/OFQ and NOP receptor gene expression may offer novel opportunities in the field of

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personalized therapies and highlights this system as a good "druggable target" for different pathological conditions.

Keywords
DNA methylation · Epigenetics · Gene expression · Histone marks · N/OFQ · $NOP^{-/-}$ · NOP-eGFP · NOP receptor · Polymorphisms · ppN/OFQ

1 Nociceptin/Orphanin FQ (N/OFQ): Gene, Transcriptional Regulation, and Neuropeptide Precursor

Nociceptin/orphanin FQ (N/OFQ) is a 17-amino acid peptide classified as an endogenous opioid peptide, isolated in 1995 (Meunier et al. 1995; Reinscheid et al. 1995) as natural ligand for the opiate-like receptor 1 termed ORL-1 and now called N/OFQ peptide (NOP) receptor (Cox et al. 2015). NOP is the fourth member of opioid G protein-coupled receptor family (Bunzow et al. 1994; Mollereau et al. 1994). Mollereau and colleagues mapped the location of the preproN/OFQ (ppN/OFQ) gene in the 8p21 region of the human chromosome 8 (see Mollereau et al. 1996); cDNAs encoding for the gene have been cloned also in other mammalian species such as rat, mouse (Saito et al. 1995, 1997; Nothacker et al. 1996), bovine (Okuda-Ashitaka et al. 1998), and porcine (Osinski et al. 1999). Among these species, the entire C terminus of the precursor protein including N/OFQ is 100% conserved (see Reinscheid et al. 2000). All neuropeptides are generated from inactive precursor proteins, and their bioactivity can be regulated by a variety of posttranslational modifications, including proteolytic processing (Sossin et al. 1989). In this regard, the heptadecapeptide N/OFQ is synthesized as a part of a larger polypeptide precursor (176 amino acids in humans), and its sequence is flanked by Lys-Arg proteolytic excision motifs (Meunier et al. 1995; Nothacker et al. 1996).

The polypeptide precursor, containing additional pairs of basic amino acid residues, can encode for other putative biologically active peptides, one of 35 and the other of the 17 amino acids, located upstream and downstream of the heptadecapeptide N/OFQ, respectively (Meunier et al. 1995).

For these two peptides, no binding or activation of intracellular signaling has been found (Neal et al. 1999), even though it has been demonstrated that the 17-amino acid peptide (termed NocII or OFQ II) seems to exhibit some effects on locomotion and pain perception (Florin et al. 1997; Rossi et al. 1998). The third mature peptide produced from the same polypeptide precursor was called nocistatin (NST), which recognizes specific binding sites distinct from the NOP receptors and not yet fully characterized, in the mouse brain and spinal cord (Okuda-Ashitaka et al. 1998).

As reported in Ensembl genome database, the human ppN/OFQ gene has four transcripts or splice variants (Fig. 1) (Arjomand and Evans 2001) and undergoes alternative splicing between exons 3 and 4 to generate two transcripts (N23K and N27K) which in turn encode for protein with different C terminus (Saito et al. 1996). In particular, N23K seems to work as a neuropeptide precursor but also as a crucial factor in neuronal differentiation (Saito et al. 1995); the exon 2, instead, contains the

Name	Transcript ID	bp	Protein	Biotype
PNOC-201	ENST00000301908.7	1200	176aa	protein coding
PNOC-204	ENST00000522209.1	1007	112aa	protein coding
PNOC-202	ENST00000518479.5	554	154aa	protein coding
PNOC-203	ENST00000519592.5	1310	No protein	Retained intron

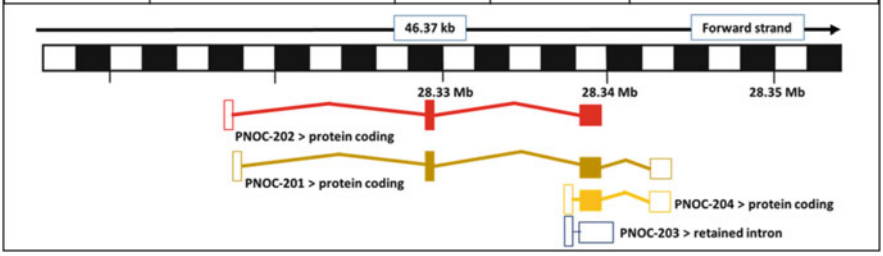

Fig. 1 Human gene encoding the ppN/OFQ. Location Chromosome 8: 28,316,986-28,343,355. This gene has four transcripts (splice variants) reported in the figure as PNOC-201, PNOC-204, PNOC-202, and PNOC-203 (http://www.ensembl.org/Homo_sapiens/Gene/Splice?db=core;g=ENSG00000168081;r=8:28316986-28343355)

N/OFQ:	Phe-**Gly**-**Gly**-**Phe**-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-**Lys**-Leu-Ala-**Asn**-**Gln**
DYNORPHIN A:	Tyr-**Gly**-**Gly**-**Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-**Lys**-Trp-Asp-**Asn**-**Gln**

Fig. 2 Amino acid sequences of N/OFQ and dynorphin A opioid peptides

translational start site for ppN/OFQ and encodes the signal peptide sequence (Arjomand and Evans 2001).

Despite the information regarding the processing of ppN/OFQ gene is still incomplete, it has been reported that the subtilisin-like prohormone convertase (PC) family may play a crucial role in neuroendocrine precursor processing (Rouillé et al. 1995). In particular, the lack of functional PC2 member of this family might cause the increase of unprocessed ppN/OFQ in the amygdala and in the hypothalamus of mice reducing, as a consequence, the production of mature N/OFQ (Allen et al. 2001).

The human ppN/OFQ precursor is largely expressed throughout the brain, but it is also present in some peripheral tissues and in the immune system (Mollereau et al. 1996; Nothacker et al. 1996; Peluso et al. 1998) sharing some similarities with precursors coding for the other opioid peptides (preproenkephalin, preprodynorphin, and proopiomelanocortin) (Mollereau et al. 1996; Nothacker et al. 1996). Likewise its precursor, also the N/OFQ neuropeptide sequence resembles those of opioid peptide dynorphin A showing a high sequence homology (Fig. 2). Notwithstanding this homology, N/OFQ is physiologically and pharmacologically different from the other opioid peptides (Butour et al. 1997; Reinscheid et al. 1996, 1998).

Over the years, a number of studies investigated the regulation of the ppN/OFQ gene in neurons, astrocytes, and immortalized cell lines. Different groups demonstrated that the ppN/OFQ precursor synthesis, as well as its processing and the N/OFQ peptide secretion, is stimulated by the activation of the cAMP-signaling pathway (Saito et al. 1995; Buzas et al. 1998; Sirianni et al. 1999; Xie et al. 1999a) and that ppN/OFQ transcription can also be modulated by other cellular mediators such as steroid hormones and neurotrophic factors (Buzas et al. 1999; Xie et al. 1999a). In particular, the ppN/OFQ mRNA levels in glial cells are dramatically increased (approximately ~30-fold) by high levels of intracellular cAMP (Buzas et al. 1998). Further investigations showed that high intracellular cAMP levels in astrocytes are associated with inflammatory conditions, which in turn promote the transcription of the ppN/OFQ gene (Buzas et al. 2002). Other studies have reported that the ppN/OFQ gene expression regulation appears to be mediated by ERK and p38 activation (Rosenberger et al. 2001; Buzas et al. 2002) through the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) on Ser^{133} (Yamamoto et al. 1988; Zaveri et al. 2006). Zaveri and co-workers (2000), by cloning the 5′-untraslated region of the human ppN/OFQ gene, characterized the ppN/OFQ promoter region indicating the elements responsible for its transcriptional regulation. Consistent with previous studies (Xie et al. 1999a), two cAMP response element (CRE) sites were identified in the promoter region of human ppN/OFQ, unlike only one present in the mouse (Zaveri et al. 2002), and the transcriptional activation is mediated by a stretch of 110 bases adjacent to the first intron (Zaveri et al. 2000). It is worth noting that the promoter sequence adjacent to the first intron exhibits a remarkable homology between human and mouse ppN/OFQ genes, up to 360 bp in the 5′-UTR. Exactly in this region is located the transcription starting site (TSS, ATG-start codon) for the human ppN/OFQ (Zaveri et al. 2000) which may act as binding site of different transcription factors. In particular, the presence of a cis-acting regulatory element of ~30 bp which binds the Sp1 transcription factor has been highlighted in this portion of promoter (Zaveri et al. 2002). The deletion of this DNA region or mutation at the Sp1-binding site may cause significant loss of human ppN/OFQ transcription (Zaveri et al. 2002). In addition, the Sp1 inhibitor mithramycin A seems able to inhibit both basal- and cAMP-induced stimulations of the ppN/OFQ transcription in rat cortical neurons (Zaveri et al. 2006).

2 NOP Receptor: Gene and Transcriptional Regulation

NOP receptor, originally named ORL-1, is a member of the seven transmembrane G protein-coupled receptor family (GPCR). Likewise its endogenous ligand, NOP amino acid sequence shares significant homology with the other opioid receptors (see Chen et al. 1994; Bunzow et al. 1994; Fukuda et al. 1994; Mollereau et al. 1994). The NOP sequence similarity with the μ-, δ-, and κ-opioid receptors is around 65%, and a higher level of homology was found in some transmembrane domains (~85%) and also in the region interacting with G proteins. Several parallel studies, performed by means of different techniques and by different groups, indicate that

NOP is largely distributed in the central nervous system (Bunzow et al. 1994; Fukuda et al. 1994; see Mollereau and Mouledous 2000). NOP presence has been reported in different species: in rat as "rat opioid receptor-C" (ROR-C in Fukuda et al. 1994; LC132 in Bunzow et al. 1994; Lachowicz et al. 1995), in mouse as "mouse opioid receptor-C" (MOR-C in Nishi et al. 1994) or "κ3-related opioid receptor" (KOR 3 in Pan et al. 1996), and in humans as "opioid receptor-like 1" (ORL-1; Mollereau et al. 1994). Pharmacological and receptor-ligand binding studies suggested that alternative splice variants of this receptor may exist in rat (Wang et al. 1994; Xie et al. 1999b, 2000) and mouse (Pan et al. 1996, 1998; Mathis et al. 1997) and in humans as well (Wick et al. 1995).

Rat NOP gene is located on the chromosome 3 (instead of chromosome 20 in humans), and it exceeds 10 kb in length and contains six exons ranging from 34 to 524 bp, interrupted by five introns. Sequence analysis of rat NOP gene revealed the following features: the ATG translation initiation codon in the exon 2, an open reading frame consisting of 1,283 bp, the presence of two putative RNA polymerase II binding sites (TATA-box), and two major transcription initiation sites (in the 5′ flanking region and in intron 1) (see Currò et al. 2001). Primer extension analysis revealed the expression of different splice variants in different tissues, suggesting that the rat NOP gene can be alternatively spliced to give multiple mRNAs. Notably, four splice variants lacking exon 1 are expressed only in the brain. In contrast, five isoforms containing the exon 1 are expressed in various tissues, such as the brain, testes, and gastrointestinal tract (Currò et al. 2001). Mouse NOP gene is instead located on the distal region of chromosome 2 and contains five exons even though the protein coding region starts in exon 2 and ends in exon 4. Two alternative splice variants differing only in their 5′-untranslated regions were described in this animal species (Pan et al. 1996).

As currently reported in the Ensembl genome database, the human NOP gene has three different splice variants (Fig. 3).

Name	Transcript ID	bp	Protein	Biotype
OPRL1-202	ENST00000349451.3	3369	370aa	protein coding
OPRL1-201	ENST00000336866.6	3321	370aa	protein coding
OPRL1- 203	ENST00000355631.8	3147	370aa	protein coding

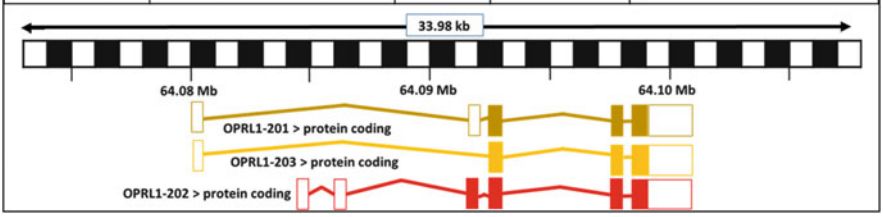

Fig. 3 Human gene encoding the NOP receptor (or OPRL1). Location Chromosome 20: 64,080,173-64,100,643 forward strand. This gene has three transcripts (splice variants) reported in the figure as OPRL1-202, OPRL1-201, and OPRL1-203 (http://www.ensembl.org/Homo_sapiens/Gene/Splice?db=core;g=ENSG00000125510;r=20:64080173-64100643)

Interestingly, it has been proposed that human NOP gene can be regulated by two alternative promoters which are lacking of TATA-box and rich in GC dinucleotides (Ito et al. 2000).

In other words, an alternative promoter mechanism responsible for the transcription and alternative splicing of human NOP gene exists. Notably, cDNA sequencing revealed that human NOP gene initiates transcription with two alternative promoters, 1A and 1B. The alternate promoter usage results in three NOP transcripts, according with the current information reported in the Ensembl genome database. The 1A promoter has been so far reported exclusively in human genomic sequence, and some homologies have been detected between the human NOP promoter 1B and the mouse NOP promoter (Pan et al. 1996). This aspect could suggest that NOP transcription regulation may be quite different in human and mouse.

Despite all these genomic studies, the transcription regulation of NOP gene is still not completely clear, even though the transcription factor response elements Sp1, AP-2, EGR, Krox-20, ETF, and CP1 or GCF sites are also found in the promotor region of the human NOP gene (Toll et al. 2016).

3 Genetic Manipulation of the N/OFQ-NOP Receptor System

Genetically manipulated animal models have significantly contributed to deepen the knowledge about the specific functions of the N/OFQ-NOP receptor system. In 1997, the first model of mice lacking NOP was generated by deletion of exons 2 and 3 using the gene targeting technique confirming the successful of NOP deletion through the complete absence of N/OFQ binding (Nishi et al. 1997). In the brain of these mutant mice, the quantitative autoradiographic mapping of NOP, μ, δ, and κ receptors indicated no compensatory changes of classical opioid receptors in the absence of NOP across multiple brain regions (see Clarke et al. 2001), even though few regionally specific changes were detected. Several studies utilized this mouse model revealing several interesting functions ascribed to NOP regarding memory, mood, locomotion, nociception, and addiction (Manabe et al. 1998; Gavioli et al. 2003, 2007; Marti et al. 2004; Sakoori and Murphy 2008; Marquez et al. 2008; Toll et al. 2016). Through genetic manipulation, Homberg and collaborators generated a knockout (KO) rat carrying a premature stop codon in the NOP gene using target-selected N-ethyl-N-nitrosourea (ENU)-driven mutagenesis, thus engendering a valuable complementary model to existing $NOP^{-/-}$ mice. Likewise mutant mice model, NOP-KO rats did not show compensatory changes in μ-, δ-, and κ-opioid receptors (Homberg et al. 2009). This new animal model has been behaviorally characterized by different groups demonstrating that it exhibits resilience to depressive-like behaviors (Gavioli and Calò 2013; Rizzi et al. 2011) but also resilience to the development of drug addiction showing a reduced propensity to self-administer cocaine, heroin, and alcohol (Kallupi et al. 2017). However, other studies also reported a greater susceptibility to morphine rewarding effects (Rutten et al. 2011).

Another genetic manipulation has knocked eGFP into exon 5 of mouse NOP gene producing mice expressing a functional NOP-eGFP in place of the native NOP receptor, with the aim to study location, trafficking, and plasticity of NOP receptor in the brain (Ozawa et al. 2015). First, they demonstrated that this kind of manipulation did not alter NOP gene transcription neither the binding properties of the receptor, even though the homozygous knock-in mice ($NOP^{eGFP/eGFP}$) exhibit a significant increase in the number of receptors and a more efficient signal transduction versus $NOP^{+/+}$ and $NOP^{+/eGFP}$ genotypes. The NOP-eGFP mouse model was further utilized to analyze the distribution of the engineered receptor in the spinal cord and dorsal root ganglia (DRG) after spinal nerve ligation (SNL, as chronic, neuropathic pain model) (Ozawa et al. 2018). Results showed that SNL decreases immunoreactivity for NOP-eGFP in the spinal lamine I and II outer (crucial for the mediation of noxious stimuli) and in a considerable number of primary afferents in the L4 DRG (Ozawa et al. 2018; but see also Briscini et al. 2002).

4 ppN/OFQ and NOP Receptor Gene Polymorphisms

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide in the genome sequence is altered. SNPs may influence disease susceptibility, drug sensitivity/resistance, and clinical outcomes. For these reasons, their knowledge can be useful to get more information about several disorders.

In 2008, Xuei and colleagues examined whether alterations in the genes encoding the ppN/OFQ and NOP receptor were associated with alcoholism or illicit drug dependence in a cohort of 1923 subjects (from 219 multiplex alcohol dependent families). Results identified two adjacent markers, rs6512305 and rs6090043, in the intron 1 region of NOP gene, as having a marginal association with opioid dependence (Xuei et al. 2008). Recently, the involvement of human NOP gene in post-traumatic stress disorder (PTSD) has been demonstrated in a cohort of highly traumatized subjects, showing that the SNP rs6010719 is associated with an increased PTSD symptomatology and also with the amygdala-insula functional connectivity alteration (Andero et al. 2013). Studies of human NOP gene polymorphisms also pointed out a relationship with alcohol dependence. Indeed, another SNPs, rs6010718 has been significantly associated with alcohol dependence in a cohort of 757 subjects belonging to the Scandinavian population (Huang et al. 2008). Finally, the relevance of the rs2229205 SNP within human NOP gene has been reported as a genetic factor contributing to individual vulnerability to smoking development in Japanese population (Kasai et al. 2016).

5 N/OFQ-NOP Receptor System Gene Expression Alterations in Pathological Conditions

During the past two decades, a wide series of studies highlighted the contribution of the ppN/OFQ-NOP receptor system to the regulation of a broad spectrum of physiological functions, including nociception (Darland et al. 1998; Meunier 2003; Kiguchi et al. 2016), stress and memory (Bodnar 2014; Witkin et al. 2014; Andero 2015; Rekik et al. 2017), locomotor activity (Florin et al. 1996; Marti et al. 2004), and the modulation of the reward circuitry (Ciccocioppo et al. 2000, 2004a; Lutfy et al. 2002; Kuzmin et al. 2003; Witkin et al. 2014; Kallupi et al. 2017). The role played by the N/OFQ-NOP receptor system in the abovementioned functions has been strengthened by employing different pharmacological tools (i.e., peptide or non-peptide ligands), experimental animal models, and the study of gene expression. It is worth noting that mRNA level alterations of genes encoding the ppN/OFQ and NOP receptor may exert a considerable influence on the development of several pathological conditions. Moreover, given the relevance of epigenetic regulatory events in modulating gene expression, the study of posttranslational modifications at ppN/OFQ and NOP receptor gene promoters could be crucial for the development of new therapeutic strategies.

5.1 Substance Use Disorders

Much evidence indicates that alterations in gene expression play a central role in addiction-related neuroplasticity (Maze et al. 2015; Mews and Calipari 2017; Palmisano and Pandey 2017), even though the mechanisms by which addictive drugs remodel brain circuits remain unclear. In this regard, a relevant role is revealed by epigenetic mechanisms (Szutorisz and Hurd 2016; Kim et al. 2017; Walker and Nestler 2018). Notably, different addictive drugs are able to modify ppN/OFQ and NOP gene expression through chromatin remodeling. Recently, 3, 4-methylenedioxymethamphetamine (MDMA) exposure has been found capable to modify epigenetic marks at the promoter region of ppN/OFQ and NOP genes (Caputi et al. 2016). Particularly, data showed that acute MDMA treatment results in a significant downregulation of ppN/OFQ and NOP gene expression in the rat nucleus accumbens (NAc), and these alterations are still present after 7 days of repeated exposure, suggesting that MDMA may attenuate the anti-reward function of this neuropeptidergic system. Consistent with ppN/OFQ gene expression reduction, chromatin immunoprecipitation (ChIP) analysis revealed a significant reduction in histone 3 lysine 9 acetylation (H3K9ac) levels, a marker of transcriptional activation, at the ppN/OFQ promoter. A similar reduction in H3K9ac levels was also observed at NOP promoter (Caputi et al. 2016) (see Table 1). The same MDMA experimental protocol did not cause gene expression alterations for ppN/OFQ and NOP genes in the brainstem region (Di Benedetto et al. 2011), suggesting a region-specific effect induced by this psychostimulant drug.

Table 1 Epigenetic changes at ppN/OFQ and NOP gene promoters in different protocols of drug addiction and in stressful conditions

Target	Drug of abuse and stressful condition	Gene promoter and tissue	Key finding	References
Histone modifications	*MDMA* (acute exposure)	ppN/OFQ in NAc	H3K9ac reduction	Caputi et al. (2016)
			H3K4me3 increase	
			H3K27me3 reduction	
		NOP in NAc	H3K9ac reduction	
			H3K4me3 increase	
			H3K27me3 increase	
	MDMA (repeated exposure)	ppN/OFQ in NAc	H3K9ac reduction	
	Cocaine (chronic subcutaneous infusion)	ppN/OFQ in NAc	H3K4me3 decrease	Caputi et al. (2014b)
		ppN/OFQ in 1CPu	H3K27me3 increase	
		NOP in NAc	H3K4me3 increase	
			H3K27me3 decrease	
		NOP in 1CPu	H3K4me3 decrease	
	Alcohol (binge paradigm)	ppN/OFQ in AMY	H3K27me3 decrease	D'Addario et al. (2013b)
			H3K9ac increase	
	Alcohol (repeated binge paradigm)		H3K9ac increase	
DNA methylation	*Alcohol*	ppN/OFQ in AMY	No changes	D'Addario et al. (2013b)
	Psychosocial stress/ binge drinking	NOP (human peripheral blood) (rat NAc)	Hypomethylated	Ruggeri et al. (2018)
	Childhood adversity	NOP (human peripheral blood)	Hypermethylated	Zhang et al. (2013b)

AMY amygdala, *H3K4me3* histone 3 lysine 4 trimethylation, *H3K9ac* histone 3 lysine 9 acetylation, *H3K27me3* histone 3 lysine 27 trimethylation, *lCPu* lateral caudate putamen, *NAc* nucleus accumbens

The relevance of epigenetic regulatory events in mediating addictive drug effects has been highlighted also in cocaine addiction since the alteration of global DNA methylation pattern has been observed to modulate cocaine intake (Fonteneau et al.

2017). Studies evaluating histone modifications at ppN/OFQ and NOP gene promoters indicated that chronic cocaine infusion causes a significant ppN/OFQ-NOP receptor gene expression downregulation triggered by specific histone changes. Indeed, according to the ppN/OFQ gene downregulation, the increase in histone 3 lysine 27 trimethylation (H3K27me3), a marker of transcriptional repression, and the simultaneous reduction in histone 3 lysine 4 trimethylation (H3K4me3), an activating marker, have been reported at ppN/OFQ promoter (see Table 1) (Caputi et al. 2014b).

Epigenetic mechanisms driving gene expression changes were also analyzed in alcohol dependence. In particular, the acetylation of specific H3 lysine residues at the promoter regions of certain genes regulates the transcription and contributes to alcohol-induced changes in the expression of genes associated with synaptic plasticity (Pascual et al. 2012; Moonat et al. 2013). In this frame, ethanol exposure evokes ppN/OFQ gene expression alterations in the rat amygdala complex (D'Addario et al. 2013a; Peana et al. 2017) through consistent epigenetic changes at ppN/OFQ promoter (D'Addario et al. 2013b) (see Table 1).

Interesting relationships between DNA methylation profile and stress responses have been recently evaluated (McGowan et al. 2009, 2011). In particular, epigenetic changes induced by childhood adversity might represent the molecular mechanisms predisposing to alcohol disorders. In this regard, the hypermethylation of about ten CpG sites at the promoter of different genes, including NOP, was reported by Zhang and co-workers in a cohort of 239 European Americans alcoholic patients (Zhang et al. 2013b). Other studies, evaluating psychosocial stress in relation to alcohol consumption in adolescents, reported a NOP gene promoter hypomethylation in both humans and alcohol-preferring rats (Ruggeri et al. 2018).

Others studies evaluated ppN/OFQ and NOP gene expression alterations in different paradigms of addictive drug exposure. An increase of both their mRNA levels following 1–3 weeks of ethanol withdrawal (Aujla et al. 2013) was observed in rat limbic regions. Conversely, a significant reduction of ppN/OFQ and NOP mRNA levels in the hippocampus and in the central amygdala of alcoholics (Kuzmin et al. 2009) was reported, thus suggesting different responses of this neuropeptidergic system during the distinct phases of alcohol use disorder. Interestingly, rats prenatally exposed to ethanol exhibit ppN/OFQ-NOP gene expression alterations during infancy and adolescence (Wille-Bille et al. 2018). Finally, a modulation of NOP mRNA levels was reported in an in vitro model of Δ(9)-THC exposure (Cannarsa et al. 2012).

5.2 Eating Disorders

The abundance of NOP receptor in the hypothalamus, particularly within the ventromedial nucleus (Gehlert et al. 2006), implies the existence of a crucial control for the N/OFQ-NOP system in feeding and metabolism (Pomonis et al. 1996; Economidou et al. 2006). Food deprivation causes a decrease of NOP mRNA levels in several forebrain regions and of ppN/OFQ mRNA levels in the central amygdala

(Rodi et al. 2002) in rats. Accordingly, central injection of N/OFQ induces feeding in satiated rats (Pomonis et al. 1996) and counteracts stress-induced feeding inhibition acting as corticotropin-releasing factor functional antagonist (Ciccocioppo et al. 2002, 2004b). Based on these observations, treatment with NOP antagonist may result in a significant dose-dependent reduction of food consumption in binge-eating disorder (Hardawaya et al. 2016; Raddad et al. 2016; Statnick et al. 2016). Recently, it has been evaluated whether cycles of intermittent food restriction could promote changes at N/OFQ-NOP system level in a binge-eating animal model. Authors reported that food restriction itself decreases the ppN/OFQ and NOP mRNA levels in the hypothalamus (Pucci et al. 2016) and suggest that the hypofunctionality of this peptidergic system might contribute to the maintenance of binge-eating disorder.

5.3 Parkinson Disease

The involvement of N/OFQergic system in the pathophysiology of some neurological disorders, such as Parkinson's disease, has been suggested by different studies. In particular, N/OFQ affects the nigrostriatal dopamine neurons influencing locomotion; accordingly, the pharmacological blockade of NOP receptor alleviates parkinsonian signs (Marti et al. 2005, 2010; Arcuri et al. 2016). In support of this hypothesis, rats lesioned with dopaminergic neurotoxins exhibit an increase of ppN/OFQ gene expression in the substantia nigra (Di Benedetto et al. 2009; Gouty et al. 2010), and the N/OFQ levels are ~3.5-fold higher in the cerebrospinal fluid of parkinsonian patients compared to control subjects (Marti et al. 2010). A ppN/OFQ gene expression upregulation has been also reported in the substantia nigra of rats exposed to pesticides, proposed as risk factors for Parkinson signs development, together with α-synuclein upregulation (Bastías-Candia et al. 2015; Caputi et al. 2015). However, Collins et al. (2016) reported a significant reduction of ppN/OFQ mRNA levels in the midbrain of parkinsonian patients. Authors interpreted this result as a compensatory mechanism to protect residual dopamine neurons from an excessive N/OFQ stimulation.

5.4 Epilepsy

Opioid neuropeptide alterations are relevant for other neurological disorders such as epileptic syndromes, and experimental models of epilepsy have suggested the involvement of ppN/OFQ and NOP receptor in seizures (Bregola et al. 1999; Tallent et al. 2001; Feng et al. 2004). Notably, 6 and 24 h after kainate administration, rats exhibit high ppN/OFQ mRNA levels in the thalamus, and, in addition, the pharmacological blockade of NOP receptor or N/OFQ$^{-/-}$ mice displayed reduced susceptibility to kainate-induced seizures (Bregola et al. 2002a, b; Turunc Bayrakdar et al. 2013). Together with the increase of N/OFQ mRNA and peptide release, a reduction of NOP receptor density has been observed in kainate-treated rats (Aparicio et al. 2004) as well as in neuroblastoma cell cultures (Cannarsa et al. 2008).

5.5 Pain-Related Conditions

The involvement of ppN/OFQ-NOP receptor system in pain modulation has been carefully investigated at both spinal and supraspinal levels, highlighting that NOP-mediated modulation appears more complex than μ-opioid receptor activation (Schröder et al. 2014; Kiguchi et al. 2016). Indeed, depending on route, concentration, and pain model, NOP receptor activation could potentially lead to either pronociceptive or antinociceptive effects (Calò et al. 2011; Kiguchi et al. 2016). Several studies evaluated the ppN/OFQ and NOP receptor levels in different pain conditions in terms of gene expression alterations.

In carrageenan-induced peripheral inflammation, a marked ppN/OFQ gene expression increase has been observed in the rat DRG (Andoh et al. 1997; Itoh et al. 2001).

Rats subjected to the sciatic nerve chronic constriction injury (CCI) exhibit an increased NOP synthesis in selected brain nuclei (dorsal raphe and raphe magnus nuclei, ventrolateral periaqueductal gray) (Ma et al. 2005) and show significant ppN/OFQ and NOP mRNA upregulation in the spinal tissue (Briscini et al. 2002; Wu and Liu 2018). In this regard, some controversial results exist; in fact other studies demonstrated that NOP mRNA level in the spinal cord was unchanged after CCI nerve injury, even though NOP protein levels were upregulated (Popiolek-Barczyk et al. 2014). As regards supraspinal CNS areas, the same neuropathic experimental condition produces ppN/OFQ and NOP mRNA levels reduction in the thalamus and hypothalamus of mice, together with an increase of NOP receptor gene expression in the anterior cingulate cortex (Palmisano et al. 2017).

Several studies supported the existence of comorbidity between chronic pain and stress disorder or mood alteration, suggesting the ppN/OFQ-NOP receptor system as a crossroad of these pathological conditions. Indeed, rats exposed to single-prolonged stress showed high level of NOP gene expression in the amygdala and hippocampus together with an increased pain sensitivity. Daily treatment with the NOP receptor antagonist JTC-801 mitigates pain and anxiety as well (Zhang et al. 2015).

The involvement of N/OFQergic system in herpetic and postherpetic allodynia was showed by a significant increase of ppN/OFQ mRNA levels observed in the dorsal horn of $NOP^{-/-}$ mice, 6 days after the inoculation with herpetic simplex virus type-1; this mRNA increase was almost completely suppressed after gabapentin administration (Sasaki et al. 2008).

Male mice treated with staphylococcal enterotoxin A exhibited hyperalgesia together with an increase of ppN/OFQ mRNA levels in limbic regions, with a maximum upregulation in the hypothalamus and amygdala (Kawashima et al. 2002), supporting the strict relationships between the immune system activation and nociception modulation. In this regard, the involvement of N/OFQ-NOP system in the regulation of immune responses has been ascertained. Indeed, NOP receptor is expressed in all mature leucocytes where its activation modulates inflammatory responses (Arjomand et al. 2002; Miller and Fulford 2007). In 2013, Zhang et al. investigated NOP and ppN/OFQ mRNA expression in human peripheral blood under inflammatory

conditions showing that lipopolysaccharide and cytochines suppressed mainly NOP and in part ppN/OFQ expression in human whole blood (Zhang et al. 2013a). However, previous studies reported an upregulation of ppN/OFQ mRNA induced by the same inflammatory mediators in rat astrocyte cultures (Buzas et al. 1999, 2002).

Other studies reported that NOP receptors are also expressed in the gastrointestinal tract; in this regard, recent evidence demonstrated that patients suffering from painful inflammatory bowel disease exhibit a significant decrease of NOP mRNA levels (Sobczak et al. 2014). In addition, DNA microarray profiling reported selected gene expression alteration in bladder inflammatory conditions, showing a significant ppN/OFQ upregulation in response to different inflammatory antigens (Saban et al. 2002).

NOP and ppN/OFQ mRNA analysis carried out in peripheral blood cells from end-stage cancer patients suffering from chronic pain showed significantly higher NOP levels in both cancer and postoperative patients compared with healthy controls. At the same time, the ppN/OFQ gene expression was downregulated in cancer but not in postoperative patients (Stamer et al. 2011).

In cancer patients suffering from chronic pain, analgesic tolerance is developed due to the prolonged use of opiates. Several cellular mechanisms are responsible for tolerance development, including gene expression alterations (Romualdi et al. 2002; Caputi et al. 2014a, 2018; Tapocik et al. 2016; Micheli et al. 2018). In this frame, NOP receptor gene expression alterations might represent a crucial point of interest since recent results showed that fentanyl as well as the 14-O-methylmorphine-6-sulfate, two potent analgesic agents endowed with lower tolerance to the analgesic effect than morphine, did not modify NOP receptor gene expression (Caputi et al. 2013; Kiraly et al. 2015). Accordingly, $NOP^{-/-}$ mice exhibit lower morphine tolerance compared to wild-type animals (Ueda et al. 1997, 2000), and the NOP receptor antagonism may prevent tolerance development (Zaratin et al. 2004).

Electroacupuncture (EA) has been adopted as an adjuvant in some analgesic clinical treatments. Particularly, the EA increases the ppN/OFQ and NOP mRNA levels in the spinal dorsal horn of chronic inflammatory pain suffering rats, indicating the involvement of the N/OFQ-NOP system in the effectiveness of EA procedure (Fu et al. 2007). According with the opposite action played by this peptidergic system on nociception in spinal and supraspinal CNS regions, the analgesia induced by a combination of EA and melatonin is accompanied by a decrease of ppN/OFQ mRNA levels in certain pain-related supraspinal nuclei and in the periaqueductal gray matter of inflammatory pain suffering rats (Zhou et al. 2001).

6 Conclusions

Accumulating evidence indicates the usefulness of gene expression control in different pathological conditions. Given the remarkable relevance of the N/OFQ-NOP receptor system in mediating several physiological and pathological processes, the control of its gene expression appears crucial. In this view, the observations here collected may offer the knowledge for the development of new pharmacological tools and therapeutic strategies.

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NOP-Related Mechanisms in Pain and Analgesia

Lawrence Toll, Akihiko Ozawa, and Andrea Cippitelli

Contents

Abstract

Since the discovery of the NOP receptor and N/OFQ as the endogenous ligand, evidence has appeared demonstrating the involvement of this receptor system in pain. This was not surprising for members of the opioid receptor and peptide families, particularly since both the receptor and N/OFQ are highly expressed in brain regions involved in pain, spinal cord, and dorsal root ganglia. What has been surprising is the complicated picture that has emerged from 25 years of research. The original finding that N/OFQ decreased tail flick and hotplate latency, when administered i.c.v., led to the hypothesis that NOP receptor antagonists could have analgesic activity without abuse liability. However, as data accumulated, it became clear that not only the potency but the activity per se

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was different when N/OFQ or small molecule NOP agonists were administered in the brain versus the spinal cord and it also depended upon the pain assay used. When administered systemically, NOP receptor agonists are generally ineffective in attenuating heat pain but are antinociceptive in an acute inflammatory pain model. Most antagonists administered systemically have no antinociceptive activity of their own, even though selective peptide NOP antagonists have potent antinociceptive activity when administered i.c.v. Chronic pain models provide different results as well, as small molecule NOP receptor agonists have potent anti-allodynic and anti-hyperalgesic activity after systemic administration. A considerable number of electrophysiological and anatomical experiments, in particular with NOP-eGFP mice, have been conducted in an attempt to explain the complicated profile resulting from NOP receptor modulation, to examine receptor plasticity, and to elucidate mechanisms by which selective NOP agonists, bifunctional NOP/mu agonists, or NOP receptor antagonists modulate acute and chronic pain.

Keywords
Chronic pain · Dorsal root ganglia · Immunohistochemistry · N/OFQ · Nociceptin · NOP-eGFP · NOP receptor

1 NOP Receptors and N/OFQ, the Endogenous Ligand

The fourth member of the opioid receptor family, which was initially called ORL1, LC132, XOR1, kappa 3, ROR-C, C3 (Bunzow et al. 1994; Fukuda et al. 1994; Wang et al. 1994; Lachowicz et al. 1995; Meunier et al. 1995; Pan et al. 1995), was identified by homology with the other three opioid receptors, mu, delta, and kappa. Although this receptor had homology to the other receptors, basically as high as they had to each other, it did not bind either peptide or small molecule opiates with high affinity, nor was activity blocked by naloxone at normal opioid concentrations. Therefore, it was concluded that this fourth receptor, now called the NOP (*N*ociceptin/*O*rphanin FQ *P*eptide) receptor, is in the opioid receptor family, but is not an opioid receptor (Cox et al. 2015). Initial in situ hybridization studies demonstrated receptor mRNA in many brain regions, particularly those involved in emotion and cognition (Mollereau et al. 1994, 1996; Neal et al. 1999a). Nevertheless, because of the obvious opioid connection, when the endogenous agonist nociceptin/orphanin FQ (N/OFQ) was discovered to be the fourth member of the opioid peptide family, the first experiments conducted by Meunier et al. and Reinscheid et al. had to do with pain (Meunier et al. 1995; Reinscheid et al. 1995). The logical assumption was that N/OFQ would have antinociceptive activity, like the other members of the opioid peptide family. Both groups found the opposite, N/OFQ reduced hotplate (Meunier et al. 1995) and tail flick (Reinscheid et al. 1995) latency in mice, when administered i.c.v. Naturally, it was this apparently nociceptive property that led to the perhaps misleading name nociceptin given by Meunier and colleagues.

The idea that N/OFQ was actually nociceptive was somewhat short-lived. In two important publications, Grandy and colleagues demonstrated that N/OFQ could block antinociceptive activity induced by agonists to all three opioid receptors (Mogil et al. 1996b). In fact, N/OFQ didn't turn out to be nociceptive per se, as much as it reduced the increase in tail flick latency induced by the i.c.v. injection itself (Mogil et al. 1996a). In other words, it blocked the injection-mediated stress-induced analgesia. Subsequent studies by a number of researchers demonstrated conclusively that N/OFQ, and small molecule NOP agonists, could block stress-induced analgesia, induced by a variety of stressors (Rizzi et al. 2001; Reiss et al. 2008; Xie et al. 2008).

All of the initial studies on N/OFQ were subsequent to i.c.v. administration of the peptide. Opiates also have actions in the spinal cord, and interestingly, when N/OFQ was administered intrathecally, it failed to block morphine antinociceptive activity but, in fact, potentiated morphine and had antinociceptive activity on its own (Tian et al. 1997). However, in other studies, N/OFQ had no activity when administered into the spinal cord (Vanderah et al. 1998). In a comprehensive study of several mouse strains, Mogil et al. demonstrated considerable mouse strain differences in both stress-induced analgesia and the anti-opioid actions of N/OFQ (Mogil et al. 1999). Additional early studies led to confusion about the actions of N/OFQ, as well as N/OFQ metabolites and other peptides derived from the prohormone. Not only do differences in route of administration lead to different actions of N/OFQ, this peptide was found to induce a pronociceptive response at very low doses (atto to femtomole) after intraplantar or intrathecal (i.t.) administration (Inoue et al. 1998). This could be blocked by NK1 receptor antagonists and therefore appeared to be due to stimulation of substance P release (Inoue et al. 1998; Sakurada et al. 1999). However, at higher doses (nanomole, i.t.), N/OFQ blocked substance P-induced pain response (Inoue et al. 1999). In addition, Pasternak and colleagues reported that i.c.v. administered N/OFQ was initially pronociceptive, but then over time, this developed into a naloxone-reversible antinociceptive action (Rossi et al. 1997), as if N/OFQ was being metabolized to a peptide that activates opioid receptors. In addition, this group also reported that two N/OFQ N-terminal fragments N/OFQ(1–7) and N/OFQ(1–11) had naloxone-reversible antinociceptive activity. It has never been made clear how this works since neither peptide has high affinity for the NOP receptor nor any of the classical opiate receptors (Rossi et al. 1997; Mathis et al. 1998). Studies in rats were also problematic, as Vanderah et al. could find no nociceptive or antinociceptive actions of N/OFQ in either the brain or spinal cord (Vanderah et al. 1998). These significant differences in reasonably straightforward experiments clearly indicate that NOP receptor-mediated analgesia is dependent upon strain, species, and the particular assay being conducted. Species differences in NOP receptor activity with respect to pain are probably most evident when comparing rodents and nonhuman primates (Ko et al. 2009; Ding et al. 2016).

Although initial in situ hybridization studies suggested NOP receptor activity being related to emotion and cognition, subsequent immunohistochemistry, in vitro autoradiography, and in situ hybridization studies by Watson and coworkers fully characterized the location of both NOP receptors and N/OFQ (Neal et al. 1999a, b).

These experiments clearly demonstrated the presence of both NOP receptors and N/OFQ in brain regions involved in pain and analgesia. In particular, both peptide and receptor could be found in high concentrations in the ventral lateral periaqueductal gray (vlPAG) and the rostral ventromedial medulla (RVM), the brainstem descending analgesic pathway, as well as in the spinal cord and dorsal root ganglia. Electrophysiological studies in a variety of brain regions, including the vlPAG, demonstrated that NOP receptors acted like other members of the opioid receptor family and opened inwardly rectifying potassium (GIRK) channels, thereby hyperpolarizing and reducing the activity of neurons after receptor activation (Connor et al. 1996; Heinricher et al. 1997; Vaughan et al. 1997). In fact, NOP receptors appear to be more ubiquitous than the opioid receptors. In the vlPAG, the mu receptor is on about half of the neurons, while N/OFQ activated NOP receptors on virtually every neuron tested. The presence of NOP receptors in the vlPAG led Grandy to hypothesize that the anti-opiate actions of N/OFQ could be due to inactivation of this antinociceptive pathway. Mu opioid receptor activation on vlPAG neurons leads to antinociceptive activity, and this activity can be blocked by naloxone and also by N/OFQ if administered together with morphine directly into the vlPAG (Morgan et al. 1997). This was taken an important step further by Fields and colleagues (Pan et al. 2000).

The descending pain modulatory pathway travels from the PAG to the brainstem RVM to the dorsal horn of the spinal cord, and morphine can block the pain signal at any locus. Fields had proposed a model whereby activation of primary (OFF) cells leads to analgesia, while activation of secondary (ON) cells leads to pain or hyperalgesia (Pan and Fields 1996). Morphine acts as an analgesic in this brain region by both inhibiting the ON cells and at the same time disinhibiting the OFF cells via inhibition of GABA interneurons. Kappa receptor activation inhibits the OFF cells, which leads to increased pain or hyperalgesia (Pan et al. 1997). In electrophysiological experiments, Fields and colleagues found that N/OFQ activated NOP receptors and therefore hyperpolarized both primary (OFF) and secondary (ON) cells. By this mechanism, N/OFQ blocked opioid analgesic activity by inactivating the analgesic OFF cells. Conversely, in the condition of opioid withdrawal, the pain-inducing ON cells are activated, leading to hyperalgesia, and these cells are also hyperpolarized by N/OFQ activation of NOP receptors. In this case N/OFQ would block this pain signal, leading to a net analgesic effect. These results clearly demonstrated that the actions of N/OFQ or other NOP agonists can be dependent upon the state of the animal (Pan et al. 2000).

2 Expression of NOP Receptors in Brain, Spinal Cord, and DRG

There have been several publications that have described the localization of NOP receptors and N/OFQ in the brain, spinal cord, and dorsal root ganglia (DRG) that give clues to the involvement of this receptor in pain and analgesia. The NOP receptor can be found throughout the brain, in large amounts in brain

regions involved in anxiety, memory, reward, and pain. It can also be found in the dorsal horn of the spinal cord and in DRG, which obviously suggests a connection to pain. The location of NOP receptors was determined by using multiple histological approaches including in situ hybridization, immunohistochemistry, and in vitro autoradiography (Anton et al. 1996; Neal et al. 1999a; Florin et al. 2000; Chen and Sommer 2006). Each method has its own benefits and challenges. In situ hybridization is specific and sensitive but shows only the mRNA-containing cell bodies. In vitro autoradiography can be very specific, but is not particularly sensitive, nor does it provide cellular resolution. Immunohistochemistry can be sensitive, with excellent resolution, but immunostaining on accurate tissue regions is hugely affected by the selectivity of the antibodies. In fact, antibodies for the NOP receptor have been problematic. Although several papers have been published, one paper was retracted after the same antibodies identified virtually the same receptor localization in NOP receptor knockout mice (Anton et al. 1996; Corrigendum 1999). G protein-coupled receptor (GPCR) antibodies cannot be considered reliable until they are tested with knockout animals. At this point, there are no NOP receptor antibodies that have been validated in this manner.

2.1 NOP-eGFP Receptors in Brain

In order to develop a new tool for NOP receptor identification, Brigitte Kieffer developed a mouse with eGFP attached to the C-terminal tail of NOP receptors (Ozawa et al. 2015). These knock-in mice are similar to delta receptor-eGFP and mu receptor-mCherry mice also developed by Kieffer and colleagues (Scherrer et al. 2006). We have used the NOP-eGFP mice to explore the localization of the NOP receptor in brain, spinal cord, and DRG. Although these mice provide a sensitive method to identify the NOP receptor, the tagged receptor presents certain problems. First of all, receptor number is higher in the knock-in mouse than the wild type. Because this knock-in receptor maintains the normal NOP receptor promotor, the regional location should be identical to the wild type; however the large fusion protein may affect degradation and potentially trafficking. This has been a controversial issue for the delta-eGFP receptor (Wang et al. 2010), though ultimately many in situ hybridization studies in wild-type animals seem to confirm the delta-eGFP findings (Scherrer et al. 2009; Bardoni et al. 2014). With respect to the NOP-eGFP receptor, localization of this fusion protein is generally consistent with previous in situ hybridization and in vitro autoradiography studies (Neal et al. 1999a).

Location of the NOP-eGFP receptor in brain is similar to what was expected. Receptor level is very high in the PAG, locus coeruleus, and RVM, regions important to the ascending and descending pain pathways, as well as in the anterior cingulate cortex, a brain region important to the affective component of pain (Ozawa et al. 2015). NOP receptors are also on virtually every cell in the trigeminal ganglia and trigeminal nucleus caudalis, suggesting that the NOP system could be involved in migraine (Fig. 1). Interestingly, in the trigeminal ganglia, as with the dorsal root ganglia, there is some overlap with CGRP in the small diameter neurons. However,

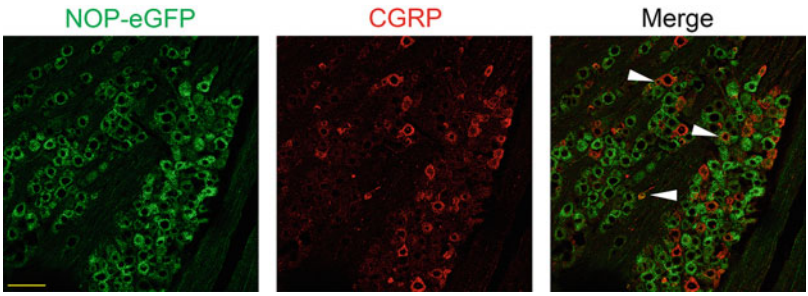

Fig. 1 NOP-eGFP expression in the trigeminal ganglion. White arrows depict the cells co-expressing NOP-eGFP and calcitonin gene-related peptide (CGRP). Scale bar 100 μm

overall there are relatively few neurons that are double stained for NOP receptor and CGRP, a neuropeptide known to be involved in migraine (Edvinsson 2003). This is consistent with publications demonstrating that N/OFQ is dramatically decreased in the cerebral spinal fluid and blood during migraine (Ertsey et al. 2005), inhibits neurogenic dural vasodilatation (Bartsch et al. 2002; Capuano et al. 2007), and clearly suggests NOP receptors as potential target for treatment of migraine. As discussed previously, NOP-eGFP receptors are also highly expressed in the amygdala and hippocampus, regions involved in stress and learning, as well as nucleus accumbens, ventral tegmental area, and medial habenula, regions involved in reward and drug abuse.

2.2 NOP Receptors in Spinal Cord

In addition to the NOP-eGFP expression in the brain, NOP-eGFP receptors are highly expressed in the spinal cord (Ozawa et al. 2018). Consistent with an involvement in pain after intrathecal administration, NOP receptors are mostly distributed between laminae I through III in the spinal dorsal horn, regions important for the regulation of pain, itch, and touch. NOP-eGFP receptor immunoreactivity was very high in spinal laminae I, II_{outer}, and the dorsal border of lamina II_{inner}, where peptidergic (CGRP-positive) and non-peptidergic (isolectin B4 (IB4)-positive) nociceptive primary afferents project. The intense immunoreactivity extended into the ventral border of laminae II_{inner} and III, which are characterized by the presence of excitatory interneurons that express the gamma isoform of the protein kinase C – (PKCγ-positive interneurons). This region of the dorsal horn has been demonstrated to be important to injury-induced chronic mechanical allodynia (Malmberg et al. 1997). In addition to NOP receptor distribution in the dorsal horn, in general agreement with the location of the receptors by in vitro autoradiography in rats (Neal et al. 1999a), strong immunoreactivity was also detected in lamina X, and a moderate fluorescent signal was observed throughout the intermediate zone and ventral horn.

2.3 NOP Receptors in Dorsal Root Ganglia

NOP-eGFP receptors are also highly expressed in DRG (Ozawa et al. 2018). Interestingly, in situ hybridization studies indicated that NOP receptor mRNA was very abundant but NOP receptors were not detected in DRG in an initial in vitro autoradiographic study (Neal et al. 1999a). However, their presence of NOP receptors in DRG had been detected by electrophysiological and immunohistochemical studies (Chen and Sommer 2006; Murali et al. 2012; Anand et al. 2016). Furthermore, the type of DRG neurons that express NOP receptors is consistent with both the location of receptors in the spinal cord and the antinociceptive activity of NOP receptor agonists. DRG neurons are cell bodies for specialized cells that send axons both to the spinal cord and the periphery. Subtypes of DRG neurons are generally electrophysiologically distinguished by their conduction velocity, as well as their cell body size combined with histochemistry; fast-conducting, thickly myelinated, A-beta fibers (large diameter neurons); slower-conducting thinly myelinated A-delta (medium diameter neurons); and slowly conducting unmyelinated C-fiber (small diameter neurons) (Gebhart and Schmidt 2013).

Immunohistochemical studies with NOP-eGFP mice indicated that the receptors are expressed in various subpopulations of DRG neurons and are co-expressed with many known cell markers (Fig. 2; Ozawa et al. 2018). These studies have demonstrated that a small percentage of small NOP-eGFP positive cells are IB4-positive (non-peptidergic), with a larger number co-expressing CGRP and mu receptors (and therefore are peptidergic). This is consistent with an electrophysiological study, which demonstrated that 85% of peptidergic C-nociceptors (small IB4-negative DRG neurons) are responsive to both N/OFQ and DAMGO, while a smaller percentage of small IB4-positive neurons were responsive to N/OFQ (Murali et al. 2012). These data suggest that the NOP-N/OFQ system can function by inhibition of peptidergic nociceptors, which are essential to acute heat pain and heat hyperalgesia. These neurons project to laminae I and II_{outer} of the spinal cord and are consistent with high expression of NOP receptors, as described above. In addition to heat stimuli-responsive NOP-eGFP-positive peptidergic

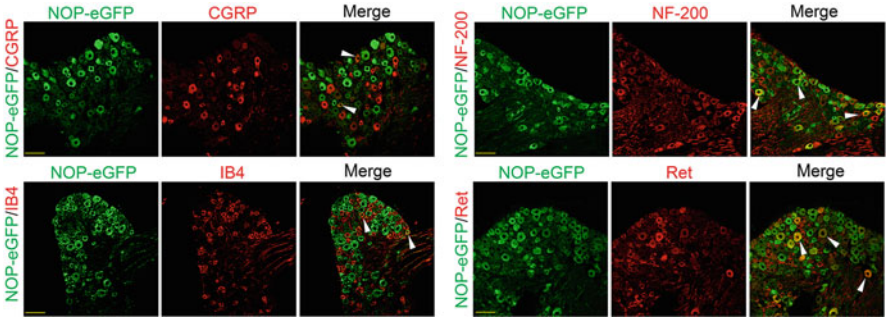

Fig. 2 NOP-eGFP expressing DRG neurons. White arrows depict the cells co-expressing NOP-eGFP and cellular markers. Scale bar 100 μm. Reprinted with permission from Ozawa et al. (2015)

C-nociceptors, the presence of NOP receptors on small IB4+ C-fibers suggests that NOP receptor activation may also modulate acute mechanical pain, similar to delta receptors, as described by Scherrer and colleagues (Scherrer et al. 2009; Bardoni et al. 2014). There are also a large number of myelinated DRG neurons expressing NOP-eGFP. Medium myelinated primary afferents express NOP-eGFP receptors in the absence of CGRP. These are not typical A-delta nociceptors but may represent myelinated low-threshold mechanoreceptors (LTMRs) that are important for touch (Luo et al. 2009; Abraira and Ginty 2013; Bardoni et al. 2014). These low-threshold primary afferents project to the ventral border of lamina II_{inner} and III, which also express NOP-eGFP immunoreactivity and are known to be involved in the development of injury-related allodynia (Malmberg et al. 1997; Neumann et al. 2008). Our recent studies have demonstrated that NOP receptors seem to be on large A-beta fibers that are also important for touch. All of these results are consistent with known actions of spinal and peripheral NOP receptor activation in both acute and chronic pain models (Khroyan et al. 2011b; Ozawa et al. 2018). Further molecular characterization using a variety of additional markers will be required to fully resolve the identity of these primary afferents. When considered together with the effects of N/OFQ discussed above when administered directly into the vlPAG or RVM, it becomes clear that the location of the NOP receptor and circuity explain the dichotomy of N/OFQ blocking opioid analgesia in the brain but being analgesic when administered into the spinal cord.

3 NOP Receptor Knockout Studies

NOP receptors were deleted by homologous recombination by Nishi et al. (1997), and Pintar and colleagues, who deleted both the receptor (Clarke et al. 2001) and the peptide (Kest et al. 2001). Pain mechanisms were studied extensively in these animals. Although these animals have no apparent difference in pain sensitivity itself, the homozygous KO animals developed morphine tolerance at a reduced rate compared with either wild-type or heterozygous animals (Ueda et al. 1997, 2000). In fact, physical signs of morphine dependence were also reduced in the NOP receptor KO mice. These results suggest that both tolerance and physical dependence develop due to an upregulation of the anti-opioid, NOP, system (Ueda et al. 2000). However, in another report, the effect of NOP receptor deletion on physical dependence, but not tolerance, could be reproduced (Mamiya et al. 2001). There was no change in the development of either thermal or mechanical pain due to chronic constriction injury (CCI) in NOP knockout versus wild-type mice (Bertorelli et al. 2002). A similar result was found when the gene for preproN/OFQ was deleted. In these animals, there was no difference in sensitivity to acute pain. However, there was increased nociceptive response during prolonged stimulation which occurs during the second phase of the formalin test (Depner et al. 2003). These results were confirmed both in NOP(−/−) mice (Rizzi et al. 2006) and rats (Rizzi et al. 2011). Overall, these results suggest that endogenous N/OFQ contributes the control of pain during prolonged nociceptive stimulation and that NOP receptor plasticity is likely involved in the development of tolerance and dependence to opiates.

4 NOP Receptor-Active Compounds as Analgesics

NOP receptor agonists and antagonists and both peptide and small molecules have been recently reviewed (Toll et al. 2016). However, a brief discussion here is necessary for a historical prospective on the investigations into and understanding of the actions of NOP receptors on pain itself.

4.1 Antagonists

After the discovery of N/OFQ, the initial theory was that antagonists could have antinociceptive or analgesic activity. Since N/OFQ blocked opioid analgesia, an antagonist could be analgesic per se by reducing the endogenous tone of N/OFQ. The first partial agonists and antagonists, developed by Guerrini, Calo, and colleagues, were peptides that were tested for antinociceptive activity in mice after i.c.v. administration. The first "antagonist" discovered, [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1–13)-$NH_2$, turned out to be a partial agonist, which had anti-opiate actions in vivo (Guerrini et al. 1998; Bertorelli et al. 1999). However, subsequent compounds, including [$Nphe^1$]N/OFQ (1–13)-NH_2 and [$Nphe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101), were discovered to be very selective competitive antagonists (Calo et al. 2000, 2002). Both of these compounds had potent and direct antinociceptive activity in the warm water tail withdrawal assay and potentiated morphine analgesia when administered i.c.v. These studies demonstrated that endogenous N/OFQ in the brain must be either activating pain pathways or blocking the action of endogenous opioids, probably enkephalin, from reducing pain signals.

Interestingly, the profiles of small molecule NOP receptor antagonists appear to be different than the peptides. The first selective NOP receptor antagonist discovered, J-113397, had no agonist activity in vitro, and when given systemically in vivo, it blocked the hyperalgesic activity of N/OFQ (Ozaki et al. 2000). This is consistent with data using SB-612111, a higher affinity and more selective NOP receptor antagonist. SB-612111 has no direct antinociceptive activity but reverses N/OFQ inhibition of morphine analgesia and morphine tolerance and other behavioral actions of N/OFQ (Zaratin et al. 2004; Rizzi et al. 2007). Similar results, i.e., no effect per se but blocking of N/OFQ actions, were obtained with the potent and selective NOP antagonist comp 24 (Goto et al. 2006; Fischetti et al. 2009). However, this is inconsistent with a different NOP receptor antagonist JTC-801, which appears to have potent analgesic activity in both acute and chronic pain models that was not reversed by naloxone (Yamada et al. 2002; Mabuchi et al. 2003; Suyama et al. 2003). This compound was tested in people and ultimately dropped after Phase II clinical trials. The reason why this antagonist but not other NOP receptor antagonists has antinociceptive activity in acute pain models is not clear. JTC-801 is less selective than other antagonists tested, which might account for the different behavioral actions. The difference between peptides and small molecule antagonists is also not clear. It is conceivable that the difference has to do with the site of administration, since the peptides are uniformly administered by an i.c.v. route, while the small molecules were administered systemically.

4.2 Small Molecule Agonists

4.2.1 Selective Agonists

Naturally, the first selective agonist to be tested in pain assays was N/OFQ itself. As a 17 amino acid peptide, it required direct injection into the brain or spinal cord to reach the CNS. The first selective small molecule NOP receptor agonist, Ro 64-6198, was developed by Roche originally as a potential anxiolytic (Jenck et al. 2000). In their original publication, when given systemically, Ro 64-6198, which has subnanomolar affinity for NOP receptors and full agonist activity in the [^{35}S]GTPγS binding assay, had potent anxiolytic activity but had neither antinociceptive activity in the tail flick assay, nor did it induce allodynia (Jenck et al. 2000). This has often been the case, with selective small molecule agonists, such as SR16835 and SCH 225288, having anti-allodynic and antitussive activity, respectively, but no direct acute antinociception in rodents (McLeod et al. 2009; Khroyan et al. 2011b). However, as with many properties of NOP receptor activation, acute antinociceptive activity can be dependent upon the pain model. There are data demonstrating that selective NOP receptor agonists can have antinociceptive activity in rodents in the formalin and inflammatory pain test (Byford et al. 2007; Rizzi et al. 2016, 2017) and Ro 64-6198 has modest antinociceptive activity in hot plate, but not tail flick assay (Reiss et al. 2008). However, it should be taken into consideration that each of these pain assays is reflexive, in response to a painful stimulus. NOP agonists are often sedative, and it is possible that some of this "antinociceptive" activity could be a function of sedation rather than analgesia.

This appears to be quite different in nonhuman primates, where selective NOP agonists have very potent antinociceptive activity that is blocked by NOP receptor antagonists, but not by naloxone (Ko et al. 2009).

4.2.2 Nonselective Agonists

Many studies from several investigators had demonstrated the N/OFQ and NOP receptor agonists could modulate opiate activity. As described above, NOP receptor agonists, when given i.c.v. or systemically, had the ability to block opioid analgesia, diminish opioid reward, and block opioid tolerance and dependence. This led to the hypothesis that a compound with appropriate efficacy at NOP and mu opioid receptors might retain antinociceptive activity but with other side effects, such as abuse liability, reduced. Zaveri, Toll, and colleagues explored this hypothesis extensively, publishing results of several nonselective compounds with differing ratios of NOP to mu affinity and efficacy, attempting to titrate these parameters to design an analgesic with reduced side effects. They proved the initial hypothesis to be correct, as a compound such as SR16435, a high-affinity partial agonist at NOP and mu receptors, had naloxone-reversible antinociception and was rewarding (induced a conditioned place preference (CPP)) but with reduced tolerance development (Khroyan et al. 2007) and SR16507, a potent full agonist at both NOP and mu receptor, had potent antinociceptive activity but was rewarding. Presumably in these compounds, the mu agonist activity was too high for the rewarding aspect

to be blocked by the NOP partial or even full agonist activity. However, SR14150, a weak partial agonist at mu receptors and a potent partial agonist at NOP receptors, had naloxone-reversible antinociceptive activity but did not induce a CPP (Toll et al. 2009). This compound clearly demonstrated that with the correct parameters of affinity and efficacy in a single compound, the NOP agonist activity could reduce the reward but still leave antinociceptive actions of the mu component. Interestingly, buprenorphine itself has high affinity at opioid receptors and moderate affinity at NOP receptor (Wnendt et al. 1999; Lester and Traynor 2006). Both the antinociceptive and rewarding aspects of buprenorphine appear to be reduced by its inherent NOP agonist activity (Ciccocioppo et al. 2007; Khroyan et al. 2009; Lutfy et al. 2003). These results led to additional compounds by Husbands and colleagues in which the NOP receptor activity was increased in buprenorphine-type compounds, once again leading to compounds with antinociceptive activity but reduced reinforcing effects (Khroyan et al. 2011a; Ding et al. 2016). Recently, two additional nonselective NOP/mu agonists have proven interesting in nonhuman primate and human studies. Cebranopadol, a full mu/full NOP agonist from Grunenthal, is a very potent analgesic, which is particularly potent in chronic pain assays and is in Phase III clinical trials (Linz et al. 2014; Scholz et al. 2018). AT-121, from Zaveri, is a mixed partial agonist with potent antinociceptive activity in nonhuman primates and appears devoid of unwanted opioid side effects (Ding et al. 2018).

5 Effect of N/OFQ on Opioid Tolerance

Because N/OFQ has anti-opiate activity, it was hypothesized that the NOP receptor system might be involved in the development of tolerance to opiates. One could imagine that if the NOP system is upregulated with chronic opiate treatment, this could functionally inhibit further actions of the opiate, which would result as tolerance to the drug. This was initially tested in NOP receptor knockout mice, for which morphine tolerance was reduced (Ueda et al. 1997). Conversely, chronic morphine treatment led to an upregulation of NOP receptor mRNA in the spinal cord, and morphine tolerance was reduced by a subcutaneous or intrathecal administration of the NOP receptor antagonist J-113397 or the more selective antagonist SB-612111 suggesting that the reduction in antinociceptive activity (tolerance) after chronic morphine can, at least partially, be attributed to an upregulation of the NOP system. (Ueda et al. 2000; Zaratin et al. 2004) In fact, administration of J-113397 directly into the vlPAG alone was sufficient to block the expression of tolerance after chronic morphine administration (Scoto et al. 2010). In the knockout mice for the N/OFQ precursor protein (preproN/OFQ), there is no consensus, as one group found no development of morphine tolerance with up to 3 weeks of morphine treatment (Chung et al. 2006), while another group using a similar genotype found the development of tolerance to morphine equivalent to wild type (Kest et al. 2001). However, naloxone-induced withdrawal jumping was significantly reduced, indicating that in the absence of the peptide, morphine

dependence is reduced (Kest et al. 2001). Interestingly, although NOP receptor antagonists block the expression of tolerance, N/OFQ itself, when administered into the brain daily after systemic morphine treatment, blocks the development of tolerance (Lutfy et al. 2001). The explanation for this phenomenon is not perfectly clear, but might suggest that the presence of N/OFQ concurrently with morphine blocks the ability of morphine to upregulate the NOP system, thereby attenuating the development of tolerance. Overall, perhaps it is not surprising that a receptor that opposes the actions of morphine in the brain can modulate tolerance development as well.

6 Chronic Pain

It turns out the effects of NOP receptor agonist activation appear to be considerably clearer with respect to chronic than acute pain (Schroder et al. 2014). Early studies examining the effects of N/OFQ on pain induced by inflammation or sciatic nerve injury suggested potential neuroplasticity, as the peptide was very effective in inducing anti-allodynic and anti-hyperalgesic activity in these chronic pain models (Hao et al. 1998; Bertorelli et al. 1999). In the rat chronic constriction injury (CCI) model, spinally administered N/OFQ inhibited thermal hyperalgesia as well as mechanical allodynia, while it had no effect on mechanical pain thresholds in naïve rats (Courteix et al. 2004). Similarly, the selective NOP receptor agonist Ro 64-6198 inhibited mechanical and cold allodynia after peripheral and spinal administration in CCI rats, while it had no effect on naïve animals (Obara et al. 2005). Similar results were obtained with less selective NOP/mu agonists. SR14150, a partial agonist at both receptors, has mu-mediated (naloxone-reversible) antinociceptive activity in the tail flick test but anti-allodynic activity in SNL mice that was blocked by the NOP antagonist SB612111. As with Ro 64-6198, the more selective NOP full agonist SR16835 had no activity in the tail flick in naïve mice but potent anti-allodynic activity, which was reversed by SB612111, in SNL mice (Khroyan et al. 2011b).

One explanation for changes in antinociceptive activities of NOP receptor agonists in chronic pain animals could be changes in gene expression and subsequent changes in NOP receptor or N/OFQ levels. The level of NOP receptors, NOP receptor mRNA, and N/OFQ have all been examined subsequent to both peripheral or spinal nerve injury and to inflammatory pain models. Initial studies using semiquantitative rtPCR indicated a pain-induced increase in mRNA of both NOP receptors and N/OFQ (Andoh et al. 1997; Briscini et al. 2002; Ma et al. 2005). This appears to be consistent with an increase in efficacy of NOP agonists for chronic as opposed to acute pain. More recent studies using NOP-eGFP and wild-type mice produced different results. In these animals, spinal nerve ligation (SNL) to induce chronic pain caused a dramatic decrease in NOP receptors in specific spinal cord laminae and in DRG (Figs. 3 and 4; Ozawa et al. 2018). As seen in Fig. 3, SNL greatly decreased NOP-eGFP receptors in the ipsilateral but not contralateral spinal

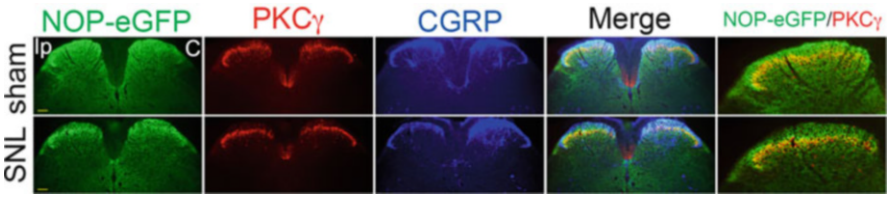

Fig. 3 NOP-eGFP receptor distribution in the spinal cord in sham and SNL mice. *Ip* ipsilateral, *C* contralateral. Scale bar 100 μm. Reprinted with permission from Ozawa et al. (2018)

dorsal horn laminae I and II_{outer}, with no apparent change in PKCγ-stained region of lamina II_{inner} and lamina III. As discussed above, these regions containing PKC-γ-positive neurons are thought to be important for peripheral nerve injury-induced mechanical pain (Neumann et al. 2008). NOP receptor changes in the dorsal horn are consistent with a corresponding decrease in NOP receptor mRNA levels in spinal cord of wild-type SNL mice (Ozawa et al. 2018). Similar results were found in DRG, a large decrease in both NOP-eGFP receptors and NOP receptor mRNA levels in SNL mice (Fig. 4). Furthermore, the DRG neurons that were most greatly diminished were the small diameter CGRP and mu receptor-containing cells that correspond to C-nociceptors with axon terminals in laminae I and II_{outer}.

Based upon these changes in NOP receptors, it was hypothesized that N/OFQ, when administered i.t., would attenuate mechanical allodynia induced by SNL, measured by using von Frey filaments poked into the injured paw, but be less effective on heat pain in the hotplate test, since many of these NOP receptor-containing cells are missing in DRG and spinal cord of SNL mice. This would be similar to analgesic actions demonstrated for delta opioid receptors in mice treated with complete Freund's adjuvant (CFA) (Scherrer et al. 2009). Surprisingly, N/OFQ was actually more effective in blocking heat pain than it was attenuating cold pain or mechanical allodynia. This suggests two possibilities. One possibility is that there are C-nociceptors that modulate heat and cold pain that contain NOP receptors but do not co-express CGRP, which are not affected by SNL, allowing N/OFQ to maintain effectiveness; or more likely, NOP receptors can be found on spinal projection neurons that transmit the heat pain signal to the brain. This along with our hypothesis as to how NOP receptors reduce pain signals in the spinal cord of naïve and SNL mice is shown in Fig. 5. In this way, intrathecally administered N/OFQ would still block hotplate-induced heat pain despite the fact that NOP receptors are missing on a significant portion of potential nociceptors. In naïve animals, NOP receptor agonists presynaptically inhibit C-fiber-evoked responses in the spinal dorsal horn in order to mediate an antinociceptive response (Wang et al. 1996; Lai et al. 1997; Liebel et al. 1997; Carpenter et al. 2000). In SNL mice, spinal NOP receptor activation apparently directly inhibits heat- and cold-specific projection neurons in the dorsal horn to induce an analgesic response. Electrophysiological experiments should directly address this issue.

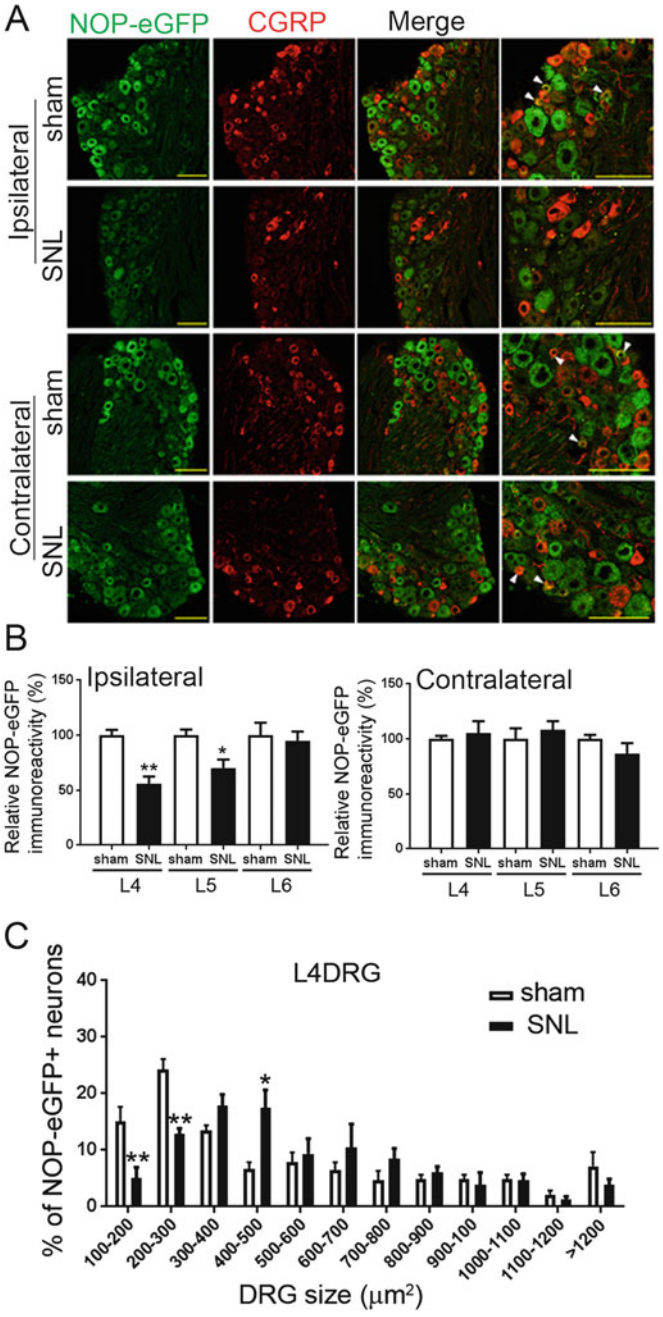

Fig. 4 NOP-eGFP expression in DRG under a chronic pain condition. (**a**) Representative images of L4 DRG neurons derived from sham-operated and SNL mice. (**b**) NOP-eGFP expression level in DRG neurons. (**c**) Population of NOP-expressing DRG neurons in sham and SNL mice. Scale bar 100 μm. Reprinted with permission from Ozawa et al. (2018)

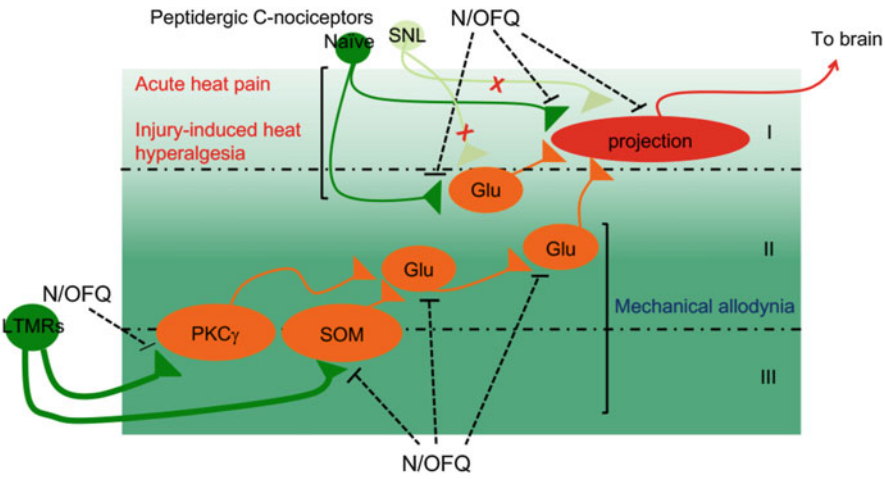

Fig. 5 Schematic of hypothesized NOP receptor-mediated inhibition of pain signaling in spinal cord

7 Conclusions

The involvement of NOP receptors and N/OFQ in pain transmission has been definitively proven, with a mixed NOP/mu agonist now in clinical trials. Furthermore, selective NOP agonists seem poised for development as analgesics as well. Nevertheless, there are many significant issues and hurdles remaining. NOP agonists have demonstrated the potential for considerable side effects. NOP receptor agonists are often very sedative (Higgins et al. 2001; Byford et al. 2007), although this seems to be diminished in cebranopadol and AT-121, for reasons that are not clear (Linz et al. 2014; Ding et al. 2018). One possibility for reduced sedation is ligand bias, as cebranopadol seems to internalize NOP receptors to a lesser extent than other agonists and is less efficacious at β-arrestin activation than G protein coupling (Rizzi et al. 2016). NOP agonists also block long-term potentiation, as well as spatial memory (Sandin et al. 1997; Bongsebandhu-Phubhakdi and Manabe 2007; Kuzmin et al. 2009). This will have to be examined carefully in people. It may be of great value that NOP agonists are more effective in blocking chronic than acute pain since that is certainly a bigger problem, particularly since long-term administration of opiates is greatly discouraged. The mechanism by which NOP receptor agonists have increased efficacy in chronic pain is still unknown and upon further investigation could not only uncover actions of the NOP system but could lead to a better understanding of the transition to chronic pain.

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NOP-Related Mechanisms in Substance Use Disorders

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Contents

Abstract

Nociceptin/orphanin FQ (N/OFQ) is a 17 amino acid peptide that was deorphanized in 1995 and has been widely studied since. The role of the N/OFQ system in drug abuse has attracted researchers' attention since its initial discovery. The first two scientific papers describing the effect of intracranial injection of N/OFQ appeared 20 years ago and reported efficacy of the peptide in attenuating alcohol intake, whereas heroin self-administration was insensitive.

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Since then more than 100 scientific articles investigating the role of the N/OFQ and N/OFQ receptor (NOP) system in drug abuse have been published. The present article provides an historical overview of the advances in the field with focus on three major elements. First, the most robust data supportive of the efficacy of NOP agonists in treating drug abuse come from studies in the field of alcohol research, followed by psychostimulant and opioid research. In contrast, activation of NOP appears to facilitate nicotine consumption. Second, emerging data challenge the assumption that activation of NOP is the most appropriate strategy to attenuate consumption of substances of abuse. This assumption is based first on the observation that animals carrying an overexpression of NOP system components are more prone to consume substances of abuse, whereas NOP knockout rats are less motivated to self-administer heroin, alcohol, and cocaine. Third, administration of NOP antagonists also reduces alcohol consumption. In addition, NOP blockade reduces nicotine self-administration. Hypothetical mechanisms explaining this apparent paradox are discussed. Finally, we focus on the possibility that co-activation of NOP and mu opioid (MOP) receptors is an alternative strategy, readily testable in the clinic, to reduce the consumption of psychostimulants, opiates, and, possibly, alcohol.

Keywords
Addiction · Drug-seeking · N/OFQ · Nociceptin · NOP · Orphanin FQ · Relapse

1 Introduction

The 17 amino acid peptide nociceptin/orphanin FQ (N/OFQ) was discovered by screening brain extracts as the natural ligand for the orphan G protein-coupled receptor (GPCR) opioid receptor-like 1 (ORL1), now known as NOP (Meunier et al. 1995; Reinscheid et al. 1995). N/OFQ and its cognate receptor exhibit a high degree of sequence identity to dynorphin and kappa opioid receptors (KOP), respectively. However, N/OFQ does not activate any of the classical mu (MOP), delta (DOP), and kappa (KOP) opioid receptors. Based on structural similarities between N/OFQ and dynorphin A, a general consensus has been reached so that the N/OFQ-NOP system is now considered the fourth member of the opioid superfamily (Cox et al. 2015; Toll et al. 2016).

Since the very beginning, neuroanatomical studies in rodents revealed a high degree of distribution of N/OFQ and NOP receptors in major mesolimbic structures including the central amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the ventral tegmental area (VTA). Moderate expression was also detected in the nucleus accumbens (Nac) and striatum. In addition, like all classical opioid peptides and receptors, the N/OFQ-NOP system is widely represented in cortical regions (Sim and Childers 1997; Neal et al. 1999; Letchworth et al. 2000; Slowe et al. 2001; Sim-Selley et al. 2003; Gehlert et al. 2006). More recent studies in

dogs and humans replicated these findings confirming that the neuroanatomy of the system is highly conserved among species (Witta et al. 2004; Kimura et al. 2011; Lohith et al. 2012; Witkin et al. 2014; Narendran et al. 2018). Due to the similarities between the N/OFQ and the other opioid systems, one of the first scrutinized areas of the system's neural function was that of pain and drug abuse. Indeed, the name nociceptin (Meunier et al. 1995) was derived from observations of pro-nociceptive actions following supraspinal administration of the peptide. Subsequent studies have revealed that the modulation of pain pathways by N/OFQ is complex, with NOP receptors mediating analgesia in the spinal cord and hyperalgesia in the brain (see for review) (Darland et al. 1998; Fioravanti and Vanderah 2008; Lambert 2008; Kiguchi et al. 2016). In July 1998 and in January 1999, the first two studies linking the N/OFQ-NOP receptor system to drug abuse were published. In original work, Walker and colleagues showed that intracerebroventricular (ICV) microinjection of N/OFQ did not affect operant heroin self-administration in the rat (Walker et al. 1998). In the other study, however, it was demonstrated that acute ICV administration of the peptide increased alcohol consumption in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats (Ciccocioppo et al. 1999). However, repeated administration of N/OFQ markedly reduced alcohol drinking and prevented alcohol-induced conditioned place preference (Ciccocioppo et al. 1999). After these two earlier studies, several reports were published over the years with more than 100 articles currently listed in PubMed. The largest body of available data support the hypothesis that activation of NOP by its endogenous ligand or by highly selective synthetic small-molecule agonists attenuates drug abuse-related behaviors (for review, see also Witkin et al. 2014). However, as in the case of pain, the pharmacology of the N/OFQ system appears more complex than originally thought, and recent rapidly accumulating evidence points to the possibility that drug abuse-related behaviors are inhibited by NOP antagonists rather than agonists (Post et al. 2016; Rorick-Kehn et al. 2016). Here, we will summarize the major findings generated over 20 years of research on N/OFQ and drug abuse, findings that were largely guided by the general hypothesis that activation of NOP attenuates the motivation for drugs of abuse. We then will review more recent data showing that attenuation of N/OFQ transmission has a protective role for the development of drug dependence and that NOP antagonism attenuates the consumption of substances including alcohol and nicotine. Finally, we will focus on a series of clinically available molecules such as buprenorphine and cebranopadol that activate both NOP and MOP receptors and that have shown promising features relevant for the treatment of drug abuse (Wnendt et al. 1999; Bloms-Funke et al. 2000; Huang et al. 2001). To facilitate the analysis of the large number of papers published to date, the effects of NOP agonists, antagonists, and mixed MOP/NOP compounds on different drugs of abuse will be described in separate paragraphs. Additional discussion on the role of N/OFQ-NOP system in drug abuse can be found in several recent reviews (Zaveri 2011; Witkin et al. 2014; Lutfy and Zaveri 2016).

2 The N/OFQ System and Alcohol Abuse

2.1 NOP Agonism

Together with nicotine, alcohol is the most commonly used drug of abuse in the world, with about 240 million people suffering from alcohol use disorder (Gowing et al. 2015). Alcoholism follows a pattern similar to other abused drugs, characterized by binges of alcohol consumption consisting either of daily episodes or prolonged days of heavy drinking (Koob 2013). Alcoholism, like other forms of substance abuse, can be conceptualized as a disorder that includes a progression from impulsivity (positive reinforcement) to compulsivity (negative reinforcement) where both genetic and environmental risk factors drive the progression to alcohol addiction (Goldman et al. 2005; Koob 2013; Costin and Miles 2014; Spanagel et al. 2014; Spanagel 2009).

The first study scrutinizing the role of the N/OFQ in alcohol abuse was published in 1999 (Ciccocioppo et al. 1999). In that study, it was shown that repeated ICV administration of N/OFQ attenuated voluntary two-bottle choice alcohol drinking (choice between 10% alcohol and water) in genetically selected Marchigian Sardinian alcohol-preferring (msP) rats. Over the following years, this initial finding was replicated using different experimental procedures and NOP selective agonists. For instance, it was demonstrated that activation of NOP by peptidic N/OFQ analogues as well as by small synthetic agonists blunted the reinforcing and motivating effects of alcohol as measured in conditioned place preference (CPP) experiments in mice (Kuzmin et al. 2003, 2007; Zaveri et al. 2018b) and operant and home cage alcohol self-administration or relapse models in rats (Martin-Fardon et al. 2000; Kuzmin et al. 2007; Aziz et al. 2016; Ciccocioppo et al. 2002c, 2003a; Economidou et al. 2006, 2008). Most of the drinking studies were carried out in msP rats, but efficacy of these compounds in nonselected Wistar rats has also been documented (Kuzmin et al. 2007; Aziz et al. 2016). However, in studies in which msP and Wistar rats were tested in parallel, it was always found that suppression of alcohol drinking and relapse was more pronounced in the msP line (Economidou et al. 2008; de Guglielmo et al. 2015; Martin-Fardon et al. 2010). Compared to Wistar rats, the msP line exhibits overexpression of the corticotropin-releasing factor (CRF) system, likely driven by two single nucleotide polymorphism at CRF1 receptor locus (Hansson et al. 2006; Ayanwuyi et al. 2013; Cippitelli et al. 2015; Logrip et al. 2018). As a consequence of this overexpression, msP rats are more sensitive to stress, have a high anxiety-like phenotype, and show depression-like symptoms that are all improved by alcohol consumption (Ciccocioppo et al. 2006; Ciccocioppo 2013; Egervari et al. 2018). In this rat line, 2 weeks of voluntary alcohol drinking reduced the overexpression of CRF1R receptors in various brain areas, which points to the possibility that these animals drink to self-medicate negative affect associated with their overactive stress system (Hansson et al. 2007). Considering the possibility that activation of NOP receptors mediates a potent anxiolytic and anti-stress effect

and that N/OFQ acts as a functional antagonist of the CRF1R system (Griebel et al. 1999; Jenck et al. 2000a, b; Ciccocioppo et al. 2001, 2002a, b, 2003b, 2004a, 2014a), it is possible that in msP rats the effect on alcohol drinking produced by N/OFQ was due to its ability to alleviate the negative affective state (triggering excessive drinking) associated with heightened CRF1R transmission. Gene expression studies showed that msP rats are also characterized by an innate overexpression of the N/OFQ-NOP receptor system in several stress-regulatory brain regions, including the CeA. On one hand, this may represent a compensatory reorganization of N/OFQ neurotransmission to counteract the overactivity of the stress system (Economidou et al. 2008). On the other hand, the overexpression of NOP receptors may contribute to conferring higher sensitivity to NOP agonists that, when microinjected into the CeA, blocked both excessive alcohol intake and anxiety in this rat line (Economidou et al. 2008; Aujla et al. 2013). Wistar rats with a history of chronic alcohol exposure exhibit neuroadaptive changes of the N/OFQ-NOP and CRF1R systems resembling the innate dysregulation of these systems in msP rats. For instance, Wistar rats made dependent on alcohol through chronic intermittent ethanol vapor exposure showed increased anxiety, enhanced sensitivity to stress, overexpression of the CRF1R receptors in the CeA, and enhanced sensitivity to CRF1R antagonists (Gehlert et al. 2007; Sommer et al. 2008; Ciccocioppo et al. 2009). Interestingly, administration of NOP agonists in alcohol-dependent rats attenuated the expression of acute withdrawal signs (Economidou et al. 2011). Moreover, following protracted abstinence, NOP activation reduced anxiety, excessive alcohol drinking, and stress-induced relapse triggered by the postdependent state (Martin-Fardon et al. 2010; Economidou et al. 2011; Aujla et al. 2013; Ciccocioppo et al. 2014a; de Guglielmo et al. 2015). Additional evidence for alcohol-induced neuroadaptive changes of the N/OFQ-NOP receptor system comes from electrophysiological studies in CeA slice preparations. This work showed that N/OFQ attenuated alcohol-evoked facilitation of $GABA_A$ neurotransmission and that this effect was significantly more pronounced in msP and in alcohol-dependent rats (Roberto and Siggins 2006; Cruz et al. 2012; Herman et al. 2013). In addition, it was shown that, in the CeA, NOP receptor agonism diminished glutamatergic neurotransmission per se but at the same time occluded the inhibitory effect of alcohol on glutamate (Kallupi et al. 2014). Altogether these findings support two major conclusions: First, chronic exposure to high doses of alcohol (i.e., following passive alcohol intoxication) leads to neuroadaptive overexpression of the N/OFQ system in mesolimbic regions. Second, NOP agonism appears to be more efficacious in inhibiting alcohol-related behaviors when drinking is associated with high anxiety and enhanced stress sensitivity (i.e., elicited by innate or environmentally evoked overexpression of the extrahypothalamic CRF system).

2.2 NOP Antagonism

As discussed above, a wealth of studies suggests that activation of NOP attenuates alcohol drinking and seeking (Table 1). However, evidence is emerging supporting

Table 1 Compounds targeting the N/OFQ-NOP receptor system, relative developmental stage, and effects on alcohol abuse

	Chemical entity	Effects	Dev. phase	Ref.
Agonist				
N/OFQ	Peptidic	↓ Alcohol intake	Preclinical	Ciccocioppo et al. (1999) and Economidou et al. (2006)
		↑ Alcohol intake		Cifani et al. (2006)
		↓ Alcohol self-administration		Ciccocioppo et al. (2004b) and Economidou et al. (2008)
		↓ Somatic withdrawal signs		Economidou et al. (2011)
		↓ Stress-induced reinstatement		Martin-Fardon et al. (2000)
		↓ Cues-induced reinstatement		Ciccocioppo et al. (2004b)
		↓ Alcohol-induced CPP		Kuzmin et al. (2003)
		↓ Alcohol intake		Ciccocioppo et al. (2014b)
MT-7716	Small molecule	↓ Somatic withdrawal signs		Ciccocioppo et al. (2014b) and de Guglielmo et al. (2015)
		↓ Stress-induced reinstatement		Ciccocioppo et al. (2014b) and de Guglielmo et al. (2015)
		↓ Cues-induced reinstatement		Ciccocioppo et al. (2014b)
SR-8993	Small molecule	↓ Alcohol intake; alcohol self-administration; progressive ratio; stress- and cues-induced reinstatement		Aziz et al. (2016)
AT-312	Small molecule	↓ Alcohol-induced CPP		Zaveri et al. (2018b)
Ro 64-6198	Small molecule	↓ Alcohol self-administration		Kuzmin et al. (2007)
		↑ Alcohol intake		Economidou et al. (2006)
UFP-112	Peptidic	↓ Alcohol intake		Economidou et al. (2006)
UFP-102	Peptidic	↓ Alcohol intake		Economidou et al. (2006)
OS-462	Peptidic	↓ Alcohol intake		Economidou et al. (2006)

(continued)

Table 1 (continued)

	Chemical entity	Effects	Dev. phase	Ref.
Antagonist				
UFP-101	Peptidic	↓ Alcohol self-administration	Preclinical	Ciccocioppo et al. (2007)
LY2940094	Small molecule	↓ Alcohol intake; alcohol self-administration; progressive ratio; stress-induced reinstatement		Rorick-Kehn et al. (2016)
LY2817412	Small molecule	↓ Alcohol self-administration		Kallupi et al. (2017)
J-113397	Small molecule	↑ Alcohol intake		Miranda-Morales et al. (2013)
SB-612111	Small molecule	↓ Alcohol self-administration		Cippitelli et al. (2016) and Kallupi et al. (2017)
Nphe	Peptidic	— Alcohol intake	Clinical	Ciccocioppo et al. (2002c)
LY2940094	Small molecule	↓ Alcohol intake		Post et al. (2016)

the possibility that these effects can also be achieved with NOP antagonists (Table 1). For instance, in a study with LY2940094 (aka BTRX-246040), a selective and potent NOP antagonist recently developed by Eli Lilly (Toledo et al. 2014), we found that this agent reduced alcohol consumption in two different lines of genetically selected alcohol-preferring rats, including the msP line (Rorick-Kehn et al. 2016). The same molecule, tested in a small clinical trial with 88 patients diagnosed with alcohol use disorder (AUD), showed efficacy in reducing the number of heavy drinking days which provided important proof of principle for the translational potential of NOP antagonism (Post et al. 2016). Indirect evidence supporting the putative therapeutic potential of NOP antagonism comes from studies in genetically modified NOP knockout rats. Compared to wild-type controls, these engineered animals self-administer significantly smaller amounts of alcohol, cocaine, and heroin but show unimpaired motivation for saccharin, a natural reward (Kallupi et al. 2017).

Why both NOP agonists and antagonists reduce the motivation for alcohol is still unclear. However, a critical analysis of historical data with NOP agonist may be of help to reconcile these apparently contrasting findings and to formulate new hypothesis on the role of the N/OFQ system in AUD. The first possibility to consider is that in pharmacological studies, exogenous administration of nonphysiological doses of NOP agonists may have depressed N/OFQ transmission through receptor desensitization. If so, NOP receptor agonism may have resulted in paradoxical antagonistic effects. It is known, in fact, that NOP receptors are subject to rapid desensitization that may occur within minutes after administration of a high dose of an agonist or after chronic agonist treatment (Toll et al. 2016). Most importantly, in a recent study

that investigated the effect of chronic administration of the potent and selective NOP agonist MT-7716, it was shown that alcohol drinking was not affected acutely, progressively decreased during repeated drug administration and, compared with the control group, remained lower for several days after treatment discontinuation (Ciccocioppo et al. 2014b). Indirectly, the NOP desensitization hypothesis is also supported by data demonstrating that compared to Wistar controls, high alcohol drinking msP rats have higher expression of N/OFQ and NOP receptor mRNA in numerous mesolimbic brain areas, including the CeA and NAc (Economidou et al. 2008; Ciccocioppo et al. 2014a). Moreover, in an earlier study in msP rats in which a low constant dose of N/OFQ was delivered ICV for 7 consecutive days by means of osmotic mini-pumps, a significant increase in alcohol intake was observed (Cifani et al. 2006). At that time, this finding was interpreted as a consequence of the ability of N/OFQ to stimulate feeding and caloric intake. In msP rats, increase in alcohol drinking following acute administration of Ro64-6198 was also observed; intake decreased after repeated dosing (Economidou et al. 2006). In light of the NOP desensitization hypothesis, it is tempting to speculate that the increase in drinking following chronic N/OFQ was due to receptor stimulation under conditions in which the system did not undergo desensitization. Additional evidence supporting the possibility that NOP activation facilitates rather than decreases drinking comes from studies in Wistar rats exposed to chronic intoxicating concentrations of alcohol. These animals, in fact, show upregulation of the NOP receptor transcript in the CeA and BNST that is associated with enhanced propensity to excessive drinking (Sommer et al. 2008; Aujla et al. 2013). At the mechanistic level, an intriguing hypothesis is that overexpression of the NOP system in msP and postdependent Wistar rats may have been induced by a "physiological" attempt to counteract the pathological (genetically or environmentally determined) overactivity of the extrahypothalamic CRF system (Hansson et al. 2006; Gehlert et al. 2007; Sommer et al. 2008; Ciccocioppo et al. 2009; Aujla et al. 2013; Ayanwuyi et al. 2013; Cippitelli et al. 2015). However, stimulation of NOP receptors in the mesolimbic circuitry may lead to a hypodopaminergic and hypohedonic state that can increase the motivation for drugs of abuse. It is known, in fact, that activation of NOP following intra-VTA administration of N/OFQ attenuates dopamine (DA) release in the NAc (Murphy and Maidment 1999). Consistently, studies using NOP knock-out mice showed that N/OFQ transmission facilitated chronic responses to alcohol and methamphetamine by suppressing the animals' basal hedonic state. Based on this finding, the authors concluded that the N/OFQ-NOP system may play a permissive role in the development of drug abuse (Sakoori and Murphy 2008a).

3 The N/OFQ System and Opioid Abuse

The first study investigating the effect of N/OFQ manipulation on opioid abuse was published two decades ago (Walker et al. 1998). Results were negative as ICV administration of N/OFQ did not reduce operant heroin self-administration in the rat. This finding was unexpected because pain studies showed that N/OFQ, despite

Table 2 Compounds targeting the N/OFQ-NOP receptor system, relative developmental stage, and effects on opioids abuse

Agonist	Chemical entity	Effects	Dev. phase	Ref.
N/OFQ		↓ Morphine-induced CPP	Preclinical	Ciccocioppo et al. (1999), Murphy et al. (1999), Ciccocioppo et al. (2000), Sakoori and Murphy (2004, 2008b) and Economidou et al. (2006)
		— Heroin self-administration		Walker et al. (1998)
		↓ Morphine-induced somatic withdrawal signs		Kotlinska et al. (2000)
SR-8993	Small molecule	↓ Morphine-induced CPP		Zaveri et al. (2018a)
Ro 64-6198	Small molecule	↓ Morphine-induced reinstatement		Shoblock et al. (2005)
Ro 65-6570	Small molecule	↓ Oxycodone-induced CPP		Rutten et al. (2010)
		↓ Tilidine-induced CPP		
		↓ Morphine-induced CPP		
		↓ Heroin-induced CPP		

being an opioid-like peptide, acted in the brain as a functional anti-opioid (Grisel et al. 1996; Mogil et al. 1996a, b). In contrast to this earlier finding, later self-administration studies (Table 2) in rats and monkeys showed reductions in opioid intake following administration of Ro 64-6198 and SCH221510, two small synthetic NOP agonists (Ko et al. 2009; Podlesnik et al. 2011; Sukhtankar et al. 2014). These effects were systematically blocked by pretreatment with the selective NOP antagonist J-113397 (Podlesnik et al. 2011; Sukhtankar et al. 2014). The ability of NOP agonists to block opioid reward was further demonstrated in place conditioning experiments in which ICV administration of N/OFQ blocked the acquisition and the expression of morphine CPP (Murphy et al. 1999; Ciccocioppo et al. 2000; Sakoori and Murphy 2004). In CPP experiments, opioid reward was also blocked following administration of the potent and selective NOP agonists Ro 64-6198, Ro 65-6570, and AT-312 (Shoblock et al. 2005; Rutten et al. 2010; Zaveri et al. 2018a).

A key neurochemical correlate of these behavioral findings was identified in a microdialysis experiment showing that ICV administration of N/OFQ reduced morphine-induced dopamine (DA) release in the nucleus accumbens (NAcc) of conscious rats (Di Giannuario and Pieretti 2000). Further support for this potential

mechanism comes from immunohistochemistry experiments indicating that N/OFQ blocked the expression of c-fos, a marker of neuronal activation, induced by morphine in the shell of the NAc (Ciccocioppo et al. 2000). In fact, rewarding stimuli, including morphine, potently increase c-fos expression in this area, reflecting activation of dopamine (DA) receptor-containing neurons (Barrot et al. 1999).

Few studies investigated the hypothesis that N/OFQ contributes to the development of tolerance to the analgesic effect of opioids. This possibility was supported by an early study showing that repeated morphine injections increased the brain levels of this anti-opioid peptide (Yuan et al. 1999). Consistent with this hypothesis, it was also shown that treatment with selective NOP receptor antagonists prevented the development and expression of opioid tolerance (Scoto et al. 2007, 2009). Moreover, NOP knockout mice showed a 50% reduction in tolerance to the analgesic effect of morphine (Ueda et al. 1997). Based on these data, the possibility that N/OFQ may also influence the development of tolerance to other central effects of opiates (i.e., reward) cannot not be excluded. In this respect, it would be interesting to test the effect of NOP receptor antagonists for their potential in preventing the escalation of opioid self-administration.

Another behavioral outcome associated with drug addiction is locomotor sensitization, a phenomenon in which repeated intermittent administration of drugs of abuse leads to a progressive increase in locomotor activity (Robinson and Berridge 1993). According to the incentive sensitization theory of addiction, this phenomenon may reflect an increase in drug "wanting" that occurs following repeated drug experiences (Robinson and Berridge 1993). The effect of N/OFQ on morphine-induced sensitization has been studied, but results remained unclear. In fact, either no effect was reported following N/OFQ administration or, when Ro 64-6198 and Ro 65-6570 were tested, these agents reduced the expression of morphine-induced locomotor sensitization, but these effects were impervious to blockade by the selective NOP antagonist [Nphe^1]N/OFQ(1–13)-NH_2 (Ciccocioppo et al. 2000; Kotlinska et al. 2005). Finally, as in the case of alcohol, activation of NOP has been shown to prevent the expression of somatic opioid withdrawal signs in morphine-dependent rats (Kotlinska et al. 2000).

4 The N/OFQ System and Psychostimulant Abuse

The reinforcing properties of psychostimulants are linked to their ability to facilitate dopaminergic neurotransmission within the mesocorticolimbic circuit as a result of stimulating neurotransmitter release or blocking its reuptake (Di Chiara and Imperato 1988; Nicolaysen and Justice 1988; Wise and Rompre 1989; Jones et al. 1999). However, chronic exposure to these drugs leads to several neurobiological adaptations that occur at different stages of the addiction cycle and involve various transmitter systems (Nestler and Aghajanian 1997; Nestler 2001; Koob et al. 2004; Koob and Le Moal 2008; Koob and Volkow 2016).

Among these, the endogenous opioid system plays a primary role related to its modulation of the reinforcing effects of psychostimulants (Corrigal and Coen 1991; Contet et al. 2004; Le Merrer et al. 2009).

The anti-opioid nature of N/OFQ and its ability to reduce DA and glutamatergic transmission in mesolimbic regions have prompted the hypothesis that activation of NOP may counteract the effects of psychostimulants (Murphy and Maidment 1999; Di Giannuario and Pieretti 2000; Meis and Pape 2001). Based on these considerations, several studies investigated the involvement of N/OFQ transmission in the acquisition of psychostimulant sensitization and place preference, with attention to the distinction between endogenous and exogenous N/OFQ actions in influencing the incentive proprieties of cocaine and amphetamines (Table 3).

In particular, CPP studies showed that exogenous N/OFQ reduced the rewarding effects of cocaine and amphetamines (Kotlinska et al. 2003; Sakoori and Murphy 2004), and these findings were replicated with peripheral administration of brain-penetrating synthetic agonists (Rutten et al. 2010; Zaveri et al. 2018a).

Consistent with these findings, it was shown in a microdialysis study that ICV administration of N/OFQ attenuated cocaine-induced increase in extracellular DA in the NAc (Lutfy et al. 2001). In a similar study, it was found that reverse dialysis of N/OFQ into the NAc shell significantly reduced cocaine-induced increase in extracellular DA levels in the same area and this effect of N/OFQ was prevented by administration of the selective NOP receptor antagonist SB-612111 (Vazquez-DeRose et al. 2013).

On the other hand, administration of UFP-101, another selective NOP antagonist, did not significantly modify basal DA levels, suggesting a limited role of endogenous N/OFQ in modulating physiological DA transmission (Koizumi et al. 2004; Calo et al. 2005). However, UFP-101 was able to elicit modest CPP and enhanced methamphetamine-induced place preference (Sakoori and Murphy 2008a). Moreover, mice lacking NOP exhibited enhanced cocaine CPP compared to their wild-type littermates (Marquez et al. 2008b).

These findings are consistent with the hypothesis that endogenous N/OFQ may contribute to producing a hypodopaminergic and hypohedonic state that, as suggested above (see Sect. 2), may contribute to enhancing the motivation for drugs of abuse.

A recent study reported that NOP receptor activation by the NOP agonist SR-8993 did not affect cocaine CPP nor reinstatement elicited by cocaine priming or administration of the pharmacological stressor yohimbine (Sartor et al. 2016).

Few studies explored the effects of N/OFQ manipulation on psychostimulant-induced locomotor sensitization. Evidence available to date shows that administration of the peptide blocks the development of cocaine and amphetamine-induced psychomotor sensitization (Lutfy et al. 2002; Kotlinska et al. 2003; Lutfy and Zaveri 2016). This effect was not observed in NOP KO mice, further confirming that it is mediated by NOP activation (Bebawy et al. 2010).

Very little is known about the effect of NOP modulation on psychostimulant self-administration. One early study showed that ICV administration of the peptide

Table 3 Compounds targeting the N/OFQ system, relative developmental stage, and effects on psychostimulants and on nicotine abuse

Agonist	Chemical entity	Effects	Dev. phase	Ref.
N/OFQ	Peptidic	↓ Cocaine-induced CPP	Preclinical	Kotlinska et al. (2002) and Sakoori and Murphy (2004)
		↓ Methamphetamine-induced CPP		Kotlinska et al. (2003) and Zhao et al. (2003)
		— Stress-induced cocaine reinstatement		Martin-Fardon et al. (2000)
		— Cocaine-induced CPP		
		— Reinstatement induced by stress or cocaine		
		↓ Cocaine-induced CPP		Sartor et al. (2016)
		↓ Morphine-induced somatic withdrawal signs		Kotlinska et al. (2000)
		↓ Cocaine-induced locomotor sensitization		Lutfy et al. (2002)
		↓ Amphetamine-induced locomotor sensitization		Kotlinska et al. (2003)
AT-202	Small molecule	— Cocaine self-administration		Kallupi et al. (2018)
		↑ Nicotine self-administration		Cippitelli et al. (2016)
AT-312	Small molecule	↓ Morphine-induced CPP		Zaveri et al. (2018a)
		↓ Cocaine-induced CPP		
Ro 65-6570	Small molecule	↓ Cocaine-induced CPP		Rutten et al. (2010)
Ro 64-6198	Small molecule	↓ Morphine-induced CPP		Shoblock et al. (2005)

attenuated stress-induced reinstatement of extinguished lever pressing for alcohol but not for cocaine (Martin-Fardon et al. 2000).

5 The N/OFQ System and Nicotine Abuse

Nicotine is the primary psychoactive component of tobacco and, like most drugs of abuse, acts upon the mesocorticolimbic reward system of the brain to initiate dependence (Pontieri et al. 1996). So far, very few studies have investigated the significance

of N/OFQ-NOP neurotransmission in the regulation of nicotine-related behaviors (Table 3). In one of the first published studies, it was demonstrated that mice lacking the NOP receptor show higher voluntary drinking of a low concentration of nicotine solution compared to wild-type mice (Sakoori and Murphy 2009). NOP KO mice show increased hippocampal acetylcholine release, providing additional evidence of the modulatory role of N/OFQ on acetylcholine transmission (Uezu et al. 2005).

More recently Cippitelli and colleagues investigated the role of the NOP system in a model of nicotine and alcohol co-administration. The NOP receptor agonist AT-202 increased nicotine self-administration in nicotine postdependent and nondependent rats. Conversely, the specific NOP antagonist SB-612111 reduced nicotine self-administration in both groups of animals, suggesting that NOP receptor antagonists rather than agonists may show potential as treatments for nicotine dependence (Cippitelli et al. 2016).

Additional studies will be necessary before drawing conclusions about the therapeutic potential of NOP antagonists in nicotine addiction. However, considering that NOP antagonists are efficacious in attenuating alcohol drinking and that alcohol and nicotine are among the most frequently co-abused drugs, it will be a priority to evaluate the therapeutic potential of this approach in future studies.

6 The N/OFQ System: Co-activation of NOP and MOP Receptors

Growing evidence suggests that compounds that co-activate MOP and NOP opioid receptors (Table 4) have potential for the treatment of drug abuse (Ciccocioppo et al. 2007; Toll et al. 2009; Toll 2013; Kallupi et al. 2018).

Molecules with mixed MOP/NOP agonist properties were first investigated with the aim to develop successful analgesics with reduced tendency to evoke tolerance and low abuse liability compared to classical MOP agonists (Khroyan et al. 2011b; Toll 2013; Ding et al. 2016). These compounds, in addition to analgesic activity, preserve most of the classical MOP agonist effects including relaxation, feeling of pleasure, and respiratory depression but at lower intensity compared to heroin, morphine, methadone, and other traditional opioid agonists (Lambert et al. 2015; Calo and Lambert 2018; Gunther et al. 2018; Ruzza et al. 2018).

The potential of MOP/NOP agonists or partial agonists in drug abuse treatment was initially suggested by studies with buprenorphine. This drug is classically viewed as a partial agonist at MOP and antagonist at DOP and KOP receptors (Huang et al. 2001). However, more recent studies demonstrated that it also acts as a low-affinity partial agonist at NOP (Wnendt et al. 1999; Bloms-Funke et al. 2000; Huang et al. 2001). Most importantly, activation of NOP by buprenorphine appears to have distinct pharmacological consequences (Lutfy et al. 2003; Marquez et al. 2008a; Khroyan et al. 2009). For instance, activation of NOP contributes to attenuating the analgesic effects of high doses of buprenorphine (Lutfy et al. 2003; Marquez et al. 2008a; Khroyan et al. 2009). Moreover, in a study with Marchigian Sardinian alcohol-preferring (msP) rats, buprenorphine produced a bidirectional effect on

Table 4 Mixed MOP/NOP receptors compounds, relative developmental stage, and effects on substance abuse

Agonist	Chemical entity	Effects	Dev. phase	Ref.
Buprenorphine	Small molecule	↓ Alcohol intake through NOP receptors (high doses)	Preclinical	Ciccocioppo et al. (2007)
		↑ Alcohol intake through MOP receptors (low doses)		
		↓ Cocaine self-administration		Lukas et al. (1995) and Kallupi et al. (2018)
		↓ Heroin seeking during extinction		Sorge et al. (2005)
		↓ Cocaine seeking during extinction		
		↓ Heroin-induced reinstatement		
		↓ Cocaine-induced reinstatement		
		↓ Cocaine intake		
		— Heroin intake		Sorge and Stewart (2006)
Cebranopadol	Small molecule	↓ Escalation of cocaine self-administration		de Guglielmo et al. (2017)
		↓ Conditioned reinstatement of cocaine-seeking behavior		
		↓ Cocaine self-administration		Shen et al. (2017)
AT-034	Small molecule	↓ Cocaine self-administration		Kallupi et al. (2018)
AT-201	Small molecule	↓ Cocaine self-administration		Kallupi et al. (2018)

alcohol drinking. Low doses increased alcohol consumption, while high doses reduced it. Buprenorphine-induced increases of alcohol drinking were blocked by naltrexone, suggesting that this effect was mediated by MOP receptors. On the other hand, reductions of alcohol intake were selectively blocked by the NOP agonist UFP-101 but not by naloxone (Ciccocioppo et al. 2007). These findings demonstrate that inhibition of drinking by buprenorphine was specifically mediated by NOP receptors, for which the drug has low affinity, and these observations may also explain why anti-alcohol effects occurred at high buprenorphine doses. Interestingly, in an earlier clinical study in heroin addicts abusing alcohol, it was shown that high buprenorphine doses were also able to reduce alcohol consumption in this population (Kakko et al. 2003). In another clinical study conducted in heroin-addicted patients co-abusing cocaine, it was also shown that high doses of buprenorphine reduced the consumption of the psychostimulant. Interestingly, this effect was evident only at

highest doses of buprenorphine and appeared to be independent from reductions in heroin use. This clinical study replicated evidence from a number of preclinical investigations that systematically demonstrated the efficacy of buprenorphine in attenuating cocaine self-administration in rats and monkeys and humans (Lukas et al. 1995; Montoya et al. 2004; Sorge et al. 2005; Sorge and Stewart 2006; Kallupi et al. 2018). In some circumstances, the "therapeutic" effect of buprenorphine on cocaine intake was attenuated by administration of naltrexone (Mello et al. 1993; Wee et al. 2012). However, in a more recent investigation, administration of naltrexone was not sufficient to prevent buprenorphine-induced inhibition of cocaine self-administration in rats (Kallupi et al. 2018). In this latter study that attempted to more precisely characterize the mechanism of action of buprenorphine on cocaine self-administration, buprenorphine's effects were tested against naltrexone, the selective NOP antagonist SB-612111, or a combination of both drugs. The results showed that buprenorphine-induced reduction of cocaine self-administration was prevented only if NOP and MOP receptors were simultaneously blocked by co-administration of the two antagonists (Kallupi et al. 2018). Based on this finding, the authors concluded that reduction of cocaine self-administration by buprenorphine requires actions at both MOP and NOP receptors and is only achieved at high drug doses due to its low affinity for NOP. Support for the co-activation hypothesis came from a study with AT-034 and AT-201, two new molecules specifically designed to activate MOP and NOP, with much weaker affinity for DOP and KOP that, like buprenorphine, reduce operant cocaine self-administration (Zaveri et al. 2013; Journigan et al. 2014). Noteworthy, NOP binding affinity and efficacy of these three molecules are different, which opens questions on the intimate mechanism responsible for their effect on cocaine.

An interesting development was the recent discovery of cebranopadol, a pan-opioid agonist that activates MOP and NOP receptors with similar potency and efficacy and with slightly lower affinity, also DOP and KOP (Linz et al. 2014; Schunk et al. 2014; Lambert et al. 2015). Recently, two independent studies demonstrated that cebranopadol is efficacious in attenuating the motivation for cocaine in drug self-administration studies while leaving unaffected (or slightly increased) the consumption of natural rewards (de Guglielmo et al. 2017; Shen et al. 2017). Most importantly, in one of these studies replicating earlier findings with buprenorphine, the authors demonstrated that the effect of cebranopadol was blocked by co-administration of naltrexone and SB-612111, but not when these two antagonists were given separately (Shen et al. 2017).

Cebranopadol is currently under clinical development for chronic pain (Linz et al. 2014; Schunk et al. 2014; Lambert et al. 2015; Christoph et al. 2017; Scholz et al. 2018). At pharmacological effective doses, it exhibits low tendency to produce respiratory depression and produces no impairment of motor coordination (Dahan et al. 2017; Gunther et al. 2018). Moreover, cebranopadol appears to have lower abuse potential compared to classical opioid agonists (Shen et al. 2017; Tzschentke et al. 2017; Ruzza et al. 2018; Gohler et al. 2019).

Based on these findings, it is tempting to hypothesize that co-activation of MOP and NOP receptors may represent a novel highly promising strategy to treat drug abuse. The advanced stage of development of cebranopadol allows for rapid translation of these preclinical findings into clinical trials in addicted patients. Moreover, there are other molecules under development that selectively activate NOP and MOP without affecting other opioid receptors that are promising candidates for development in the field of drug abuse (Ding et al. 2016, 2018).

7 Conclusions

Two decades of research on N/OFQ and drug abuse provided significant evidence supporting the therapeutic potential of NOP agonists in the treatment of drug abuse. The most robust evidence has been generated in the field of alcoholism, followed by the psychostimulant and opioid fields. Very little is known about the role of N/OFQ in nicotine abuse. However contrary to what was observed with other substances of abuse, activation of NOP appears to have a permissive role for nicotine reward as it increases nicotine consumption. New studies with selective NOP antagonists that have been recently become available are revealing more complicated scenarios. For instance, it was shown that, similar to NOP agonism, NOP receptor blockade reduced alcohol drinking and seeking in laboratory animals and in humans. To reconcile these contrasting findings, we proposed here the hypothesis that high basal N/OFQ-NOP transmission is responsible for inducing a hypohedonic state that can ultimately motivate drug consumption. This is why animals with innate (msP rats) or alcohol-induced (postdependent Wistar rats) overexpression of NOP show higher motivation for alcohol, whereas rats with genetic deletion of NOP self-administer less alcohol cocaine and heroin (Table 5). Derived from these observations, we then proposed that the effect of NOP agonists on behavior motivated by alcohol and on other substances of abuse may depend upon rapid desensitization of the N/OFQ-NOP system following agonist administration. This hypothesis is supported by at least three main elements: First, NOP receptors are subject to rapid desensitization following exogenous administration of NOP agonists. Second, in few studies acute administration of

Table 5 Genetic deletion of NOP receptor in rat and mice and related effects on drug abuse-related behaviors

NOP (−/−)	Effects	Ref.
Rat	↓ Cocaine self-administration	Kallupi et al. (2017)
	↓ Progressive ratio for cocaine	
	↓ Heroin self-administration	
	— Cocaine-induced CPP	
	↓ Alcohol self-administration	
Mouse	↓ Cocaine-induced locomotor sensitization	Marquez et al. (2013)
	↓ Amphetamine-induced locomotor sensitization	Marquez et al. (2008b)
	↑ Cocaine-induced CPP	Sakoori and Murphy (2009)
	↑ Nicotine intake	

N/OFQ increased rather than decreased alcohol drinking. Third, the effect of NOP agonists increases during chronic drug administration and is maintained for several days after the treatment is stopped.

A final consideration concerns mixed MOP/NOP agonists. Buprenorphine is the prototype of this class of molecules, but recently other compounds with higher potency and efficacy for NOP have been synthesized. Cebranopadol is an example of this new class of molecules, but it binds to all four opioid receptors. However, other recently developed compounds, like BU08070, BU08028, AT-121, and SR16435 activate only MOP and NOP receptors (Khroyan et al. 2007, 2011a; Ding et al. 2016, 2018). Considering the efficacy of buprenorphine and cebranopadol on alcohol, cocaine, and opioid self-administration, it is tempting to hypothesize that combinations of MOP/NOP agonists may provide an additional strategy to treat drug abuse.

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NOP Receptor Ligands and Parkinson's Disease

Daniela Mercatelli, Clarissa Anna Pisanò, Salvatore Novello, and Michele Morari

Contents

Abstract

Nociceptin/Orphanin FQ (N/OFQ) and its NOP receptor are highly expressed in motor areas of the rodent, nonhuman, and human primate brain, such as primary motor cortex, thalamus, globus pallidus, striatum, and substantia nigra. Endogenous N/OFQ negatively regulates motor behavior and dopamine transmission through NOP receptors expressed by dopaminergic neurons of the substantia nigra compacta. Consistent with the existence of an N/OFQ tone over dopaminergic transmission, blockade of NOP receptor antagonists increases striatal

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dopamine release. In this chapter, we will review the evidence linking the N/OFQ-NOP receptor system to Parkinson's disease (PD). We will first discuss data showing that the central N/OFQ-NOP receptor system undergoes plastic changes in different basal ganglia nuclei following dopamine depletion. Then we will show that NOP receptor antagonists relieve motor deficits in different rodent and nonhuman primate models of PD. Mechanistically, NOP receptor blockade in substantia nigra reticulata results in rebalancing of the inhibitory GABAergic and excitatory glutamatergic inputs impinging on nigro-thalamic GABAergic neurons, leading to thalamic disinhibition. We will also present data showing that, in addition to motor symptoms, N/OFQ also plays a role in the parkinsonian neurodegeneration. In fact, NOP receptor antagonists possess neuroprotective/neurorescue properties in in vitro and in vivo models of PD.

Keywords
Compound 24 · J-113397 · L-DOPA · Nociceptin/Orphanin FQ · NOP · Parkinson's Disease · SB-612111 · Trap-101

1 Expression of ppN/OFQ and NOP in PD

The first evidence of changes of pre-proN/OFQ (ppN/OFQ) and NOP gene expression in models of PD came from the in situ hybridization (ISH) study of Watson and collaborators (Norton et al. 2002) who reported a threefold increase of ppN/OFQ mRNA levels and a ~80% reduction of NOP mRNA levels in the substantia nigra compacta (SNc) after unilateral injection of the parkinsonian toxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (mfb) of the rat (Table 1). Considering that also a ~70% reduction of N/OFQ-stimulated [^{35}S]GTPγS binding was simultaneously detected in SNc, it was concluded that dopamine (DA) depletion results in upregulation of ppN/OFQ and downregulation of NOP receptor expression in SNc (Norton et al. 2002). We have later confirmed these findings (Marti et al. 2005), reporting a threefold increase of ppN/OFQ and a ~60% reduction of NOP receptor mRNA in the DA-depleted rat SNc showing, in addition, similar changes in SN reticulata (SNr; twofold increase of ppN/OFQ and ~25% loss of NOP receptor mRNA). Romualdi and collaborators confirmed an increase of ppN/OFQ in SNc after 6-OHDA using RT-PCR (di Benedetto et al. 2009), and also showed a reduction of ppN/OFQ in striatum (−50%), indicating that opposite changes of N/OFQ transmission might occur in these areas. Consistently, we reported an increase and a reduction of N/OFQ autoradiographical binding in striatum and SNc/SNr, respectively, in rats hemilesioned with 6-OHDA (Marti et al. 2012). Interestingly, chronic treatment with L-DOPA and dyskinesia onset did not change this pattern (Marti et al. 2012). These adaptive changes seemed to specifically affect the nigro-striatal pathway since ppN/OFQ levels were unaffected in other brain areas such as cerebral cortex, nucleus accumbens, thalamus, and globus pallidus (Marti et al. 2010). Studies using MPTP, its active metabolite MPP+, or even a combination of the pesticides paraquat and maneb as

Table 1 Summary of the studies on ppN/OFQ and NOP gene expression

Model	Species	Technique	Readout	Area	% change	Extent of lesion	Reference
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	SNc VTA	+200 +60	>95[a]	Norton et al. (2002)
			NOP mRNA	SNc VTA	−85 −85		
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	SNc SNr	+200 +100	>90[a]	Marti et al. (2005)
			NOP mRNA	SNc SNr	−60 −20		
6-OHDA (icv)	Rat	RT PCR	ppN/OFQ mRNA	SN Striatum	+50 −50	50[b] 60[b]	di Benedetto et al. (2009)
			NOP mRNA	SN Striatum	n.e. −50		
MPP+ (icv)	Rat	RT PCR	ppN/OFQ mRNA	SN Striatum	+60 −50	75[b] 65[b]	
			NOP mRNA	SN Striatum	−30 −50		
MPTP (systemic)	Mouse	ISH	ppN/OFQ mRNA	SNc SNr VTA Striatum STN	+150[c] >400 n.c. n.c. n.c.	58–80[d]	Gouty et al. (2010)
6-OHDA (mfb)	Rat	ISH	ppN/OFQ mRNA	SNc SNr STN striatum M1/M2 NAcc GP VPL/M	+150 +105 +45 −25 n.c. n.c. n.c. n.c.	>90[a]	Marti et al. (2010)

(continued)

Table 1 (continued)

Model	Species	Technique	Readout	Area	% change	Extent of lesion	Reference
6-OHDA (mfb), dyskinetic	Rat	Autoradiography on slices	N/OFQ binding	Striatum SNr/SNc STN M1/M2	+50 −50 n.c. n.c.	>95[a]	Marti et al. (2012)
Paraquat + maneb (systemic)	Rat	RT PCR	ppN/OFQ mRNA	Striatum SN	n.c. +50	35–40[b]	Bastias-Candia et al. (2015)
			NOP mRNA	Striatum SN	n.c. −40		
PD patients	–		ppN/OFQ mRNA	SN			Collins et al. (2015)

ISH in situ hybridization, *mfb* medial forebrain bundle, *n.c.* no change
[a]Striatal TH immunohistochemistry
[b]TH levels in striatum or SN (western blot)
[c]Strain-dependent
[d]Dopamine neuron count in SNc

DA-depleting parkinsonian toxins substantially replicated what observed with 6-OHDA. An increase of ppN/OFQ in SNc was captured with RT-PCR following i.c.v. injection of MPP+ (di Benedetto et al. 2009) or systemic administration of paraquat/maneb (Bastias-Candia et al. 2015) in rats, and MPTP administration in mice (Gouty et al. 2010), although in this case such effect was strain-dependent. Nonetheless, in this study, BM Cox and collaborators (Gouty et al. 2010) reported a strong elevation of ppN/OFQ in SNr, in line with that found with 6-OHDA. A careful immunohistochemical analysis showed that this elevation occurred in neurons and, specifically, in a subset of GABAergic neurons spanning throughout the SNr (Gouty et al. 2010). Altogether, morphological studies consistently showed that DA loss is accompanied by area-dependent changes of the N/OFQ-NOP receptor system in the basal ganglia: upregulation of ppN/OFQ expression associated with reduction of NOP receptor binding in SNc/SNr and downregulation of ppN/OFQ expression associated with increase of NOP receptor binding in striatum. This picture seems to be confused by the postmortem analysis of the SN of PD patients that instead revealed a downregulation of ppN/OFQ gene expression (Collins et al. 2015). This finding does not easily reconcile with preclinical data and might indicate the occurrence of compensatory mechanisms to prevent excessive NOP receptor activation.

2 N/OFQ Release in PD

In vivo microdialysis showed that the increase in nigral ppN/OFQ mRNA observed after DA depletion was actually accompanied by an elevation of N/OFQ release in the extracellular space (Marti et al. 2005). Radioimmunoassay of dialysates from the microdialysis probes simultaneously implanted in the lesioned and unlesioned SNr of 6-OHDA hemilesioned rats revealed that N/OFQ-like immunoreactivity at baseline was threefold elevated in the lesioned compared to the unlesioned side. Interestingly, when the rat was placed on a rotating cylinder (rotarod test), N/OFQ levels rose in the SNr of both sides, indicating a functional role of N/OFQ during exercise. In these conditions, however, the side difference became larger (>3.5-fold) with highest levels attained in the lesioned SNr. An increase of extracellular N/OFQ levels in SNr was observed not only after destruction of DA neurons but also during functional inhibition of DA transmission caused by haloperidol treatment (Marti et al. 2010). Although the increase was milder (50%), it suggested the existence of an ongoing tonic inhibitory control mediated by striatal DA, likely via the indirect striato-nigral pathway, on nigral N/OFQ release. In striking agreement with studies in models of PD, an increase of N/OFQ levels was observed in the CSF of parkinsonian patients (Marti et al. 2010). In fact, we measured N/OFQ levels in the CSF of PD patients subjected to surgical implantation of electrodes for deep brain stimulation, in comparison with non-PD neurological controls. Both cohorts were balanced for number (17 controls subjects against 20 PD patients), age, and gender. PD patients were affected by advanced and severe PD (Hoehn&Yahr stage 2.9, UPDRS II score 46.1) with a long history of disease (11.8 years). In line with that found in the DA-depleted rat SNr, we reported a 3.5-fold elevation of N/OFQ

levels in the CSF from PD vs non-PD patients. Since human CSF is in equilibrium with parenchymal fluids, this study suggests that N/OFQ might play a role in PD.

3 NOP Receptor Ligands in PD Models: Symptomatic Efficacy

In line with the hypothesis that the increase of N/OFQ expression and release contributes to PD, NOP receptor antagonists proved effective in reducing motor deficits and neurodegeneration in PD models. Preliminary studies in naïve rats somewhat predicted the effectiveness of NOP antagonists in PD models. In fact, while N/OFQ was capable of elevating glutamate (GLU) levels in SNr, the NOP receptor peptide antagonist [Nphe^1]N/OFQ(1–13)NH_2 ([Nphe^1]) inhibited it (Marti et al. 2002). Moreover, while exogenous N/OFQ injected in SNr reduced DA levels in striatum and motor activity, the NOP receptor peptide antagonist UFP-101 injected in SNr and the NOP receptor small molecule J-113397 administered systemically caused opposite effects (Marti et al. 2004b). The symptomatic efficacy of NOP receptor antagonists was consistently shown in different models of PD, as detailed below.

3.1 Models of Functional Parkinsonism: Genetic and Pharmacological Blockade of NOP Receptor

The first evidence that an NOP receptor antagonist could reverse parkinsonian symptoms was obtained in a rat model of neuroleptic-induced parkinsonism (Marti et al. 2004a). In that study, UFP-101 was injected (30 nmol) through a cannula implanted in the SNr of a rat made cataleptic by systemic haloperidol (0.8 mg/kg). Akinesia was assessed with the bar test and GLU levels in SNr were simultaneously monitored via a microdialysis probe. Haloperidol blocks striatal D2 receptors, which causes disinhibition of the striato-pallidal and subthalamo-nigral pathways, leading to stimulation of the nigral output and motor inhibition. Indeed, haloperidol elevated both akinesia and nigral GLU levels whereas UFP-101 transiently reduced akinesia and normalized GLU levels. Moreover, UFP-101 triggered contralateral rotations, more intensely in haloperidol-treated rats than in naïve rats. The role of endogenous N/OFQ in neuroleptic-induced parkinsonism was also investigated in mice (Mabrouk et al. 2010). In this model, the antiakinetic effectiveness of UFP-101 (10 nmol, i.c.v.) was confirmed. In addition, we showed that similar effects were produced also by systemic administration of J-113397 (0.1–10 mg/kg). The role of endogenous N/OFQ in modulating neuroleptic-induced parkinsonism was further supported by the finding that NOP receptor knockout ($NOP^{-/-}$) mice were less prone to develop motor impairment after administration of low doses (0.3–0.8 mg/kg) of haloperidol (Marti et al. 2005).

The effectiveness of NOP receptor antagonists was also proven in reserpinized animals. Reserpine depletes vesicular stores of monoamines by blocking the vesicular monoamine transporter type 2 (VMAT2). Reserpine administration (0.1–3 mg/

kg) in mice caused a dose-dependent and long-lasting impairment of motor activity lasting for >4 days (Volta et al. 2010a). Repeated daily administration of J-113397 (1 mg/kg) caused acute improvement of motor activity (which, however, underwent tolerance within 2 days) and accelerated recovery of motor function that reached (drag test), approximated (bar test), or even exceeded (rotarod test) baseline values within 4 days. Again, studies in $NOP^{-/-}$ mice substantiated these findings. In fact, in line with that found with haloperidol, $NOP^{-/-}$ mice were less prone to develop motor impairment after administration of reserpine 1 mg/kg. Finally, acute administration of SB-612111 (0.1–10 mg/kg) also dose-dependently improved motor activity in reserpinized mice, an effect partly replicated by the NOP antagonist NiK-21273 (Marti et al. 2013).

3.2 Models of Neurotoxic Parkinsonism: 6-OHDA Hemilesioned Rats

The proof-of-concept that NOP receptor antagonists possess symptomatic antiparkinsonian properties was provided by Morari lab in 2005 (Marti et al. 2005), testing UFP-101 and J-113397 in comparison with a fixed-doses of L-DOPA (1 mg/kg) as a positive control, in 6-OHDA hemilesioned rats, a well-validated model of neurodegenerative PD (Schwarting and Huston 1996a, b). UFP-101 (0.1–30 nmol, intranigral) and J-113397 (0.1–3 mg/kg, i.p.) reduced akinesia in the bar test and akinesia/bradykinesia in the drag test, and improved overall motor performance in the rotarod test, replicating the pattern of responses to L-DOPA. Other small molecules NOP antagonists given i.p. were later proven effective in this model: Trap-101 (a non-chiral analog of J-113397) (Marti et al. 2008), GF-4 (a derivative of Trap-101) (Volta et al. 2010b), NiK-21273 (Marti et al. 2013), and the more potent and selective NOP antagonists Compound 24 (Volta et al. 2011) and SB-612111 (Marti et al. 2013).

All NOP antagonists improved motor function in the three different tests (bar, drag, and rotarod) with no major difference in efficacy. Nonetheless, differences in potency were observed, Compound 24 being the most potent antagonist (effective in all three tests already at 0.1 mg/kg) and Trap-101 the least potent one (effective at 1 mg/kg in the drag test and at 10 mg/kg in the drag and rotarod test).

What also emerged from these studies was the bell-shaped profile of the dose–response curve of NOP receptor antagonists. In fact, NOP receptor antagonists lost their positive effect or even caused overt motor inhibition when given at high doses. This applied to J-113397, GF-4, and Compound 24 administered at 30 mg/kg. We could not observe such a phenomenon for Trap-101 (tested up to 30 mg/kg) and SB-612111 (tested up to 1 mg/kg), although we cannot rule out that this might occur at higher doses. Indeed, Trap-101 induced motor inhibition when administered at 30 mg/kg to naïve rats or mice (Marti et al. 2008). The mechanisms underlying motor facilitation and inhibition induced by NOP receptor antagonists were investigated (Viaro et al. 2013) using selective DA receptor antagonists, and mice constitutively lacking the D2 receptor ($D2R^{-/-}$ mice) (Baik et al. 1995) or its long

(L) isoform ($D2L^{-/-}$ mice) (Usiello et al. 2000), which is preferentially expressed at the postsynaptic level (Picetti et al. 1997; Usiello et al. 2000). Motor inhibition induced by UFP-101 30 mg/kg or J-113397 10 mg/kg was abolished (and in some cases reversed into motor facilitation) by low doses of the atypical D2 antagonist amisulpride but not by the typical D2 antagonist raclopride. In addition, it was abolished in $D2R^{-/-}$ mice but remained unchanged in $D2L^{-/-}$ mice. Considering the affinity of low doses of amisulpride for presynaptic D2 receptors (Scatton et al. 1997; Schoemaker et al. 1997), these data suggested that motor inhibition induced by high doses of NOP receptor antagonists is mediated by activation of presynaptic D2 receptors. To support this view, also motor inhibition induced by high doses of dopaminergic agonists (L-DOPA 100 mg/kg or pramipexole 0.1–1 mg/kg) was reversed by amisulpride and/or cancelled in $D2R^{-/-}$ mice. The most parsimonious explanation is that blockade of presynaptic NOP receptors located on dopaminergic terminals elevates DA release (Marti et al. 2004b), and extracellular DA binds to presynaptic D2 receptors activating the inhibitory auto feed-back.

In one study, we addressed the question whether the antiparkinsonian effect of SB-612111 undergoes tolerance (Marti et al. 2013). SB-61211 was chronically administered for 16 days at a low, ineffective (0.01 mg/kg) or high, maximally effective (1 mg/kg) dose. Motor function was assessed before and after acute administration. Essentially, there was no chronic effect of SB-612111 over motor function over time (i.e., no changes of baseline motor activity) and the acute effect of the drug remained unchanged during the study, indicating there was no development of tolerance. We also repeated the experiment using ineffective (0.5 mg/kg) and effective (1.5 mg/kg) doses of NiK-21273. In this case, we noticed a late improvement of motor function at baseline in the bar and drag tests (12–16 days after the onset of administration), but a rapid tolerance (within 4 days) to the acute effects.

One particular aspect of the symptomatic antiparkinsonian effect of NOP antagonists that was investigated in 6-OHDA rats was the ability to interact with L-DOPA. The first study addressing this issue (Marti et al. 2007) showed that fully effective doses of J-113397 (1 mg/kg) caused additive improvement of motor activity (in the drag and rotarod tests) when challenged with a submaximal dose of L-DOPA (1 mg/kg). Interestingly, we found an additive stimulation of the nigro-thalamic pathway as a neurochemical correlate of this behavior (discussed below). The ability of NOP receptor antagonists to positively interact with L-DOPA was later confirmed using maximally effective doses of Trap-101 (10 mg/kg) in combination with subthreshold (ineffective) doses of L-DOPA (0.1 mg/kg) (Marti et al. 2008). In this case, the combination produced greater antiakinetic effect (bar test) and improvement of overall gait ability (rotarod test) than that produced by each compound alone. Interestingly, this effect was reproduced when L-DOPA was administered systemically and Trap-101 was perfused in SNr via a microdialysis probe. In fact, simultaneous monitoring of GLU and GABA levels in SNr and ipsilateral ventromedial thalamus (VMTh) in these animals establishes that the neurobiological substrate of the positive interaction was the nigro-thalamic pathway (discussed below). Finally, a marked synergistic interaction between subthreshold doses of SB-612111 (0.01 mg/kg) and L-DOPA (0.1 mg/kg) was demonstrated (Marti et al. 2013), an effect replicated by subthreshold doses of NiK-21273 (0.5 mg/kg).

Whether an NOP antagonist, in addition to potentiating the therapeutic effect of L-DOPA, would also exacerbate L-DOPA-induced dyskinesia (LID) was specifically assessed (Marti et al. 2012) in the classical 6-OHDA rat model of L-DOPA-induced dyskinesia (Cenci et al. 1998). In fact a previous study in MPTP-treated nonhuman primates (marmosets) reported that a high dose of J-113397 (30 mg/kg s.c.) potentiated the effect of L-DOPA, at the cost of exacerbating dyskinesia (Visanji et al. 2008). Consistently, when 10 nmol UFP-101 (i.c.v.) or 3 mg/kg J-113397 (i.p.) were co-administered with L-DOPA to L-DOPA-primed dyskinetic rats, exacerbation of abnormal involuntary movements (AIMs), i.e., the rodent correlate of dyskinesia, was observed. In this model, however, the effect was mild and limited to the limb subgroup of AIMs (50% with UFP-101, 20% with J-113397), the axial and orolingual (facial) muscles being spared. Interestingly, in that study we found that intranigral but not intrastriatal UFP-101 injection replicated the effect of i.c.v. injections, suggesting that NOP antagonists act where NOP tone is elevated (i.e., in SNr) but not where N/OFQ is reduced (i.e., striatum). Consistently, in that study we provided the proof-of-concept that NOP receptor agonists exert antidyskinetic actions.

3.3 Models of Neurotoxic Parkinsonism: MPTP-Treated Mice

MPTP-treated mice are not routinely used as a model for studying the symptomatic antiparkinsonian effects perhaps due to the inconsistencies of MPTP effects across different labs and protocols, though motor deficits can be captured and quantified in these mice (Sedelis et al. 2000). Using a classical protocol of acute MPTP administration (4 × 20 mg/kg, 90 min apart) causing ~60% loss of striatal dopaminergic terminals, we were able to capture a robust increase in the immobility time (from <1 to 26 s, bar test) and a ~30% reduction in stepping activity (drag test) and time on rod (rotarod test) a week after MPTP administration. J-113397 reversed these changes just as our positive control, L-DOPA. Indeed, J-113397 caused a reduction of immobility time and an increase in stepping activity and rotarod performance in the dose range of 0.01–0.03 mg/kg, that were quantitatively similar, although not as prolonged as those evoked by 10 mg/kg L-DOPA. In addition, we observed a reversal of action at 1 mg/kg, again pointing out the bell-shaped nature of the dose–response curve of this molecule.

3.4 Models of Neurotoxic Parkinsonism: MPTP-Treated Nonhuman Primates

We investigated the effect of J-113397 in MPTP-treated, stably parkinsonian macaques (Viaro et al. 2008), the gold standard in preclinical models of PD. Four macaques were enrolled in this study: their motor performance was assessed and quantified via computerized time reaching tasks (MAP test, i.e., the platform and straight rod tests) or by post hoc videotape analysis (UPDRS scale) by a neurologist

blind to the experiment. Preliminary dosing in these animals indicated a positive effect of 0.01 mg/kg J-113397 in the MAP test. This dose also caused symptomatic benefit in all four animals, improving various motor parameters such as hypokinesia, bradykinesia, tremor, balance, and rigidity. J-113397 was overall 50% less effective than L-DOPA (30 mg/kg i.m.), although it was as effective as L-DOPA on hypokinesia. Very much like that observed in rodent models, higher doses of J-113397 negatively affected behavior; in particular, 1 mg/kg J-113397 caused long episodes of freezing. A study in MPTP-treated marmosets (Visanji et al. 2008) revealed that 30 mg/kg J-113397 (s.c.) was capable of potentiating the effect of a low subtherapeutic dose (12.5 mg/kg) of L-DOPA, at the cost, however, of inducing dyskinesia. These authors raised the concern that the L-DOPA sparing effect of NOP antagonists would be counterbalanced by the appearance of dyskinetic movements.

3.5 Models of α-Synucleinopathy

The discovery that mutations in the SNCA gene coding for α-synuclein were associated with autosomal dominant PD (Polymeropoulos et al. 1997), and that Lewy Bodies, a neuropathological feature of PD, are mainly composed by α-synuclein (Spillantini et al. 1997), paved the way for the research on the genetics of PD. It is well accepted that the fibrillization process of α-synuclein that leads to LB formation also determines the formation of toxic species that cause neuronal death. Thus, overexpression of native or mutated α-synuclein has been used to replicate synucleinopathy observed in PD brains. To extend the previous findings in functional and neurodegeneration models of PD, we tested SB-612111 in a synucleinopathy model. In this model, a recombinant adeno-associated virus of serotype 2/9, carrying human p.A53T α-synuclein transgene (AAV2/9-hα-syn), a mutated, toxic form of α-synuclein (α-syn), under the promotor of synapsin 1, was injected into the SNc of naïve rats. Although the primary endpoint of the study was the assessment of the neuroprotective properties of SB-612111, motor behavior was monitored along with chronic administration of the compound. Transgene expression was associated with nigro-striatal degeneration and progressive motor deficits, namely reduction of stepping activity (Arcuri et al. 2016). SB-612111, administered daily for 8 weeks, starting a week after virus injection, was able to attenuate nigro-striatal degeneration (see below) and prevent motor impairment.

4 Mechanisms of Symptomatic Action of NOP Receptor Antagonists

The mechanism underlying the motor promoting action of NOP receptor antagonists was well defined. Several lines of evidence converge in suggesting that NOP receptor antagonists act to reset the balance between excitatory and inhibitory inputs

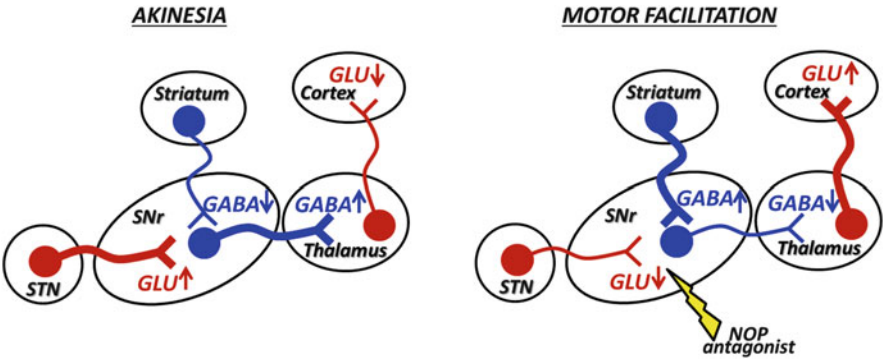

Fig. 1 Neurochemical and functional consequences of NOP receptor blockade in the substantia nigra reticulata (SNr) of parkinsonian rats. Parkinsonian akinesia is characterized by disinhibition of the activity of the glutamatergic neurons located in subthalamic nucleus (STN), and reduction of the activity of the striatal GABAergic neurons projecting to SNr (left panel). Microdialysis studies in 6-OHDA hemilesioned rats revealed that NOP receptor antagonists oppose these changes, reducing glutamate (GLU) and increasing GABA levels in SNr (right panel). This causes overinhibition of the nigro-thalamic pathway and thalamic disinhibition, ultimately resulting in motor facilitation

impinging on nigro-thalamic GABAergic neurons, thus causing disinhibition of thalamo-cortical pathways and movement facilitation (Fig. 1). According to the classical model of basal ganglia functioning (Albin et al. 1989; Alexander et al. 1990), the parkinsonian condition is associated with an increased activity of the glutamatergic projections from the subthalamic nucleus to the output nuclei of the basal ganglia, namely SNr and the pars interna of the globus pallidus (GPi). Simultaneously, the inhibitory projection from striatal GABAergic medium-sized spiny neurons (MSNs) to the SNr/GPi (the so-called "direct pathway") becomes hypoactive. This causes a net increase of the excitatory inputs over the tonically active nigrofugal GABAergic neurons, leading to further inhibition of thalamo-cortical projections.

4.1 NOP Receptor Antagonists and GLU Release

Microdialysis studies in rodents consistently pointed out that NOP receptor antagonists are capable of reducing GLU release in SNr. Haloperidol-induced catalepsy/akinesia is associated with elevation of nigral GLU release (Mabrouk et al. 2010; Marti et al. 2004a). This is due to the disinhibition of subthalamo-nigral pathway as a consequence of blockade of inhibitory D2 receptors expressed on striato-pallidal MSNs. UFP-101 (10 nmol, i.c.v.) and J-113397 (1 mg/kg, i.p.) reversed haloperidol-induced nigral GLU release in the rat (Marti et al. 2004a, 2005). J-113397 was also effective in the mouse (Mabrouk et al. 2010). In both species, normalization of haloperidol-elevated GLU release was accompanied by reversal of akinesia. Consistently, genetic deletion of the NOP receptor attenuated

haloperidol-induced catalepsy and its neurochemical correlate. In fact, $NOP^{-/-}$ mice did not show the typical rise of GLU levels observed in $NOP^{+/+}$ and wild-type mice following haloperidol (0.3 mg/kg), actually showing a reduction, and were also insensitive to the cataleptic action of this dose of haloperidol. Since there was no difference in basal GLU levels between $NOP^{+/+}$ and $NOP^{-/-}$ mice, we conclude that there is a close relationship between catalepsy and endogenous N/OFQ and glutamate in SNr. To confirm this view, a microdialysis study showed that $NOP^{-/-}$ mice treated with reserpine 1 mg/kg developed a milder increase in nigral GLU levels than $NOP^{+/+}$ controls, which was accompanied by a 50% reduction of catalepsy severity (Volta et al. 2010a).

Microdialysis studies in 6-OHDA rats substantially confirmed the ability of NOP receptor antagonists to reduce nigral GLU. In fact, in a microdialysis study where probes were simultaneously implanted in the lesioned and unlesioned SNr, UFP-101 (1–10 μM through the probe) or J-113397 (0.1–3 mg/kg, i.p.) reduced GLU levels (20–30%) in both hemispheres, although more potently in the lesioned one. We should recall that GLU levels detected at baseline via microdialysis only minimally (~20%) derive from neuronal sources (Morari et al. 1996), so it is possible that a "floor effect" prevented to detect further reduction. Similar reductions of basal GLU release were observed administering Trap-101 (10 mg/kg) (Marti et al. 2008) or GF-4 (1 mg/kg) (Volta et al. 2010b) systemically, or Trap-101 (10 μM) (Marti et al. 2008) and Compound 24 (0.03 μM) (Volta et al. 2011) through a microdialysis probe implanted in SNr. Also, during these studies microdialysis was coupled to behavioral monitoring (the immobility time in the bar test), confirming that these procedures led to significant attenuation of akinesia. Consistently, combined administration of Trap-101 and L-DOPA caused slightly more profound (~30%) and/or faster inhibition of nigral GLU release (depending on the way Trap-101 was administered) which was associated with additive attenuation of akinesia.

Overall, these data suggest that DA loss amplifies a tonic excitatory action of endogenous N/OFQ over nigral GLU terminals (Marti et al. 2002). Since an increase of excitatory input over nigro-thalamic GABA neurons causes thalamic inhibition and impairment of motor initiation, it is plausible that NOP receptor antagonists reduce akinesia by blocking this action.

4.2 NOP Receptor Antagonists and GABA Release

The resetting of GLU inputs in SNr is perhaps not the only mechanism through which NOP receptor antagonists ameliorate parkinsonian motor symptoms. In fact, a positive effect of NOP receptor antagonists on GABA levels in SNr was disclosed using microdialysis. Elevation of GABA levels in SNr might mimic the physiological inhibitory control operated by the striato-nigral direct pathway over nigro-thalamic neurons, which is reduced due to the loss of nigro-striatal dopaminergic innervation (Albin et al. 1989; Alexander et al. 1990). The first evidence was obtained in 6-OHDA hemilesioned rats where both L-DOPA and J-113397 (1 mg/kg) elevated nigral GABA levels, and their combination caused an additive effect,

which correlated with an additive antiakinetic effect (Marti et al. 2007). Interestingly, the additive increase of nigral GABA was prevented by perfusion of the voltage-dependent sodium channel blocker tetrodotoxin (TTX) indicating that GABA levels monitored by microdialysis were indeed the result of neuronal activity. This GABA facilitating effect was later replicated by systemic administration of Trap-101 (10 mg/kg) (Marti et al. 2008) and GF-4 (1 mg/kg) (Volta et al. 2010b) as well as by reverse dialysis of Trap-101 (10 μM) (Marti et al. 2008) and Compound 24 (3 μM) (Volta et al. 2011) in SNr. This latter approach, in particular, proved that NOP receptors located in SNr tonically inhibit GABA release in this area.

4.3 NOP Receptor Antagonists and Nigro-Thalamic GABAergic Transmission

Whether the changes of GLU and GABA levels induced by NOP antagonists in SNr effectively impacted over the activity of nigro-thalamic GABAergic neurons and, through them, on motor function, was specifically addressed in dual probe microdialysis studies coupled to behavioral testing (Marti et al. 2007, 2008; Volta et al. 2010b, 2011) where one probe was implanted in the lesioned SNr and another in the ipsilateral ventro-medial thalamus (VMTh), which is a target of nigral projections. We first showed that GABA release in VMTh was reduced (~30%) by intranigral perfusion with TTX, indicating that GABA levels were partly due to nigro-thalamic neuron activity (Marti et al. 2007). Consistently, blockade of nigro-thalamic activity with TTX also reduced the immobility time, since it disinhibited thalamo-cortical projections (Marti et al. 2007). A similar effect was produced by the combination of L-DOPA plus J-113397 that, in addition to elevating GABA and reducing GLU in SNr (see above), also reduced GABA in VMTh (Marti et al. 2007). These effects were occluded by TTX, suggesting that these neurochemical changes reflected ongoing neuronal activity and the involvement of nigro-thalamic neurons. To confirm this view, TTX also prevented the reduction of nigral GLU release induced by the L-DOPA/J-113397 combination (Fig. 2). The involvement of the nigro-thalamic pathway in the motor promoting action of NOP antagonists was further proven by reverse dialysis of the $GABA_A$ receptor antagonist bicuculline in SNr (Marti et al. 2007). In fact, we reasoned that if an elevation of GABA in SNr was responsible for the inhibition of nigro-thalamic neurons, blockade of nigral $GABA_A$ receptors, which are expressed by nigro-thalamic neurons, would prevent this effect. In fact, bicuculline did not block the rise of nigral GABA induced by L-DOPA plus J-113397 but prevented its inhibitory effect over nigro-thalamic activity and thalamic GABA levels, and also abolished its behavioral correlate (i.e., the antiakinetic effect) (Marti et al. 2007). Bicuculline also delayed, although it did not abolish, the inhibitory effect on GLU release, possibly indicating that the potentiation of nigral GABA rather than the inhibition of nigral GLU was instrumental for the antiakinetic effect (Marti et al. 2007).

The role of nigral NOP receptors on nigro-thalamic transmission was further proven by directly perfusing the NOP antagonists Trap-101 (Marti et al. 2008) and

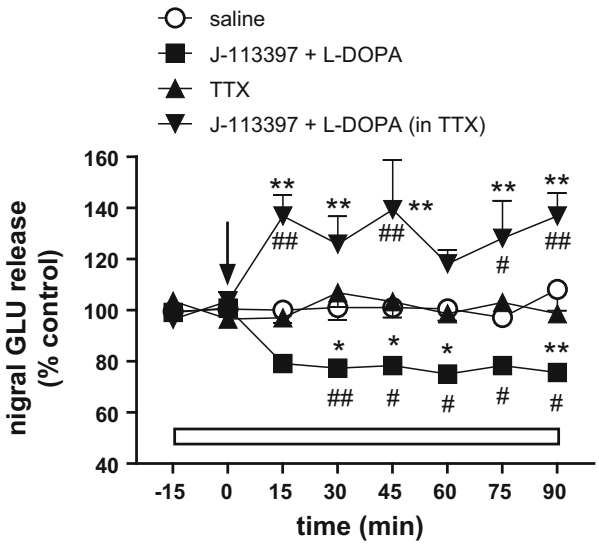

Fig. 2 Tetrodotoxin (TTX) prevented the reduction of GLU release in SNr induced by combined administration of L-DOPA and J-113397. Perfusion with TTX (1 μM; open bar) in the SNr started 90 min before systemic (i.p.) co-administration (arrow) of J-113397 (1 mg/kg) and L-DOPA (1 mg/kg plus benserazide 15 mg/kg). Data are means ± SEM of 5–6 experiments per group. Statistical analysis was performed by two-way ANOVA with repeated measures followed by the Bonferroni test. $^{*}p < 0.05$, $^{**}p < 0.01$ different from saline; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ different from TTX

Compound 24 (Volta et al. 2011) in SNr. In both studies, not only did the NOP antagonists reduce nigral GLU and elevate nigral GABA (see above) but they also reduced thalamic GABA along with akinesia, directly proving that blockade of nigral NOP receptors promotes movement by overinhibiting the nigro-thalamic input. Consistently, when combined with L-DOPA, Trap-101 produced a larger reduction of thalamic GABA (Marti et al. 2008).

Reverse dialysis of Compound 24 in SNr also provided valuable information on the mechanisms underlying the *motor inhibiting* effect caused by high doses of NOP receptor antagonists (Volta et al. 2011). In fact, perfusion with a high concentration (3 μM) of Compound 24 increased akinesia and evoked neurochemical changes opposite to those associated with a 100-fold lower, antiakinetic concentration, i.e., reduction of GABA release in SNr and elevation of GABA release in VMTh (a tendency for an elevation of GLU release in SNr was also evident). Reverse dialysis of the D2 receptor antagonist raclopride in combination with Compound 24 demonstrated that nigral DA was involved in the inhibitory action. In fact, when nigral D2 receptors were blocked, 3 μM of Compound 24 reversed its action, reducing akinesia, it also elevated nigral GABA and reduced nigral GLU and thalamic GABA, very much like 0.03 μM of Compound 24 in the absence of raclopride. Conversely, the lower concentration of Compound 24 became behaviorally and neurochemically ineffective in the presence of raclopride (a significant

reduction of GABA release in SNr was detected, though). Therefore, nigral DA regulates the responsiveness of nigro-thalamic GABA neurons to the NOP antagonists. Using a combined pharmacological and genetic approach, we have next demonstrated that motor facilitation induced by NOP antagonists involves D2 postsynaptic receptors and motor inhibition D2 presynaptic receptors (Viaro et al. 2013).

5 NOP Receptor Ligands in PD Models: Neuroprotective Efficacy

Cox and collaborators provided the first evidence that endogenous N/OFQ contributes to parkinsonian degeneration (Marti et al. 2005). In fact, ppN/OFQ$^{-/-}$ mice were reported to be more resistant than ppN/OFQ$^{+/+}$ mice to the neurotoxic action of acute MPTP, showing a greater number of DA neurons and striatal DA terminals spared a week after acute MPTP administration. Since no changes of MPTP metabolism or uptake were observed in ppN/OFQ mice (Marti et al. 2005), the authors attributed the different responsiveness to MPTP to a possible neurotoxic role of endogenous N/OFQ. Interestingly, N/OFQ was not effective against methamphetamine-induced neurotoxicity, which is mainly targeted to striatal terminals, suggesting that N/OFQ could attenuate MPTP-induced toxicity acting at the nigral level (Brown et al. 2006). Since ppN/OFQ codes for other two biologically active neuropeptides beyond N/OFQ, namely, N/OFQ II and nocistatin, we thought mandatory to confirm the toxicity of endogenous N/OFQ in NOP$^{-/-}$ mice (Arcuri et al. 2016). Indeed, NOP$^{-/-}$ mice responded to acute MPTP (4 × 25 mg/kg i.p., every 90 min) exactly as ppN/OFQ$^{-/-}$ mice, showing greater resistance to the toxin than NOP$^{+/+}$ mice (50 vs 75% dopamine neuron loss, respectively). The greater resistance to MPTP was also accompanied by better motor performances in the bar and drag tests (Arcuri et al. 2016). The idea that endogenous N/OFQ could play a neurotoxic role in PD was further corroborated using a clinically driven study design in more progressive models, which allow a window for therapeutic intervention. In these experiments, the NOP receptor antagonist SB-612111 was used, and its administration was delayed with respect to the neurotoxic insult, as it occurs in the clinics where the patient comes to the attention of the neurologist when motor symptoms appear, i.e., far later the disease has started its course. Thus, SB-612111 (10 mg/kg, twice daily for 10 days starting at the 4th day after the onset of MPTP) was capable of preventing the nigro-striatal degeneration induced by subacute MPTP administration (25 mg/kg, i.p., once daily for 7 days) (Arcuri et al. 2016). Moreover, SB-612111 (1 mg/kg, twice daily for 8 weeks, commencing a week after AAV2/9 h-α-syn injection) attenuated the nigro-striatal neurodegeneration induced by α-syn overexpression. The percentage of DA cells spared was significantly greater in SB-612111-treated (50%) than in saline-treated (25%) rats. Considering that about 50% of nigral DA cells die a week after AAV2/9 hα-syn injection, i.e., at the time when SB-612111 administration is commenced, this is a remarkable result.

The proof that exogenous N/OFQ is harmful for DA neurons was provided by our laboratory in collaboration with O'Keeffe laboratory (Collins et al. 2015). N/OFQ (1 μM) and the NOP agonist UFP-112 (3 μM), ineffective alone, were able to potentiate the toxic action of 6-OHDA on SH-SY5Y cell viability, this effect being reversed by NOP antagonists, UFP-101 and SB-612111. Remarkably, N/OFQ alone exerted detrimental effects on neuronal survival and complexity (neurite length and branching) in primary cultures of DA neurons, being as effective as the parkinsonian toxins MPP^+ and 6-OHDA (Collins et al. 2015). In addition, N/OFQ caused additive effects when combined with either parkinsonian neurotoxin. N/OFQ effects were observed at relatively low concentrations (10–500 nM) and were specifically reversed by SB-612111, indicating that they were mediated by NOP receptors.

Studies are ongoing to identify the mechanisms underlying the neurotoxic pathways activated by N/OFQ. Since exogenous N/OFQ elevates whereas NOP receptor antagonists reduce glutamate release in the rodent SNr (see Sect. 4.1), we first hypothesized that endogenous N/OFQ can cause DA neuron degeneration through GLU-mediated excitotoxicity (Brown et al. 2006; Marti et al. 2005). Indeed, changes in mitochondrial potential due to inhibition of complex I by MPP+ (the active metabolite of MPTP) lead to oxidative stress and GLU-mediated excitotoxicity, which contribute to degeneration of DA neurons (Meredith and Rademacher 2011; Serra et al. 2002). In addition, NOP receptor antagonists can modulate N/OFQ-induced microglial activation (Laudenbach et al. 2001). This is particularly relevant for PD, since neuroinflammation plays an important role in neurodegeneration (Nolan et al. 2013; Poewe et al. 2017). N/OFQ modulates the inflammatory and microglial responses, although both pro- and anti-inflammatory effects of N/OFQ have also been described (Mallimo and Kusnecov 2013). Indeed, NOP receptor seems to bidirectionally modulate the expression and release of cytokines. In particular, it has been proven that N/OFQ inhibits the production of pro-inflammatory cytokines such as IL-6, IL1β, and TNFα in different tissues and cell types, including glial cells. On the contrary, prolonged activation of the NOP receptor causes a dramatic activation of NF-kB, a key modulatory transcription factor of the pro-inflammatory response (Toll et al. 2016). How NOP receptor exerts its action on microglia is not clear. Since cytokine activation and NOP receptor signaling share a common transduction pathway, i.e., the mitogen activated protein kinase (MAPK) pathway, we can speculate a cross-talk between NOP and cytokine signals in the modulation of the inflammatory response. Preliminary data in support of this hypothesis come from the study of O'Keeffe and colleagues (Collins et al. 2015), showing that N/OFQ inhibits the survival and growth of DA neurons in cultures through the p38-MAPK cascade. The p38-MAPK signaling is known to be implicated in different neurodegenerative diseases through its regulatory action on apoptosis and inflammation (Cuenda and Rousseau 2007; Zarubin and Han 2005) and increased phospho-p38 levels have been shown in the SNc DA neurons of PD patients (Karunakaran et al. 2008).

6 Conclusions

Major unmet clinical needs in the field of PD are a disease-modifying therapy, a good pharmacological control over non-motor symptoms, among which cognitive impairment, depression, pain, dysautonomias (e.g., orthostatic hypotension and stipsis) and drugs preventing the development of motor complications associated with L-DOPA therapy (e.g., dyskinesia). Preclinical data strongly suggest that the N/OFQ-NOP receptor system is a novel target in PD therapy and, in particular, that NOP receptor antagonists might provide both symptomatic and neuroprotective/neurorescue benefits, also acting as L-DOPA sparing agents. Although the risk of worsening L-DOPA-induced dyskinesia in advanced, complicated PD patients, or to accelerate dyskinesia development in de novo PD patients treated with L-DOPA, should be weighed, this might be overcome by careful titration of the dose of NOP receptor antagonist. Alternatively, we have proposed NOP receptor partial agonists as a possible alternative to NOP receptor antagonists (Marti et al. 2013). In fact, we proved that N/OFQ or NOP receptor agonists Ro 65-6590 or AT-403 improve established dyskinesia in L-DOPA-primed rats or nonhuman primates (Arcuri et al. 2018; Marti et al. 2012), acting on NOP receptor expressed in striatum where N/OFQ tone is low (Marti et al. 2012). Therefore, an NOP receptor partial agonist would improve motor deficits acting on NOP receptors in SNr, where N/OFQ is abnormally elevated, without exacerbating, or perhaps even ameliorating, L-DOPA-induced dyskinesia through stimulation of up-regulated striatal NOP receptors. Interestingly, the therapeutic benefit afforded by an NOP antagonist might extend over non-motor symptoms of PD. In fact, endogenous N/OFQ also contributes to depression (Gavioli and Calo 2013; Post et al. 2016), cognitive impairment (Khan et al. 2018; Redrobe et al. 2000) as well as to impairment of cardiovascular (bradycardia and hypotension) (Malinowska et al. 2002) and gastrointestinal (reduced motility) (Sibaev et al. 2015) functions. Consistently, NOP receptor antagonists were shown to improve depression in a number of preclinical tests and also in humans (Gavioli and Calo 2013; Post et al. 2016). The availability of the first orally active NOP receptor antagonist (LY2940094) (Toledo et al. 2014) has opened the way for the first clinical trial in PD, which has been press launched early 2018. We are eagerly awaiting for the results of this study to confirm that NOP receptor antagonists might really represent a new hope for PD patients (Arcuri et al. 2017).

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NOP Ligands for the Treatment of Anxiety and Mood Disorders

Elaine C. Gavioli, Victor A. D. Holanda, and Chiara Ruzza

Contents

Abstract

Many studies point toward the nociceptin/orphanin FQ (N/OFQ) and the N/OFQ peptide receptor (NOP) as targets for the development of innovative drugs for treating anxiety- and mood-related disorders. Evidence supports the view that the activation of NOP receptors with agonists elicits anxiolytic-like effects, while its blockade with NOP antagonists promotes antidepressant-like actions in rodents. Genetic studies showed that NOP receptor knockout mice display an antidepressant-like phenotype, and NOP antagonists are inactive in these animals.

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In contrast, the genetic blockade of NOP receptor signaling generally displays an increase of anxiety states in the elevated plus-maze test. In this chapter we summarized the most relevant findings of NOP receptor ligands in the modulation of anxiety and mood disorders, and the putative mechanisms of action are discussed.

Keywords
Animal behavior · Anxiety · Depression · Nociceptin/orphanin FQ · NOP receptor · Stress

Abbreviations

ACTH	Adrenocorticotropic hormone
BDNF	Brain-derived neurotrophic factor
BNST	Bed nucleus of the stria terminalis
CRF	Corticotropin-releasing factor
DRL	Differential reinforcement of low rate schedule
DRN	Dorsal raphe nucleus
FGF-2	Fibroblast growth factor
HPA	Hypothalamus-pituitary-adrenal axis
Icv	Intracerebroventricular
IL-6	Interleukin-6
J-113397	1-[(3R,4R)-1-(cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one
JTC-801	N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl) benzamide hydrochloride
LPS	Bacterial lipopolysaccharide
LY2940094	[2-[4-[(2-chloro-4,4-difluoro-spiro[5Hthieno[2,3-c]pyran-7,4′-piperidine]-1′-yl)methyl]-3-methylpyrazol-1-yl]-3-pyridyl] methanol
N/OFQ	Nociceptin/orphanin FQ
NOP(−/−)	Mice knockout for the NOP receptor
ppN/OFQ	N/OFQ precursor
ppN/OFQ(−/−)	Mice knockout for the N/OFQ precursor
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus of hypothalamus
Ro 64-6198	(1S,3aS)-8-(2,3,3a,4,5,6-Hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one
Ro 65-6570	(RS)-8-(1,2-Dihydro-1-acenaphthylenyl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one
SB-612111	(5S,7S)-7-[[4-(2,6-dichlorophenyl)-1-piperidinyl]methyl]-6,7,8,9-tetrahydro-1-methyl-5H-benzocyclohepten-5-ol
SNP	Single-nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor

TNF-α	Tumor necrosis factor-α
UFP-101	[Nphe^{1}, Arg^{14}, Lys^{15}] N/OFQ-NH_2

1 Introduction

In this chapter we will summarize the most relevant literature findings which give support to the role played by nociceptin/orphanin FQ (N/OFQ) and its receptor NOP in the modulation of anxiety- and mood-related disorders. This peptidergic system is highly expressed in brain areas relevant to the processing of emotions, such as hippocampus, prefrontal cortex, hypothalamus, amygdala, and the monoaminergic nuclei into the brainstem (Boom et al. 1999; Mollereau and Mouledous 2000; Neal et al. 1999a, b). This suggests the NOP receptor as innovative pharmacological target for the treatment of stress-related disorders, including anxiety, major depression, post-traumatic stress disorder (PTSD), and others. Briefly, a growing body of evidence suggests that the N/OFQ system plays opposite roles in the modulation of anxiety and mood disorders (Gavioli and Calo' 2006). In fact, the activation of the NOP receptor signaling consistently evokes anxiolytic-like effects, while its blockade robustly causes antidepressant-like actions. The literature findings supporting this view are summarized in this chapter.

2 N/OFQ-NOP Receptor System and Anxiety

Fear and anxiety are physiological responses evoked during stressful events or under a real threat in order to cope with a harmful situation, thus elevating the chances of survival. Still in this context, both states overlap, being fear conceptualized as a set of autonomic and behavioral responses to the imminent real or perceived threat, while anxiety is the anticipation of a future threat (Steimer 2002). In some individuals anxiety responses may become uncontrollable, excessive, and inappropriate, even after withdrawal of the stimulus, lacking any adaptive value and negatively influencing the quality of everyday life (APA 2013). A meta-regression analyses estimated the 12-month prevalence for "any" anxiety disorder in 7.3% in the world population, being twice more common in females than males (Baxter et al. 2013). One direct implication of anxiety disorders is the reduction in work capacity and rise in labor absences that lead to direct and indirect social and economic costs. Given the current imperfections in pharmacological therapies, there is a crucial need for studies focusing on understanding the pathophysiological aspects of anxiety and at the same time, those aimed at the design of novel alternatives for the treatment of these disorders. In this context, the N/OFQ-NOP receptor system is a compelling and novel pharmacological target for the treatment of anxiety and stress-related disorders.

2.1 Clinical Studies

Only few clinical data support the idea of a relationship between the N/OFQ-NOP system and anxiety states. In particular, one study reported a link between the NOP receptor and PTSD. In fact, Andero and colleagues reported that, in humans, a single-nucleotide polymorphism (SNP) of the NOP receptor is associated with a self-reported history of childhood trauma and PTSD symptoms after a traumatic event. This polymorphism was also associated with altered fear learning and fear discrimination mechanisms, and magnetic resonance imaging analysis revealed differential amygdala-insula functional connectivity in those individuals expressing the alternative protein (Andero et al. 2013). It is widely known that amygdala nuclei are activated by fear stimuli and it is a core area related to the processing of emotions (Bornhövd et al. 2002).

2.2 NOP Ligands: Behavioral Studies

2.2.1 NOP Agonists

Few years after the discovery of N/OFQ, Jenck and colleagues from Hoffman-La Roche pharmaceutics reported that intracerebroventricular (icv) administration of N/OFQ (0.1–3 nmol) reduced anxiety-associated behaviors in rodents assessed in several assays, including light-dark and elevated plus-maze tests, exploratory behavior in a unfamiliar environment, urocortin-induced anxiogenic-like state, and operant conflict test (Jenck et al. 1997). This study showed for the first time that the activation of the NOP receptor by an agonist may elicit acute anxiolytic effects. Jenck's findings were confirmed and extended in the following years by different research groups. N/OFQ reduced anxiety in mice when directly confronted with a natural threat (a rat) in the defense test battery (Griebel et al. 1999). Anxiolytic-like effects were also obtained in mice in the elevated plus-maze (Gavioli et al. 2002; Asth et al. 2016), hole-board (Kamei et al. 2004), and elevated T-maze (Asth et al. 2015) tests and in rats in the conditioned defensive burying and elevated plus-maze test (Vitale et al. 2006; Aujla et al. 2013; Filaferro et al. 2014).

Despite the large amount of evidence supporting anxiolytic effects due to the activation of NOP receptor signaling, there are two preclinical studies sustaining an increase of anxiety-related behaviors for N/OFQ injected in the lateral ventricle, amygdala, or BNST in rats (Fernandez et al. 2004; Green et al. 2007) in the elevated plus-maze, open-field, and light-dark aversion tests, concomitant with reduction in the spontaneous locomotion. Similarly, Vitale and colleagues described that N/OFQ ($>$1 nmol, icv) reduced exploration to open arms in the rat elevated plus-maze test. However, they interpreted the effects of N/OFQ as inhibition of locomotion (Vitale et al. 2006) instead of increase of anxiety states. Thus, the effects of N/OFQ on anxiety, mainly at higher doses, may be confounded by locomotor impairments.

With the aim to identify innovative anxiolytic drugs, an explosion of drug discovery efforts was directed at the identification of small-molecule NOP ligands (for a review of non-peptide NOP ligands, see Mustazza and Bastanzio (2011) and

Toll et al. (2016)). The available non-peptide NOP agonists which display anxiolytic-like properties are summarized in Table 1. The most widely studied compound is Ro 64-6198 that promoted, after systemic administration, anxiolytic-like effects in rats, mice, and guinea pigs subjected to a variety of animal models of anxiety (Jenck et al. 2000; Varty et al. 2005; Nicolas et al. 2006, 2007; Chang et al. 2015). Additionally, Ro 64-6198 produced marked reduction of anxiety in response to a variety of mild to strong anxiogenic stimuli, such as in the Vogel conflict punished drinking test in Sprague Dawley rats, in the social approach-avoidance test in Lewis rats, in the novelty-induced hypophagia in C57BL/6J mice, and in stress-induced hyperthermia in NMRI mice (Goeldner et al. 2012). The anxiolytic action of Ro 64-6198 is due to the NOP receptor activation, since it was inactive in NOP receptor knockout (NOP(−/−)) mice (Varty et al. 2005). Additionally, the administration of Ro 64-6198 lacked abuse liability in a self-stimulation paradigm (Le Pen et al. 2002), and no signs of tolerance to the anxiolytic-like effects were detected following 15 days of treatment (Dautzenberg et al. 2001).

As far as PTSD is concerned, intra-central amygdala injections of N/OFQ significantly and selectively reduced anxiety-like behavior evoked by restraint in rats assessed in the elevated plus-maze test (Ciccocioppo et al. 2014). Similarly, the systemic and central amygdala infusion of SR-8993, a new highly selective NOP agonist, impaired fear memory consolidation, when injected 30 min before or immediately after fear conditioning, in mice exposed to a single severe stress, a model of PTSD-like behavior (Andero et al. 2013). These data suggest that activation of the NOP receptor signaling may be useful as prevention for PTSD after a stressful event.

It is worth of mention that NOP agonists can impair motor performance and locomotor activity (Marti et al. 2004; Sandin et al. 1997). This effect can bias the interpretation of behavioral studies and, more importantly, can represent a side effect limiting the development of NOP agonists as anxiolytics. Of note, Ro 65-6570, an analogue of Ro 64-6198, caused anxiolytic-like effects in the elevated plus maze at doses tenfold lower than those able to modify motor performance of mice (Asth et al. 2016). This suggests that this behavioral effect of NOP agonists is genuine. Anyway, while developing NOP agonists as innovative anxiolytic drugs, the possibility of a narrow therapeutic index should be taken into account.

2.2.2 NOP Antagonists

While the anxiolytic effects of NOP agonists are robust and consistent among different laboratories, species, and models, the effects of NOP antagonists on anxiety states are debated and strongly assay-dependent. In the majority of the studies, NOP antagonists were inactive (Gavioli and Calo' 2006; Lu et al. 2011; Varty et al. 2005, 2008; Vitale et al. 2006; Uchiyama et al. 2008a, b). In the elevated T-maze test, the icv administration of UFP-101 (1–10 nmol), a peptide NOP antagonist, reduced the latency of inhibitory avoidance, indicating an anxiolytic-like effect. N/OFQ (0.3 nmol) prevented the UFP-101 (1 nmol)-anxiolytic actions, demonstrating that this action occurs via central NOP receptors (Duzzioni et al. 2011). Recently, the profile of the orally active NOP antagonist LY2940094 developed by the

Table 1 Effects of non-peptide NOP receptor agonists in preclinical models of anxiety

Assay	Compound	Species and strain	Effects	References
Elevated plus-maze test	Ro 64-6198	Wistar and SD rat	↑ Time spent and distance moved in open arms	Jenck et al. (2000) and Dautzenberg et al. (2001)
	SCH 221510	CD-1 mouse	↑ Time spent in open arms	Varty et al. (2008)
	Compound 1c	Rat	↑ Time spent and distance moved in open arms	Wichmann et al. (1999)
	Compound 3c	Rat	↑ Time spent and distance moved in open arms	Wichmann et al. (2000)
	SCH 221510	Gerbil	↑ Time spent in open arms	Varty et al. (2008)
	Compound 1	Long-Evans and Hooded rat	↑ Time spent in open arms	Ross et al. (2015)
	Ro 65-6570	CD-1 mouse and NOP (−/−) mouse	↑ Time spent and number of entries in open arms; no effects in NOP(−/−) mice	Asth et al. (2016)
	SR-8993	Wistar rat	↑ Time spent in open arms in naive and after chronic alcohol consumption	Aziz et al. (2016)
	AT-090	CD-1 mouse and NOP (−/−) mouse	↑ Time spent and number of entries in open arms; no effects in NOP(−/−)	Asth et al. (2016)
Isolation-induced vocalizations	Ro 64-6198	CD-1 mouse	↓ Number and duration of vocalization	Varty et al. (2005)
	Ro 64-6198	Hartley guinea pig	↓ Number of vocalization	Varty et al. (2005)
	SCH 221510	Dunkin-Hartley guinea pig	↓ Separation-induced vocalizations in pups	Varty et al. (2008)
	Compounds 15 and 16	Hartley guinea pig	↓ Number of vocalization	Yang et al. (2009)
	SCH 655842	Dunkin-Hartley guinea pig	↓ Separation-induced vocalizations in pups	Lu et al. (2011)

Conditioned lick suppression	Ro 64-6198	CD-1 mouse	↑ Number of punished licks	Varty et al. (2005)
	SCH 221510	CD-1 mouse	↑ Number of punished licks	Varty et al. (2008)
	Compounds 15 and 16	Rat	↑ Number of punished licks	Yang et al. (2009)
	Compound 24	Rat	↑ Number of punished licks	Ho et al. (2009)
	SCH 655842	CD-1 mouse	↑ Number of punished licks	Lu et al. (2011)
	Ro 64-6198	Wistar rat	↑ Number of punished responses	Jenck et al. (2000)
	Ro 64-6198	SD rat	↑ Drinking time	Goeldner et al. (2012)
	SCH 221510	CD-1 mouse	↑ Number of punished licks	Varty et al. (2008)
	MCOPPB	ddY mouse	↑ Number of punished responses	Hirao et al. (2008b)
	PCPB	ddY mouse	↑ Number of punished responses	Hirao et al. (2008a)
Marble-burying test	Ro 64-6198	C57BL/6J mouse	↓ Marble-burying behavior	Nicolas et al. (2006)
	SCH 655842	C57BL/6J mouse	↓ Marble-burying behavior	Lu et al. (2011)
Ultrasound-induced defensive behaviors	Ro 64-6198	Lister-hooded rat	↓ Freezing behavior	Nicolas et al. (2007)
Fear-potentiated auditory startle	Ro 64-6198	Wistar rat	↓ Startle responses	Jenck et al. (2000)
Panic-like anxiety test	Ro 64-6198	Wistar rat	No effects	Jenck et al. (2000)
Open-field test	Ro 64-6198	Mouse	↑ Time spent in the center	Chang et al. (2015)
Social approach-avoidance	Ro 64-6198	Lewis rat	↑ Time spent in the social compartment	Goeldner et al. (2012)
Novelty-induced hypophagia	Ro 64-6198	C57BL/6J mouse	↓ Latency to drink and increase milk intake	Goeldner et al. (2012)
Stress-induced hyperthermia	Ro 64-6198	NMRI mouse	↓ Stress-induced hyperthermia	Goeldner et al. (2012)

AT-090 1-(1-((1s,4s)-4-iso-propylcyclohexyl)piperidin-4-yl)indoline-2,3-dione, *MCOPPB* 1-[1-(1-methylcyclooctyl)-4-piperidinyl]-2-[(3R)-3-piperidinyl]-1H-benzimidazole, *PCPB* 2-(3,5-dimethylpiperazin-1-yl)-1-[1-(1-methylcyclooctyl)piperidin-4-yl]-1H-benzimidazole, *Ro 64-6198* (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, *Ro 65-6570* (RS)-8-(1,2-dihydro-1-acenaphthylenyl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, *SCH 221510* 3-endo-8-[bis(2-methylphenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octan-3-ol, *SCH 655842* endo-8-[bis(2-chlorophenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octane-3-carboxamide, *SR-8993* 1-[1-(cyclooctylmethyl)-4-piperidinyl]-5-fluoro-2-(3R)-3-pyrrolidinyl-1H-benzimidazole

pharmaceutical industry (Eli Lilly) was investigated in several animal models of anxiety. LY2940094 attenuated fear-conditioned immobility in mice and stress-induced hyperthermia in rats, with a minimal effective dose of 30 mg/kg (Witkin et al. 2016). However, this compound was inactive in other behavioral assays in which conventional antidepressants and benzodiazepines were active, such as the rat-conditioned emotional response, the mouse four-plate test, and rat novelty-suppressed feeding assay (Witkin et al. 2016). Additionally, LY2940094 did not display any behavioral alteration when evaluated under the Vogel conflict test in rats or marble-burying in mice (Post et al. 2016). Taken together, the NOP antagonist, LY2940094, produced anxiolytic effects in behavioral assays that only partially overlap with the effects evoked by conventional anxiolytics and selective 5-HT reuptake inhibitors. Further studies aimed to evaluate the anxiolytic effects of NOP antagonists after repeated administrations are needed to investigate the efficacy of these compounds.

NOP antagonists were also tested as potential pharmacological interventions for PTSD. The repeated administration of the NOP antagonist JTC-801 (6 mg/kg, ip, 14 days) reversed the anxiety-like, nociceptive-related behaviors and hypocortisolism induced by a single-prolonged stress (Zhang et al. 2015). In addition, elevated N/OFQ levels in serum, cerebrospinal fluid, periaqueductal gray matter, and hippocampus at day 21 of single-prolonged stress were blocked by 14 days of JTC-801 administration (Zhang et al. 2015). Repeated injections of JTC-801 treatment also reversed NOP receptor protein and mRNA up-regulation in amygdala and periaqueductal gray matter (Zhang et al. 2015). More recently, Genovese and Dobre (2017) showed that predator exposure produced a relatively long-lasting deficit in the exploration of the open arms of the elevated plus maze. Acute administration of J-113397 mitigated this performance deficit in a dose-dependent manner. Importantly, the largest dose of J-113397, administered in animals without predator exposure, was essentially devoid of effects on anxiety (Genovese and Dobre 2017). Thus, NOP antagonists can significantly mitigate the effects of a stressful event and may provide effective treatment for PTSD and stress-induced anxiety states.

2.3 Genetic Blockade of the NOP Receptor Signaling

Evidence from the genetic blockade of the NOP receptor signaling, by using knockout animals, also implicates the N/OFQ-NOP receptor system in the control of anxiety. The first study comes from mice lacking the N/OFQ precursor (ppN/OFQ) gene (ppN/OFQ(−/−) mice). These animals spent less time in open or unprotected areas compared to wild-type mice in the open-field, plus-maze, and light-dark aversion tests, suggesting an anxiogenic-like phenotype (Koster et al. 1999). Additionally, ppN/OFQ(−/−) mice displayed increased emotional responses when individually housed mice were crowded together (5/cage). Under these conditions, mice lacking ppN/OFQ gene developed greater anxiety-like behaviors in the light-dark box and acoustic startle test (Ouagazzal et al. 2003). To interpret

these findings, it is relevant to mention that the ppN/OFQ gene encodes other bioactive peptides, such as nocistatin and nociceptin II; thus, the anxiogenic phenotype of ppN/OFQ(−/−) mice cannot be exclusively attributed to the depletion of N/OFQ.

As far as the blockade of the NOP receptor is concerned, both studies with antisense oligonucleotides and NOP(−/−) mice and rats are available. The infusion of antisense oligonucleotides targeting the NOP receptor significantly reduced expression of NOP in some brain areas, as the paraventricular nucleus, prefrontal cortex, and septum (Blakley et al. 2004). These rats showed increased anxiety-related behaviors in the elevated plus-maze test (Blakley et al. 2004). Concerning the NOP(−/−) rodents, several studies have been performed with animals using distinct genetic background, and the main robust findings are that NOP(−/−) mice and rats display increased anxiety-related behavior in the elevated plus-maze test (Gavioli et al. 2007; Sakoori and Murphy 2009; Rizzi et al. 2011). Moreover, NOP(−/−) mice showed anxiolytic-like phenotype in the novelty-suppressed feeding behavior and elevated T-maze tests, while no behavioral phenotype was found in the open-field, hole-board, marble-burying, and stress-induced hyperthermia assays (Gavioli et al. 2007). No data are available about the anxiety levels of NOP(−/−) mice under stressful conditions.

2.4 Mechanisms of Action of NOP Agonists on Anxiety

A large amount of data demonstrated that the NOP agonist-induced relief of anxiety states is prevented by the co-administration of NOP antagonists (Gavioli and Calo' 2006; Varty et al. 2005, 2008; Vitale et al. 2006; Uchiyama et al. 2008a, b; Lu et al. 2011). Likewise, antianxiety-like effects of NOP agonists are not produced in NOP(−/−) mice (Asth et al. 2016; Varty et al. 2005). These findings demonstrate that the mechanism by which this class of compounds exerts anxiolytic effects is the selective activation of the NOP receptor. Additionally, the microinfusion of N/OFQ into the central amygdala, but not in the dorsal hippocampus, reduced anxiety in naive and stressed rodents (Uchiyama et al. 2008b; Goeldner et al. 2010: Ciccocioppo et al. 2014). Therefore, the NOP receptors expressed in the central amygdala are particularly relevant for the anxiolytic effects of NOP agonists.

Another putative mechanism underlying the anxiolytic-like effects of NOP agonists is the ability of this peptide to functionally counteract the corticotropin-releasing factor (CRF) actions on CRF1 receptors (Jenck et al. 1997; Rodi et al. 2008; Ciccocioppo et al. 2014; Filaferro et al. 2014). Evidence coming from microinjection studies indicate that the bed nucleus of the stria terminalis (BNST) is the brain area where this functional antagonism operates (Rodi et al. 2008). Serotonin (5-HT) is a neurotransmitter that displays an essential role in the regulation of emotion. A close relationship between the 5-HTergic and the N/OFQergic systems can be suggested based on the fact that NOP receptors are located on 5-HTergic neurons in the dorsal raphe nucleus (Le Maitre et al. 2005). N/OFQ delivered into the dorsal raphe reduced the 5-HT outflow (Tao et al. 2007), produced

an increase of K^+ currents in neurons (Vaughan and Christie 1996), and inhibited their firing rates (Nazzaro et al. 2010). The activation of 5-HT signaling from the dorsal raphe nucleus to the BNST, via actions at 5-HT_{2C} receptors, engages a CRF inhibitory microcircuit into the BNST that silences anxiolytic BNST outputs to the ventral tegmental area and lateral hypothalamus, therefore increasing anxiety (Marcinkiewcz et al. 2016). Thus, it may be hypothesized that N/OFQ could evoke anxiolytic effects by reducing the 5-HT availability and firing rates in the dorsal raphe and concomitantly by counteracting the anxiogenic CRF actions into the BNST (Fig. 1). It is worth mentioning that the BNST contains high levels of NOP mRNA and injection of N/OFQ during ex vivo slice electrophysiological analyses of BNST neurons confirmed that more than half of BNST neurons express functional NOP receptors (Dawe et al. 2010). Future studies specifically aimed at understanding the effects of NOP agonists in the dorsal raphe nucleus in anxiety states are needed.

From an intracellular point of view, little is known about the biochemical pathways leading to the NOP agonist-induced anxiolytic effects. A recent study by Asth and colleagues demonstrated that only NOP agonists able to recruit the β-arrestin 2 protein evoked anxiolytic-like behavior in mice in the elevated plus-maze test (Asth et al. 2016). This study suggests that the β-arrestin 2-dependent signal, rather than the G protein-dependent cascade, is important for the NOP receptor-mediated anxiolytic effects. In the near future, studies with NOP-biased agonists and β-arrestin knockout mice will confirm or deny this interesting hypothesis.

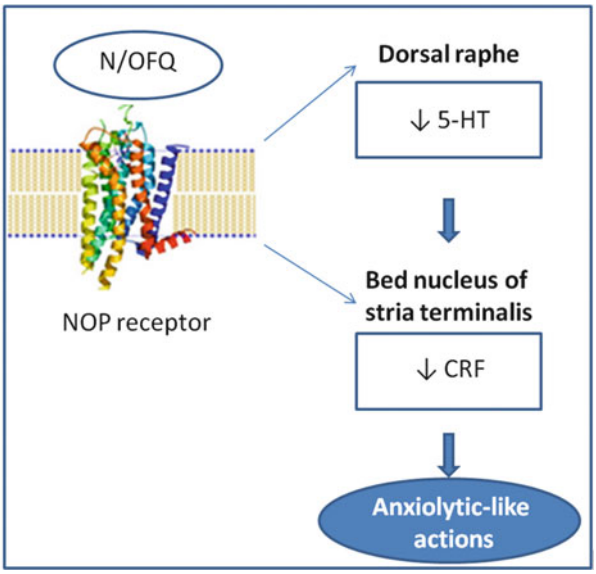

Fig. 1 Putative mechanisms of the anxiolytic-like effects of NOP agonists. The activation of the NOP receptor induces anxiolytic effects by reducing the 5-HT availability and firing rates in the dorsal raphe and concomitantly by counteracting the anxiogenic CRF actions into the bed nucleus of stria terminalis

3 N/OFQ-NOP Receptor System and Mood Disorders

Depression is the second most prevalent psychiatric disorder, after anxiety, leading to substantial negative impact on the quality of life. A meta-analysis study estimated the 12-month prevalence for major depression in about 5% in the world population (Ferrari et al. 2013). Major depression affects people at any age, and it is twofold more prevalent in women than in men (Van de Velde et al. 2010). Depressed mood and loss of interest in daily activities (anhedonia) are the key symptoms observed in depressed patients. Moreover, altered motivational behavior, appetite, and sleep, for example, may be experienced by a patient with depression (APA 2013).

The pharmacotherapy of depression is costly and widely prescribed by physicians. However, significant limitations were reported, such as considerable side effects, delayed onset of antidepressant action, and low efficacy after pharmacological treatment (Berton and Nestler 2006). These claims emphasize the need to identify innovative antidepressants. Neuropeptidergic systems represent an important target for the development of antidepressants (Werner and Coveñas 2010).

3.1 Clinical Studies

The N/OFQ-NOP receptor system is considered a potential candidate to modulate mood-related states. Evidence from humans suggests an association of depressive mood disorders and elevated plasma N/OFQ (Gu et al. 2003; Wang et al. 2009). In fact, plasma N/OFQ levels were significantly elevated in women with postpartum depression and in bipolar depression patients. In subjects with bipolar mania, N/OFQ plasma levels were significantly lower than those of the control group. These pilot clinical studies suggest that during a depressive state, N/OFQ levels are consistently increased. However, larger studies are required to confirm and extend these preliminary findings. Regarding NOP ligands, recently, an 8-week, double-blind, placebo-controlled trial evaluated the NOP antagonist LY2940094, developed by Eli Lilly and Company (Toledo et al. 2014), as a novel oral treatment for major depression (Post et al. 2016). Once-daily oral dosing of LY2940094 at 40 mg for 8 weeks vs placebo provided some evidence for an antidepressant effect assessed by the GRID-Hamilton Depression Rating Scale (Post et al. 2016). The authors reported that the onset of the LY2940094 antidepressant actions in humans are quite similar to conventional monoaminergic drugs, since weeks of treatment are required to promote the therapeutic efficacy (Post et al. 2016). These findings constitute the first human data providing evidence that the blockade of NOP receptor signaling represents an innovative strategy for the treatment of major depression.

3.2 NOP Ligands: Behavioral Studies

3.2.1 NOP Antagonists

In 2002, Redrobe and colleagues reported for the first time the potential antidepressant effects of two chemically unrelated NOP receptor antagonists. In this study, the peptide [Nphe^1]N/OFQ(1–13)NH_2 and the non-peptide J-113397 reduced the immobility time of mice in the forced swimming test (Redrobe et al. 2002). As summarized in Table 2, these initial findings were confirmed and extended with the peptide NOP receptor antagonist UFP-101 and the non-peptide SB-612111, which has been reported to induce antidepressant-like effects in different species (rat and mouse) and behavioral despair assays, i.e., forced swimming and tail suspension tests (Gavioli et al. 2003, 2004; Rizzi et al. 2007). Another structural distinct NOP antagonist LY2940094 was studied for the treatment of major depression. This compound when acute orally administered in rats reduced immobility time in the forced swimming test (Post et al. 2016). At lower doses LY2940094 also augmented the behavioral effects of fluoxetine without changing target occupancies (NOP and serotonin reuptake transporter) (Post et al. 2016).

To increase the translationality of preclinical findings to human, in the last years, studies aiming to evaluate the effects of NOP antagonists in animal models of depression, which mimic in rodents, some of the depressive symptoms reported by patients were performed. In this context, the mouse learned helplessness model was used, which is characterized by uncontrollable, unpredictable, and inescapable electric footshocks. After such exposure, most of them fail to escape from the electrified chamber upon subsequent presentation of shock (these animals are named helpless); antidepressant treatment reverses this behavior (Pryce et al. 2011). In helpless mice, acute treatment with UFP-101 (3–10 nmol) and SB-612111 (3–10 mg/kg) significantly and selectively reduced escape latencies and escape failures. In fact, no effects of drug treatments were observed in mice subjected to the controllable electric footshocks and non-stressful situations (Holanda et al. 2016).

Various medical conditions that involve activation of the immune system are associated with psychological and neuroendocrine changes that resemble the characteristics of depression. In particular the "inflammatory theory of depression" points to the immune system and the inflammatory response as potentially important contributors to the pathophysiology of depression. In this context, increasing evidence has indicated that immune challenge by bacterial lipopolysaccharide (LPS) induces a depressive-like state and neuroinflammatory responses that are restored by antidepressants (for a review, see Dantzer et al. (2008)). Medeiros et al. (2015) showed that the acute treatment with distinct NOP antagonists, UFP-101 and SB-612111, when injected 24 h after LPS, reversed LPS-induced depressive-like behavior in mice, measured as immobility time in the tail suspension test (Medeiros et al. 2015). However, when the NOP receptor antagonist SB-612111 was injected 30 min prior LPS, it did not modify LPS-induced sickness signs and depressive-like behavior.

Table 2 Effects of NOP receptor antagonists in preclinical models of depression

Assay	Compound	Species and strain	Effects	References
Forced swimming test	UFP-101	Swiss and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Gavioli et al. (2003, 2004)
	UFP-101	Wistar rat	↓ Immobility time	Gavioli et al. (2004)
	[Nphe^1]N/OFQ(1–13)-NH_2	CD-1 mouse	↓ Immobility time	Redrobe et al. (2002)
	UFP-113	Swiss mouse	↓ Immobility time	Asth et al. (2016)
	[F/G]N/OFQ(1–13) NH_2	Swiss mouse	↓ Immobility time	Asth et al. (2016)
	J-113397	CD-, Swiss, and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Redrobe et al. (2002) and Gavioli and Calo' (2006)
	SB-612111	Swiss and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Rizzi et al. (2007)
	LY2940094	NIH-Swiss and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Post et al. (2016) and Witkin et al. (2016)
Tail suspension test	UFP-101	Swiss mouse	↓ Immobility time	Gavioli et al. (2004)
	SB-612111	Swiss mouse	↓ Immobility time	Rizzi et al. (2007)
DRL-72	LY2940094	SD rat	No effect	Witkin et al. (2016)
Chronic mild stress	UFP-101	Wistar rat	↑ Sucrose solution intake and ↓ immobility time after 21 days of treatment	Vitale et al. (2009, 2017)
Learned helplessness	SB-612111 and UFP-101	Swiss mouse	↑ Escapes and ↓ escape latencies	Holanda et al. (2016, 2018)
LPS-induced depressive-like behavior	SB-612111 and UFP-101	Swiss and CD-1 mouse	↓ Immobility time	Medeiros et al. (2015)

DRL differential reinforcement of low rate schedule, *J-113397* 1-[(3R,4R)-1-(cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one, *LPS* bacterial lipopolysaccharide, *LY2940094* [2-[4-[(2-chloro-4,4-difluoro-spiro[5Hthieno[2,3-c]pyran-7,4′-piperidine]-1′-yl)methyl]-3-methylpyrazol-1-yl]-3-pyridyl]methanol, *SB-612111* (5S,7S)-7-[[4-(2,6-dichlorophenyl)-1-piperidinyl]methyl]-6,7,8,9-tetrahydro-1-methyl-5H-benzocyclohepten-5-ol, *UFP-101* [Nphe^1, Arg^{14}, Lys^{15}] N/OFQ-NH_2

Until now, two studies have reported the effects of the repeated treatment with a NOP antagonist in rodents subjected to a chronic mild stressful situation (Vitale et al. 2009, 2017). Prolonged exposure to mild stressors promotes changes in animal behavior related to the core symptoms of depression, i.e., anhedonia, which are assessed by evaluating the reduction of sucrose preference consumption in rodents (Willner 1997). Vitale et al. (2009, 2017) have demonstrated that 21 days of icv UFP-101 restored sucrose preference consumption and reversed the increase of immobility time in the forced swimming test, which were blocked by the co-administration of N/OFQ (Vitale et al. 2009). Of note, UFP-101 also reversed the misbalance in 5-HT turnover rates in the frontal cortex and pons and the elevation in serum corticosterone levels induced by unpredictable chronic stress (Vitale et al. 2009). More recently, Vitale et al. (2017) showed that chronic mild stress reduced neural stem cell proliferation and neurogenesis in adult rat hippocampus. Repeated treatment with UFP-101 did not affect the reduced cell proliferation in stressed rats, which was restored by fluoxetine. However, similar to the standard antidepressant fluoxetine, UFP-101 increased the number of doublecortin-positive cells, thus restoring neurogenesis. UFP-101 and fluoxetine also significantly increased fibroblast growth factor (FGF-2) expression, reduced by chronic stress (Vitale et al. 2017). These findings support the view that blockade of NOP receptors produces antidepressant-like effects associated with restoration of neurogenesis and FGF-2 expression highly impacted by chronic mild stress.

Despite the promising antidepressant effects of the NOP antagonist LY2940094 in rats and humans (Post et al. 2016), distinct from imipramine, LY2940094 was inactive under a differential reinforcement of low rate schedule (DRL-72) (Witkin et al. 2016). The DRL schedules have been used to evaluate the effects of a wide variety of drugs, including amphetamines, cannabinoids, and antidepressants. To earn a reinforcer, organisms operating under a DRL schedule are required to withhold a response for a predetermined amount of time before responding, and therefore this schedule maintains a low rate of responding and can be viewed as a response-inhibition task (Kirshenbaum et al. 2008). Thus, lack of effects for LY2940094 in the DRL-72 model may suggest that the blockade of the NOP receptor might not control impulsivity (Marek et al. 2016). These findings indicate that the blockade of the NOP receptor signaling overlaps biologically with some, but not all, of the substrates underlying the antidepressant-like effects of monoamine antidepressants (Witkin et al. 2016).

3.2.2 NOP Agonists

The effects of the NOP receptor activation were also investigated in behavioral despair assays. N/OFQ given icv did not induce any behavioral change in mice (Redrobe et al. 2002), but when co-administered, it reversed the antidepressant-like effect induced by the NOP receptor antagonists UFP-101, SB-612111, and J-113397 (Gavioli et al. 2003, 2004; Gavioli and Calo' 2006; Rizzi et al. 2007). Similarly, the non-peptide NOP receptor agonists Ro 64-6198 (Goeldner et al. 2012) and Ro 65-6570 (Holanda et al. 2018), given systemically, did not change rodent behavior in the rat forced swimming and mouse tail suspension tests and in the

learned helplessness model, respectively. Additionally, Ro 65-6570 completely blocked the antidepressant effects of NOP antagonists in the learned helplessness model (Holanda et al. 2018). These observations suggest that the selective block of the NOP receptor is the mechanism by which NOP antagonists promote antidepressant-like effects. Additionally, our research group recently reported that the NOP receptor activation inhibits the acute antidepressant effects of nortriptyline and fluoxetine, but not those of *R*-ketamine, assessed in the forced swimming and learned helplessness model (Holanda et al. 2018). We hypothesized that NOP agonists impair central monoaminergic transmission under stressful conditions neutralizing the antidepressant effects of drugs with monoaminergic-dependent mechanisms of action. These observations are important to reinforce the hypothesis of inhibitory effects on the monoaminergic neurotransmission subsequent to the activation of the NOP receptor. Additionally, considering the high rate of comorbidity between anxiety and mood disorders and the complexity of these diseases that often required a multitarget therapy, these data may be relevant in the future for the clinical use of NOP ligands.

3.3 Genetic Blockade of the NOP Receptor Signaling

In agreement with the antidepressant profile evoked by the pharmacological blockade of the NOP receptor, the genetic depletion of the NOP receptor gene also elicits an antidepressant phenotype in mice and rats. NOP(−/−) mice displayed reduced baseline immobility time in the forced swimming and tail suspension tests when compared to wild-type mice (Gavioli et al. 2003, 2004). Similar results were observed for NOP(−/−) rats which displayed antidepressant-like phenotype in the forced swimming test (Rizzi et al. 2011). Knockout studies have also shown that the effects of NOP receptor antagonists were mediated by the NOP receptor. Treatment with UFP-101, J-113397, SB-612111, and LY2940094 reduced immobility time in wild-type, but not in NOP(−/−), mice (Gavioli et al. 2003; Gavioli and Calo' 2006; Rizzi et al. 2007; Witkin et al. 2016).

Our research group also evaluated the behavior of NOP(−/−) mice in LPS-induced sickness and depressive-like states. LPS evoked similar sickness signs and significantly increased tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) plasma levels 6 h post-injection in wild-type and NOP(−/−) mice. However, LPS treatment evoked depressive-like effects in mice expressing the NOP receptor but not in NOP(−/−) mice (Medeiros et al. 2015), thus supporting an antidepressant-like phenotype for LPS-challenged NOP(−/−) mice. As mentioned by Medeiros et al. (2015), the resistance of mice lacking the NOP receptor to the effects of LPS can be attributed to the different responsiveness to the LPS-induced neurobiological modifications that lead to the development of the depressive-like phenotype. Further studies aimed at investigating the behavioral phenotype of NOP(−/−) in the learned helplessness model are in progress in our laboratories. Taken together, genetic findings from preclinical studies suggest that the blockade of N/OFQ signaling induces robust antidepressant-like effects.

3.4 Mechanisms of Action of NOP Antagonists on Mood

A considerable amount of data demonstrates that the mechanism by which NOP antagonists evoke antidepressant effects is the exclusive blockade of the NOP receptor. In fact, these effects were reversed by NOP agonists (Gavioli et al. 2003, 2004; Rizzi et al. 2007; Vitale et al. 2009; Asth et al. 2016), and the effects of the antagonists were not observed in NOP(−/−) mice (Gavioli et al. 2003, 2004; Post et al. 2016). As far as the brain areas involved in the antidepressant actions of NOP antagonists is concerned, a behavioral study suggested the hippocampal formation as a relevant site for the antidepressant actions of this class of molecules. In fact, the bilateral administration of UFP-101 into the dorsal hippocampus reduced immobility time in behavioral despair tests (Goeldner et al. 2010).

Regarding the neurochemical mechanisms, a link between the N/OFQergic system and the monoaminergic transmission is now well documented. A considerable amount of in vitro and in vivo data supports an inhibitory effect of endogenous N/OFQergic signaling on monoaminergic neurotransmission in brain areas relevant to mood disorders. In vitro N/OFQ and NOP agonists reduce monoamine release in synaptosomes and brain slice preparations (Schlicker and Morari 2000). Postsynaptic inhibitory effects of N/OFQ on noradrenergic and serotonergic neurons have been reported in whole-cell patch clamp studies (Connor et al. 1996; Vaughan and Christie 1996). In this assay, the NOP antagonist UFP-101 is able to prevent the N/OFQ-induced increase of K^+ currents, being inactive per se (Gavioli et al. 2004). Thus, these cellular actions of N/OFQ result in reduced neuronal firing of the monoaminergic neurons projecting to the cortex. In agreement with these ideas, a microdialysis study performed in freely moving rats has shown that the NOP agonist N/OFQ-NH_2 injected into rat locus coeruleus inhibited noradrenaline release from the prefrontal cortex (Okawa et al. 2001). Additionally, N/OFQ injected into the dorsal raphe nucleus reduced 5-HT outflow in the dorsal raphe and nucleus accumbens (Tao et al. 2007). Furthermore, treatment with NOP antagonists may restore the chronic mild stress-induced misbalance of 5-HT turnover in the pons and cerebral cortex (Vitale et al. 2009). Taken together, the literature data strongly suggest that NOP receptor antagonists might elicit antidepressant-like effects by counteracting the inhibitory effects of the endogenous N/OFQ on monoaminergic systems at both pre- and postsynaptic levels, thus potentiating the monoaminergic signal (Fig. 2). In line with this, microdialysis studies showed that the single oral administration of the NOP receptor antagonist LY2940094 increased 5-HT, but not dopamine and noradrenaline, levels in the rat prefrontal cortex (Post et al. 2016). [$Nphe^1$]N/OFQ(1–13)NH_2 injected in the dorsal raphe nucleus increased extracellular 5-HT in the same area (Tao et al. 2007). Indeed, systemic administration of J-113397 also increased noradrenaline efflux in the amygdala, which was suppressed by local infusion of N/OFQ (Kawahara et al. 2004). In this context, a slight but significant increase of monoamine availability is reported in microdialysis studies after administration of NOP antagonists.

The hypothalamus-pituitary-adrenal (HPA) axis has been implicated in the pathogenesis of affective disorders (Leonard 2005). A long-lasting desensitization of the

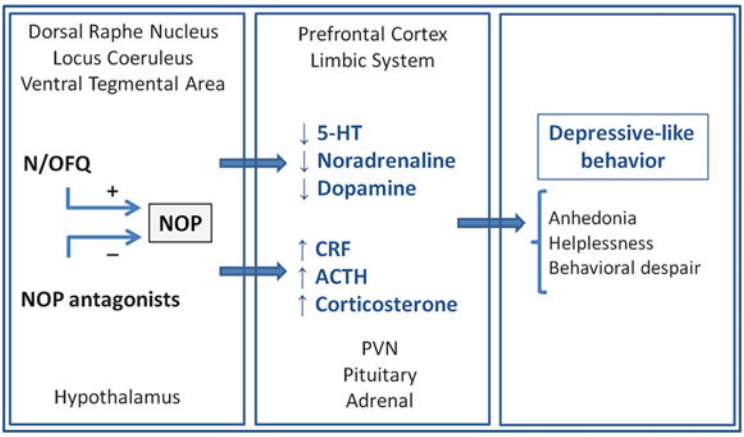

Fig. 2 Putative mechanisms by which NOP antagonists induce antidepressant actions. The activation of the NOP receptor signaling in monoaminergic neurons contributes to the reduction of monoamine levels in the prefrontal cortex and limbic areas. In the hypothalamus-pituitary-adrenal (HPA) axis, NOP receptor activation increases corticotropin-releasing factor (CRF) into the paraventricular nucleus of hypothalamus (PVN) and circulating adrenocorticotrophic hormone (ACTH) and corticosterone levels. NOP antagonists counteract the endogenous activation of the NOP receptor, thus restoring monoamines and stress hormone levels

negative feedback of the HPA axis has been reported both in chronically stressed rats and clinically depressed patients (Aguilera et al. 1994; Tafet and Bernardini 2003). Therefore, as discussed in Gavioli and Calo' (2013), maladaptive physiological responses due to stress could upregulate N/OFQergic signaling in the brain. Of note, Vitale et al. (2009) showed increased corticosterone levels in rats chronically exposed to mild stress situations, and the chronic administration of UFP-101 reduced the stress-induced increase in serum corticosterone levels. It agrees with the notion that classical antidepressants can restore elevated serum corticosterone and adrenal hypertrophy induced by chronic stress (Reul et al. 1993; McEwen 2005). The effects of stress on N/OFQergic signaling could involve a close relationship between 5-HT, CRF, and N/OFQ neurotransmission. Additionally, inhibitory effects of N/OFQ on dorsal raphe 5-HT neurons were significantly increased after acute forced swim stress as showed by in vitro neurochemical and in vivo electrophysiological studies (Nazzaro et al. 2010). The effects of stress-induced potentiation of N/OFQergic signaling were abolished by a CRF1 receptor antagonist (Nazzaro et al. 2010). Still regarding the relationship between N/OFQ and HPA axis, the central (icv) and intra-BNST injection of N/OFQ in naïve rats increases the activation of the HPA axis, as suggested by the increase in corticosterone and adrenocorticotropic hormone plasma levels (Devine et al. 2001; Nicholson et al. 2002; Fernandez et al. 2004; Vitale et al. 2006; Green et al. 2007) and CRF mRNA in paraventricular hypothalamic nucleus and pro-opiomelanocortin mRNA in the anterior pituitary (Leggett et al. 2006). Thus, it seems that during stressful events, the endogenous N/OFQergic transmission is tonically activated and contributes to increase the HPA axis besides decrease

monoaminergic transmission; altogether these actions are underlying a depressive-like state (Fig. 2).

A link between depression and impaired adult neurogenesis in the hippocampus is now well documented. A reduced hippocampal volume has been observed in depressed patients, and altered monoaminergic brain levels, resulting from classical antidepressant treatment, are shown to have strong reinforcing effects on adult neurogenesis. Based on the Vitale's findings (Vitale et al. 2017), it is conceivable that NOP antagonists can evoke antidepressant actions by restoring hippocampal neurogenesis and increasing the expression of neuronal factors such as FGF-2. These actions are in some manner similar to the effects of the conventional antidepressant fluoxetine (Vitale et al. 2017).

From an intracellular point of view, Asth et al. (2016) demonstrated that the blockade of NOP mediated β-arrestin is required for the induction of the antidepressant-related behavioral effects of NOP antagonists. β-arrestin dependence is similar to what was reported for the anxiolytic effects of NOP agonists (Asth et al. 2016). Thus, for the emotional effects of NOP ligands, the β-arrestin-dependent signal seems to be more relevant than the G protein-dependent cascade. Of note, the involvement of the blockade of β-arrestin 2 signal has been already reported for the antidepressant effects of classical mood stabilizer drugs (Golan et al. 2010). Anyway, as stated for NOP receptor agonists, further studies are needed to better elucidate the role played by β-arrestin 2 in the antidepressant effects of NOP antagonists and, more in general, to understand the role of this protein in the etiopathology of stress-related disorders.

4 Concluding Remarks and Further Perspectives

A growing body of preclinical and clinical studies from different research groups suggested that the activation of NOP receptors with agonists induces anxiolytic-like effects, while their blockade with antagonists promotes antidepressant-like actions. Additionally, preclinical data support the idea that NOP antagonists can be useful in some particular anxiety states, i.e., PTSD. The mechanism(s) by which NOP receptor signaling regulates anxiety and depression is still unknown, and some potential explanations were discussed in this chapter. Further studies are needed to better elucidate the mechanisms by which the endogenous N/OFQ regulates anxiety states and mood. In particular the recent findings about the role of β-arrestin 2-dependent pathways in the anxiolytic and antidepressant actions of NOP ligands open the street toward new lines of research and raise questions about the use of NOP-biased ligands as innovative, more effective/safer anxiolytic and antidepressant drugs. Another question to be addressed is if NOP antagonists can induce antidepressant actions by acting on neurotransmitter systems different from monoamine, such as glutamate or other modulators of neuronal plasticity (i.e., BDNF, NogoA, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein).

It is well known that anxiety and mood disorders are very complex diseases, triggered by a number of intrinsic and/or environmental factors and that often

required a combination therapy. In this view issues should be addressed regarding the possible clinical advantages of NOP ligands compared to classical anxiolytic and antidepressant drugs and the possibility of co-administering NOP ligands with classical drugs to improve their profile of pharmacological effects. An important question in this field is if NOP antagonists are able to evoke faster antidepressant effects than SSRI and tricyclic antidepressant, thus overcoming one of the major limitations of this therapeutics.

In conclusion, with the availability of potent and selective NOP ligands (agonists and antagonists) and of animals lacking ppN/OFQ or the NOP receptor, a large amount of preclinical studies now delineate the NOP protein as a pharmacological target for the generation of innovative anxiolytics or antidepressants. In the near future, preclinical studies will better elucidate some biochemical aspects, still not clear, on the ways by which the N/OFQ-NOP system and NOP ligands modulate emotional states. Nevertheless, some issues will be addressed only with the identification of good small molecules (i.e., LY2940094) suitable to be tested in humans. In fact, only clinical studies can firmly answer questions about the time course of effects and provide insight into the therapeutic advantages of the use of NOP ligands, alone or in combination, for ameliorating the conditions of anxious and depressed patients.

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The Nociceptin/Orphanin FQ System and the Regulation of Memory

Lionel Moulédous

Contents

Abstract

Nociceptin/orphanin FQ (N/OFQ) is an endogenous neuropeptide of 17 amino acids, related to opioid peptides but with its own receptor, distinct from conventional opioid receptors, the ORL1 or NOP receptor. The NOP receptor is a G protein-coupled receptor which activates Gi/o proteins and thus induces an inhibition of neuronal activity. The peptide and its receptor are widely expressed in the central nervous system with a high density of receptors in regions involved in learning and memory. This review describes the consequences of the pharmacological manipulation of the N/OFQ system by NOP receptor ligands on

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learning processes and on the consolidation of various types of long-term memory. We also discuss the role of endogenous N/OFQ release in the modulation of learning and memory. Finally we propose several putative neuronal mechanisms taking place at the level of the hippocampus and amygdala and possibly underlying the behavioral amnestic or promnesic effects of NOP ligands.

Keywords
Amygdala · Drug-induced amnesia · Hippocampus · Long-term memory · Nociceptin/orphanin FQ · Promnesic compound

1 Introduction

Nociceptin, also called orphanin FQ (N/OFQ), is an endogenous peptide involved in numerous physiological functions at the level of the nervous, cardiovascular, respiratory, gastrointestinal, urinary, and immune systems (Lambert 2008). Its receptor, ORL1 for opioid receptor-like 1 or NOP, was first cloned by homology with opioid receptors (Mollereau et al. 1994). It is a G protein-coupled receptor of the rhodopsin family that has very strong homologies with classical mu (MOP), delta (DOP), and kappa (KOP) opioid receptors. However, it has a very low affinity for conventional opioid ligands such as morphine or enkephalins which initially made it an orphan receptor. A 17-amino-acid peptide corresponding to N/OFQ was soon purified from rat and pig brain (Meunier et al. 1995; Reinscheid et al. 1995). The discovery of this system is therefore one of the first examples of reverse pharmacology. The peptide is very similar, in terms of sequence and charge, to the endogenous KOP agonist dynorphin A. It is derived from a protein precursor capable of releasing other peptides whose function remains unknown (Mollereau et al. 1996). The binding of N/OFQ to the NOP receptor leads to the activation of Gi/o inhibitory G proteins, with consequent inhibition of adenylyl cyclase and voltage-gated calcium channels, and activation of GIRK (inwardly rectifying) potassium channels (New and Wong 2002).

The development of ligands specific for the NOP receptor made it possible to study in preclinical models the major physiological functions and pathologies in which it is involved. At the level of the nervous system, the most promising, in terms of therapy, are the following (Lambert 2008): pain, drug dependence, Parkinson's disease, anxiety, depression, and memory. Indeed the NOP receptor has a very wide distribution in the central nervous system (Mollereau and Mouledous 2000). It is present in the cortex, the thalamus, and the limbic system [including the hippocampus (HPC), the septum, the bed nucleus of the stria terminalis (BNST), the amygdaloid complex, the hypothalamus, and monoaminergic nuclei (raphe nucleus, locus coeruleus, ventral tegmental area, substantia nigra)]. The neurons producing the precursor have a slightly more restricted distribution (Reinscheid et al. 2000), with strong expression in the BNST, the medial preoptic area, the lateral septum, and the medial and central amygdala (CeA). This distribution strongly suggests a role of the N/OFQ system at the interface between the control of stress and emotions

Table 1 Main behavioral paradigms used to assess the memory-modulating properties of the N/OFQ system

Name	Description of the task	References
Morris water maze (MWM)	Used to assess spatial memory. The mouse is placed in a pool of water where it must learn to use spatial cues located in the room to navigate to a submerged platform. The time to reach the platform decreases across trials, and during the probe test, when the platform is removed, animals spend more time in the quadrant where the platform was located. The visible platform version of the test allows to assess nonspatial components such as swimming ability and procedural memory	Higgins et al. (2002), Koster et al. (1999), Kuzmin et al. (2009), Manabe et al. (1998), Redrobe et al. (2000), Sandin et al. (1997, 2004)
Fear conditioning (FC)	Used to assess aversive associative memory. It is a form of Pavlovian conditioning based on the association of an aversive stimulus (an electric shock) with a conditioned stimulus, the context in which the shock was received (contextual FC), or a discrete cue such as a sound (tone FC). During the retention test, the freezing behavior (conditioned response) triggered by the presentation of the context or the sound is measured	Andero et al. (2013), Fornari et al. (2008), Goeldner et al. (2009), Mamiya et al. (2003), Ouagazzal (2015), Rekik et al. (2017)
Inhibitory avoidance (IA)	Used to assess aversive associative memory. Also called passive avoidance. The mouse receives a foot shock when it enters a dark compartment (step-through version) or steps down a platform (step-down version). During the retention test, the animal has to inhibit its natural tendency to enter the secure dark environment or leave the aversive platform. If it remembers receiving the electric shock, the step-through or step-down latency should increase	Adem et al. (2017), Hiramatsu and Inoue (1999, 2000), Hiramatsu et al. (2008), Liu et al. (2007), Manabe et al. (1998), Miwa et al. (2009, 2010), Roozendaal et al. (2007)
Object recognition (OR)	Used to assess recognition memory. During the learning phase, the mouse is allowed to explore two identical objects in an open field. During the test phase, one of the objects is replaced by a new one. If the animal detects the change, and thus recognizes only the familiar object, it will spend more time exploring the new one	Goeldner et al. (2008)

(continued)

Table 1 (continued)

Name	Description of the task	References
Y-maze, spontaneous alternation	Used to assess spatial working memory. The mouse is put in the center of a Y-maze and allowed to explore it freely without any reward. If its spatial working memory is intact, an animal is supposed to alternate regularly between the three arms in order to optimize its exploration strategy	Hiramatsu and Inoue (1999, 2000), Mamiya et al. (1999), Miwa et al. (2009), Ouagazzal (2015)

(Fulford 2015; Gavioli and Calo 2013; Witkin et al. 2014) and memory processes (Andero 2015; Noda et al. 2000; Ouagazzal 2015) that are the main focus of this review article.

2 Pharmacological Modulation of Learning and Memory by NOP Agonists

2.1 N/OFQ Affects Different Types of Long-Term Memory

The first study on the effect of N/OFQ on memory was performed in rats and focused on spatial memory. It showed that the intra-hippocampus administration (in the CA3 region) of 10 nmol of the peptide almost completely blocked the acquisition in the Morris water maze (MWM) (see Table 1 for a description of the behavioral paradigms). However, the possibility of confounding effects, notably related to a disturbance of the exploratory behavior of the animal by the peptide, was not totally ruled out (Sandin et al. 1997). It was subsequently shown that a lower dose of 3.3 nmol injected into the HPC produced the same inhibition of learning without negative effect on exploration (Sandin et al. 2004). Normal learning in the visible platform version of the test also enabled the authors to rule out other confounding effects related to sensory perception or motivation. In addition, the co-administration of the NOP antagonist [Nphe^1]N/OFQ(1–13)-NH_2 (Calo et al. 2000) showed that this deleterious action on spatial learning was indeed mediated by the NOP receptor (Redrobe et al. 2000). The same negative impact on memory acquisition, specifically in the spatial version of the MWM, was observed in mice at doses of 5 and 10 nmol after intra-cerebroventricular (ICV) and 1 nmol after intra-CA3 injection (Kuzmin et al. 2009). Here again the consequences of N/OFQ injection were prevented by the administration of the [Nphe^1]N/OFQ(1–13)-NH_2 antagonist.

Other types of memory are also affected by the ICV or intracerebral administration of the peptide. This was the case for contextual memory in the contextual fear conditioning test (CFC) for ICV doses of 0.01–1 nmol in mice (Mamiya et al. 2003) and 1–2.5 nmol in rats (Fornari et al. 2008). The latter study also demonstrated that this amnestic effect was not due to a phenomenon of state dependence, meaning

an integration of the interoceptive properties of the drug in the memory trace, since the memory was not restored when the test was performed in the presence of N/OFQ. On the other hand, N/OFQ was shown to be less active in the tone fear conditioning (TFC) paradigm (Mamiya et al. 2003) except at high dose (5 nmol) in rats (Fornari et al. 2008). Inhibitory avoidance (IA) is another aversive memory paradigm in which animal performances are affected by N/OFQ. In mice, 0.5–5 nmol administered ICV during the acquisition produced a decrease in the step-down latency during the retention test (Hiramatsu and Inoue 1999). A similar effect was observed for a dose of 0.5 nmol in the step-through version of the test in rats, this amnestic action being blocked by the co-administration of 1 nmol of [Nphe^1]N/OFQ(1–13)-NH_2 (Hiramatsu et al. 2008). Moreover ICV doses of 1 and 4 nmol delayed the acquisition in a multi-trial version of IA in mice, and this effect was again prevented by [Nphe^1]N/OFQ(1–13)-NH_2 (Liu et al. 2007). Signs of amnesia were also observed when the inhibitory avoidance phenomenon was evaluated in the elevated T-maze test (Asth et al. 2015). In rats, N/OFQ has also been injected intra-basolateral amygdala (BLA) in an IA paradigm, and doses of 1–100 pmol have been shown to negatively affect memory retention performance (Roozendaal et al. 2007). The last type of memory on which the effect of N/OFQ has been tested is recognition memory. In the mouse object recognition (OR) test, the peptide injected ICV (from 1 nmol) or intra-HPC (3 nmol, dorsal HPC) before learning induced memory deficits when retention was evaluated 24 h later (Goeldner et al. 2008).

2.2 Amnestic Effects of Systemic Administration of NOP Agonists

Since the discovery of the N/OFQ system, several small systemically active NOP receptor agonists have been identified (Toll et al. 2016; Zaveri 2003). In the context of learning and memory, the vast majority of studies have been based on systemic administration of the NOP agonist Ro 64-6198 [(1S, 3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triazaporo[4.5]decan-4-one], a compound developed by Roche (Wichmann et al. 2000). Overall, all the effects of ICV administration of N/OFQ described above could be reproduced in rodents by intraperitoneal (IP) administration of Ro 64-6198 in a dose ranging from 0.3 to 3 mg/kg. Specifically, in mice, the compound impaired spatial learning in the MWM (Higgins et al. 2002; Kuzmin et al. 2009), fear conditioning to the context, but not to the tone (including in an immediate shock deficit paradigm which eliminates a possible confounding role of the anxiolytic properties of the NOP agonist) (Goeldner et al. 2009), learning in inhibitory avoidance (only at high dose) (Adem et al. 2017), as well as object recognition memory (Goeldner et al. 2008).

It is worth noting that the interpretation of the results obtained with Ro 64-6198 is complicated by the appearance of a sedative action for high doses with an impairment in motor performances (Jenck et al. 2000). This confounding effect has been excluded in some studies, for example, by showing that learning was unaltered in the visible platform version of the MWM (Higgins et al. 2002;

Kuzmin et al. 2009) or that short-term memory was unaffected in the object recognition test (Goeldner et al. 2008). Beyond this putative nonspecific neurological impairment, it must also be taken into account that the selectivity of Ro 64-6198 for the NOP receptor is not optimal and that it interacts in particular, although with a 100-fold lower affinity, with the other members of the opioid receptor family (Jenck et al. 2000). Thus the inhibitory effect of the compound at 3 mg/kg in IA learning was not blocked by the [Nphe^1]N/OFQ(1–13)-NH_2 antagonist (Adem et al. 2017). Overall, it can be concluded that the effective doses are slightly higher than the doses producing anxiolytic effects and slightly lower than those provoking sedation (Jenck et al. 2000; Varty et al. 2005), indicating a relatively narrow therapeutic window for the amnestic action of the reference small molecule NOP agonist. It is therefore necessary to continue to improve the catalog of small NOP receptor agonist molecules, especially in terms of selectivity. In this framework, a recent study using the new compound SR-8993 (3 mg/kg IP) in the fear conditioning paradigm gave results partially in agreement with the reported effects of Ro 64-6198. Like the latter, SR-8993 inhibited context conditioning, but contrary to Ro 64-6198, it also attenuated tone conditioning (Andero et al. 2013). This latest report also showed that the amnestic properties of the NOP agonist were conserved in a mouse model of dysregulated fear (Andero et al. 2013).

2.3 Different Phases of Long-Term Memory Can Be Targeted

In most of the studies mentioned so far, treatment with NOP agonists was carried out before learning, and it was therefore difficult to know whether the amnestic effects observed were due to an inhibition of memory acquisition (encoding), consolidation (stabilization of the memory trace), or both. In the paradigms based on multiple trial learning like the MWM, it has been clearly demonstrated that the activation of NOP receptors interferes with the acquisition phase of the task (Higgins et al. 2002; Kuzmin et al. 2009; Redrobe et al. 2000; Sandin et al. 2004). This inhibition of acquisition could be linked to a perturbation of spatial working memory. Indeed, ICV administration of 0.5–5 nmol of N/OFQ decreased the performances, evaluated by spontaneous alternation, in the Y-maze (Hiramatsu and Inoue 1999). Similarly, using a multi-trial IA protocol, it was shown that ICV N/OFQ delayed the acquisition of the task in mice (Liu et al. 2007). For the other paradigms for which NOP agonists have been tested, the data suggest also an impairment of the memory consolidation phase. N/OFQ injected ICV in mice after conditioning inhibited long-term memory retention in FC (Mamiya et al. 2003). The SR-8993 and Ro 65-6570 agonists also exhibited amnestic properties in FC when administered immediately after conditioning (Andero et al. 2013; Rekik et al. 2017). Similarly in IA in rats, intra-BLA injection of 1–100 pmol of N/OFQ immediately or 3 h (but not 6 h) post-training impaired retention performance (Roozendaal et al. 2007). Finally, in the mouse OR paradigm, pretreatment with Ro 64-6198 disrupted the long-term memory tested 24 h after learning but did not affect the short-term memory tested at 3 h, which also suggests an action on the consolidation phase (Goeldner et al. 2008).

It therefore seems that, depending to the type of memory considered, systemic or central activation of NOP receptors may interfere with the acquisition phase of memory, especially in spatial tasks and/or in procedures based on multi-trial learning, or with its consolidation, especially for aversive and recognition memory.

The effects of NOP receptor activation on the later phases of long-term memory processes have been poorly studied. At doses known to affect the acquisition or consolidation processes, the agonists Ro 64-6198 and Ro 65-6570 did not inhibit memory retrieval in the object recognition and contextual fear conditioning paradigms, respectively, in mice (Goeldner et al. 2008; Rekik et al. 2017). Under certain circumstances, memory retrieval can cause a destabilization of the memory trace. The memory must then go through a process called reconsolidation to be stabilized again over time (Alberini and Ledoux 2013; Nader 2015). It has recently been shown that NOP agonists administered immediately after memory reactivation inhibit the reconsolidation of contextual fear memory in mice (Rekik et al. 2017). This effect was produced by both N/OFQ (3 nmol ICV) and small molecule agonists Ro 65-6570 (1 mg/kg IP) and AT-403 (0.1 mg/kg IP), a recently discovered compound showing a high affinity and selectivity for NOP receptors (Ferrari et al. 2017). On the other hand, at the same doses, the two small agonist molecules were ineffective in interfering with the reconsolidation of tone fear memory suggesting that, as with fear memory consolidation, NOP receptor activation is more effective in interfering with contextual than cued fear memory reconsolidation (Rekik et al. 2017).

2.4 Promnesic Effects of NOP Agonists

Some studies have shown that very low doses of ICV N/OFQ (10–100 fmol) could prevent the deleterious action of scopolamine in models of working memory (spontaneous alternation in Y-maze) and IA (Hiramatsu and Inoue 2000). Such promnesic effects have even been reported for doses as low as 1 fmol after intra-HPC injection (Miwa et al. 2009). However, it has since been shown that these properties were not mediated by the NOP receptor as they persisted in receptor KO mice and the involvement of a metabolite of the peptide has been suggested (Miwa et al. 2010).

Other reports have demonstrated biphasic effects of ICV (Adem et al. 2017) and intra-HPC (Sandin et al. 2004) injection of N/OFQ. Thus, contrary to the amnestic actions obtained for the 3.3 nmol intra-HPC dose in rats, intermediate doses of 0.33–1 nmol facilitated learning in the MWM (Sandin et al. 2004). In addition, these promnesic effects were reversed by a NOP antagonist. Similarly in mice, it has been recently shown that ICV administration of 1 or 10 nmol of N/OFQ inhibited performance in the IA test but that the 0.01 nmol dose had a facilitating role (Adem et al. 2017). Thus, even though the majority of studies suggest that intermediate doses of NOP agonist are inactive in learning and memory paradigms, the abovementioned work encourages further investigation of potential promnesic consequences of NOP receptor activation.

3 Modulation of Learning and Memory by Endogenous N/OFQ

In view of the amnestic effects produced by the administration of NOP receptor agonists, it may be proposed that under certain circumstances, the release of endogenous N/OFQ could inhibit learning and memory processes. A set of data from the study of NOP receptor or peptide precursor knockout (KO) mice suggest that this is indeed the case.

3.1 Evidence from the Study of Receptor or Precursor KO Mice

The first constitutive NOP receptor knockout (NOP(−/−)) mouse line showed enhanced performances in terms of learning and memory. On the one hand, memory acquisition was facilitated in the MWM test, NOP(−/−) mice learning faster than the NOP(+/+) mice, but showing no improvement in terms of retention of the spatial memory (Manabe et al. 1998). Similarly, in a KUROBOX system that makes it possible to test spatial learning with less stress than MWM, NOP(−/−) performed better than NOP(+/+) mice (Nagai et al. 2007). On the other hand, it is the memory retention that was increased in IA, with NOP(−/−) mice showing extended retention time compared to NOP(+/+) mice (Manabe et al. 1998). In the same way, in fear conditioning, contextual memory (but not the association of the electric shock with an auditory cue) was more durable in NOP(−/−) mice (Mamiya et al. 2003). Also, in the water-finding test, the same mouse line showed an enhancement of latent learning, compared to NOP(+/+) mice, that might be related to a decrease in dopamine content in the frontal cortex (Mamiya et al. 1998). Finally, NOP(−/−) mice showed no working memory improvement when evaluated by the alternation behavior in the Y-maze (Mamiya et al. 1999).

In contrast to the NOP KO (Manabe et al. 1998), the first study of ppN/OFQ precursor KO (ppN/OFQ(−/−)) mice showed that they had wild-type-like performances in the MWM (Koster et al. 1999). This discrepancy could be due to differences in the genetic background of the two lines or to a ceiling effect linked to differences in task difficulty between the two studies. It is also possible that the lack of performance improvement was due to the anxious phenotype of the ppN/OFQ(−/−) line, which is not observed in NOP(−/−) mice in the EPM test (Mamiya et al. 1998). Indeed, ppN/OFQ(−/−) mice showed abnormalities of response and adaptation to stress (Koster et al. 1999). These phenotypic differences between receptor and precursor KO mice could be linked to the deletion of the other two peptides present in the precursor sequence and whose target and function remain elusive (Mollereau et al. 1996). Anyway subsequent studies have managed to highlight an improvement in learning and memory processes in ppN/OFQ(−/−) lines. The same N/OFQ peptide-deficient mice showed improved acquisition of the water maze task provided that the mice were single-housed, thus reducing chronic social stress (Higgins et al. 2002). ppN/OFQ(−/−) animals also performed better during reversal training in the MWM (Kuzmin et al. 2009). In terms of aversive

memory, mice showed an increase in memory retention in FC and IA (Adem et al. 2017; Higgins et al. 2002), which is consistent with the NOP(−/−) mouse phenotype (Mamiya et al. 2003; Manabe et al. 1998).

The results obtained with the KO lines for the peptide or the receptor are globally consistent with the hypothesis of an inhibitory role of the N/OFQ system on various forms of learning and long-term memory. The study of constitutive KO, however, does not exclude the involvement of developmental adaptations in these animals and makes it difficult to identify the temporal phase of learning that is affected by the absence of receptor or peptide (learning rate vs memory retention). It is also possible that some of the apparent promnesic effects observed in constitutive KO mice do not result from a direct improvement of memory processes. A general increase in the level of arousal of the animals could, for example, indirectly increase acquisition and retrieval performances. The generation of conditional mutant mice could help addressing these questions. These limitations of genetic models can also be overcome by the use of pharmacological approaches based on NOP antagonists.

3.2 Evidence from the Study of the Effect of NOP Antagonists

There are very few studies specifically designed to test the promnesic properties of NOP antagonists. In the majority of cases, the antagonists were used to reverse the amnestic effects of NOP agonists and thus to demonstrate that these properties were specific for the NOP receptor. These reports, however, included a control group treated by the antagonist alone, and in the vast majority of cases, this treatment was shown to have no effect on learning and memory. This was, for example, the case for the [Nphe^1]N/OFQ(1–13)-NH_2 antagonist at the dose of 50 nmol intra-HPC in rats (Redrobe et al. 2000) and 10 nmol ICV in mice (Kuzmin et al. 2009) on memory acquisition in the MWM. Similarly 10 nmol of [Nphe^1]N/OFQ(1–13)-NH_2 ICV in mice did not improve acquisition in IA (Liu et al. 2007). Another antagonist, UFP-101 (Calo et al. 2002), at the dose of 5 nmol intra-HPC did not improve performances in the OR paradigm (Goeldner et al. 2008). On the contrary, it has been shown in the rat that post-training intra-BLA injection of 10 pmol of [Nphe^1]N/OFQ(1–13)-NH_2 increased memory retention in IA (Roozendaal et al. 2007). In addition, preliminary results suggested that the J-113397 antagonist (Kawamoto et al. 1999) IP at doses of 3 and 10 mg/kg in mice favored contextual learning in the immediate shock deficit paradigm and improved spontaneous alternations reflecting spatial working memory in the Y-maze (Ouagazzal 2015). The study of NOP antagonists therefore only partly confirms the hypothesis suggested by the characterization of KO mice, namely, the possibility of improving memory performance by blocking the N/OFQ system. It must be emphasized, however, that most of the studies cited above were not aimed at the validation of the promnesic properties of NOP antagonists. In most cases, a single dose has been tested. In addition, the high performance of untreated control groups leaved little room for improved learning or memory retention in these studies. It seems therefore important to characterize further the potential promnesic effects of NOP antagonists, particularly in models in which the learning and memory capacities are altered.

4 Sites and Mechanisms of Action Associated with the Modulation of Learning and Memory by the N/OFQ System

The N/OFQ system presents such a wide distribution in the brain that its effects on memory are probably mediated by a multitude of mechanisms involving many regions such as the hippocampus, the extended amygdala, the prefrontal cortex, some aminergic nuclei, some thalamic nuclei, and the habenula (Gavioli and Calo 2013; Mollereau and Mouledous 2000; Witkin et al. 2014). In the following chapter, we will focus on the direct actions of the peptide in two regions which are key for the types of long-term memory that have been discussed in the previous sections, namely, the hippocampus and the amygdala.

4.1 The N/OFQ System in the Hippocampus

The hippocampus is probably a major site of action of the N/OFQ system for the modulation of learning and memory as evidenced by the amnestic effects of intra-HPC N/OFQ injections described above (Goeldner et al. 2008; Kuzmin et al. 2009; Sandin et al. 1997; Sandin et al. 2004). Numerous N/OFQ-containing interneurons are found in the dentate gyrus (DG) and CA1, CA2, and CA3 subregions of the rodent hippocampus (Ikeda et al. 1998; Neal et al. 1999b). By contrast the NOP receptor is expressed primarily on principal neurons in this area (Neal et al. 1999a). [^{3}H]N/OFQ binding to rat and mouse brain sections is high in the stratum radiatum and oriens of the CA1 field and moderate in the corresponding areas of the CA3 region and the DG molecular layer. It is much lower in the pyramidal and granular and in the lacunosum moleculare layers (Higgins et al. 2002). This inhibitory system is therefore ideally placed to negatively modulate transmission and synaptic plasticity at the major relays of the hippocampal circuit.

Thus, on slices of rat DG, N/OFQ has been shown to inhibit synaptic transmission at the level of the lateral perforant path-granule cell synapse by a mechanism involving postsynaptic hyperpolarization linked to activation K^+ currents (Yu and Xie 1998). The peptide also inhibited the induction of long-term potentiation (LTP) by the high-frequency stimulation of the lateral perforant path as well as the NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) evoked by stimulation of this pathway. Here again the phenomenon seems postsynaptic since N/OFQ attenuated the inward currents evoked by focal application of NMDA (Yu and Xie 1998). N/OFQ-induced changes in synaptic strength may actually be bidirectional since, at the same synapse in the mouse, another study has shown that the peptide also inhibited depotentiation and NMDA-dependent long-term depression (LTD) (Wei and Xie 1999).

In the principal cells of the CA3 region of the hippocampus, N/OFQ inhibited N-, L-, and P/Q-type voltage-gated calcium channels (Knoflach et al. 1996) and activated GIRK-type potassium channels (Ikeda et al. 1997). In rat CA3 slices, the peptide showed inhibitory actions on epileptiform activity, with both presynaptic

and postsynaptic sites of action (Tallent et al. 2001). In particular, it inhibited EPSCs generated by stimulation of mossy fibers but also associational/commissural fibers. At the postsynaptic level, the increase of K^+ currents moved neurons away from their threshold for firing. But, unlike in the DG, presynaptic actions were also demonstrated, with a decrease in the frequency of miniature EPSCs (Tallent et al. 2001).

Finally, N/OFQ also increased K^+ currents in the principal cells of the CA1 region of the rat hippocampus (Madamba et al. 1999) and could therefore interfere with pyramidal cell activation and synaptic plasticity in this area. It is in fact at the Schaffer collateral/CA1 synapse that the electrophysiological properties of N/OFQ have been studied the most, especially by comparing the effects of the exogenous application of N/OFQ to those produced by the release of endogenous peptide. In rat hippocampal slices, exogenous N/OFQ inhibited synaptic transmission at the Schaffer collateral/CA1 level, probably by a presynaptic mechanism, as suggested by the increased paired-pulse facilitation (Yu et al. 1997). Another study also showed potentiation of feed-forward inhibition at the same synapse (Gutierrez et al. 2001). Subsequent work in the mouse also showed a depression of evoked population spikes but suggested a postsynaptic mechanism related to hyperpolarization of pyramidal cells via GIRK channel activation (Bongsebandhu-phubhakdi and Manabe 2007; Higgins et al. 2002). Regarding LTP, studies in rats and mice showed an inhibition of NMDA-dependent LTP induced by theta burst-type high-frequency stimulations by exogenous N/OFQ (Higgins et al. 2002; Yu et al. 1997). This inhibition could be due to the hyperpolarization phenomena described above but could also involve a more direct regulation of NMDA receptor activity and signaling, and in particular an inhibition of kinases such as CamKII (Mamiya et al. 2003) and ERK (Goeldner et al. 2008). The role of endogenous N/OFQ was first studied in NOP(−/−) mice. In these animals, LTP induced by 100 Hz high-frequency tetanic stimulation of Schaffer collaterals was favored (Manabe et al. 1998). A subsequent study confirmed these results by showing that it was a form of NMDA-dependent LTP. The increase in LTP in these KO mice was probably of postsynaptic origin and was not found for lower frequency stimulation trains (20 and 50 Hz) (Taverna et al. 2005). Bongsebandhu-phubhakdi and Manabe subsequently confirmed these results by showing an increase in 100 Hz tetanic stimulation induced LTP produced by the antagonist UFP-101 (Bongsebandhu-phubhakdi and Manabe 2007). In this case, UFP-101 opposed the inhibitory action of endogenous N/OFQ released at least in part from enkephalin-sensitive GABAergic interneurons. In contrast, no effect of the antagonist on basal synaptic transmission was demonstrated, suggesting the absence of basal N/OFQ tone. It is also interesting to note that UFP-101 did not affect theta burst-induced LTP suggesting that there was no N/OFQ release under these conditions of Schaffer collateral stimulation (Bongsebandhu-phubhakdi and Manabe 2007).

Overall, all of these investigations on hippocampus slices allow to draw several conclusions: (1) exogenous N/OFQ inhibits synaptic transmission and NMDA-dependent LTP by hyperpolarizing all types of principal cells, (2) the contribution of a presynaptic site of action is variable depending on the synapse and the species considered, and (3) endogenous N/OFQ may have similar inhibitory effects, but it

appears to be released only under particular stimulation conditions. Points 1 and 2 agree with the above behavioral data showing inhibitory actions of intra-HPC injection of N/OFQ on learning and memory (Goeldner et al. 2008; Kuzmin et al. 2009; Sandin et al. 1997, 2004) and synergistic effects between the peptide and an NMDA antagonist (Goeldner et al. 2008, 2009). Point 3 implies that it will be very important to better characterize the physiological and pathological conditions of N/OFQ release in the hippocampus to identify the circumstances under which NOP antagonists might exert promnesic effects.

By acting mainly on the principal cells in the HPC, N/OFQ differs from conventional μ and δ opioids, which act indirectly by inhibiting GABAergic transmission (Bramham and Sarvey 1996). However, N/OFQ could still have an indirect mechanism of action but rather via the regulation of the release of cholinergic or monoaminergic mediators (Schlicker and Morari 2000). In this context, at the level of the hippocampus, only the modulation by N/OFQ of cholinergic signaling and its role in memory has been studied. Thus, it has been reported that N/OFQ inhibited the efflux of [^{3}H]choline on electrically stimulated rat hippocampal slices (Cavallini et al. 2003) and that the ICV injection of 0.5 nmol of the peptide induced a sharp fall in acetylcholine release in the rat HPC (Hiramatsu et al. 2008). In addition, NOP KO mice had an increased baseline level of acetylcholine in the hippocampus, associated with enhanced (higher power) theta rhythms during wake and REM sleep (Uezu et al. 2005). However, at the behavioral level, no synergy could be demonstrated between the amnestic effects of N/OFQ and the cholinergic nicotinic receptor antagonist, mecamylamine, nor with the muscarinic receptor antagonist, scopolamine in the object recognition test, which suggests that the two systems do not interact in this paradigm (Reiss et al. 2012). Further studies will be needed to demonstrate a possible contribution of inhibition of acetylcholine release to the amnestic properties of the peptide in spatial and contextual memory paradigms.

A final way by which the hippocampal N/OFQ system could affect learning and memory is through the modulation of structural plasticity processes, i.e., adult neurogenesis in the DG or the plasticity of mature neurons. This hypothesis has not been studied in detail yet, but some indications suggest that it could be valid. Work done in vitro on primary cultures of embryonic hippocampal neurons produced conflicting results. Initially one study showed a positive effect of N/OFQ on the number and length of dendrites (Ring et al. 2006). On the contrary Alder et al. have described more recently an inhibitory action of exogenous N/OFQ on dendritic growth, via an enhancement of the activity of RhoA, a small GTPase involved in cytoskeleton regulation (Alder et al. 2013). In vivo data are in agreement with this inhibitory effect of the peptide. Thus, an increase in the length of the primary dendrites and the number of spines of the granular cells of the DG was observed in ppN/OFQ(−/−) mice (Alder et al. 2013). In addition, a recent study has shown that repeated administration of the antagonist UFP-101 was able to increase the number of immature neurons positive for doublecortin in the DG of rats under chronic stress (Vitale et al. 2017). It can therefore be suggested that endogenous N/OFQ has a negative impact on the structural plasticity of mature neurons, but also on the generation of new neurons in the adult DG that contribute to spatial memory (Marin-Burgin and Schinder 2012).

N/OFQ therefore has negative effects on neuronal excitability and synaptic plasticity in the hippocampus. Its mechanism of action is not fully elucidated but may involve presynaptic inhibition of glutamate release and postsynaptic hyperpolarization, both processes being characteristic of Gi-coupled receptors. Finally the influence of N/OFQ on adult neurogenesis at the DG level and more generally on neuronal structural plasticity deserves further investigation.

4.2 The N/OFQ System in the Amygdala

Concerning the N/OFQ-sensitive aversive memory paradigms (FC, IA), the key region is the amygdala and in particular the basal and lateral nuclei (BLA) and the central nucleus (CeA). The BLA is the brain region where the processes of plasticity underlying emotional associative memory take place (association between the unconditioned stimulus, here the electric shock, and the conditioned stimulus, here the context or the tone), whereas the CeA is rather an output structure triggering conditioned behaviors (Johansen et al. 2011). The BLA and CeA contain N/OFQ labeled cell bodies and fibers (Neal et al. 1999b), and the NOP receptor is expressed in both regions (Neal et al. 1999a). As already mentioned systemic or ICV administration of NOP agonists was more efficient in inhibiting the acquisition, consolidation, and reconsolidation of hippocampus-dependent contextual aversive memory than that of amygdala-dependent cue aversive memory (Fornari et al. 2008; Goeldner et al. 2009; Mamiya et al. 2003; Rekik et al. 2017). However two studies have reported amnestic effects of intra-amygdala injection of NOP ligands. In the rat, in the IA paradigm, 1–100 pmol of N/OFQ administered in the BLA post-training impaired retention performance (Roozendaal et al. 2007). On the contrary, N/OFQ injection in the CeA was inactive. In the same report, it was shown that intra-BLA administration of the NOP antagonist [Nphe^1]N/OFQ(1–13)-NH_2 increased memory performances and that this improvement was prevented by atenolol (an antagonist of the β1-adrenergic receptor) (Roozendaal et al. 2007). This result suggests that endogenous N/OFQ prevents aversive memory consolidation by interfering with noradrenalin (NA) signaling. The second study, using the TFC paradigm in mice, demonstrated that intra-CeA injection of the new NOP agonist SR-8993 inhibited memory consolidation (Andero et al. 2013). This data contrasts with the lack of effect of intra-CeA injection of N/OFQ reported by Roozendaal (Roozendaal et al. 2007). This apparent discrepancy could be explained by differences in species and behavioral paradigms or by the relatively high dose used in the mouse study that might have allowed diffusion of the drug from the CeA to the BLA. In any case, these behavioral data are in good agreement with the cellular actions of the peptide that have been described in this brain region. Similarly to the hippocampus, both pre- and postsynaptic actions have been reported. In rat brain slices, N/OFQ diminished evoked EPSCs in CeA neurons by a presynaptic mechanism (Kallupi et al. 2014). Moreover the opposite effect of the NOP antagonist [Nphe^1]N/OFQ(1–13)-NH_2 suggested that endogenous N/OFQ may tonically regulate basal spontaneous CeA glutamatergic activity (Kallupi et al. 2014).

N/OFQ was also shown to inhibit presynaptically GABAergic synaptic transmission in CeA neurons (Roberto and Siggins 2006). Finally, also in the rat, N/OFQ hyperpolarized a fraction of CeA neurons projecting to the periaqueductal grey by enhancing an inwardly rectifying potassium conductance (Chen et al. 2009). A similar spectrum of actions has been described in the rat BLA with a partial suppression of evoked EPSCs and inhibitory postsynaptic currents (IPSCs) as well as spontaneous miniature EPSCs and IPSCs (Meis and Pape 2001), and a reduction of the excitability of the majority of class I projecting cells (Meis and Pape 1998). Besides glutamatergic and GABAergic transmission, and in agreement with the behavioral study cited above (Roozendaal et al. 2007), the modulation of the release of NA by the N/OFQ system has been described. Local infusion of the peptide in the BLA decreased NA levels measured by microdialysis by around 30%, whereas systemic administration of the NOP antagonist J-113397 doubled basal levels of the adrenergic transmitter (Kawahara et al. 2004).

5 Conclusion: Future Directions

Both exogenous and endogenous N/OFQ clearly have a negative impact on learning and memory. These impairments appear to mainly affect context-dependent learning, to involve multiple regions including the HPC and the BLA, and to be mediated through pre- and postsynaptic inhibition of NMDA and noradrenergic signaling. So far three types of long-term memory have been investigated: spatial memory in the MWM, aversive memory in the FC and IA paradigms, and recognition memory in the OR test. Therefore an outstanding issue is the generality of the involvement of NOP receptor function in various forms of learning. Given its wide distribution, the NOP receptor could be involved in a number of memory-related brain functions, not limited to hippocampus-dependent memory. Thus two forms of memory deserve further investigation, in particular because they have a major therapeutic interest. The first one is short-term memory and especially working memory. Studies suggest that N/OFQ could disrupt working memory evaluated by spontaneous alternation in the Y-maze (Hiramatsu and Inoue 1999) and delayed matching or delayed nonmatching to position tasks (Higgins et al. 2002), but the active doses are relatively high. This work should be completed to better characterize these effects and in particular their specificity. Similarly preliminary data suggest that the administration of NOP antagonists may favor working memory (Ouagazzal 2015) but here again more research is needed. The second form of memory for which the role of the N/OFQ system remains to be characterized is reward memory. The peptide was shown to prevent the development of conditioned place preference induced by abuse drugs such as opioids, stimulants, and alcohol (Zaveri 2011). This inhibitory effect was proposed to be due to the anti-reward properties of the system. Indeed N/OFQ has been shown to reduce morphine- and cocaine-induced release of dopamine in the nucleus accumbens (Di Giannuario et al. 1999; Lutfy et al. 2001). However, in order to develop a place preference, the animals have to learn the association between the rewarding properties of the drug and the context in

which the drug is experienced. It is therefore possible that part of the inhibitory effect of N/OFQ in this task is due to an attenuation of associative contextual memory. NOP agonists could thus be useful to decrease the rewarding properties of drugs of abuse but also to weaken maladaptive drug-associated memories that can promote relapse (Milton and Everitt 2012).

This last point brings us to the question of the therapeutic perspectives of the N/OFQ-NOP receptor system in the field of learning and memory. Autoradiographic localization of N/OFQ binding sites in macaque brain demonstrated that similarly to rodents the NOP receptor is highly expressed in the hippocampus and the amygdala in primates, suggesting a conservation of memory-modulating properties of the peptide across species (Bridge et al. 2003). A moderate to high expression of NOP receptors has also been demonstrated in principal cells of the DG, CA1, and CA3 in the human brain (Berthele et al. 2003). Polymorphisms or changes in NOP receptor expression have been associated with various neuropsychiatric conditions in human such as PTSD (Andero et al. 2013), alcohol dependence (Huang et al. 2008), opiate addiction (Briant et al. 2010), and suicide (Lutz et al. 2015) but, so far, not with pathologies characterized by deficits in learning and memory. Based on the preclinical data, one might suggest that NOP agonists could be useful as amnestic drugs for disorders associated with maladaptive memories such as PTSD and addiction. This hope, however, must be tempered by the fact that NOP agonists can be predicted to interfere more efficiently with hippocampus-dependent episodic memories than amygdala-dependent emotional memories. It is also important to note that, although several clinical trials have been performed, no NOP-selective agonist has been advanced into phase II (cebranopadol, a phase III analgesic compound is a mixed NOP-MOP agonist) (Zaveri 2016), one main concern being the narrow therapeutic window before sedative effects are observed in patients. More promising may be the use of NOP antagonists as memory enhancers. A recent study reported the NOP antagonist LY2940094 to be safe and well tolerated and to show some efficacy in reducing symptoms of depression in major depressive disorder patients (Post et al. 2016). Another phase II study is underway with a higher dosage of the compound for the same pathology (ClinicalTrials.gov Identifier: NCT03193398). Provided that the promnesic properties of NOP antagonists are better characterized in preclinical models, it seems therefore realistic to envision testing such molecules in the future to improve learning and memory in patients suffering from cognitive deficits associated with neuropsychiatric or neurodegenerative diseases.

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N/OFQ-NOP System in Food Intake

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Contents

Abstract

While lifestyle modifications should be the first-line actions in preventing and treating obesity and eating disorders, pharmacotherapy also provides a necessary tool for the management of these diseases.

However, given the limitations of current anti-obesity drugs, innovative treatments that improve efficacy and safety are needed.

Since the discovery that the activation of the Nociceptin/Orphanin (N/OFQ) FQ peptide (NOP) receptor by N/OFQ induces an increase of food intake in laboratory animals, and the finding that this effect can be blocked by NOP antagonists, many NOP agonists and antagonists have been synthesized and tested in vitro and in vivo for their potential regulation of feeding behavior. Promising results seem to suggest that the N/OFQergic system may be a potential

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therapeutic target for the neural control of feeding behavior and related pathologies, especially in binge-like eating behavior.

Keywords
Eating disorders · Food intake · N/OFQ · Nociceptin/orphanin FQ · Nociceptin/orphanin FQ peptide (NOP) receptor · NOP agonist · NOP antagonist · Obesity

1 Introduction

Obesity is a multifactorial and chronic disease, recognized as a serious public health problem; however, the management of obesity remains to be a major challenge. For this reason, this chapter will examine the role of nociceptin/orphanin (N/OFQ) system that may represent a novel obesity treatment approaches as revealed by the numerous papers where it was described so far.

The groundwork for appreciating the role of the endogenous opioid system in mediating food intake regulation, and the value of N/OFQ in this context, began in the late 1970s, when Margules et al. demonstrated that the opiate antagonist naloxone abolished overeating in obese mice (ob/ob) and rats (fa/fa) and suggested that pituitary beta-endorphin could be involved in overeating and in the obesity syndrome (Margules et al. 1978). A few years later, Steve Woods reported that injection of beta-endorphin into the brain ventricle increased food intake in rats (McKay et al. 1981). Later, Leibowitz demonstrated that the brain paraventricular nucleus was very sensitive to the effect of beta-endorphin to induce feeding behavior in rats (Leibowitz and Hor 1982). In the 1990s, it was shown that the opiate antagonists naloxone and naltrexone reduced feeding in some particular situations associated with obesity (Bodnar 1998; Giugliano and Lefebvre 1991).

Thus, various studies confirmed that the opioid system plays a role in regulation of short-term induced feeding in rats. However, others observed an opposite effect in humans with the discovery that elevated levels of opioid activity (in β-endorphin equivalents) were present in the cerebrospinal fluid of patients with anorexia nervosa (Kaye et al. 1982). As research on opioids continued, some authors proposed them as a link between the reward system and the feeding profile of the animal and formulated important hypothesis about the animal opioid-sensitive feeding system (McLean and Hoebel 1983; Morley et al. 1983). Among the opioid peptides, not only beta-endorphin but also dynorphin and its fragments were found to stimulate feeding behavior which may indicate that kappa opiate receptors are important for the expression of ingestive behavior (Morley and Levine 1983). A further study with selective agonists for kappa, mu, and delta opioid receptors confirmed that kappa agonists were the most potent agents in stimulating feeding behavior but Gosnell and coworkers found that other opioid receptors were involved as well (Gosnell et al. 1986). Since the discovery of N/OFQ and de-orphanization of its receptor (opioid receptor-like 1, ORL1), together with the fundamental paper (Pomonis et al. 1996), the field of N/OFQ and food intake developed quickly, and the results will be presented in the following paragraphs.

2 N/OFQ and NOP Agonists: Stimulation of Food Intake

In the late 1990s, a new 17-amino acid neuropeptide with high analogy to endogenous opioid peptides, especially to dynorphin, was simultaneously identified by two groups of investigators (Meunier et al. 1995; Reinscheid et al. 1995). This neuropeptide was named "nociceptin" by Meunier, while Reinscheid named it "orphanin FQ." After the identification of nociceptin/orphanin FQ (N/OFQ), the ORL1 was renamed N/OFQ peptide (NOP) receptor based on the nomenclature guidelines recommended by the International Union of Basic and Clinical Pharmacology (Cox et al. 2015; Meunier et al. 1995; Reinscheid et al. 1995). There was a marked homology between NOP and classical opioid receptors, especially the kappa opioid receptor. The name "nociceptin" was chosen because the new peptide induced delayed analgesia, as well as rapid hyperalgesia that was not blocked by opioid antagonists. It was clear from the beginning that N/OFQ elicited both analgesia and hyperalgesia through pharmacologically distinct receptors that do not correspond to the traditional opioid receptors. Many other biological effects were described, which the reader can explore in the original papers reporting its discovery. In the composite name "orphanin FQ," orphanin referred to the fact that endogenous receptor of the peptide was unknown, while the letters FQ indicated first amino acid of the peptide ("F" for phenylalanine) and the last one ("Q" for glutamine) (Reinscheid et al. 1995). Soon after, studies to understand the role of this new peptide in feeding behavior were undertaken. Pomonis et al. were the first to report that N/OFQ stimulated food intake in rats when injected into the animals' brain right lateral ventricles (Pomonis et al. 1996); they also found this effect could be blocked by peripheral administration of the opioid antagonist naloxone.

This hyperphagic effect of N/OFQ after right lateral ventricle injection in satiated rats was also confirmed by other research labs (Polidori et al. 2000a). The range of doses used for the intracerebroventricular (icv) injections to elicit feeding was from 1 to 10 nmol/rat. These icv injections of N/OFQ produced a transient hypolocomotion within the first minutes, but the effect on food intake was selective (not elicited as a consequence of water intake), taking place 7 and 15 min after icv injection and lasting up to 45 min.

Other authors reported that N/OFQ exerted not only an acute effect but also a long-lasting one. In fact, in one study, continuous 12-day icv infusion of N/OFQ in mice brains, through subcutaneously implanted pumps, produced a significant increase of food intake and body weight in mice fed with regular chow or moderately high-fat diet (Matsushita et al. 2009). In addition in a pair-fed experiment, in which N/OFQ-treated mice were fed with the same amount of food as the vehicle group, obviously the pair feeding prevented the increase of body weight caused by N/OFQ, but surprisingly, in the treated mice, compared to the vehicle mice, there was an increase of the mass of white adipose tissue, as well as higher expression levels of lipogenic genes (Matsushita et al. 2009). Moreover, in the pair-fed experiment, N/OFQ produced hyperinsulinemia and hypercholesterolemia, suppressing brown adipose tissue function. Interestingly, N/OFQ also caused a reduction of body temperature, indicating that there may be another mechanism involved in eliciting

Table 1 Orexigenic effects triggered by NOP receptor activation

Compounds	Species	Tested dose and route of administration	References
N/OFQ	Rat	1–10 nmol; LV	Pomonis et al. (1996)
[FG]N/OFQ(1–13) NH	Mouse	0, 0.3–3 nmol; icv	Olszewski et al. (2000)
N/OFQ	Rat	0.21–4.2 nmol; LV, 3V, ARC	Polidori et al. (2000a)
N/OFQ(1–13)-NH2	Rat	0.21–4.2 nmol; LV, 3V	Polidori et al. (2000b)
Ro 64-6198	Rat	0.3–2.5 mg/kg; ip	Ciccocioppo et al. (2002)
[(pF)Phe4]NC(1–13) NH2	Rat	0.1–2 nmol/rat; icv	Rizzi et al. (2002)
OS-500 and OS-462	Rat	1.21–12.15 nmol; icv	Economidou et al. (2006)
Ac-RYYRIK-ol	Mouse	0.001–0.1 nmol; supraspinal administration	Gunduz et al. (2006)
N/OFQ	Mouse	1–100 nmol; icv	Rizzi et al. (2007b)
UFP-112	Mouse	2.5–250 pmol; icv	Rizzi et al. (2007b) Calo et al. (2011)
PWT2-N/OFQ	Mouse	2.5–250 pmol; icv	Guerrini et al. (2014)

LV lateral cerebroventricle injection, *icv* intracerebroventicular injection, *3V* third cerebroventricle injection, *ARC* hypothalamic arcuate nucleus injection, *ip* intraperitoneal administration

feeding behavior. These findings suggested that N/OFQ could play a role in the development of obesity not only by inducing hyperphagia but also by affecting energy homeostasis (Matsushita et al. 2009).

Since the discovery of the NOP receptor, there has been progress in the development of NOP agonists and antagonists (Table 1 lists the former and Table 2 lists the latter). In addition, research using transgenic animals, which will be presented below, has enabled a better understanding of the role of the NOP receptor and has led to new pharmacological treatments for human diseases.

In the late 1990s, N/OFQ analogues became available, and a number of feeding behavior studies were conducted with them (Calo et al. 1998). The first fragment of the N/OFQ peptide was the 1–13 portion, which showed agonist properties. Indeed, central injection of the N-terminal fragment N/OFQ(1–13)-NH_2 proved to be the active sequence, inducing hyperphagic activity in sated rats and inhibiting the release of pro-opiomelanocortin (POMC)-derived peptides. N/OFQ(1–12)-NH_2 and N/OFQ(1–9)-NH_2 were inactive in stimulating food intake (Polidori et al. 2000b). The hypothalamic arcuate nucleus (ARC) proved to be the most sensitive site among the brain regions investigated.

Later studies compared the effects of these analogues with those of the natural N/OFQ peptide. The orexigenic effect of the NOP agonist UFP-112 was found to be 100-fold more potent than that of N/OFQ (Calo et al. 2011; Rizzi et al. 2007b) and PWT2-N/OFQ 40-fold more potent compared to N/OFQ (Guerrini et al. 2014).

Table 2 Antagonist effect on N/OFQ-induced feeding behavior

Compounds	Species	Dose antagonist/N/OFQ; route of administration	References
[Nphe1]N/OFQ(1–13)-NH2	Rat	16.80/1.68 nmol; 3V	Polidori et al. (2000b)
NC-797	Rat	0.4–17/1.68 nmol; icv	Economidou et al. (2006)
UFP-101	Rat	1.80–7.40/1.68 nmol; icv	Economidou et al. (2006)
SB-612111	Mouse	1 mg/kg; i.p./1 nmol; icv	Rizzi et al. (2007a)
	Rat	10 or 30 mg/kg orally	Witkin et al. (2014)
LY2940094	WT or KO mice	3 or 30 mg/kg orally	Statnick et al. (2016)
	Rat	10 or 30 mg/kg orally	
	DIO mice	20 mg/kg orally	

3V third cerebroventricle injection, *icv* intracerebroventicular injection, *ip* intraperitoneal administration, *WT* wild-type mice, *KO* NOP knockout mice, *DIO* dietary induced obese

Moreover, the effect of UFP-112 appeared to be much longer lasting than that of N/OFQ. In fact, Calo et al. (2011) reported that the orexigenic effect of N/OFQ lasted 1 h, while the hyperphagic effect of UFP-112 lasted 6 h (Calo et al. 2011).

Other NOP receptor ligands were characterized in vivo assays in rodents to study how they may stimulate food intake: the hexapeptide Ac-RYYRIK-ol (Gunduz et al. 2006) or [(pF)Phe4]NC(1–13)NH2 compared to NC(1–13)NH2 (Rizzi et al. 2002) or [Phe^1 Ψ(CH-NH)Gly]-nociceptin(1–13)NH, which is a synthetic pseudopeptide (Olszewski et al. 2002). The latter showed short-lasting hyperphagic effect but was able to induce c-Fos expression in the same brain areas activated by N/OFQ administration (Olszewski et al. 2002). In subsequent studies, Olszewski et al. also showed that N/OFQ does not increase preferred diets/macronutrients but rather produces a general increase in food intake (Olszewski et al. 2002, 2010).

Three other new compounds, OS-500, OS-462, and OS-461, were investigated in doses ranging from 1.2 to 12.2 nmol/rat, injected into the brain lateral ventricle (Economidou et al. 2006). The order of potency in eliciting food intake was OS-500 > OS-462, while the OS-461 was practically inactive at all doses tested (Economidou et al. 2006).

Several groups have synthesized other peptide and non-peptide opiate ligands to bind to N/OFQ (Zaveri 2003). Among the non-peptide NOP receptor ligands, the agonist Ro 64-6198, discovered by Jenck et al. (2000), was found to elicit hyperphagia in freely feeding rats or in food-deprived rats, after the administration of this by intraperitoneal injection at a dose 2.5 mg/kg. Though the other doses tested (0.3 and 1 mg/kg) were not effective in inducing hyperphagia, other studies indicated that the 0.3 mg/kg dose was active in reducing the effect of both corticotropin-releasing factor (CRF) and restraint-induced anorexia in rats (Ciccocioppo et al. 2002).

In fact, it has been found that N/OFQ exhibits stress-reducing properties (Gavioli and Calo 2006; Vitale et al. 2006) and that its activation blocks stress-induced inhibition of feeding, an effect mediated by functional inhibition of the CRF system (Ciccocioppo et al. 2001, 2002, 2003) which is the primary mediator of stress responses in mammals (Schank et al. 2012). The CRF system initiates the neuroendocrine response to stress via the hypothalamic–pituitary–adrenal (HPA) axis and coordinates several behaviors via actions on extra hypothalamic sites. Moreover, N/OFQ antagonizes the anxiogenic-like effect of CRF in key brain regions (Filaferro et al. 2014; Rodi et al. 2008) that are involved in excessive consumption of palatable food in eating disorders, namely, the bed nucleus of the stria terminalis and the central amygdala (Blasio et al. 2013; Cottone et al. 2009; Micioni Di Bonaventura et al. 2014, 2017). This interesting interaction and the alteration of N/OFQ and CRF mechanisms may contribute to escalating intake, binging, and alterations in brain stress and reward pathways. It is also hypothesized that N/OFQ and CRF system interact in intestinal pathological conditions (see the review by Agostini and Petrella 2014) and thus that NOP agonists may prove to be valuable for the treatment of intestinal disorders. For example, one study reported that the oral administration of NOP receptor (SCH 221510) decreased inflammation in a mouse model of colitis (Sobczak et al. 2014). Similarly, N/OFQ has been found to be involved in the reduction of gut inflammation (Brookes et al. 2007; Petrella et al. 2013) and to modulate gastrointestinal function and pain (Agostini et al. 2009).

3 NOP Receptor Antagonists: Inhibition of Food Intake

While opioid agonists are involved in eliciting food intake in animals, antagonists are involved in blocking it. Indeed, in naïve 24-h food-deprived rats, the opioid antagonist naloxone reduced regular chow intake in animals acutely injected (0.1, 1.0, and 10.0 mg/kg) immediately before food presentation (Sanger and McCarthy 1982). On the other hand, this anorexigenic action of naloxone which was attenuated when the animals became used to a food-deprivation schedule (Sanger and McCarthy 1982). Interestingly, the nonselective phenylpiperidine opioid antagonist, LY255582, was shown to reduce food consumption, water, and body weight gain of obese Zucker rats, when tested in a single subcutaneous dose (0.31 mg/kg) (Shaw et al. 1991).

Given this evidence of the involvement of opioid antagonists in the control of feeding, when NOP antagonists became available, it was a natural progression to test them in similar studies of food intake.

The first discovered functional antagonist of N/OFQ coming from the same precursor was nocistatin (Costentin et al. 1998; Joseph et al. 2006). This neuropeptide centrally administered in the doses from 1 to 3 nmol significantly reduced food intake in 24-h food-deprived rats during the first and second hour postinjection. Furthermore, in the same range of doses, it suppressed N/OFQ-induced feeding (Olszewski et al. 2000).

Structural modification of the first amino acid of the N/OFQ neuropeptide led to the discovery of a new synthetic peptide [Nphe^1]N/OFQ(1–13)-NH_2 10725267 (Guerrini et al. 2001; Hashimoto et al. 2000) which showed an antagonist effect on NOP receptor-mediated inhibition of cAMP formation in Chinese hamster ovary cells. Soon after this study, it was tested against N/OFQ-induced feeding behavior. At an antagonist/agonist molar ratio of 10/1, [Nphe^1]N/OFQ(1–13)-NH_2 significantly inhibited the hyperphagic effect of N/OFQ (1.68 nmol/rat) (Polidori et al. 2000b). Two other NOP receptor antagonists, the first a fragment of the peptide N/OFQ denominated UFP-101 ([Nphe^1, Arg^{14}, Lys^{15}]N/OFQ-NH_2) at the doses between 1.8 and 7.4 nmol/rat and the second the synthetic product called NC-797 ((1*R*,2*S*)-*N*-amidino-2-[2-(4-chlorobenzoylamino)-6-methoxyquinazolin-4-yl]aminocyclohexylamine dihydro-chloride) at the doses between 0.4 and 17 nmol/rat, were tested on N/OFQ-induced feeding behavior. Both were found to dose dependently reduce this induced feeding behavior (Economidou et al. 2006). Moreover UFP-101 produced antidepressant-like effects in animal model of chronic mild stress in male Wistar rats (Vitale et al. 2017).

A further antagonist is SB-612111: this potent NOP receptor non-peptide antagonist (Zaratin et al. 2004) had no effect on food consumption in Swiss sated mice but significantly reduced the orexigenic effect of N/OFQ (Rizzi et al. 2007a). In addition, SB-612111 has been shown to inhibit dose dependently the food intake and body weight gain in Long–Evans rats under a high-fat/high-sugar diet. Recently, LY2940094, a novel potent and selective N/OFQ antagonist of non-peptidergic origin, was found to reduce feeding in 15-h fasted NOP(+/+) but not NOP(−/−) mice orally administered this molecule at doses 3 or 30 mg/kg (Statnick et al. 2016). The effect of LY2940094 on food intake and body weight was also studied in diet-induced obesity (DIO) mice. Given by oral gavage at 20 mg/kg (8 mL/kg) to DIO C57Bl/6 mice just before the onset of the dark photoperiod, this dose reduced fasting-induced food intake in mice and their body weight as recorded up to 24 h after administration (Statnick et al. 2016).

Antagonistic properties against N/OFQ-induced hyperphagia have also been demonstrated with antisense oligodeoxynucleotides directed against either exons 1, 2, or 3 of the NOP gene, while a missense probe demonstrated to be ineffective (Leventhal et al. 1998).

These works suggest that the inhibition of NOP receptor signaling will decrease food intake and might therefore be useful in the treatment of obesity and eating disorders.

The microstructure of food intake may be also affected by the N/OFQ-NOP receptor system. Studies with a model of NOP receptor knockout (NOP(−/−)) mice showed a reduced preference for sucrose and a lower intake of high-fat diet under no-choice conditions (Koizumi et al. 2009). The deletion of the NOP receptor did not affect the conditioned place preference or operant responding, suggesting that reward responses and motivation for food were unaltered in these knockout mice.

Indeed, fasted NOP(−/−) mice, in the first 2 h of free access to food, ate less (0.9 versus 1.3 g) and less frequently (17 versus 22 meals/h) than the wild-type mice (Farhang et al. 2010). As mentioned before (Rizzi et al. 2007a), N/OFQ given icv at 1 nmol significantly stimulated food intake in wild-type mice, whereas it was completely inactive

in NOP(−/−) mice. Moreover, Witkin and colleagues reported that NOP knockout mice exhibit a reduction in fasting-induced feeding (Witkin et al. 2014), and this reduction was also replicated in Statnick et al. (2016).

4 N/OFQ and Food Preference

As well-known from previous studies, opioids are involved in the modulation of reward and palatability in feeding behavior.

One study to explore this further found that N/OFQ induced hyperphagia in fat-preferring rats, but not in sucrose-preferring animals or those that opt for a neutral diet. Also, the researchers observed that its effect on palatability differs from that of opioids, which lead to increased intake of preferred diets (Olszewski et al. 2002). In more detail, the study used a two-choice food availability test (50% sucrose in high-sucrose diet and 50% fat in high-fat diet). N/OFQ was injected in doses ranging from 0.1 to 1 nmol into the lateral ventricle of rats that showed a preference for high-fat diet or for sucrose diet or for a neutral diet. In the fat-preferring rats, N/OFQ injection was not followed by a selective increase in high-fat diet or sucrose diet but by an increase of the intake of both diets. In the sucrose-preferring rats, N/OFQ did not modify the intake of either diet.

In NOP knockout mice, the motivational properties on food resulted in not altered (Koizumi et al. 2009). The authors observed that NOP knockout mice showed no changes in conditioned place preference to high-fat diet under food-deprived conditions. Similarly, testing a number of conditions in operant food self-administration experiments or taste reactivity to sucrose test, they found that the genotype did not determine differences and suggested thus there was no effect related to the motivational properties of food. Surprisingly, body weight and plasma leptin were substantially disrupted in NOP knockout mice, particularly in fasted mice.

5 Brain Sites Involved in Nociceptin-Induced Food Intake and Its Possible Mechanisms of Action

N/OFQ can elicit food intake in sated rats not only when injected into the lateral cerebroventricle (LV) but also when it is administered into the third (3V) but not into the fourth (4V) brain ventricle (3V > LV > 4V) at doses of 2.1 nmol into the LV, 0.42 nmol into the 3V, and no response into the 4V up to 4.2 nmol (Stratford et al. 1997). This suggests that the hypothalamus could be a key brain area of N/OFQ orexigenic activity. Also brain areas such as the ventromedial hypothalamic nucleus (VMH) or the shell of the nucleus accumbens (NAc) (at a range of doses between 2.5 and 25 nmol) are very sensitive to its effect. The NAc was found to be more sensitive than the VMH (Stratford et al. 1997). Among the brain areas studied, ARC neurons have been shown to be the most sensitive to the hyperphagic effect of N/OFQ, since doses as low as 0.2 nmol/rat induced eating behavior. On the other hand, areas such

as the paraventricular nucleus (PVN) and the amygdala (AMY) have been shown to be insensitive to N/OFQ (respectively, up to 0.42 nmol in the PVN and up to 2.1 nmol into the AMY) (Polidori et al. 2000b). Overall, these results correlate well in indicating the presence of the NOP receptor and the effect of N/OFQ in these brain areas. The only difference was observed with the PVN, where a high density of NOP receptors is described but no effect of N/OFQ up to 2.1 nmol was observed (Anton et al. 1996; Neal et al. 1999). Regarding the mechanism involved in N/OFQ-induced hyperphagia, the literature indicates that activation of opioid and NOP receptors produces a modulation of neuronal excitability and synaptic communication by hyperpolarizing neurons through the activation of G protein-gated K+ channels (Emmerson and Miller 1999). Furthermore, in a study using intracellular recording from coronal slices, Wagner et al. showed that N/OFQ exerts an inhibitory effect on ARC neurons which represent a group of anorexigenic POMC neurons (Wagner et al. 1998). In addition, another study found that N/OFQ attenuates c-Fos expression in α-melanocyte-stimulating hormone (α-MSH) immune-positive neurons at meal termination (Bomberg et al. 2006). It can be hypothesized that N/OFQ exerts inhibitory control of brain areas involved in eating; this is possibly the mechanism by which the neuropeptide acts to initiate feeding behavior. Farhang and collaborators showed that N/OFQ exerts its hyperphagic effect by acting both pre- and postsynaptically modulating glutamatergic and G protein-gated K+ channels, respectively, via activation of the NOP receptor (Farhang et al. 2010). Data on N/OFQ-induced feeding support the hypothesis that it produces its effects through the prolonging of meal duration, while conversely antagonists reduce feeding through shortening the meal duration of the animals (Farhang et al. 2010). One additional aspect of the N/OFQ mechanism described above is that it has been shown to diminish the effects of a toxin agent, such as lithium chloride, on anorexigenic and aversive responsiveness (Olszewski et al. 2010). Central systems involved in termination of feeding that seem to be influenced by N/OFQ encompass oxytocin, α-MSH, and CRF (Olszewski and Levine 2004).

6 N/OFQ Interaction with Other Neurotransmitters Involved in Regulating Feeding Behavior

Feeding behavior is controlled by many modulators, the most significant of which seem to be the brain melanocortin and the opioid system.

Indeed, early studies by Ferrari (1958) based on anatomical, neurochemical, and functional evidence suggested that many behaviors are under the balanced control of opioid and antiopioid systems and melanocortins were the most important antiopioids (Lee et al. 2008).

Several studies showed that the α-MSH, a product of POMC, is synthesized in the ARC and it produces a strong anorexigenic effect when injected into different loci of the brain (Vergoni and Bertolini 2000; Voisey et al. 2003).

Studying the site of action of N/OFQ-induced feeding effect in different brain areas, it was clear that the ARC was a very important component of the neural

network that mediates N/OFQ-dependent food consumption (Polidori et al. 2000b). It was later observed with immunohistochemical experiments that N/OFQ could produce a reduction of α-MSH which is revealed to be present at high concentrations at meal termination. Therefore a functional interaction has been suggested in the regulation of food intake (Bomberg et al. 2006).

The acute exposure to various stressors produces inhibition of food intake in rats (Stengel and Tache 2014), and this inhibitory effect is mainly mediated by the involvement of CRF signaling pathways in the brain. Further, this inhibitory effect has been observed in different species (Wang et al. 2011). Therefore, with the discovery of N/OFQ, many studies have been performed to evaluate the interaction between N/OFQ and CRF in the regulation of feeding behavior and in particular where in the brain it occurs. In this regard, it has been shown that N/OFQ reduces both stress and CRF-induced (0.1–1.0 μg/rat) anorexia in rats. In particular, N/OFQ (0.1–2.0 μg/rat) completely abolished the hypophagic effect induced by electric footshock stress or by icv CRF injection. The effect on CRF-induced anorexia is selective since it does not reduce eating both in food-deprived and lipopolysaccharide-treated rats (Ciccocioppo et al. 2001). Further data to support this functional antagonism to the CRF system comes from the selective NOP agonist Ro-64-6198 which reversed the effect on both stress and CRF-induced anorexia in rats (Ciccocioppo et al. 2002).

The brain cannabinoid system is also involved in feeding behavior since it stimulates appetite in humans and in rats (Foltin et al. 1986; Williams et al. 1998). Indeed, Δ^9-Tetrahydrocannabinol (THC) injection into the PVN of the rats at the dose of 5 μg/μL reliably stimulates feeding. Considering the cannabinoid system, it has been shown that subcutaneous or intraperitoneal injection of the selective CB_1 receptor antagonist (0.2–2 mg/kg) SR141716 (Rinaldi-Carmona et al. 1994) blocks the N/OFQ-induced feeding (Pietras and Rowland 2002).

7 N/OFQ-Induced Feeding in Obesity Models

There is sufficient evidence to say that the feeding response elicited by orexigenic and anorexigenic neuropeptides differs in lean and obese animals (Cusin et al. 1996). Wistar Ottawa Karlsburg W (WOKW) rats, obtained by cross-breeding procedures between hypertensive and diabetic rats, develop the main features of metabolic syndrome, such as moderate hypertension, dyslipidemia, hyperinsulinemia, obesity, and impaired glucose tolerance (van den Brandt et al. 2000a). In contrast, Dark Agouti (DA) rats seem to be resistant to metabolic syndrome and have been considered as a control lineage for the WOKW lineage (van Den Brandt et al. 2000b). These two strains showed a different response to the central injection of N/OFQ. In DA rats, doses of N/OFQ between 2.1 and 8.4 nmol injected into the lateral brain ventricle produced a statistically significant increase of food intake dose-dependently within the first hour of icv injection, an effect that was not observed in the WOKW rats (Filippetti et al. 2007).

8 Nociceptin Effects in Binge Eating Models

Binge eating in humans is a dysregulated form of feeding behavior that occurs in multiple eating disorders, and it is characterized by consumption of a large amount of food in a short period of time, during which the person has a sense of loss of control of eating (Amianto et al. 2015; D'Addario et al. 2014; Dingemans et al. 2002). This human eating behavior is often preceded by anxiety or stress and is accompanied by feelings of guilt or shame. It is also moderately heritable (50–60%). Genetic factors as well as environmental factors contribute to its development (Bulik et al. 1998, 2003). The finding that stress-induced feeding in rodents leads to preferential sucrose ingestion which is blocked by opiate antagonists and intraventricular injections of beta-endorphin suggests a connection between sugar ingestion, increased eating, and increased production of beta-endorphin (Fullerton et al. 1985). Animal binge eating models were developed by Boggiano (Placidi et al. 2004) and by Cifani (Cifani et al. 2009, 2013; Micioni Di Bonaventura et al. 2012) in which periods of food restrictions, high palatable food, and acute stress are combined in female rats to elicit the binging behavior for the palatable food.

There is evidence that the endogenous opioid system is involved in the expression of binge eating disorders. Indeed, the opioid antagonist naloxone reduced the consumption of sweet high-fat foods in obese and lean female binge eaters, though not in non-binging controls (Drewnowski 1995). Evidence of the N/OFQ-NOP receptor system in binge eating behavior came from studies of NOP agonists and antagonists (Hardaway et al. 2016; Micioni Di Bonaventura et al. 2013). Indeed, N/OFQ at the dose of 1 nmol increased food intake in female rats of the Cifani's binge eating model, in which cycles of food restriction increased the animals' sensitivity to the hyperphagic effect of N/OFQ for palatable food (Micioni Di Bonaventura et al. 2013). In this model, the food restriction seems to be responsible in the hypothalamus for the downregulation on messenger RNA levels of N/OFQ and its receptor NOP, and these alterations might be due to selective histone modification changes (Pucci et al. 2016). On the contrary the selective N/OFQ antagonist SB-612111 reduced the binge eating behavior when given intraperitoneally at the dose of 0.1, 1, 3, and 10 mg/kg 30 min prior to access to 1 h sessions of high-fat (60%) palatable food (Hardaway et al. 2016).

Another selective NOP antagonist, LY2940094, given intraorally to male rats at doses of 10 and 30 mg/kg, strongly inhibited intake of a high-energy diet for a full 5 h (Statnick et al. 2016). This finding provides further grounding for the idea that N/OFQ-NOP receptor system plays a role in this dysregulated behavior. At the 2018 Neuroscience conference, Hardaway et al. reported a preliminary characterization of how the nociception system influences the intake of highly palatable food (Hardaway et al. 2018). In their electrophysiology study, they found that bath application of N/OFQ led to a reduction in evoked GABAergic transmission in the central amygdala and that this reduction was blocked by pre-application of new selective NOP antagonist: BTRX-246040. Further study will investigate the brain neural circuits that drive preference for palatable food consumption and the role of BTRX-246040 on them through the new iDISCO-CLEARMAP technique.

9 Conclusion

An abundance of scientific studies indicate that food intake is an integrated behavior and involves factors such as the energetic value of the food for the survival of the species, the cultural and learned cues associated with it, and its hedonic value (Novelle and Dieguez 2018). The latter factor is somehow regulated by the opioid system.

Characterization of the orexigenic action of N/OFQ on ingestive behavior in rats has opened a new avenue of research. Given the harmful effects of obesity, findings that may help people regulate their food intake are welcome. In this regard, future studies should build upon the promising results obtained in studies of NOP antagonists in different models of animal obesity. In addition, given the stimulatory properties of N/OFQ, future research should also explore the neuropeptide's action in anorexia animal models. Pharmacological treatments for eating disorders, obesity, and related pathologies are currently limited, and the NOP receptor may be an important new molecular target for the development of novel, safe, and effective anti-obesity drugs that reduce overeating and weight gain and consequent metabolic complications.

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N/OFQ-NOP System in Peripheral and Central Immunomodulation

Salim Kadhim, Mark F. Bird, and David G. Lambert

Contents

Abstract

Classical opioids (μ: mu, MOP; δ: delta, DOP and κ: kappa, KOP) variably affect immune function; they are immune depressants and there is good clinical evidence in the periphery. In addition, there is evidence for a central role in the control of a number of neuropathologies, e.g., neuropathic pain. Nociceptin/Orphanin FQ (N/OFQ) is the endogenous ligand for the N/OFQ peptide receptor, NOP; peripheral and central activation can modulate immune function. In the periphery, NOP activation generally depresses immune function, but unlike classical opioids this is in part driven by NOP located on circulating immune cells. Peripheral activation has important implications in pathologies like asthma and sepsis. NOP is expressed on central neurones and glia where activation can modulate glial function. Microglia, as resident central 'macrophages', increase/infiltrate in pain and following trauma; these changes can be reduced by N/OFQ. Moreover, the

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interaction with other glial cell types such as the ubiquitous astrocytes and their known cross talk with microglia open a wealth of possibilities for central immunomodulation. At the whole animal level, clinical ligands with wide central and peripheral distribution have the potential to modulate immune function, and defining the precise nature of that interaction is important in mitigating or even harnessing the adverse effect profile of these important drugs.

Keywords
Astrocytes · Gliosis · Immune function · Lymphocytes · Microglia · N/OFQ receptor (NOP) · Neuropathic pain · Nociceptin/Orphanin FQ · Sepsis

1 Introduction

Classical opioids (μ: mu, MOP; δ: delta DOP and κ: kappa, KOP) are immunomodulatory; this has been known for decades. Indeed, Hussey and Katz reported in 1950 that opioid addicts were more prone to infection and this was unlikely due to the injection itself (Hussey and Katz 1950). The site of this immunomodulation can be peripheral or central with the precise targets (especially peripheral) being disputed and highly controversial. Prescribing physicians are advised to consider and discuss immune modulation in chronic use decisions. Since its first de-orphanisation N/OFQ and NOP [non-classical opioid receptor (Lambert 2008)] have also been ascribed a role in immunomodulation, and in this chapter we review their roles at peripheral and central sites.

2 Peripheral Immune Actions

2.1 Classical Opioids

Opioid receptor expression on immune cells is still highly controversial. It is widely accepted that opioids have immunomodulatory properties, for example inhibition of T-cell activity or inhibition of B-cell antibody production (Manfredi et al. 1993; Morgan 1996). However, there is significant debate as to whether this action occurs through direct or indirect mechanisms. Evidence is strongly divided regarding the detection of classical opioid receptor (MOP, DOP and KOP) expression on immune cell types (Caldiroli et al. 1999; Bidlack 2000; Cadet et al. 2001; Al-Hashimi et al. 2013, 2016; Kadhim et al. 2018b). Some have posited that the action of morphine in immune responses is via the toll-like receptors (TLR), which have been shown to possess a morphine binding domain (Madden et al. 2001; Hutchinson et al. 2012).

2.2 N/OFQ-NOP

Conversely, there is significant evidence for expression of NOP receptors on immune cell subtypes. Several studies have identified the presence of ppN/OFQ and NOP mRNA (the precursor to N/OFQ) in polymorphonuclear cells, B cells, T cells and monocytes and mast cells (Peluso et al. 1998; Arjomand et al. 2002; Williams et al. 2008a; Singh et al. 2013; Al-Hashimi et al. 2016). Interestingly, screening of phytohemagglutinin (PHA)-activated human lymphocytes identified AT7-5EU cDNA, which encodes NOP, with divergent coding of a non-translated 5′ region in comparison to neuronal tissue. This message is encoded into B and T cell NOP mRNA, and suggests tissue-specific expression of the NOP receptor. Furthermore, these experiments indicated a tenfold increase in NOP mRNA expression after induction with PHA, implying NOP has an important role in immune function (Wick et al. 1995). Further studies have demonstrated similar levels of NOP mRNA in both immune cells and neuronal tissue (Peluso et al. 1998). Expression of functional NOP receptor has been identified in numerous continuous cell lines generated from immune cells. Using [^{125}I]-N/OFQ, Horn and colleagues identified surface expression of NOP on Raji cells, a human B cell lymphoma line (Hom et al. 1999). NOP was further identified in CEM and MOLT-4 T cell leukemic lines and the monocyte lymphoma cell line U-937 using [^{3}H]-N/OFQ to identify binding sites (Peluso et al. 1998). The addition of phorbol-12-myristate-13-acetate (PMA) to Mono Mac 6 cells, a monocyte leukemic cell line, led to increases in ppN/OFQ mRNA via the inhibition of mitogen-activated protein kinase signal transduction pathways (Zhang et al. 2016). Identification of NOP expression on primary immune cells has been challenging due to poorly selective antibodies for the NOP receptor and acquiring the necessary yield of protein to undertake a radioligand binding assay. Recently, a fluorescent marker for NOP, N/OFQ$_{ATTO594}$, has been used to identify NOP receptor expression on human polymorphonuclear cells taken from healthy volunteers (Bird et al. 2018). Interestingly, not all polymorphonuclear cells expressed the NOP receptor protein; this is a cautionary note when assuming mRNA will always translate into protein.

Immune cells have also been shown to express N/OFQ. Human CD19+ B cells were amongst the first to be identified as expressing a novel N/OFQ mRNA transcript resulting in a truncated N/OFQ precursor lacking the signal peptide. Following mitogen-activation, N/OFQ mRNA transcripts, similar to that found in neuronal tissue, was upregulated in all lymphocytes (Arjomand et al. 2002). The mRNA transcript for ppN/OFQ has also been found in polymorphonuclear cells, which include neutrophils, eosinophils and granulocytes (Williams et al. 2008a). Furthermore, neutrophils stimulated with N-formyl-methionine-leucine-phenylalanine (FMLP) have been shown to release N/OFQ (Fiset et al. 2003).

The presence of both N/OFQ and NOP in immune cells would strongly indicate a role in immunological function for this ligand-receptor pairing. An area where this pairing may have significant effect is in the trafficking of immune cells, with N/OFQ

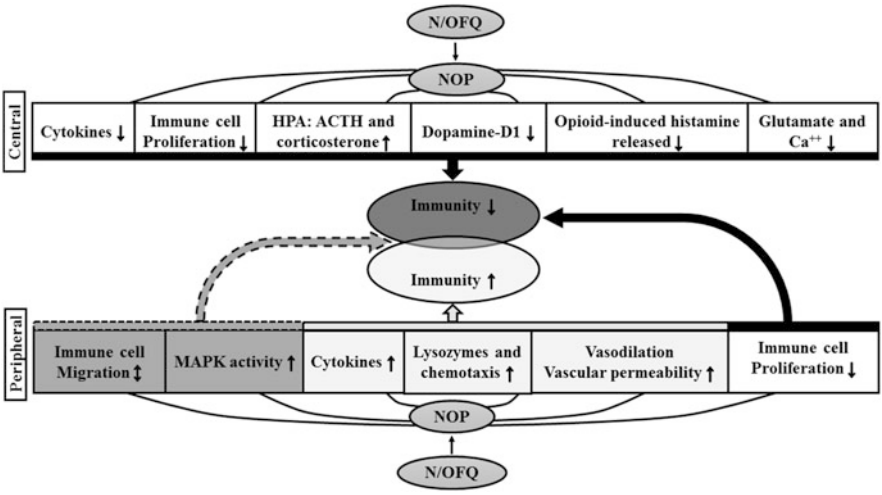

Fig. 1 Mechanisms by which N/OFQ can affect the immune system. Different central (upper panel) and peripheral (lower panel) 'targets' can inhibit (black arrows), activate (light grey arrow) or both inhibit/activate immune function (grey dotted arrow)

having significant effects on cell migration, both positive and negative. A significant example of the positive effects of N/OFQ was measured using monocytes taken from healthy volunteers. The monocytes were exposed to either FMLP or N/OFQ and chemotaxis measured (Trombella et al. 2005). FMLP caused robust migration of monocytes which was matched by N/OFQ, which displayed a high potency (pEC_{50} 11.15) in producing migration. Confirmation of action through the NOP receptor was obtained through pharmacological characterisation using several NOP selective agonists, the inability of naloxone to block the function of N/OFQ at monocytes and through antagonism of migration via the NOP antagonist UFP-101 (Trombella et al. 2005). Neutrophil chemotaxis is also positively affected by the addition of N/OFQ. N/OFQ induced chemotaxis with maximal effect at 100 pM in ex vivo migration studies, and these findings were matched in mouse in vivo models whereby N/OFQ increased neutrophil migration into ad-hoc air pouches (Serhan et al. 2001). Conversely, both lung mast cells and eosinophils have been shown to be negatively affected by N/OFQ in regards to migration (Singh et al. 2016). Both human mast cell line-1 (HMC-1) and primary human lung mast cell migration produced by stem cell factor (SCF) were significantly inhibited by the addition of N/OFQ. Clearly there is a cell and tissue specific migratory response to NOP activation. In addition, and as reviewed by Thomas et al. (2014), N/OFQ induces vasodilation and increases the vascular permeability, actions that play a central role in immune response modulation (Fig. 1).

2.3 N/OFQ-NOP in Disease

The presence of NOP and/or N/OFQ in the immune system, as well as its ability to affect immune cell movement and function, identify a potential mediator of disease-related activity in immunity. NOP and N/OFQ activity has been demonstrated to show potential roles in several immune based diseases. Both NOP and N/OFQ have been implicated in the pathogenesis of colitis, an inflammatory bowel disease (Kato et al. 2005). NOP knockout mice demonstrated significant reduction in symptoms following treatment with dextran sulphate sodium (DSS), which is capable of producing acute colitis. In further studies, administration of SB612,111 (a high affinity NOP antagonist) to DSS-induced colitis also reduced symptoms of colitis as well as a reduction of the cytokines interferon-γ (IFN-γ), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). These cytokines are all known mediators of colitis (Alt et al. 2012). Increased levels of N/OFQ have also been detected in the synovial fluid of patients suffering with rheumatoid arthritis (Fiset et al. 2003). The increased level of N/OFQ was believed to be related to the high concentration of polymorphonuclear cells usually found in synovial fluid of patients suffering with this disease.

As previously noted, both lung eosinophils and mast cells express the NOP receptor. This is particularly relevant to asthma. Asthma is the result of obstruction of airflow (airway constriction, immune infiltration and remodelling) leading to difficulty in breathing (Haldar et al. 2008; Lotvall et al. 2011; Gough et al. 2015). Initial studies indicated that activation of NOP, via N/OFQ, led to inhibition of airway contraction and the release of the inflammatory peptide, substance P (Shah et al. 1998). This initial evidence for NOP receptor function in airway constriction was verified by work in ex vivo human bronchial tissue (Basso et al. 2005). Electric field stimulation produced contractions in the tissue, which was inhibited by N/OFQ in a concentration-dependent manner. Furthermore, the actions of N/OFQ could be blocked by the NOP antagonist, UFP-101, indicating action through the NOP receptor. In a more recent work, tissues from both healthy volunteers and asthmatic patients were screened for the presence of NOP and N/OFQ via PCR. In these studies, N/OFQ was identified in lung eosinophils and, in asthmatic patients, levels were found to be increased in sputum (Singh et al. 2016). In parallel experiments, N/OFQ was found to inhibit migration of immune cells through NOP receptor activation, as well as increasing wound healing in isolated human airway smooth muscle (HASM) cells. Using the same cells, it was found that N/OFQ led to relaxation of HASM cells in spasmogen-stimulated gel contraction experiments, a finding mirrored in Ovalbumin-sensitised mice. These findings suggest that NOP agonists could be potential therapeutic agents for asthma, with a spasmolytic and immune depressor profile.

Sepsis is the result of the immune system producing an overwhelming and potentially life-threatening response to an infection. Treatment options are limited to antibiotics, fluids and supportive care. Translation from the laboratory to the clinic has been poor and there is a real need for novel therapeutics. The mechanisms by which sepsis occurs are poorly understood, but NOP and N/OFQ have been

implicated in this disease. Initial evidence for the role of N/OFQ-NOP in sepsis was found using rat models subjected to caecal ligation and puncture to induce sepsis. In these models, addition of N/OFQ to caecal ligation and puncture led to increased mortality, whereas addition of UFP-101 increased survival rates through inhibition of cell migration and modulation of pro-inflammatory cytokines and chemokines (Carvalho et al. 2008).

Both ppN/OFQ and NOP mRNA levels were decreased in peripheral blood taken from healthy volunteers exposed to varying concentrations of LPS. Furthermore, cytokines, such as TNF-α, IL-1β, IL-10 and IFN-γ, also demonstrated the ability to decrease ppN/OFQ and NOP mRNA levels in healthy volunteer blood (Zhang et al. 2013). While it was initially posited that this was a negative feedback loop downregulating N/OFQ and NOP expression, further data have demonstrated an increase in protein levels. In a small cohort of patients diagnosed with sepsis, plasma N/OFQ concentrations were measured; levels were higher in patients who died (3 pg mL^{-1}) compared to survivors (1 pg mL^{-1}) (Williams et al. 2008b). An inverse relationship was discovered with regards to ppN/OFQ mRNA in septic patients, with ppN/OFQ levels showing significant reduction when compared to healthy volunteers. Furthermore, this study demonstrated a correlation between increased levels of the septic inflammatory marker, procalcitonin and decreased levels of ppN/OFQ (Stamer et al. 2011). A larger prospective study was undertaken assessing 82 septic patients who were sex and age matched to healthy volunteers. Plasma N/OFQ was measured on the first 2 days after admission to the intensive care unit, with a follow-up sample taken in the recovery period. Radioimmunoassay and PCR data demonstrated an increase in plasma N/OFQ concentrations in Days 1 and 2 compared to recovery. Conversely, mRNA levels of ppN/OFQ and NOP decreased compared to healthy volunteers (Thompson et al. 2013).

3 Central Immune Actions

3.1 CNS Can Propagate an Immune Response Through Several Mechanisms

Despite a long history, the idea that the CNS is an immune-privileged organ is disappearing; the brain can mount immune responses and fight invading organisms (Galea et al. 2007). The meningeal lymphatic vasculature can transport cells and molecules resulting in cross-talk between the peripheral and central immune systems (Raper et al. 2016). According to clinically relevant studies, CNS innate immunity can be activated against pathogenic invasion (Carare et al. 2014). Beside microglia, resident central macrophages, meningeal macrophages and dendritic cells (namely in dura, arachnoid and pia mater, choroid plexus and perivascular spaces) can produce significant *protective* actions (Herz et al. 2017).

Several cellular components are involved in regulation of central immune response. Microglia are the central immune responders; they have specialised functions with higher reactivity and mobility than other cell populations in the

CNS and respond to antigens and neuronal damage. When activated they can release proinflammatory mediators and undergo morphological changes (from round and small cell body with long processes to amoeboid with shorter processes) (Inoue and Tsuda 2018). In addition, they migrate to the site of injury, proliferate, perform phagocytic activity and change their protein expression profile (mainly express complement receptors and major histocompatibility complex proteins). Fully activated microglia resemble other macrophages (Hanisch and Kettenmann 2007; Davoust et al. 2008; Colton and Wilcock 2010).

Astrocytes are the most abundant cell population in the CNS and the term 'astrocytes' or 'astroglia' is attributed to their star-like shape with diverse processes and morphology depending on anatomical location (Raff et al. 1983; Bailey and Shipley 1993). Their processes cover synapses, contact nodes of Ranvier and form gap junctions between the processes of neighbouring astrocytes. Astrocytes are multifunctional elements participating in local blood flow regulation (Attwell et al. 2010), supplying neuronal nutrients and controlling brain haemostasis (Mulligan and MacVicar 2004; Magistretti 2006; Araque and Navarrete 2010). They form the majority of the blood–brain barrier and control its endothelial elements (Giaume et al. 2007). They can be precursors and are involved in neurogenesis and gliogenesis (Kettenmann and Verkhratsky 2008) along with detection process and guiding the growth of axons and development of certain neuroblasts when neuronal repair is required (Powell and Geller 1999; Araque and Navarrete 2010). Due to their high number of connection sites, astrocytes have high integration capacity and important roles in the regulation of neuronal activity (Smith 2010). They have a role to play in a number of central pathologies (Bundgaard and Abbott 2008). While neurons are able to propagate action potentials, astrocytes are not, and their excitability occurs through increasing the intracellular concentration of calcium ($[Ca^{2+}]_i$) and release of glutamate, purines, Gamma-aminobutyric acid and D-serine. These transmitters might be responsible for astrocyte–astrocyte communication and/or astrocyte–neuron cross-talk (Nedergaard et al. 2003; Seifert et al. 2006). In addition, these gliotransmitters control the dynamics of the synaptic cleft (Cornell-Bell et al. 1990; Volterra and Meldolesi 2005).

Cellular changes associated with microglial or astroglial activation (gliosis; microgliosis and astrogliosis, respectively) have been reported in models of inflammation and chronic pain (Beggs and Salter 2006; Ji and Suter 2007; Inoue and Tsuda 2018; Kohno et al. 2018). Regardless of the order, the sequence and the intensity of glial activation (due to infection, chronic or neuropathic pain and/or opioid tolerance), astrocytes and microglia have been found to be involved in the pathogenesis of the immunomodulation (e.g., in neuropathic pain) in terms of initiation and progress (Raghavendra et al. 2003; Tanga et al. 2004; Ledeboer et al. 2005; Hald et al. 2009). Following activation, glial cells produce and release pain mediators such as nitric oxide and prostaglandins (Watkins and Maier 2000) and proinflammatory cytokines such as IL-1 and TNF-α (Watkins et al. 2001; Marchand et al. 2005; Charo and Ransohoff 2006; Scholz and Woolf 2007).

Oligodendrocytes are well-known myelin producing cells, providing neurone 'insulation' and a propagated action potential. In addition, these cells are sensitive

to the release of neurotransmitters and neural activity (Bakiri et al. 2009). They play an important role in the pathogenesis of different neurological diseases such as multiple sclerosis. They can release and/or respond to proinflammatory cytokines in response to brain injury (Jurewicz et al. 2005; Ramesh et al. 2012). On the other hand, and despite their specialised function, neurons have been found to release or respond to cytokines in different immunomodulatory conditions (Oh et al. 2001; Zhang et al. 2005).

In summary, complex interplay between neurons, immune cells, and glial cells are responsible for normal regulation and also initiation and maintenance of a number of neuropathologies of which neuropathic pain is an example.

3.2 The Effect of N/OFQ on the Central 'Immune System'

NOP is expressed centrally by neurons in the brain and spinal cord (Pettersson et al. 2002). In addition, a range of glial cells (astrocytes, oligodendrocytes and microglia) have been found to express NOP receptor (Eschenroeder et al. 2012; Kadhim et al. 2018a). N/OFQ is also produced and released by N/OFQ releasing neurons as well as by a wide range of glial cells (Buzas et al. 1998; Buzas 2002; Eschenroeder et al. 2012; Bedini et al. 2017). N/OFQ-NOP therefore has the potential to modulate glial function.

N/OFQ has been found to play an important role in central immunomodulation but the underlying mechanisms remain to be fully understood. Several possible mechanisms have been proposed (Fig. 1). Proinflammatory cytokines, the main immune modulating molecules, are likely modulated by N/OFQ. Intrathecal administration of N/OFQ induced antagonist-reversed down-regulation of cytokine mRNA transcripts. It has been found that pain processing is accompanied by astrocyte activation, which is characterised by an elevated level of proinflammatory cytokines (Lai et al. 2018). Hence, the antinociceptive effect of N/OFQ might be related to its ability to inhibit cytokine expression and/or release in the CNS (Fu et al. 2007; Finley et al. 2008). In addition, infiltration of peripheral immune cells is an important event in the pathophysiology of immunomodulation and pain (Boddeke 2001). Zhao et al. (2002) reported that increased numbers of microglia induced by trauma were reduced by central administration of N/OFQ. N/OFQ-induced immunomodulation may be as a result of inhibition of the proliferation and migration of infiltrating and resident immune cells (note: in the periphery N/OFQ can both promote and inhibit migration). Furthermore, in the hypothalamic-pituitary-adrenal (HPA) axis, adrenocorticotrophic hormone (ACTH) is well known as a site of immunomodulation and there is controversial evidence with classical opioids (Al-Hashimi et al. 2013). N/OFQ has been found to activate HPA axis and increase the levels of ACTH (Devine et al. 2001).

Moreover, several neurotransmitters involved in the regulation of immune function are affected by N/OFQ-NOP system; these include dopamine, histamine, noradrenaline and glutamate. Dopamine is an immunomodulatory neurotransmitter and inhibition of its release can reduce immune activity (Tsao et al. 1997; Basu and Dasgupta 2000; Nakano et al. 2009). There is an extensive literature base

demonstrating that dopamine release is inhibited by N/OFQ (Murphy et al. 1996; Murphy and Maidment 1999; Marti et al. 2004, 2005). Histamine release is an important event involved in the propagation of immune response; morphine-induced central histamine release is also affected by N/OFQ (Eriksson et al. 2000). Along with important roles in the pain pathway, noradrenaline is also an immunomodulator, and its release is inhibited by N/OFQ (Kappel et al. 1998). Given that glutamate and calcium signalling can be important players in immune activation (Watkins et al. 2001; Mattson and Chan 2003), N/OFQ-induced inhibition of glutamate (Nicol et al. 1996; Meis and Pape 2001; Kallupi et al. 2014; Meyer et al. 2017) and LPS-induced calcium signalling (Bedini et al. 2017) possibly affect the pattern of immune activation. The majority of these data are from work in neurones but as we note the brain is so much more than neurones. It can be concluded that the activation of NOP by N/OFQ can participate in central immunomodulation via multiple pathways; if there is disease specificity then this might open some new therapeutic options.

3.3 The Effect of Immunomodulation on the N/OFQ and NOP Receptor

The majority of the text above has covered immunomodulatory effect of NOP, but immune modulation can affect NOP and N/OFQ (the reverse) in the same ways as seen in the periphery in pathologies such as sepsis. As noted in Table 1, the expression profile, integrity and the activity of NOP and N/OFQ can be affected

Table 1 The effect of different immunomodulatory conditions on the expression and activity of central NOP receptor and/or N/OFQ

Cell/tissue type-species	Proinflammatory mediator/process	Effect on NOP	Effect on N/OFQ	Study
Primary rat astrocytes	LPS, IL-1β and TNF-α	–	↑ (mRNA)	Buzas (2002)
Human U87 astrocytes	LPS	↓ (mRNA and protein)	↑ (mRNA and protein)	Bedini et al. (2017)
Rat	PTSD	–	↑ (protein)	Zhang et al. (2012)
Rat	Traumatic brain injury	↔	↑	Witta et al. (2003)
Rat cortical neurons	Leukaemia inhibitory factor	–	↑ (mRNA)	Minami et al. (2001)
Mice DRG neurons	LPS	–	↑ (protein)	Acosta and Davies (2008)
Rat (in vivo) Rat primary microglia	Chronic constriction injury	↑ NOP activity	↑	Popiolek-Barczyk et al. (2014)
Rat amygdala complex	Ethanol	–	Epigenetic modulation of ppN/OFQ	D'Addario et al. (2013)

by a wide range of immunomodulatory conditions. These include bacterial products such as LPS, proinflammatory cytokines, ethanol traumatic brain injury, spinal cord injury in cultured neurons cultured glial cells or in whole animals.

4 Conclusions

Since its early description as a peptide receptor system involved in the modulation of pain processing, a plethora of biological functions, pathological indications and, importantly, therapeutic opportunities have been described. We know that classical opioids can modulate immune function and that immune pathologies can modulate opioid receptor, peptide and drug responsiveness. Here we have discussed both peripheral and central immune modulation by N/OFQ-NOP where there are similarities and differences in the brain and periphery. With the generally improved side effect profile for NOP activation with N/OFQ, and novel ligands such as cebranopadol close to the clinic, understanding the clinical consequences of the immune modulatory effects described above will be an area of research focus.

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N/OFQ-NOP System and Airways

Bruno D'Agostino, Manuela Sgambato, Renata Esposito, and Giuseppe Spaziano

Contents

Abstract

Asthma is a heterogeneous chronic inflammatory disease of the airways. The most prevalent form is atopic asthma, which is initiated by the exposure to (inhaled) allergens. Intermittent attacks of breathlessness, airways hyperresponsiveness, wheezing, coughing, and resultant allergen-specific immune responses characterize the disease. Nociceptin/OFQ-NOP receptor system is able to combine anti-hyperresponsiveness and immunomodulatory actions. In particular, N/OFQ is able to inhibit airways microvascular leakage and bronchoconstriction through a presynaptic and non-opioid mechanism of action that blocks tachykinin release. Moreover, it also acts on allergenic sensitization because it is able to modulate the immune response that triggers the development of airway hyperresponsiveness through an interaction on cell membranes of dendritic cells (DCs) that are generally responsible to start and sustain allergic T helper 2 (TH2)-cell responses to inhaled allergens in asthma. In asthmatic

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patients, sputum showed elevated N/OFQ levels that are related to increased eosinophil counts. The addition of exogenous N/OFQ in sputum obtained from patients with severe asthma attenuated eosinophils migration and release of inflammatory mediators. These observations confirmed that elevated endogenous N/OFQ levels in asthmatic sputum were lower than the ones required to exert beneficial effects, suggesting that supplementation with exogenous N/OFQ may need. In conclusion, the innovative role of N/OFQ in counteracting airways inflammation/hyperresponsiveness opens new potential targets/strategies in asthma treatment.

Keywords
Airway · Airway hyperresponsiveness · Asthma · Cough · Nociceptin

1 Asthma

Asthma is a complex heterogeneous disease caused by a combination of genetic and environmental factors. According to the world health association, approximately 330 million people worldwide suffer from asthma, which accounts for 250,000 deaths annually. Many asthma phenotypes are distinguished, all characterized by variable airflow obstruction, bronchial hyper-responsiveness, and chronic airways inflammation. The most prevalent form of asthma is atopic asthma, which is initiated by the exposure to (inhaled) allergens, which often induces intermittent attacks of breathlessness, airways hyperreactivity, wheezing, coughing, and resultant allergen-specific immune responses (Nigro et al. 2015). Airways autonomic nervous system dysfunction plays a vital role in asthma pathogenesis conducing to an abnormality in airways smooth muscle physiology (hypercontractility). The inflammatory process within the airways promotes epithelial–mesenchymal transition, airways wall thickening, sub-epithelial fibrosis, myofibroblast/myocyte hyperplasia and hypertrophy and epithelial hypertrophy, by the activation of many types of cells such as mast and dendritic cells. These structural changes (airways remodeling) lead to deterioration of airways function. The current therapy of asthma includes controller and reliever drugs. Controller drugs include inhaled or oral steroids, long-acting β2-agonists combined with steroids, leukotriene receptor antagonists, sustained release theophylline, and anti-IgE. Reliever drugs are used during acute conditions and as required. These include short-acting β2-agonists, theophylline, and anticholinergics. A combination of inhaled β2-agonists and glucocorticoids is considered the gold standard therapy for the management of asthma. Currently, available asthma therapies provide symptomatic relief but are largely ineffective in controlling the progression of the disease.

There is a need for the development of new therapeutic molecules that can provide greater efficacy than steroids and with a better safety profile. Recently, several new compounds have been developed including anti-IgE antibodies, chemokine antagonists, and immunomodulators with the objective of targeting and modulating the physiological effects of that particular individual mediator. However, targeting individual molecules might not provide the desired effect due to the

extensive redundancy present. This opens up the avenue to identify and develop compounds with a multicellular site of action.

2 Bronchomotor Tone

The human airways are innervated via efferent and afferent autonomic nerves, which regulate many aspects of airways function. The parasympathetic nervous system is the dominant neuronal pathway in the control of airways smooth muscle tone. Stimulation of cholinergic nerves causes bronchoconstriction, mucus secretion, and bronchial vasodilation. Sympathetic nerves may control tracheobronchial blood vessels, but no innervation of human airways smooth muscle has been demonstrated. Beta-adrenergic receptors, however, are abundantly expressed on human airway smooth muscle and activation of these receptors causes bronchodilation. In addition to the classical cholinergic bronchoconstrictor and adrenergic bronchodilator, neural pathways within the airways exist, which are neither adrenergic nor cholinergic: the non-adrenergic, non-cholinergic (NANC) mechanisms (Stretton 1991). With respect to airways smooth muscle tone, NANC neural responses may induce either contraction, mediated by the release of sensory neuropeptides (from a subpopulation of non-myelinated C-fiber primary afferent neurons) such as substance P (SP), neurokinin A (NKA), and the peptide calcitonin gene-related peptide (CGRP) (excitatory, e-NANC), or relaxation, mediated by vasoactive intestinal peptide and nitric oxide (inhibitory, i-NANC) (Urbanek et al. 2016). The stimulation of excitatory NANC nerves causes bronchoconstriction, mucus secretion, vascular hyperpermeability, cough, and vasodilation, a process called "neurogenic inflammation."

3 Allergic Inflammation

Allergic inflammation often is classified into three temporal phases. Early-phase reactions that are induced within seconds to minutes of allergen challenge; late-phase reactions that occur within several hours; by contrast, chronic allergic inflammation is a persistent inflammation that occurs at sites of repeated allergen exposure (Ray and Cohn 1999). To trigger the allergic inflammatory reaction, sensitization to the allergens is necessary. Allergen can be sampled by dendritic cells in the airways lumen and can enter tissues through disrupted epithelium or, for some allergens with protease activity, can gain access to submucosal dendritic cells by cleaving epithelial cell tight junctions. Activated dendritic cells present the antigen to naïve T cells that, in the presence of IL-4, acquire the characteristics of T helper 2 (TH2) cells. These cells produce IL-4 and IL-13 that, in the presence of co-stimulatory molecules, lead B cells to produce antibody of the IgE class. IgE diffuses locally and systemically and binds to the high-affinity receptor for IgE (FcεRI) on tissue-resident mast cells, thereby sensitizing them to respond when the host is later reexposed to the allergen (Galli et al. 2008).

3.1 Early-Phase Reactions

The IgE-FcεRI binding induces the receptor aggregation, which activates mast cells to secrete preformed mediators and lipid derived mediators and to increase the synthesis of many cytokines, chemokines, and growth factors. The rapidly secreted mediators result in bronchoconstriction, vasodilation, increased vascular permeability, and increased mucus production. Mast cells also contribute to the transition to the late-phase reaction by promoting an influx of inflammatory leukocytes (Brightling et al. 2012; Filosa et al. 2015).

3.2 Late-Phase Reactions

Late-phase reactions are thought to reflect the actions of innate and adaptive immune cells that have been recruited from the circulation, as well as the secretion of inflammatory mediators by tissue-resident cells. The innate immune cells include neutrophils, monocytes, eosinophils, and basophils. Other cells that secrete inflammatory mediators include mast cells and tissue-resident or recruited T cells.

3.3 Chronic Allergic Inflammation

When allergen exposure is continuous or repetitive, inflammation persists, and many innate and adaptive immune cells derived from the blood can be found in the tissues at sites of allergen challenge. The persistent inflammation promotes the airways remodeling process, characterized by changes in the structural cells at the affected sites. These changes include an increased number of goblet cells (which produce mucus), an increased production of cytokines and chemokines by epithelial cells, and increased deposition of extracellular-matrix molecules in the lamina reticularis. Finally, changes in fibroblasts increased development of myofibroblasts, vascularity, and thickness of the muscular layer of the airways (Galli et al. 2008; Holgate 2007). Similar changes occur not only in the large but also in the small airways (membranous terminal and respiratory bronchi of less than 2 mm diameter), suggesting that these changes together with persistent inflammation may compromise the lung conduction system by altering mechanical properties of the airways (Tartaglione et al. 2018).

4 N/OFQ in the Airways

About 20 years ago, several scientists decided to investigate the possible modulatory role of the nociceptin/OFQ-NOP receptor system on the airways physiology, highlighting the capacity of N/OFQ to partially inhibit acetylcholine release from nerve endings of guinea-pig isolated trachea, to inhibit substance P release from rat trachea and to inhibit excitatory NANC contractile responses induced by EFS and

capsaicin in guinea-pig isolated bronchus (Shah et al. 1998). It has been shown that N/OFQ inhibits excitatory NANC contractile response (tachykinergic contractions) in a concentration-dependent manner, an effect attributable to a presynaptic and non-opioid mediated mechanism of action. Indeed, the μ-, δ-, and k-opioid receptor antagonists, naloxone, naltrindole and nor-binaltorphimine respectively, are without effect upon the excitatory NANC responses in guinea-pig renal pelvis, supporting the hypothesis that the inhibitory effect of N/OFQ on the tachykinergic contractions occurs independently of classically defined opioid receptors (D'Agostino et al. 2002; Schröder et al. 2014). The release of sensory neuropeptides following nerve depolarization, however, is Ca^{2+} mediated via the opening of N-type channels both of which are regulated by opioids and N/OFQ. The tachykinin-containing C-fiber afferents in the guinea-pig large airways arise primarily from cell bodies in the jugular ganglion that contains the NOP-receptor-mRNA, providing evidence that an N/OFQ-NOP receptor interaction leads to inhibition of tachykinergic transmission in the airways (Singh et al. 2013). Several hypotheses have been advanced regarding the possible mechanisms by which N/OFQ inhibits capsaicin-induced bronchoconstriction in isolated guinea pigs. One of these supports a direct NOP receptor-mediated inhibition of vanilloid receptor (VR1) calcium influx, and/or an indirect effect via membrane hyperpolarization of sensory nerve terminals, leading to a decrease of tachykinin release from non-myelinated C fibers of afferent sensory terminal nerves that innervate all compartments of the pulmonary wall, from trachea to bronchiole. It is known that NOP receptor activation induces inhibition of voltage-gated Ca^{2+} channel current, and activation of inward-rectifier K+ channels in neurons, either of which may lead to inhibition of neurotransmitter release. Inward-rectifier K^+ channels are involved in the N/OFQ's inhibitory action since tertiapin, an inward-rectifier K^+ channel antagonist, reversed the N/OFQ effects on capsaicin-induced bronchial contraction in guinea pig (Jia et al. 2002). Therefore, activation of a K^+ conductance by N/OFQ leads to membrane hyperpolarization, a concomitant inhibition of neuronal firing and, presumably, attenuate neurotransmitter release. Based on this evidence, from 1998 the role of the N/OFQ-NOP receptor system in a guinea-pig experimental animal model of gastroesophageal reflux was investigated (Fischer et al. 1998; Jia et al. 2002). This model was obtained through pretreatment with atropine and propranolol to block muscarinic and ß-adrenergic receptors and phosphoramidon to reduce tachykinin metabolism to highlight sensory nerves activation in plasma extravasation induced by intraesophageal acid instillation in the trachea and main bronchi of guinea pigs. The results showed the ability of N/OFQ to inhibit, at very low doses, microvascular leakage in the airways, induced by intraesophageal instillation of HCl but not by exogenous SP, suggesting that N/OFQ does not act via a postsynaptic inhibitory effect at the level of the vasculature but exert its preventive effect on sensory nerves at presynaptic sites. The effects of NOP receptor activation on lung responses were also evaluated in gastroesophageal reflux rabbit model. In this model, besides the plasma extravasation observed in guinea pigs, a bronchoconstriction effect may be documented following intraesophageal acid instillation that were both significantly reduced by pretreatment with N/OFQ (Gallelli et al. 2003; Rouget et al. 2004). Simultaneously with these

studies, other actions of the nociceptin in the airways were investigated. Specifically, the identification of the presence of NOP receptors on the bronchial afferent nerve fibers of guinea pigs, induced to investigate the possible involvement of the N/OFQ/NOP system in the modulation of the cough reflex (McLeod et al. 2001, 2004). The ability of nociceptin to inhibit the cough induced by both citric acid and capsaicin was demonstrated by a central and peripheral mechanism of action (Bolser et al. 2001; Lee et al. 2006). These results opened the way to further evaluate the N/OFQ effects on respiratory functions in mouse animal model. The mouse model choice was favored by the possibility to use the isolated and perfused mouse lung technique (IPL-1). This model reproduces the physiological process of breathing, mimicking a rib cage, and recording changes in bronchial contractions in response to administration of a specific substance (D'Agostino et al. 2005). In particular, in isolated mouse lungs, the role of N/OFQ-NOP system in the bronchoconstriction induced by activation of sensory fibers has been documented. In knockout NOP receptor ($NOP^{-/-}$) animals, no modulation of capsaicin-induced bronchoconstriction was observed, further underlining a direct involvement of the NOP receptor in the inhibitory action exerted by N/OFQ. Moreover, $NOP^{-/-}$ mice showed airway hyperresponsiveness to capsaicin similar to $NOP^{+/+}$ mice, when treated with the NOP antagonist, suggesting, for the first time, an involvement of the endogenous N/OFQ in the modulation of bronchoconstriction induced by sensory fibers activation (D'Agostino et al. 2010). Various immune competent cells are able to synthesize mRNA for the peptide precursor of N/OFQ (ppN/OFQ). Moreover, immune cell types such as normal circulating lymphocytes, polymorphonuclear cells, and monocytes in addition to T and B cell lines express the full-length NOP receptor mRNA. Some evidence shows that the NOP receptor modulates proliferation of human lymphocytes in vitro and regulates antibody production and neutrophil chemotaxis. Therefore the role of N/OFQ has been evaluated in the mechanisms of allergenic sensitization and bronchial hyperresponsiveness (Sullo et al. 2013). In particular, Th2 prone Balb/C mice sensitized to ovalbumin were chosen, because active sensitization to OVA is a commonly used model for allergic airways diseases. Although this model may not entirely reflect the situation in human allergic asthma, many similarities are observed, including histologic features, allergen-induced eosinophilia, and early- and late-phase airways obstruction after allergen challenge. N/OFQ treatment during OVA sensitization process (prophylactic approach) as well as during OVA aerosol-challenge in sensitized animals (therapeutic approach) substantially reduced bronchoconstriction and immunocyte trafficking to the lung, in particular, mast cells and eosinophils. N/OFQ also reduced mucin production and inflammatory mediators like IL-4, IL-5, and IL-13 (Th2 cytokines), while, IFN-γ production, the principal effector of Th1-mediated inflammation, was not modified suggesting a Th2 selective immunomodulatory effect of N/OFQ (D'Agostino et al. 2010; Singh et al. 2016). Indeed, N/OFQ was able to modulate lung reactivity and pulmonary resistances only in Th2 prone Balb/C mice respect to Th1-prone CD1mice, suggesting a role of N/OFQ in the modulation of Th2-like inflammation observed in the asthmatic disease. The important role of N/OFQ in the Th2-like environment was validated via circular dichroism (CD), spectroscopic in vitro study

of the interaction between peptides and different cells. It is widely known that dendritic cells (DC) are generally responsible to start and sustain allergic T helper 2 (TH2)-cell responses to inhaled allergens in asthma. By circular dichroism (CD) the interaction between N/OFQ and different DCs from Balb/c and CD1 mice was evaluated. The spectroscopic data strongly indicated that N/OFQ was able to interact on the cell membrane of DCs obtained from Balb/c mice rather than CD1 mice. Therefore, these data further indicate a Th2 modulation of N/OFQ and at the same time show its direct activity on DCs (Spaziano et al. 2017). Since histopathological evidence clearly shown that the asthmatic inflammation also involves the small airways, the role of N/OFQ in the inflammation and remodeling of the small airways has been evaluated in very recent studies. As observed in the large airways, prophylactic and therapeutic approach with N/OFQ led to significant decrease of sC_{aw} (small airway compliance) as well as a reduction in the area of the bronchial wall in ovalbumin-sensitized mice. Furthermore, N/OFQ treatments were also associated with a reduction of airways smooth muscles (ASM) hyperplasia and to an interesting protective role against the loss of alveolar attachments caused by OVA sensitization suggesting a protective role of N/OFQ in maintaining the patency of the small airways during asthma (Singh et al. 2016; Tartaglione et al. 2018). Altogether these data further emphasize the N/OFQ efficacy in regulating mechanical properties and remodeling of small airways leaving to hypothesize prophylactic and therapeutic effects of N/OFQ on the airway immunopathology and AHR of allergic asthma with important consequences for potential treatment paradigms.

5 N/OFQ and Airways in Human

An in vitro study, conducted on isolated human bronchi obtained from 23 patients undergoing surgery for lung cancer, documented that NOP receptor activation modulates the cholinergic component of the contraction induced by the electrical field stimulation (EFS) inhibiting acetylcholine (ACh) release through stimulation of potassium currents (Basso et al. 2005). Further human data obtained from healthy and asthmatic patients show that N/OFQ expression was found to be up-regulated in the lung biopsies from asthmatic patients. In particular, N/OFQ has increased in the sub-epithelial layer and extracellular matrix. However, any significant increase in NOP receptor expression in asthma implying no disease signal was not detected, but significant NOP receptor expression has been shown in human airways structural and inflammatory cells, with ppN/OFQ expression only in eosinophils. In particular, asthmatic sputum showed elevated N/OFQ levels that are related to increased eosinophil counts. Interestingly, it has been observed that further addition of exogenous N/OFQ in sputum obtained from patients with severe asthma (which reported high levels of endogenous N/OFQ) significantly attenuated eosinophils migration and release of inflammatory key mediators involved in eosinophils recruitment. These observations confirmed that elevated endogenous N/OFQ levels in asthmatic sputum were much lower than the ones required to exert beneficial effects (Singh et al. 2016), suggesting that supplementation with exogenous N/OFQ is needed.

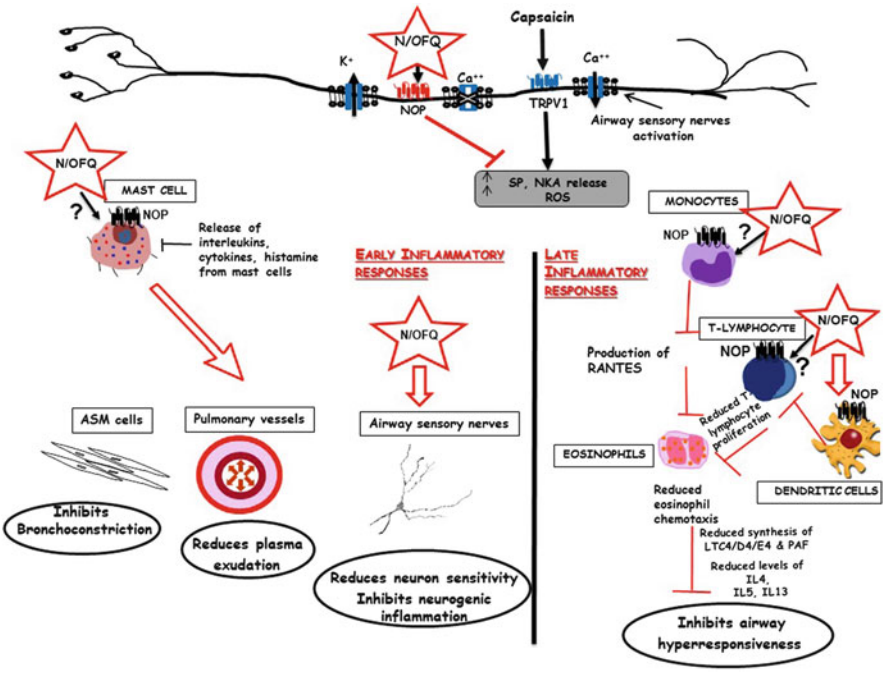

Fig. 1 N/OFQ mechanisms: dual action in the airway and immune response

It is widely recognized that N/OFQ is naturally occurring peptide and does not cross the blood–brain barrier (Lambert 2008). Moreover, the safety of local administration of N/OFQ has been already documented in a clinical trial evaluation on urodynamic effects of intravesical administration of N/OFQ in patients with neurogenic detrusor overactivity (Lazzeri et al. 2003). Therefore a simple clinical trial of nebulized N/OFQ both as a prophylactic and a therapeutic treatment during exacerbation of asthma could be warranted. The use of a nebulized formulation would reduce total body dosing, negate the likelihood of central spread, and offer the advantage of a single entity combining anti-hyperresponsiveness and immunomodulatory actions. However, it is important to underline that regarding N/OFQ possible use in cough therapy, Woodcock et al. have performed a multicenter, double-blinded, placebo-controlled, parallel-group study in patients with subacute cough showing the limited antitussive efficacy of NOP receptor agonist compared to placebo (Woodcock et al. 2010) (Fig. 1).

6 Conclusions

Based on its broad spectrum of biological effects, the N/OFQ-NOP receptor system represents an attractive target with numerous potential therapeutic utility. Far findings from animal and human studies clearly suggest that N/OFQ may

potentially play a crucial role in the pathogenesis of airway inflammation in asthma, leaving affirm that N/OFQ could have an innovative role in counteracting airways inflammatory responses and airways hyperresponsiveness. This combination of beneficial effects, rarely observed, suggests a potential role for N/OFQ in the management for both prophylaxis and exacerbations of allergic asthma.

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Effects of NOP-Related Ligands in Nonhuman Primates

Norikazu Kiguchi and Mei-Chuan Ko

Contents

Abstract

The nociceptin/orphanin FQ peptide (NOP) receptor-related ligands have been demonstrated in preclinical studies for several therapeutic applications. This article highlights (1) how nonhuman primates (NHP) were used to facilitate the development and application of positron emission tomography tracers in humans; (2) effects of an endogenous NOP ligand, nociceptin/orphanin FQ, and its interaction with mu opioid peptide (MOP) receptor agonists; and (3) promising functional profiles of NOP-related agonists in NHP as analgesics and treatment for substance use disorders. NHP models offer the most phylogenetically appropriate evaluation of opioid and non-opioid receptor functions and drug effects.

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Based on preclinical and clinical data of ligands with mixed NOP/MOP receptor agonist activity, several factors including their intrinsic efficacies for activating NOP versus MOP receptors and different study endpoints in NHP could contribute to different pharmacological profiles. Ample evidence from NHP studies indicates that bifunctional NOP/MOP receptor agonists have opened an exciting avenue for developing safe, effective medications with fewer side effects for treating pain and drug addiction. In particular, bifunctional NOP/MOP partial agonists hold a great potential as (1) effective spinal analgesics without itch side effects; (2) safe, nonaddictive analgesics without opioid side effects such as respiratory depression; and (3) effective medications for substance use disorders.

Keywords
Analgesics · Bifunctional ligands · Chronic pain · Drug abuse · Inflammatory pain · MOP receptor · NOP receptor · Opioids · Parkinson's disease · Primate · Spinal cord

1 The N/OFQ-NOP Receptor System

In 1994, several groups of scientists discovered a G protein-coupled receptor with high homology to classical opioid receptors, and this receptor was initially named opioid receptor-like 1 (ORL1) (Bunzow et al. 1994; Fukuda et al. 1994; Mollereau et al. 1994; Wang et al. 1994). A year later, two groups of scientists isolated an endogenous 17-amino acid peptide (FGGFTGARKSARKLANQ) which is selective for the ORL1 receptor. This peptide was named "nociceptin," because following intracerebroventricular injection, it produced hyperalgesia in mice (Meunier et al. 1995). The same peptide was named "orphanin FQ" based on the recognition of the ORL1 receptor and its first and last amino acid residues (Reinscheid et al. 1995). According to the nomenclature guidelines recommended by the International Union of Basic and Clinical Pharmacology, the peptide was named "nociceptin/orphanin FQ" (N/OFQ), and the ORL1 receptor was renamed "N/OFQ peptide" (NOP) receptor (Cox et al. 2015). This ligand-receptor system has been extensively studied in the past 25 years. Several articles have provided comprehensive overview about the biological actions, medicinal chemistry, pharmacology, and therapeutic applications of the N/OFQ-NOP receptor system (Calo' and Guerrini 2013; Kiguchi et al. 2016; Lambert 2008; Toll et al. 2016; Witkin et al. 2014; Zaveri 2016). This review particularly highlights the functional profiles of NOP-related ligands in nonhuman primates (NHP) and discusses the therapeutic potential of NOP receptor-targeted ligands.

1.1 Cloning of the Rhesus Monkey NOP Receptor

Similar to classical opioid receptors, NOP receptor is coupled to pertussis toxin-sensitive Gi/o proteins which inhibit adenylate cyclase and voltage-gated calcium

channels and activate inward potassium channels (Hawes et al. 2000; Margas et al. 2008; Vaughan and Christie 1996). NOP receptor activation reduces synaptic transmission by either inhibiting neuronal excitability via postsynaptically located NOP receptors or reducing neurotransmitter release via presynaptically located NOP receptors (Calo' and Guerrini 2013; Moran et al. 2000; Schlicker and Morari 2000). The NOP receptor has been implicated in numerous therapeutic applications based on burgeoning preclinical animal studies (Lambert 2008; Witkin et al. 2014). Given the species differences in receptor activation and signaling cascades between rodents and primates (Chen et al. 2013; Li et al. 2002; Schattauer et al. 2012), it is important to know if the NOP receptor functions differently between NHP and humans.

Scientists have succeeded to clone the rhesus monkey NOP receptor and found that the nucleotide sequence and amino acid sequence of the rhesus monkey NOP receptor were 95.9% and 97.8% identical to those of the human NOP receptor, respectively (Koga et al. 2009). The identified seven amino acid differences between the monkey and the human NOP receptor did not affect the potency of (+)J-113397, a NOP receptor antagonist, in the inhibition of N/OFQ-stimulated [^{35}S]GTPγS binding. There was no significant difference between the monkey and the human NOP receptor in terms of the binding affinity of ^{125}I[Tyr^{14}]N/OFQ, the [^{35}S]GTPγS binding stimulated by N/OFQ, and the antagonist activity of (+)J-113397 (Koga et al. 2009). N/OFQ seems to activate both monkey and the human NOP receptors without significant species differences.

1.2 Imaging Studies of the NOP Receptor

The distribution of ^{125}I[Tyr^{14}]N/OFQ binding sites has been optimized and determined in the brain and spinal cord of cynomolgus macaques (Bridge et al. 2003). The binding sites of ^{125}I[Tyr^{14}]N/OFQ were widespread in the NHP central nervous system and largely consistent with the mRNA expression pattern of the NOP receptor in the human central nervous system (Peluso et al. 1998). The highest levels of ^{125}I[Tyr^{14}]N/OFQ binding were detected in NHP neocortical areas (e.g., frontal cortex and cingulate cortex), hippocampus, amygdala, thalamus, and caudate putamen. There are some differences in several regions regarding low- versus high-binding levels, including the hippocampus, spinal cord, caudate putamen, ventral tegmental area, and dorsal raphe nucleus between NHP (Bridge et al. 2003) and rodents (Anton et al. 1996; Letchworth et al. 2000; Neal et al. 1999). The extensive distribution of ^{125}I[Tyr^{14}]N/OFQ binding sites in NHP not only supports the multiple functional roles of the N/OFQ-NOP receptor system (Lambert 2008; Witkin et al. 2014) but also indicates that some NOP receptor functions may be species-selective (Bridge et al. 2003).

Positron emission tomography (PET) is a powerful noninvasive in vivo imaging technique to measure the receptor occupancy and target expression and for visualization of metabolic processes (Giovacchini et al. 2011). The development of selective PET radiotracers for the NOP receptor has been successful (Hostetler et al. 2013; Pedregal et al. 2012; Pike et al. 2011). Among reported NOP PET

tracers, ^{11}C-NOP-1A was initially demonstrated as a useful radioligand to quantify NOP receptor in the rhesus monkey brain (Kimura et al. 2011). ^{11}C-NOP-1A showed good, stable brain uptake, and a selective NOP antagonist, SB-612111, decreased its distribution volume (V_T; a measure of receptor density) by approximately 50–70% in all brain regions, indicating that most brain uptake was specifically bound to NOP receptors (Kimura et al. 2011). Subsequently, ^{11}C-NOP-1A was further demonstrated as a promising PET ligand to reliably quantify NOP receptors in the human brain (Lohith et al. 2012, 2014). Whole-body scans showed radioactivity of ^{11}C-NOP-1A in the brain and peripheral organs expressing NOP receptors, such as heart, lungs, and pancreas; and its effective dose is similar to that of other ^{11}C-labeled radioligands in humans (Lohith et al. 2012). Recently, ^{11}C-NOP-1A was used to measure the in vivo binding to NOP receptors in alcohol-dependent individuals, and regional distribution volume of ^{11}C-NOP-1A was not significantly different from that of healthy individuals in the control group (Narendran et al. 2018). These findings may indicate that central NOP receptor density remains unchanged in alcohol-dependent individuals.

Another promising NOP PET tracer is [^{18}F]MK-0911 (Hostetler et al. 2013). The pattern of [^{18}F]MK-0911 binding density in the rhesus monkey brain, such as cortex, caudate putamen, hippocampus, and cerebellum, is consistent with the localization of ^{125}I[Tyr^{14}]N/OFQ binding sites in the macaque brain (Bridge et al. 2003). [^{18}F]MK-0911 displayed reversible NOP receptor-specific binding in the rhesus monkey brain, as its binding was blocked dose-dependently by selective NOP antagonists in different structures; and baseline PET scans with [^{18}F]MK-0911 in healthy humans showed similar tracer distribution and kinetics as compared to those in rhesus monkeys (Hostetler et al. 2013). Importantly, increasing doses of MK-5757, a selective NOP antagonist (Satoh et al. 2009), prior to [^{18}F]MK-0911 were associated with higher levels of the NOP receptor occupancy (Hostetler et al. 2013). Such receptor occupancy studies with selective NOP PET tracers will provide essential dose-selection guidance for future clinical development of NOP receptor antagonists. Collectively, NOP PET tracers are valuable tools to investigate the functional roles of NOP receptors and endogenous N/OFQ in humans under different disease states, such as mental disorders and substance abuse disorders, and facilitate the development of NOP-targeted ligands for different therapeutic applications.

2 Effects of N/OFQ in Nonhuman Primates

N/OFQ has been administered through different delivery routes to determine its role for modulating pain and itch in NHP. Originally, N/OFQ was co-administered with capsaicin into the monkey's tail to illustrate its peripheral antiallodynic effects, which could be blocked by a NOP receptor antagonist (Ko et al. 2002a). This early study provides the first functional evidence that activation of peripheral NOP receptors in primates could be a viable therapeutic target for alleviating peripherally elicited pain. Indeed, NOP receptors were present in most of small- and large-diameter human

dorsal root ganglion (DRG) neurons, and activation of NOP receptors inhibited capsaicin-induced calcium flux in human DRG neurons (Anand et al. 2016). NOP receptors were also found on epidermal keratinocytes and small unmyelinated and large myelinated nerve fibers in humans. The expression of NOP receptors in plantar skin affected by pachyonychia congenital was relatively lower than that of unaffected skin (Pan et al. 2018). These findings together support the notion that peripheral NOP receptor activation may be a treatment option for managing neuropathic pain.

Intrathecal delivery of mu opioid peptide (MOP) receptor agonists has become part of a routine regimen for perioperative analgesia (e.g., during caesarean section) and been used successfully in different clinical settings in the past four decades (Brill et al. 2003; Schug et al. 2006). However, itch (pruritus) is a common side effect derived from intrathecal morphine and compromises the use of spinal opioids in pain management (Ganesh and Maxwell 2007; Waxler et al. 2005). Interestingly, similar to human responses, intrathecal morphine produced long-lasting itch sensation and pain relief simultaneously in NHP (Ko and Naughton 2000). Intrathecal N/OFQ dose-dependently produced antinociception without eliciting itch scratching responses in NHP, and this effect was reversed by a NOP receptor antagonist (Ko et al. 2006). Along with the mass spectrometry, N/OFQ(2-17) was identified as the major fragment of N/OFQ in the NHP cerebrospinal fluid, and N/OFQ(2-17) did not interfere with intrathecal N/OFQ-induced antinociception (Ko et al. 2006). Given that rodents did not display robust scratching responses following intrathecal morphine (Lee et al. 2003; Sukhtankar and Ko 2013), NHP could serve as a surrogate species to build up a translational bridge for identifying novel spinal analgesics without itch side effects.

Intrathecal N/OFQ in ultralow doses (i.e., in femto moles) in mice produced pain-like behaviors manifested by biting, scratching, and licking behaviors (Sakurada et al. 1999). Unlike dual actions (i.e., pronociception in low doses and antinociception in high doses) of spinal N/OFQ in rodents (Hao et al. 1998; Inoue et al. 1999), intrathecal N/OFQ over a wide dose range, from 1 fmol to 1 μmol, only produced antinociception in NHP (Ko and Naughton 2009). More importantly, intrathecal N/OFQ did not exert anti-morphine action as N/OFQ dose-dependently enhanced intrathecal morphine-induced antinociception without attenuating morphine-induced scratching (Ko and Naughton 2009). In a NHP model of inflammatory pain, intrathecal N/OFQ was found to be the most potent peptide among all endogenous opioid-related peptides for exerting antihyperalgesia (Lee and Ko 2015). Taken together, these findings suggest that spinal N/OFQ-NOP receptor system plays a pivotal role in pain inhibition and the NOP receptor represents an attractive target as spinal analgesics (Kiguchi et al. 2016).

Supraspinal N/OFQ-NOP receptor system plays a pronociceptive role in rodents, as several studies have shown that intracerebroventricular administration of N/OFQ and NOP receptor agonists produced hyperalgesia and attenuated morphine-induced antinociception (Calo et al. 1998; Meunier et al. 1995; Reinscheid et al. 1995). With the advance of surgical techniques, an intrathecal catheter was implanted, and the catheter tip was placed in the cisterna magna of NHP for supraspinal drug delivery (Ding et al. 2015). The intracisternal administration of neuropeptides mimics the "volume transmission" of endogenous peptides transported to multiple sites in the

brain (Veening et al. 2012). Unlike substance P eliciting allodynia-/hyperalgesia-like responses, intracisternal administration of N/OFQ produced NOP antagonist-reversible antinociceptive effects, and intracisternal N/OFQ did not attenuate morphine antinociception in NHP (Ding et al. 2015). These findings provide distinct functional profiles of supraspinal N/OFQ-NOP receptor system between NHP and rodents. NHP with the intracisternal catheter could further improve our understanding of diverse neuropeptides involved in top-down, descending pain modulation in primates.

To our knowledge, NOP-related ligands have been studied in NHP in three therapeutic areas, i.e., treatment potential for (1) pain, (2) substance use disorders, and (3) Parkinson's disease. As Morari's research team has recently reviewed effects of NOP-related ligands in the NHP model of Parkinson's disease (Mercatelli et al. 2019), below we specifically discuss the effects of NOP-related ligands as analgesics and a treatment option for substance use disorders.

3 NOP-Related Agonists as Analgesics

Ample evidence indicates that NOP-related agonists exerted antinociceptive and antihypersensitive effects in rodents under a variety of pain modalities (Kiguchi et al. 2016; Schroder et al. 2014). As intrathecal and systemic administration are common drug delivery routes in the clinic, this section reviews the antinociceptive and antihypersensitive effects of NOP-related agonists following intrathecal and systemic administration in NHP.

3.1 Effects of Intrathecal Administration of NOP-Related Agonists

3.1.1 Selective NOP Receptor Agonists

The spinal dorsal horn is the major locus not only for the integration of peripheral sensory input and descending supraspinal modulation but also for regulating peripherally and centrally mediated pain (Peirs and Seal 2016). In particular, intrathecal drug delivery can provide effective, long-lasting pain relief as an alternative delivery route (Caraway et al. 2015; Smyth et al. 2015). Through chemical modification of N/OFQ by increasing its potency and decreasing its degradation, a selective NOP agonist UFP-112 exerted antinociceptive effects with higher potency and longer duration of action than N/OFQ in mice (Calo et al. 2011; Rizzi et al. 2007). Such findings can be translated to NHP as intrathecal UFP-112 was approximately ten times more potent than morphine with similar duration of action for attenuating acute pain and capsaicin-induced thermal allodynia in NHP (Hu et al. 2010).

Using an innovative chemical strategy, peptide welding technology (PWT) (Calo et al. 2018), scientists have generated different tetrabranched derivatives of N/OFQ. PWT2-N/OFQ was demonstrated to be a high-affinity, potent, and

selective NOP agonist. In particular, PWT2-N/OFQ was about 40-fold more potent than N/OFQ and produced 5 h duration of antinociception in mice (Rizzi et al. 2015). More importantly, these promising findings (e.g., largely increased potency and improved duration of action of PWT2-N/OFQ) can be translated from rodents to primates. Intrathecal PWT2-N/OFQ potently exerted full antinociceptive effects lasted for more than 24 h without eliciting scratching in NHP (Rizzi et al. 2015). For a side-by-side comparison, PWT2-N/OFQ (i.e., 3 nmol) is approximately 30-fold more potent than N/OFQ (100 nmol), and the duration of antinociceptive action of PWT2-N/OFQ (~24 h) is tenfold longer than that of N/OFQ (~2.5 h) in NHP (Ko et al. 2006; Rizzi et al. 2015). These findings indicate that PWT derivatives of N/OFQ-related peptides are viable candidates for future spinal analgesics with improved therapeutic profiles.

3.1.2 Ligands with Mixed NOP/MOP Receptor Agonist Activity

In rat neuropathic pain models, intrathecal N/OFQ not only exerted antihyperalgesia but also synergistically enhanced antihyperalgesic effects of intrathecal morphine (Courteix et al. 2004). This antinociceptive synergism by coadministration of NOP and MOP receptor agonists intrathecally has also been found in NHP (Hu et al. 2010; Ko and Naughton 2009). In order to investigate the pharmacological profile of a single molecule with mixed NOP/MOP agonist activity, scientists have identified several mixed NOP/MOP receptor agonists. [Dmt^1]N/OFQ(1-13)-NH_2 displayed similar potency and efficacy like N/OFQ in vitro, but intrathecal [Dmt^1]N/OFQ (1-13)-NH_2 was approximately 30-fold more potent than N/OFQ in producing antinociception in NHP (Molinari et al. 2013). Moreover, intrathecal PWT2-[Dmt^1]N/OFQ(1-13) exerted full antinociceptive effects with higher potency and much longer duration of action in NHP (Cerlesi et al. 2017).

Intrathecal administration of small molecules with mixed NOP/MOP partial agonist activity, such as BU08028 and SR16435, also potently and effectively attenuated hypersensitivity in mouse models of neuropathic and inflammatory pain (Sukhtankar et al. 2013). More importantly, repeated administration of intrathecal SR16435 showed slower development of tolerance to its antiallodynic effects as compared to a partial MOP agonist buprenorphine (Sukhtankar et al. 2013). Recently, scientists have identified a naltrexone-derived analog with mixed NOP/MOP partial agonist activity, BU10038, and found that intrathecal administration of BU10038 potently produced antinociception and antihypersensitivity without scratching, and intrathecal BU10038 did not cause tolerance, as compared to morphine, after chronic 4-week administration in NHP (Kiguchi et al. 2019). Collectively, these findings together strongly support the notion that mixed NOP/MOP receptor agonists display higher potency, wider therapeutic window, and slower tolerance development and such ligands should be developed as a new generation of spinal analgesics.

3.2 Effects of Systemic Administration of NOP-Related Agonists

3.2.1 Selective NOP Receptor Agonists

Behavioral effects of systemic administration of NOP-related agonists are integrated from peripheral, spinal, and supraspinal actions of NOP receptor activation. Following subcutaneous, intramuscular, or intravenous administration, selective NOP receptor agonists, such as Ro 64-6198 and SCH 221510, dose-dependently produced antinociceptive effects against different noxious stimuli in NHP (Kangas and Bergman 2014; Ko et al. 2009; Podlesnik et al. 2011; Sukhtankar et al. 2014b). In particular, systemic NOP receptor agonists effectively increased thermal nociceptive thresholds (Cremeans et al. 2012; Kangas and Bergman 2014) and attenuated capsaicin-induced allodynia (Ko et al. 2009) and carrageenan-induced hyperalgesia (Sukhtankar et al. 2014b). Compared to clinically used MOP receptor agonists, selective NOP receptor agonists did not cause adverse effects typically associated with MOP agonists, such as respiratory depression, itch, abuse liability, constipation, and physical dependence (Ding et al. 2016; Ko et al. 2009; Wladischkin et al. 2012). However, Ro 64-6198 caused sedation at a dose which was 30-fold higher than its full antinociceptive dose in NHP (Podlesnik et al. 2011). This functional profile of selective NOP agonists is still considered promising because antinociceptive doses of MOP agonists produced respiratory depression and reinforcing effects (Butelman et al. 1993; Ko et al. 2002b), kappa opioid peptide (KOP) receptor agonists produced sedation and dysphoria (Butelman et al. 2001; Ko et al. 1999), and delta opioid peptide (DOP) receptor agonists produced convulsions (Negus et al. 1994; Sukhtankar et al. 2014b) in NHP.

Selective NOP agonists did not consistently increase thermal nociceptive thresholds across different groups of NHP (Cornelissen et al. 2019; Saccone et al. 2016). It should be noted that the tail-withdrawal latency from an acute noxious stimulus, 50°C water, is not relevant to the clinical setting in which patients experience spontaneous pain and mechanical hypersensitivity (Brix Finnerup et al. 2013). This procedure has been used commonly by NHP investigators to study opioid-related ligands (Butelman et al. 2001; Ko et al. 1999; Negus et al. 1994); however, it is not useful for non-opioid analgesics with different mechanisms (Hawkinson et al. 2007; Sukhtankar et al. 2014b). Moreover, antinociceptive doses of MOP agonists measured by the NHP warm water tail-withdrawal assay impaired NHP's food-maintained operant behavior (Withey et al. 2018). These results indicate that antinociceptive doses of clinically used MOP agonists detected in NHP might be too high, i.e., no behavioral selectivity as the same antinociceptive dose has suppressed other behavioral responses. In particular, the antinociceptive dose 10 mg/kg of morphine in NHP (Cornelissen et al. 2019) was much higher than the analgesic doses of morphine (i.e., 0.1–0.2 mg/kg) used in humans (Aubrun et al. 2012), indicating that these NHP needed much higher doses of morphine to suppress their tail-withdrawal responses. As behaviorally disruptive effects of (−)Ro 64-6198 peaked at 100 min after intramuscular administration, using a 15-min inter-injection interval to assess behavioral effects of (−)Ro 64-6198 (Cornelissen et al. 2019) was a significant experimental design flaw. Without recognizing promising clinical data

of cebranopadol, a mixed NOP/opioid receptor agonist (Calo and Lambert 2018; Raffa et al. 2017; see Sect. 3.2.2), Cornelissen et al. (2019) made an inappropriate conclusion about the opioid-sparing potential of NOP agonists. Nevertheless, it is worth noting that SCH 221510 significantly produced morphine-like antinociceptive effects in a NHP "operant" nociceptive assay with behavioral selectivity (Kangas and Bergman 2014). Such findings agree with those from reflex-based assays (Podlesnik et al. 2011; Sukhtankar et al. 2014b) and support the analgesic potential of selective NOP agonists. As the functional plasticity of NOP receptors and the efficacy of NOP agonists may change along with different pain modalities (Kiguchi et al. 2016; Schroder et al. 2014), NHP studies with different outcome measures including operant behavior and hypersensitivity will advance our understanding of the analgesic potential of NOP-related ligands.

3.2.2 Ligands with Mixed NOP/MOP Receptor Agonist Activity

In addition to MOP agonist-induced antinociception enhanced by NOP agonists at the spinal level (Hu et al. 2010; Ko and Naughton 2009), the isobologram analysis demonstrates that systemic NOP receptor agonists, Ro 64-6198 and SCH 221510, synergistically enhanced buprenorphine-induced antinociception without causing respiratory depression in NHP (Cremeans et al. 2012). Buprenorphine has much lower binding affinity at NOP receptors, i.e., its K_i values range from 77 to 285 nM, and good binding selectivity for MOP over NOP receptors (i.e., from 50- to 930-fold) (Ding et al. 2018c; Khroyan et al. 2009; Spagnolo et al. 2008). In the functional assay of NOP agonist-stimulated [^{35}S]GTPγS binding, buprenorphine displayed no stimulation (Spagnolo et al. 2008) or mild stimulation (i.e., 10–15% as compared to N/OFQ) at much higher concentration (i.e., >250 nM) (Ding et al. 2018c; Khroyan et al. 2009). The receptor binding and efficacy profile of buprenorphine fits very well with its MOP partial agonist profile in NHP as the NOP receptor antagonist could not shift the dose-response curve of buprenorphine-induced antinociception (Cremeans et al. 2012).

Buprenorphine has been widely used in both humans and veterinary medicine to effectively alleviate a variety of pain conditions including neuropathic pain (Hans 2013; Raffa et al. 2014). However, buprenorphine is not completely devoid of abuse potential (Lavonas et al. 2014). Given the inhibition of dopamine neurotransmission by the NOP receptor (Flau et al. 2002; Murphy et al. 1996) and synergistic antinociception between NOP agonists and buprenorphine (Cremeans et al. 2012), we initially formed a hypothesis that coactivation of NOP and MOP receptors may potently produce analgesia with fewer side effects (Lin and Ko 2013). Despite that NOP receptor activation attenuated MOP receptor-mediated antinociception in rodents (Khroyan et al. 2009), our hypothesis that bifunctional NOP/MOP agonists may have a wider therapeutic window as compared to selective MOP or NOP agonists in primates (Lin and Ko 2013) is supported by the functional profiles of three ligands with mixed NOP/MOP agonist activity discussed below (Fig. 1).

BU08028, a recently developed buprenorphine analog, strikingly displays a similar receptor binding profile like buprenorphine (i.e., K_i: 1–10 nM for MOP, KOP, and DOP receptors) with improved binding affinity (K_i: 8 nM) and efficacy

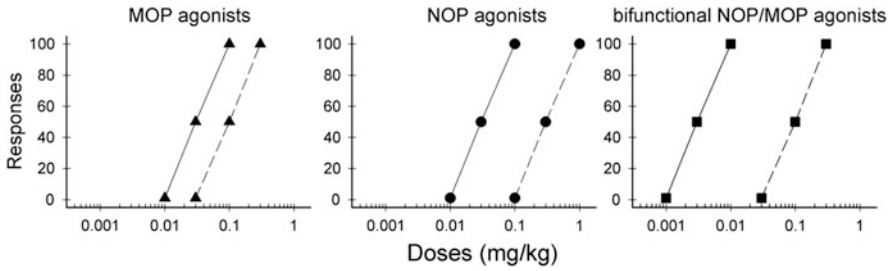

Fig. 1 A general hypothetical framework of comparison of the therapeutic windows of MOP, NOP, and bifunctional NOP/MOP agonists based on current literature. Solid lines indicate the doses at which antinociception/analgesia occurs. Dashed lines indicate the doses at which side effects, especially respiratory depression and sedation, emerge. Reprinted with permission from Lin and Ko (2013)

(~48% stimulation of [^{35}S]GTPγS binding) on NOP receptors (Khroyan et al. 2011). BU08028 exerted an extra-long duration of antinociceptive and antihypersensitive effects, up to 30 h, in NHP (Ding et al. 2016). Unlike rodent studies in which a NOP antagonist potentiated BU08028-induced antinociception (Khroyan et al. 2011), both NOP and MOP antagonists produced the same degree of the rightward shift of the dose-response curve for BU08028-induced antinociception in NHP (Ding et al. 2016). Under the progressive-ratio schedule of drug self-administration, BU08028 did not produce reinforcing effects (i.e., abuse potential) as compared to other drugs of abuse, including cocaine and buprenorphine. More importantly, unlike fentanyl which quickly caused respiratory depression, BU08028 at ~30 times higher than its antinociceptive dose did not change NHP's respiratory and cardiovascular activities. These findings provide the first functional evidence that BU08028 with mixed NOP/MOP agonist activity is a safe, nonaddictive analgesic in NHP (Ding et al. 2016).

In order to test our hypothesis by a non-morphinan chemical structure, AT-121 was identified as a bifunctional NOP/MOP partial agonist, which showed high potency (EC_{50}, 20–35 nM) and partial agonist efficacy (NOP, 41%; MOP, 14% stimulation of [^{35}S]GTPγS binding) at both NOP and MOP receptors (Ding et al. 2018c). Through a series of NHP assays, AT-121 exerted morphine-like antinociceptive and antihypersensitive effects and did not compromise respiratory and cardiovascular activities. Unlike morphine, AT-121 did not produce opioid-induced hyperalgesia and physical dependence and has a much slower development of analgesic tolerance than morphine. Slower development of tolerance to AT-121's antinociception supports the notion that coactivation of NOP and MOP receptors reserves most functional receptor reservoirs and repeated administration of a bifunctional NOP/MOP agonist may cause a smaller degree of receptor desensitization (Dumas and Pollack 2008; Lin and Ko 2013). More importantly, daily pretreatment with AT-121 attenuated reinforcing effects of oxycodone without

disrupting food-maintained operant behavior, indicative of selective inhibition of opioid-reinforced operant behavior (Ding et al. 2018c). These findings together not only support our hypothesis that bifunctional NOP/MOP agonists are safe, nonaddictive analgesics with a wider therapeutic window (Lin and Ko 2013) but also provide functional evidence that such agonists could have a dual therapeutic action for treatment of pain and opioid addiction (Ding et al. 2018c). It is worth noting that opioid and non-opioid "partial" agonists generally have proven therapeutic efficacy with favorable safety and tolerability (Kane et al. 2016; Kantola et al. 2017; van Niel et al. 2016). Similar to buprenorphine's intrinsic efficacy (e.g., ~17% stimulation of [^{35}S]GTPγS binding at MOP receptors) (Spagnolo et al. 2008), BU08028 and AT-121 are expected to exert analgesic efficacy equal to or more than buprenorphine, but with little or no abuse liability.

Cebranopadol binds to NOP, MOP, and KOP receptors with K_i values of 1–3 nM, and it has nearly full agonist activity at human NOP, MOP, and DOP receptors and partial agonist activity at KOP receptors, based on the [^{35}S]GTPγS binding assay (Linz et al. 2014). As Calo and Lambert (2018) has recently provided a comprehensive review of cebranopadol, we only briefly discuss this drug herein. Through a series of preclinical pain models in rodents, cebranopadol is highly potent (e.g., ED_{50} values, 0.5–5 μg/kg in rats with chronic pain) and fully effective in producing antinociceptive and antihypersensitive effects (Calo and Lambert 2018; Linz et al. 2014; Raffa et al. 2017). In rat models of spinal nerve ligation-induced neuropathy and arthritic pain, both NOP and MOP receptors mainly contributed to antihypersensitive effects of cebranopadol (Linz et al. 2014; Schiene et al. 2018). Cebranopadol also potently (1–5.6 μg/kg, subcutaneous) produced antinociceptive and antihypersensitive effects in NHP. After intrathecal administration, 1 μg of cebranopadol produced antinociception without eliciting scratching responses (Trapella et al. 2018). More importantly, recent clinical studies have reported promising results of cebranopadol's efficacy and tolerability. For example, an analgesic dose of cebranopadol produced respiratory depression with an estimate for minimum ventilation greater than zero l/min, which is different from full MOP agonists such as fentanyl and indicative of potential ceiling, in healthy individuals (Dahan et al. 2017). In the first clinical trial in patients with chronic low back pain, cebranopadol was effective, safe, and displayed beneficial effects, such as improved sleep and functionality, with an acceptable tolerability profile (Christoph et al. 2017). In patients experiencing moderate to severe pain following bunionectomy, cebranopadol was better tolerated and received a better overall rating than morphine controlled release (Scholz et al. 2018). In patients with moderate-to-severe cancer pain, cebranopadol was effective, safe, and well-tolerated than morphine prolonged release (Eerdekens et al. 2018). Overall, these clinical data of cebranopadol support the hypothesis that ligands with mixed NOP/MOP agonists have the improved analgesic potency and wider therapeutic window (Kiguchi et al. 2016; Lin and Ko 2013).

4 NOP-Related Ligands for Treatment of Substance Use Disorders

4.1 Effects of Selective NOP Receptor Agonists

Given that activation of NOP receptors inhibited dopamine release in the nucleus accumbens (Di Giannuario and Pieretti 2000; Murphy et al. 1996), NOP receptor agonists may not produce reward-related behaviors and may inhibit MOP receptor-mediated reward. Unlike MOP agonists, NOP agonists did not produce conditioned place preference (CPP) (Devine et al. 1996) and reinforcing effects measured by drug self-administration (Sukhtankar et al. 2014a), and they blocked MOP agonist-induced CPP in rodents (Toll et al. 2016). In NHP, the discriminative stimulus effects of Ro 64-6198 partially generalized to diazepam (Saccone et al. 2016), but Ro 64-6198 did not produce reinforcing effects as compared to alfentanil, cocaine, and methohexital (Ko et al. 2009).

Although Ro 64-6198 attenuated reinforcing effects of remifentanil, its attenuation only occurred in NHP showing sedation (Podlesnik et al. 2011). It is known that Ro 64-6198 has a limited bioavailability (Heinig et al. 2010). However, when another NOP agonist, SCH221510, was administered intracisternally, it attenuated reinforcing effects of both remifentanil and sucrose pellets, indicative of no behavioral selectivity in rodents (Sukhtankar et al. 2014a). It is not clear to what degree central NOP receptor activation can "selectively" attenuate reinforcing effects of MOP agonists or other classes of drugs of abuse without sedation in NHP. It should be noted that reinforcing effects determined by drug self-administration (operant behavior) procedures, not CPP, is considered a gold standard to assess drug's abuse liability and effective medications for substance abuse disorders (Ator and Griffiths 2003; Mello and Negus 1996). It is also important to note that MOP agonists can produce reward and reinforcing effects through mechanisms that do not require dopamine neurotransmission (Fields and Margolis 2015; Hiranita et al. 2013; Ide et al. 2017). Such evidence may explain the limited efficacy of NOP agonists for attenuating reinforcing effects of MOP agonists (Podlesnik et al. 2011; Sukhtankar et al. 2014a).

4.2 Effects of Ligands with Mixed NOP/MOP Receptor Agonist Activity

Compared to remifentanil, buprenorphine, and oxycodone, bifunctional NOP/MOP partial agonists, such as AT-121 and BU08028, did not produce reinforcing effects in NHP (Ding et al. 2016, 2018c). However, cebranopadol with full NOP and MOP agonist activity produced reinforcing effects in the fixed-ratio schedule (FR30) of reinforcement, and the reinforcing strength of cebranopadol was lower than that of fentanyl under the progressive-ratio schedule in NHP (Trapella et al. 2018). These findings are similar to a recent human study, reporting that cebranopadol produced some drug-liking effects, but cebranopadol has lower abuse potential than a MOP

agonist, hydromorphone (Gohler et al. 2019). Comparing the reinforcing effects of AT-121, BU08028, and cebranopadol under the same NHP drug self-administration procedure, NOP receptor activation seems able to attenuate reinforcing effects mediated by partial, but not full, MOP receptor agonism. Future studies using more ligands with different intrinsic efficacies at NOP versus MOP receptors will advance our understanding of the functional role of NOP receptors in modulating reinforcing effects of MOP agonists.

In a session of daily pretreatment for 5 days, AT-121 acutely attenuated and continued to attenuate reinforcing effects of oxycodone without disrupting food-maintained operant behavior; and the degree of attenuation was similar to the inhibitory effects of buprenorphine (Ding et al. 2018c). Such attenuation could be due to partial MOP agonism and/or NOP agonism. Nonetheless, AT-121 is the first ligand to demonstrate the functional efficacy of a bifunctional NOP/MOP agonist in blocking reinforcing effects of a prescription opioid oxycodone with behavioral selectivity (Ding et al. 2018c). Furthermore, BU08028 was recently found to selectively decrease alcohol drinking without altering food-maintained operant behavior following acute and chronic dosing regimens in NHP (Czoty et al. 2017). As AT-121 and BU08028 alone did not produce reinforcing effects (Ding et al. 2016, 2018c), bifunctional NOP/MOP partial agonists have opened a new avenue for developing safe, effective medications with few side effects for treating substance use disorders.

5 Conclusions

Taken together, functional profiles of NOP-related agonists in NHP have shown promising therapeutic potential for treating pain and drug abuse. NHP models offer the most phylogenetically appropriate evaluation of opioid and non-opioid receptor functions and drug effects (Chen et al. 2013; Lin and Ko 2013; Phillips et al. 2014). Often exciting findings from rodents cannot be translated to primates. For example, a recently discovered G protein signaling-biased MOP agonist, PZM21, lacked opioid rewarding effects in mice (Manglik et al. 2016). However, like oxycodone, PZM21 produced reinforcing effects in the NHP drug self-administration assay (Ding et al. 2018b). As pain and/or drug addiction is embedded in chronic diseases which cause dysregulation of multiple ligand-receptor systems in NHP and humans (Ding et al. 2018a; Ferguson et al. 2018; Kiguchi et al. 2017; Wang et al. 2011), ligands with dual or multiple targets or combined pharmacotherapy may be more effective with favorable side effect profiles. Depending upon the intrinsic efficacies for activating NOP and MOP receptors and therapeutic applications, bifunctional NOP/MOP agonists certainly provide a viable treatment option for pain and substance use disorders.

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Conflict of Interest N.K. and M.C.K. declare that there is no conflict of interest.

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Nociceptin/Orphanin FQ and Urinary Bladder

Patrizia Angelico, Marco Barchielli, Massimo Lazzeri, Remo Guerrini, and Girolamo Caló

Contents

Abstract

Following identification as the endogenous ligand for the NOP receptor, nociceptin/orphanin FQ (N/OFQ) has been shown to control several biological functions including the micturition reflex. N/OFQ elicits a robust inhibitory effect on rat micturition by reducing the excitability of the afferent fibers. After intravesical administration N/OFQ increases urodynamic bladder capacity and

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volume threshold in overactive bladder patients but not in normal subjects. Moreover daily treatment with intravesical N/OFQ for 10 days significantly reduced urine leakage episodes. Different chemical modifications were combined into the N/OFQ sequence to generate Rec 0438 (aka UFP-112), a peptide NOP full agonist with high potency and selectivity and long-lasting duration of action. Rec 0438 mimicked the robust inhibitory effects of N/OFQ on rat micturition reflex; its action is solely due to NOP receptor stimulation, does not show tolerance liability after 2 weeks of treatment, and can be elicited by intravesical administration. Collectively the evidence summarized and discussed in this chapter strongly suggests that NOP agonists are promising innovative drugs to treat overactive bladder.

Keywords
Micturition reflex · N/OFQ · NOP receptor · Overactive bladder · Rec 0438

1 The Micturition Reflex

The lower urinary tract serves two main functions: urine storage without leakage (storage phase) and release of urine (voiding phase). These two functions are dependent on central, peripheral autonomic and somatic neuronal pathways and local peripheral factors. During the storage phase, afferent impulses, which reach the central nervous system from the bladder, send information to the pons. In the pontine tegmentum, positron emission tomography studies visualized a medial region, corresponding to Barrington's nucleus or the pontine micturition center, which is involved in micturition reflex coordination, and a lateral region, which suppresses bladder contractions and improves external sphincter muscle activity during the storage phase (Blok et al. 1997). Furthermore, functional magnetic resonance imaging detected several suprapontine centers which modulate the micturition reflex in humans (Kuhtz-Buschbeck et al. 2005).

The micturition reflex involves the parasympathetic, sympathetic, and somatic systems (de Groat et al. 2015). The parasympathetic system, originating in the spinal cord sacral area, controls bladder contractions. It provides an excitatory input to the bladder through the release of acetylcholine, which excites muscarinic receptors in the detrusor smooth muscle and leads to contraction. The sympathetic system originating in the thoracolumbar cord is involved in bladder relaxation and urethral closure through the release of norepinephrine that, via β_3 receptors located in the detrusor body, leads to bladder relaxation. Norepinephrine also provides excitatory input to urethral smooth muscle, leading to rise in urethral closing pressure via α_1 receptor activation. Finally the somatic system provides excitatory input to striated urethral muscle via acetylcholine release and nicotinic receptor activation.

Immunohistochemical and morphological studies of the bladder wall demonstrated that many neuronal terminal endings do not correspond to cholinergic and adrenergic innervation (Holzer 1988). These non-adrenergic non-cholinergic nerves are peptide-containing fibers which are thought to be "silent" in normal conditions

but which might play a major role in regulating bladder functions in pathological conditions, including neurogenic bladder (Maggi and Meli 1986). Moreover, results from different laboratories have recently shown that the urothelium is involved in sensory mechanisms (i.e., the ability to express sensor molecules or to respond to thermal, mechanical, and chemical stimuli), expresses many different receptors, and can release neurotransmitters (Merrill et al. 2016).

2 Overactive Bladder and Current Therapies

Overactive bladder (OAB) is a complex clinical syndrome which the International Continence Society defines as characterized by urgency (sudden, compelling desire to pass urine which is difficult to defer), urinary incontinence (involuntary urine leakage with or without urgency), frequency, and nocturia (waking to void more than once at night), in the absence of genitourinary pathologies or metabolic factors that could explain these symptoms. OAB may be associated with, but needs to be distinguished from, detrusor overactivity (DO), which refers to an uninhibited, involuntary rise in detrusor pressure during the filling phase of filling cystometry during urodynamic assessment in a conscious cooperative patient. European and North American surveys reported OAB is found in about 16% of the general population aged 40 and over; one third of patients with a clinical diagnosis of OAB present urgency urinary incontinence (Stewart et al. 2003). As OAB-related symptoms are extremely distressing and have a significant negative impact on quality of life and healthcare costs, treatment and management remain the main challenge for healthcare professionals (Leron et al. 2018; Thiagamoorthy et al. 2016).

At present, primary pharmacological treatment for OAB is antimuscarinic agents; objective clinical data, systematic reviews, and adjusted indirect comparisons confer a high level of evidence and strong recommendations (Chapple et al. 2008). However urologists are aware that caution should be exercised when evaluating, interpreting, or prescribing antimuscarinics. Attention should focus on the natural history of OAB, choice of appropriate study design, trial duration, restricted population, economic issues, unrealistic patient expectations, high placebo response rates, and diverse methods of outcome assessment in different trials. Currently there is no consensus on how long patients should be treated, whether treatment should be continuous, intermittent, or on demand and why only relatively few patients remain on medication for more than 4–6 months (Kelleher et al. 1997). Many urologists are searching for appropriate answers to these open questions and looking for more efficacious and/or better tolerated alternatives to antimuscarinic agents (Yamada et al. 2018).

As already mentioned, stimulation of β_3 receptors relaxes the detrusor muscle; thus β_3 agonists were developed as innovative treatment for OAB, and mirabegron is the first in its class approved for this indication in 2012 (Imran et al. 2013). Mirabegron has been extensively studied in clinical trials demonstrating significant improvements in overactive bladder patients. Compared to antimuscarinics,

mirabegron displayed similar liability to induce constipation, hypertension, and tachycardia but not xerostomia. Head-to-head studies comparing efficacy and safety of β_3 selective agonists versus muscarinic antagonists would further help in defining the best strategy for treating overactive bladder (Warren et al. 2016).

Botulinum toxin (BTX) is a complex protein produced by the anaerobic bacterium Clostridium botulinum. BTX is thought to cleave SNAP-25, a synaptosome-associated protein, thereby blocking acetylcholine release at the neuromuscular junction and leading to temporary chemo-denervation and muscle relaxation (Pirazzini et al. 2017). Clinical results have shown BTX administered as multiple intramural injections is also remarkable efficacious in OAB (Kalsi et al. 2006; Patel et al. 2006) leading to FDA approval in 2013. The clinical benefit of BTX seems to last for a mean of 6–9 months, apparently independently of population and dose. Hematuria and pain are the most frequent symptoms soon after injection; systemic symptoms such as respiratory muscle weakness, extremity weakness, and hyposthenia have occasionally been reported but disappear within 4–5 weeks. Urinary retention is a main concern when BTX is given to patients with OAB as several authors reported different percentages of urinary retention over different periods of time. Nowadays there is overwhelming evidence for the efficacy, safety, and tolerability of BTX in the management of OAB (Drake et al. 2017).

Innovative drugs for the control of OAB are under development; these compounds target central and peripheral mechanisms involved in control of the micturition reflex including cyclic nucleotide metabolism, different subtypes of ion channels, receptors for prostaglandins, serotonin, vanilloids, vitamin D_3, opioids, neurokinins, and nerve growth factors (Tiwari and Naruganahalli 2006).

3 N/OFQ Preclinical Studies

Soon after the identification of N/OFQ as the endogenous ligand of the NOP receptor (Meunier et al. 1995; Reinscheid et al. 1995), a number of studies demonstrated that the N/OFQ–NOP receptor system controls several different biological functions in the central nervous system (pain transmission, food intake, learning and memory, locomotor activity, drug abuse, emotional states) as well as in the periphery (respiratory and cardiovascular systems, gastrointestinal and immune functions) (reviewed in Calo et al. 2000b). Among these, a series of elegant studies investigating the action of N/OFQ on the rat micturition reflex were performed by the research group led by CA Maggi. After i.v. administration of 10–100 nmol/kg, N/OFQ decreased the micturition frequency and increased the pressure threshold for reflex activation without modifying the amplitude of the reflex contraction (Lecci et al. 2000b). This pattern of action suggests that N/OFQ inhibits the micturition reflex by reducing excitability of bladder sensory fibers. This is corroborated by the evidence that the micturition reflex promoted by topical application of capsaicin was also inhibited by i.v. N/OFQ (Giuliani et al. 1999). Moreover N/OFQ has been reported to inhibit neurotransmitter release from sensory neuron fibers in different preparations including the guinea pig renal pelvis (Giuliani and Maggi 1996), bronchus (Rizzi et al. 1999; Shah et al. 1998), and left atrium (Giuliani and Maggi 1997) and the rat

trachea (Helyes et al. 1997) and to elicit powerful inhibitory effects on dorsal root ganglia (Abdulla and Smith 1997, 1998; Anand et al. 2016). A similar pattern of effect on micturition reflex, i.e., decreased frequency and increased threshold with no modification of contraction amplitude, was recorded in response to spinal N/OFQ at 10 nmol (Lecci et al. 2000a). Thus the inhibitory effects on micturition exerted by N/OFQ in the periphery and at spinal level can be explained by the ability of the peptide to inhibit the afferent branch of the reflex.

After supraspinal administration at 0.3–1 nmol, N/OFQ elicited a profound and naloxone-resistant inhibition of the micturition reflex by reducing the amplitude of bladder contraction and the micturition frequency and by increasing the pressure threshold (Lecci et al. 2000b). This pattern of N/OFQ effects that involves both the afferent (reduced frequency associated with increased pressure threshold) and the efferent (reduced amplitude of bladder contraction) branches of the reflex has been interpreted as due to a powerful inhibitory action of N/OFQ on the pontine micturition center (Lecci et al. 2000b). In line with this proposal, N/OFQ has been reported to elicit profound inhibitory effects via an increase in potassium conductance in different midbrain nuclei including the locus coeruleus (Connor et al. 1996), the dorsal raphe (Vaughan and Christie 1996), and the periaqueductal gray (Chiou et al. 2002; Vaughan et al. 1997).

4 N/OFQ Clinical Studies

Preclinical studies mentioned above demonstrated that N/OFQ elicits profound inhibitory effects on the micturition reflex. Based on this evidence, the effects of N/OFQ were evaluated in patients suffering from OAB. A first proof-of-concept study demonstrated that the intravesical instillation of a 1 μM N/OFQ solution did not produce significant urodynamic changes in normal subjects; however, when given to OAB patients, the peptide produced a large increase in mean bladder capacity and volume threshold for the appearance of detrusor hyperreflexia with no modifications of maximal bladder pressure (Lazzeri et al. 2001). This pattern of effects suggests that the peptide may control micturition by inhibiting the afferent branch of the reflex. The selective action of N/OFQ in patients with OAB but not in normal subjects deserves further comments. The following factors, alone or in combination, may contribute to the explanation of this finding:

1. Experimental studies demonstrated that the afferent branch of the reflex is carried by Aδ fibers in normal conditions (De Groat 1975) and by C fibers after spinal injury (de Groat et al. 1990). N/OFQ has been reported to elicit inhibitory effects on sensory neurons (see Sect. 3) that may be more powerful on C than Aδ fibers.
2. Recent immunohistochemical studies demonstrated that within the bladder suburothelium, there is a remarkable severalfold increase of NOP-positive nerve fibers in DO compared to controls (Anand et al. 2016).

3. OAB patients generally perform clean intermittent catheterization for management of incontinence, and this procedure is associated with high incidence of bacteriuria and multiple urinary tract infections (Wyndaele 2002); this leads to chronic inflammation (Schlager et al. 2004) and consequent deficit of the urothelium barrier function. Therefore the diffusion through the endothelium of a highly hydrophilic peptide such as N/OFQ can be extremely facilitated in patients performing clean intermittent catheterization than in normal subjects.

These initial findings were later confirmed in a randomized, placebo-controlled, double-blind study. Patients treated with 1 μM [desPhe^1]N/OFQ (a N/OFQ analog that lacks affinity for the NOP receptor, Calo et al. 2000a; Connor and Christie 1998; Dooley and Houghten 1996) did not show any statistically significant modification of the urodynamic parameters, while the infusion of 1 μM N/OFQ elicited a robust inhibitory effect on the micturition reflex (Lazzeri et al. 2003). In particular, N/OFQ effects on bladder capacity (173%) and volume threshold for the appearance of detrusor hyperreflexia (239%) were very similar to those reported in the previous study (183 and 248%, Lazzeri et al. 2001) underlining the robustness of the acute inhibitory effect on micturition reflex exerted by intravesical instillation of N/OFQ in OAB patients. Moreover, since the only difference between N/OFQ and its desPhe^1 derivative is the ability to bind and activate the NOP receptor, the lack of effect of [desPhe^1]N/OFQ strongly suggests that the action of N/OFQ on the micturition reflex is solely due to NOP receptor activation.

Based on these encouraging findings, the feasibility, safety, and efficacy of intravesical instillation of 1 mg N/OFQ at the first morning catheterization for 10 days were evaluated in patients who perform clean intermittent self-catheterization for neurogenic DO incontinence (Lazzeri et al. 2006). Mean daily urine leakage episodes during N/OFQ treatment were significantly reduced (0.94 vs 2.18), while no significant changes were reported in the placebo group (2.06 vs 2.43 baseline). Moreover, urodynamic parameters recorded during the study showed an increase in bladder capacity compared to baseline only in patients assigned to the N/OFQ group. It should be noted that in previous studies, urodynamic examination was performed in the presence of N/OFQ (Lazzeri et al. 2001, 2003), while in this investigation, the urodynamic assessment was always performed in the afternoon several hours after peptide instillation. Since N/OFQ is highly hydrophilic and since the patients used clean intermittent self-catheterization to control incontinence, it is unlikely that the peptide was present in the bladder during the urodynamic examination. Despite this, the urodynamic changes in response to N/OFQ were virtually superimposable in this and in previous studies. Thus a relatively short exposure to N/OFQ seems to elicit relatively long-lasting beneficial effects on DO.

Collectively the clinical findings summarized above strongly support the use of NOP receptor agonists as an innovative therapeutic approach for controlling DO incontinence.

5 Identification and Pharmacological Characterization of Rec 0438

Soon after the discovery of N/OFQ, several research groups started to investigate structure activity relationships (SAR) crucial for N/OFQ binding and activation of the NOP receptor (Guerrini et al. 2000). Some of the chemical modifications investigated increased N/OFQ binding affinity and/or functional potency (Fig. 1).

For instance the detailed investigation of SAR features of Phe^4 (Guerrini et al. 2001) allowed the identification of [(pF)Phe^4]N/OFQ(1-13)-NH_2 that behaved as a potent NOP full agonist both in vitro (Bigoni et al. 2002) and in vivo (Rizzi et al. 2002a). Another SAR study in which the Ala residues of N/OFQ were replaced with nonnatural amino acids known to induce alpha helix structure (Zhang et al. 2002) led to the identification of N/OFQ analogs, including [Aib^7]N/OFQ, showing a substantial increment of NOP agonist potency (Arduin et al. 2007; Tancredi et al. 2005). The importance of the N/OFQ pair of positively charged dipeptides Arg-Lys for NOP receptor interaction has been studied by Okada et al. (2000) leading to the identification of [$Arg^{14}Lys^{15}$]N/OFQ, a potent NOP agonist whose pharmacological features has been investigated both in vitro and in vivo (Rizzi et al. 2002b). Finally the importance of C terminal amidation has been suggested by receptor binding and in vitro functional studies (reviewed in Calo et al. 2011) that demonstrated twofold to eightfold higher potency of N/OFQ-NH_2 than the natural peptide. Interestingly bioassay studies with peptidase inhibitors suggested that C terminal amidation decreases peptide susceptibility to peptidases (Calo et al. 2000a). Thus, with the aim of increasing N/OFQ potency and duration of action, the abovementioned chemical modifications were combined in the same molecule generating Rec 0438 (aka UFP-112, Arduin et al. 2007). These chemical modifications produced a synergistic effect in terms of peptide potency: while the single modifications increased N/OFQ potency by twofold to sevenfold, their combination produced a NOP agonist 62-fold more potent than the natural peptide (see for details Table 3 of Calo et al. 2011).

The basic pharmacological profile of Rec 0438 was investigated by Rizzi et al. (2007b) who demonstrated that the peptide behaves as a highly potent and selective NOP full agonist able to produce in vivo, compared to N/OFQ, long-lasting effects. These initial findings were later confirmed in a large series of studies. In vitro the full agonist, high potency, and NOP selectivity of action of Rec 0438 were confirmed in studies performed at recombinant human NOP receptors in [^{35}S]GTPγS binding (Arduin et al. 2007), cAMP accumulation (Calo et al. 2011), calcium mobilization (Camarda et al. 2009), bioluminescence energy transfer (Malfacini et al. 2015), and dynamic mass redistribution (Malfacini et al. 2018) experiments. Similar results were obtained in bioassay studies performed using mouse, rat, and guinea pig tissues (Arduin et al. 2007; D'Agostino et al. 2010; Rizzi et al. 2007b); most importantly Rec 0438 effects were no longer evident in tissues taken from NOP receptor knockout (NOP(−/−)) mice (D'Agostino et al. 2010; Rizzi et al. 2007b). Similarly the high potency and NOP selectivity associated with long-lasting action reported in the first in vivo study of Rec 0438 on nociception, food intake, and cardiovascular

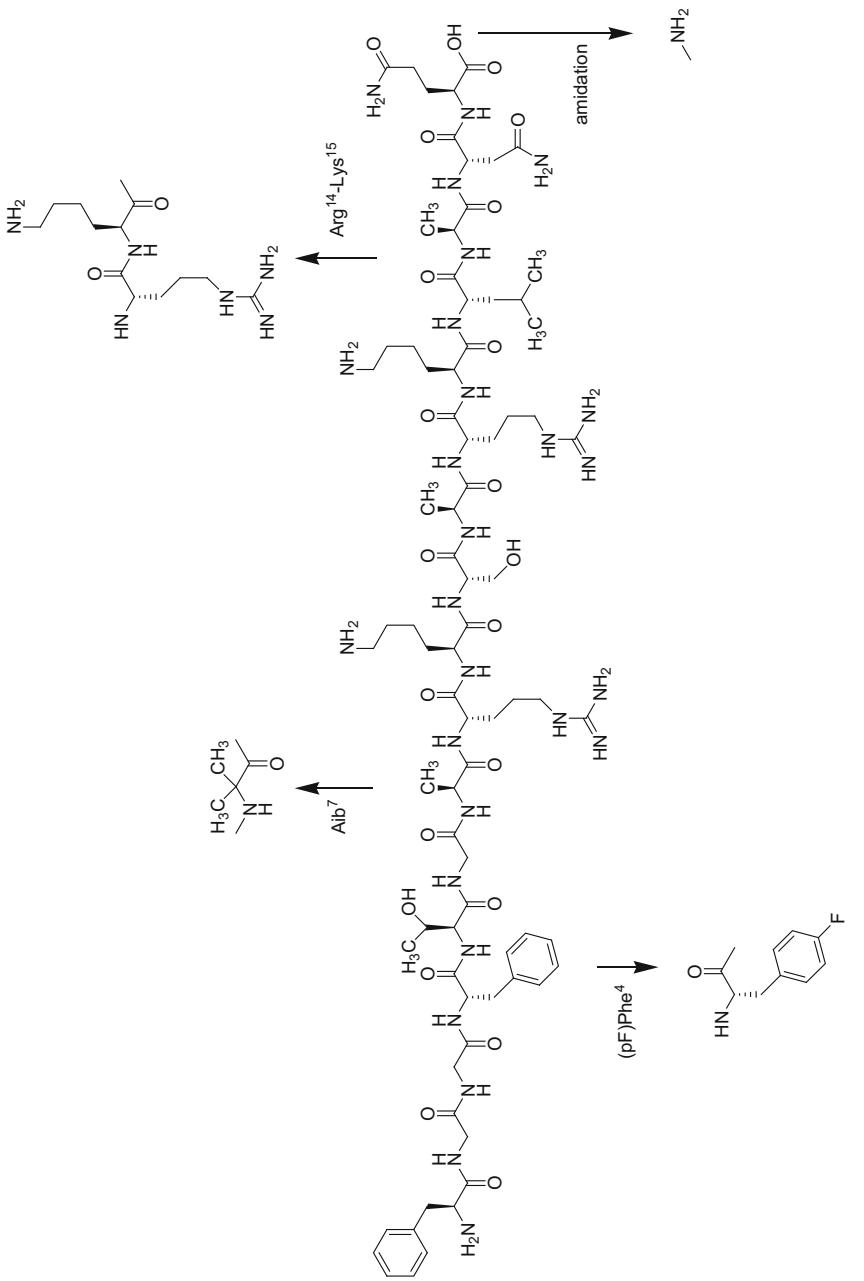

Fig. 1 Chemical structure of N/OFQ and chemical modifications used for generating Rec 0438

functions (Rizzi et al. 2007b) were later confirmed and extended in studies investigating different biological functions including gastrointestinal control (Broccardo et al. 2007, 2008; Grandi et al. 2007), ethanol drinking (Economidou et al. 2006), airway functions (D'Agostino et al. 2010; Sullo et al. 2013), and spinal analgesia in rats (Micheli et al. 2015a, b) and in nonhuman primates (Hu et al. 2010). The high NOP selectivity of Rec 0438 in vivo was confirmed in knockout studies where the effects of the peptide were no longer evident in NOP(−/−) mice (Rizzi et al. 2007b) and rats (Micheli et al. 2015a). Therefore a large body of evidence demonstrated that Rec 0438 behaved as a potent and selective NOP full agonist able to elicit long-lasting effects in vivo.

6 Rec 0438 and the Micturition Reflex

The effects of treatment with Rec 0438 in comparison with N/OFQ were studied using an animal model of cystometry in urethane anesthetized rats (for experimental details, see Angelico et al. 2005). Continuous infusion of the bladder in the anesthetized animals allows collection of multiple cystometrograms from the same rat and evaluates the drug-induced changes of cystometric parameters. After acquisition in a digitalized format, the following parameters were calculated: bladder volume capacity (BVC), defined as the volume of saline infused into the bladder necessary to induce detrusor contraction followed by micturition, and micturition pressure (MP), defined as the maximal intravesical pressure over the baseline value induced by contraction of the detrusor during micturition. Basal BVC and MP were recorded 60 min before treatment, and changes of these parameters induced by treatment were recorded for further 120 min.

Dose-response curves in response to N/OFQ (0.1, 0.3, and 3 μmol/kg, i.v. bolus) and Rec 0438 (0.003, 0.03, and 0.3 μmol/kg, i.v. bolus) are displayed in Figs. 2 and 3, respectively. N/OFQ induced a dose-related robust increase of BVC that peaked at 30 min and declined after 60–90 min. This effect was associated with a slight and transient reduction of MP that reached statistical significance with the 0.3 μmol/kg dose (Fig. 2).

Rec 0438 mimicked the action of N/OFQ on BVC being however approximately 100-fold more potent (based on ED_{50}) and eliciting slower onset and longer-lasting effects. Moreover Rec 0438 elicited a robust and dose-dependent reduction of MP (Fig. 3). These results confirmed and extended previous findings demonstrating that N/OFQ and Rec 0438 produce powerful inhibitory effects on the micturition reflex. In perfect agreement with results obtained investigating different biological activities (Calo et al. 2011), Rec 0438 compared to the natural peptide showed slower onset and longer duration of action as well as a remarkable higher potency.

In order to investigate the receptor mechanism involved in the action of N/OFQ and Rec 0438, their effects on the micturition reflex were challenged with the NOP selective antagonist SB-612111 (Spagnolo et al. 2007; Zaratin et al. 2004). The antagonist dose, i.e., 1 mg/kg, was selected based on previous studies where SB-612111 prevented the pronociceptive and orexigenic action of supraspinal

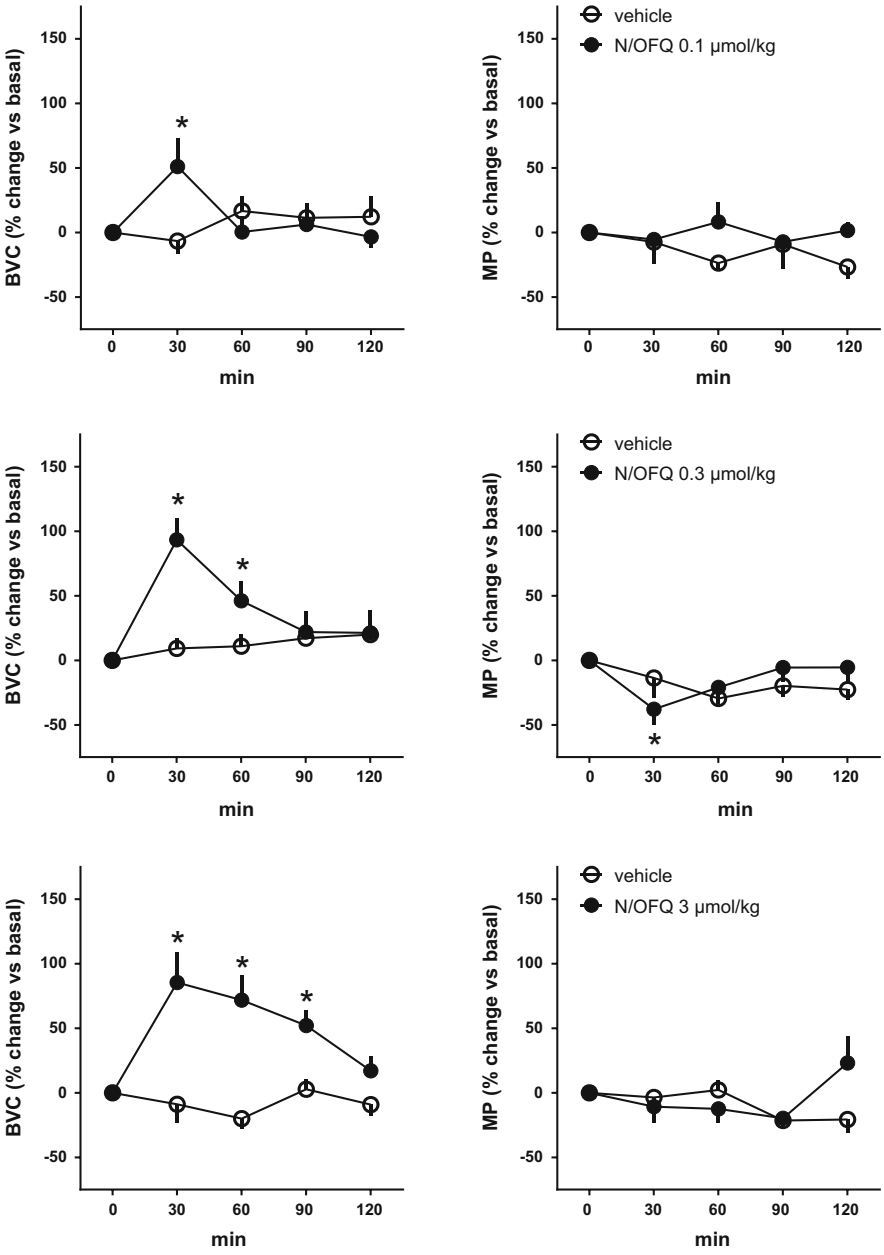

Fig. 2 Effects of increasing doses of i.v. N/OFQ on bladder volume capacity (left panels) and maximal pressure (right panels) in urethane anesthetized rats. Data are shown as mean ± sem of 7–8 animals. $*p < 0.05$ vs basal values

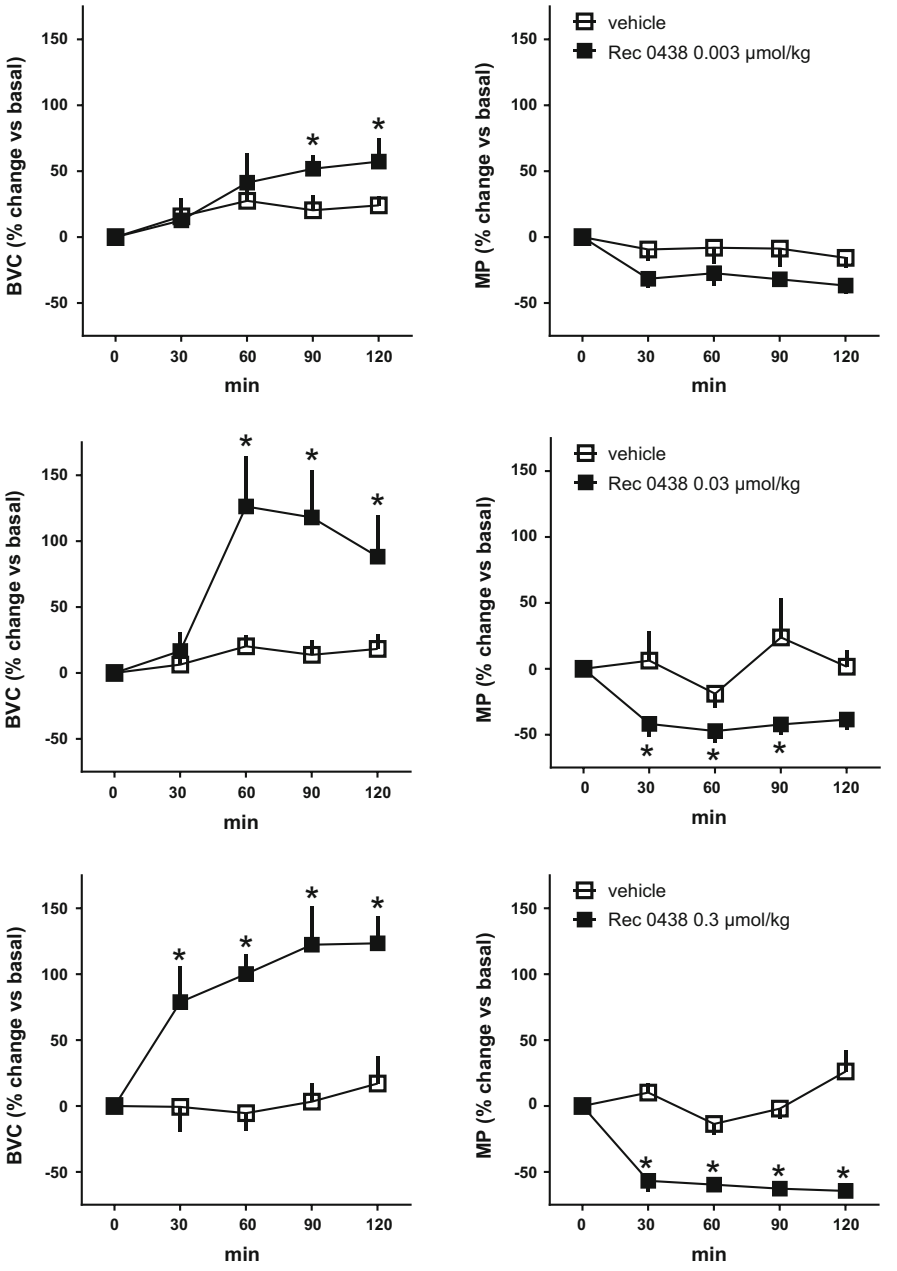

Fig. 3 Effects of increasing doses of i.v. Rec 0438 on bladder volume capacity (left panels) and maximal pressure (right panels) in urethane anesthetized rats. Data are shown as mean ± sem of 7–9 animals. $*p < 0.05$ vs basal values

N/OFQ as well as its spinal antinociceptive effect (Rizzi et al. 2007a). As shown in Fig. 4, N/OFQ 3 μmol/kg i.v. elicited a robust increase of BVC in rats pretreated with vehicle, while its action was fully prevented in animals pretreated with SB-612111. Very similar results were obtained with Rec 0438 whose action on both BVC and MP was blocked by the NOP antagonist. Thus these results demonstrated that the actions of N/OFQ as well as Rec 0438 on the rat micturition reflex are entirely dependent on their ability to activate the NOP receptor.

Tolerance liability is an important limitation regarding the use of GPCR agonists as drugs, particularly in the field of opioids (Cahill et al. 2016). To investigate this aspect, the effects of N/OFQ (3 μmol/kg, i.v.) and Rec 0438 (0.03 μmol/kg, i.v.) were assessed in animals treated for 2 weeks with a daily s.c. injection of vehicle or NOP agonist. As shown in Fig. 5, superimposable effects were recorded in response to N/OFQ in rats treated with vehicle or with N/OFQ, and similar results were obtained with Rec 0438. Therefore the similar action of N/OFQ and Rec 0438 in rats

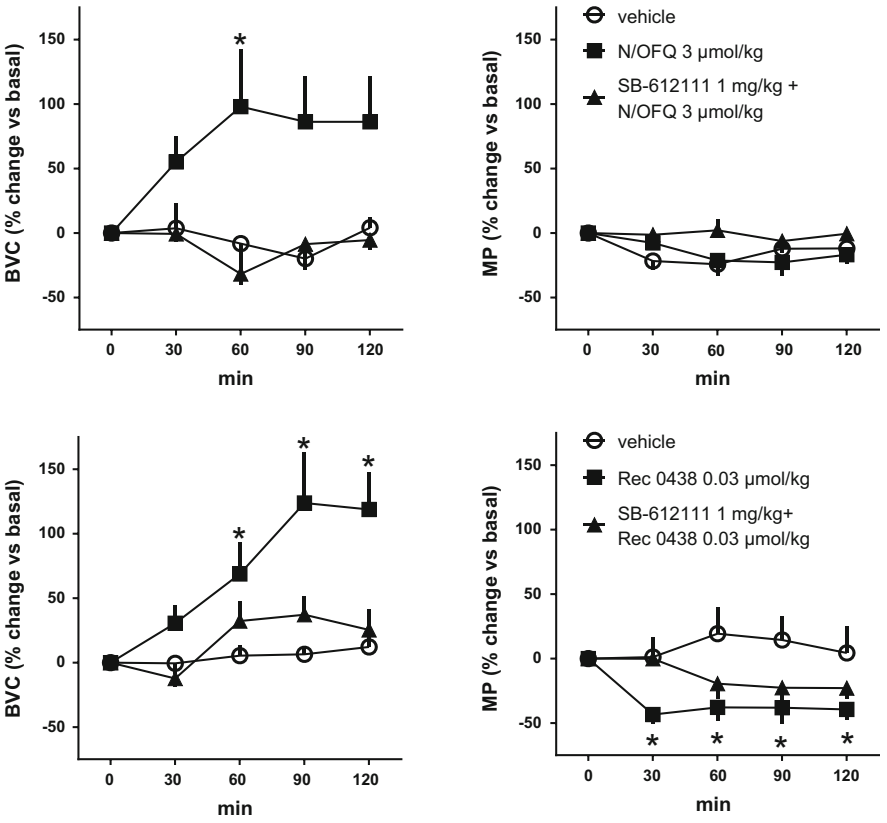

Fig. 4 Effects of SB-612111 against N/OFQ (top panels) and Rec 0438 (bottom panels) on bladder volume capacity (left panels) and maximal pressure (right panels) in urethane anesthetized rats. Data are shown as mean ± sem of 7–9 animals. *$p < 0.05$ vs basal values

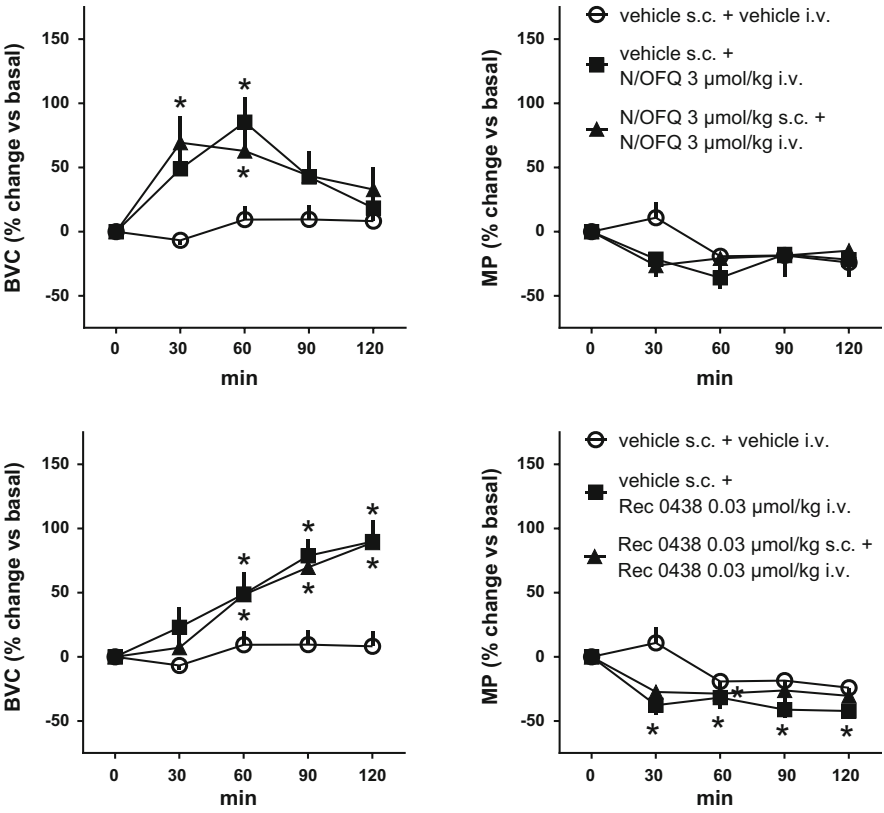

Fig. 5 Effects of i.v. N/OFQ (top panels) and i.v. Rec 0438 (bottom panels) in animals chronically (14 days) treated with s.c. vehicle or respective peptide on bladder volume capacity (left panels) and maximal pressure (right panels) in urethane anesthetized rats. Data are shown as mean ± sem of 8–11 animals. *$p < 0.05$ vs basal values

acutely and chronically treated with the two peptides suggests that the inhibitory effects on the micturition reflex due to NOP receptor activation have little if any tolerance liability. Although these results are clearly encouraging, they should not be overemphasized; in fact the period of treatment examined (2 weeks) is too short to firmly rule out tolerance as a limitation of NOP agonists in overactive bladder.

The impressive results obtained with N/OFQ in OAB patients were measured by giving the peptide intravesically. Therefore the effects of N/OFQ and Rec 0438 on rat micturition reflex were reinvestigated using this route of administration. The intravesical infusion of a 300 µM solution of N/OFQ did not elicit any significant effect on rat cystometrograms (data not shown). As discussed in the Sect. 3 one possible reason for the selective action of N/OFQ in OAB patients compared to normal subjects may be related to large differences in urothelium barrier functions. To investigate this possibility, we use protamine sulfate that is known to disrupt the tight junctions among the cells, thus making the bladder urothelium more permeable

(Chuang et al. 2004; Nishiguchi et al. 2005). Thus, 10 mg/mL protamine sulfate was infused intravesically for 1 h before testing the effects of solutions containing 300 μM N/OFQ or 30 μM Rec 0438. After protamine sulfate permeabilization, both N/OFQ and Rec 0438 induced a statistically significant increase in BVC and reduction of MP; similar to i.v. studies, Rec 0438 showed a slower onset and longer-lasting action in comparison with N/OFQ (Fig. 6).

Collectively, results obtained with N/OFQ and Rec 0438 confirm and extend previous findings demonstrating a powerful inhibitory effect on micturition reflex. In line with the literature, Rec 0438 is more potent than N/OFQ and elicits longer-lasting effects. The action of both peptides is exclusively due to NOP receptor activation, does not show tolerance liability after 2 week of treatment, and can be elicited by intravesical administration.

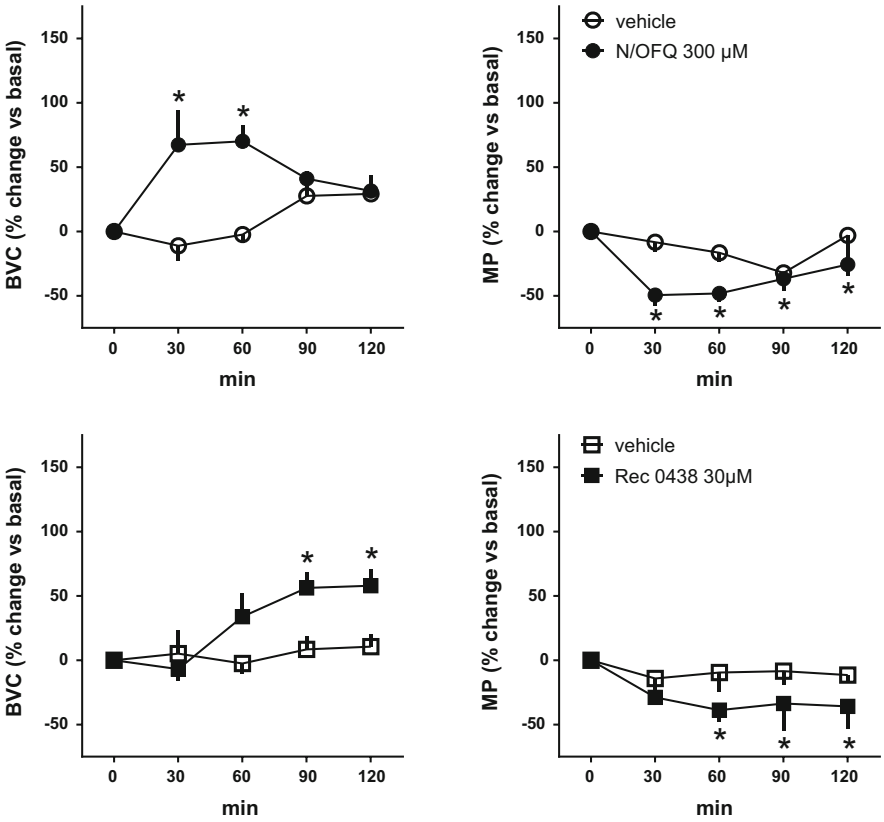

Fig. 6 Effects of intravesical administration of N/OFQ (top panels) and Rec 0438 (bottom panels) in animals pretreated with protamine sulfate on bladder volume capacity (left panels) and maximal pressure (right panels) in urethane anesthetized rats. Data are shown as mean ± sem of 5–7 animals. *$p < 0.05$ vs basal values

7 Conclusions

Animal studies demonstrated that N/OFQ elicits strong and consistent inhibitory effect on micturition reflex at supraspinal, spinal, and peripheral sites. This was confirmed in the clinic where intravesical N/OFQ promote beneficial effects in patients with OAB incontinence. Rec 0438 is a peptide NOP agonist characterized by high potency and selectivity of action and ability to elicit long-lasting effects in vivo. In rats Rec 0438 mimicked the robust inhibitory effects of N/OFQ on micturition reflex. A phase I clinical study with Rec 0438 was recently completed in normal subjects as well as in OAB patients, demonstrating that intravesical infusion of Rec 0438 is well tolerated with no leakage to the systemic circulation. A phase II study is now ongoing; if the encouraging clinical results previously obtained with N/OFQ will be confirmed with Rec 0438, a novel, possibly well-tolerated, and highly effective therapeutic option will be available for patients who perform clean intermittent self-catheterization for managing OAB incontinence.

Declaration of Interests PA and MB are employed by Recordati S.p.A. RG and GC are among the funders of the University of Ferrara spin-off company UFPeptides s.r.l., the assignee of the patent covering Rec 0438.

Details of Author Contributions PA, ML, and GC wrote the first draft of the chapter, and MB and RG critically revised it. All authors approved the final version of the article.

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Cebranopadol: A Novel First-in-Class Potent Analgesic Acting via NOP and Opioid Receptors

Thomas M. Tzschentke, Klaus Linz, Thomas Koch, and Thomas Christoph

Contents

Abstract

Cebranopadol is a novel first-in-class analgesic with highly potent agonistic activity at nociceptin/orphanin FQ peptide (NOP) and opioid receptors. It is highly potent and efficacious across a broad range of preclinical pain models. Its side effect profile is better compared to typical opioids. Mechanistic studies have shown that cebranopadol's activity at NOP receptors contributes to its anti-hyperalgesic effects while ameliorating some of its opioid-type side effects, including respiratory depression and abuse potential. Phase II of clinical

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development has been completed, demonstrating efficacy and good tolerability in acute and chronic pain conditions.

This article focusses on reviewing data on the preclinical in vitro and in vivo pharmacology, safety, and tolerability, as well as clinical trials with cebranopadol.

Keywords
Cancer pain · Chronic low back pain · Diabetic peripheral neuropathy · Nociceptin/orphanin FQ · Postoperative pain · Respiratory depression

1 Introduction

Several studies suggest that simultaneous activation of nociceptin/orphanin FQ (N/OFQ) peptide (NOP) and opioid (particularly the mu opioid peptide (MOP)) receptors may be a particularly promising approach to produce efficacious analgesia with potentially reduced side effects. In rodents, spinal administration of the endogenous NOP receptor ligand N/OFQ enhanced the analgesic effect of systemic and spinally administered morphine (Tian et al. 1997; Courteix et al. 2004). Isobolographic analysis demonstrates a synergistic interaction between NOP and MOP receptor activation at the spinal level in a neuropathic pain model (Courteix et al. 2004), and subthreshold doses of morphine and the selective NOP receptor antagonist Ro 64-6198 produced robust analgesic effects when given together in an acute pain model (Reiss et al. 2008). Importantly, these rodent findings were confirmed by studies in nonhuman primates, where spinally applied NOP receptor agonists strongly potentiated the analgesic effects of morphine and systemically administered NOP and MOP receptor agonists produced supra-additive, i.e., synergistic analgesic effects (Ko and Naughton 2009; Hu et al. 2010; Cremeans et al. 2012). Taken together, this evidence strongly suggests that mixed NOP/opioid receptor agonists may have promising therapeutic potential as novel analgesics. As will be outlined below, for cebranopadol, in line with these studies, an intrinsic synergistic interaction between its activities at NOP and opioid receptors has also been established (see Sect. 3).

In the literature, a number of mixed NOP/MOP receptor agonists have been described (Ding et al. 2016, 2018; Zaveri et al. 2013, 2015; Journigan et al. 2014; Khroyan et al. 2011, 2017; Spagnolo et al. 2008; Toll et al. 2009, 2016). However, none of these compounds matches the affinity and efficacy profile of cebranopadol across the four opioid receptors. Most of the described compounds are partial agonists at NOP or MOP or both receptors. A detailed review of these compounds is beyond the scope of this chapter. The interested reader is referred to the original literature and reviews covering this topic (Toll 2013; Bird and Lambert 2015; Toll et al. 2016).

Cebranopadol (trans-6′-fluoro-4′,9′-dihydro-*N,N*-dimethyl-4-phenylspiro[cyclohexane-1,1′(3′H)-pyrano[3,4-b]indol]-4-amine; MW 378.5) (Fig. 1) has been discovered by Grünenthal, Aachen, Germany. Cebranopadol has already been the topic of some previous reviews and commentaries. Lambert et al. (2015), Sałat et al.

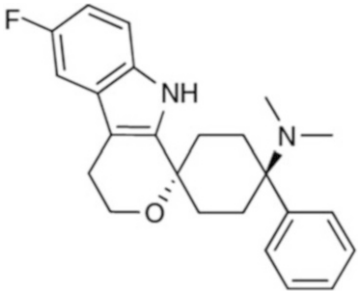

Fig. 1 Chemical structure of cebranopadol

(2015), Raffa et al. (2017), and Calo' and Lambert (2018) provide short introductions to NOP receptor pharmacology and overviews of preclinical data and clinical trials on cebranopadol available at the time. An alternative synthesis route of cebranopadol has been described (Fantinati et al. 2017).

The aim of this article is to provide a comprehensive overview on cebranopadol, based on published data but also including some hitherto unpublished data.

2 In Vitro Pharmacology

The in vitro pharmacological profile of cebranopadol was described by Schunk et al. (2014) and further elaborated by Linz et al. (2014). Subsequently, potency and efficacy of cebranopadol at NOP and MOP receptors have also been studied by Rizzi et al. (2016), largely reproducing the previously reported data. These authors also examined G-protein vs. β-arrestin signaling of cebranopadol at NOP and MOP receptors and found evidence for a moderate G-protein bias at the MOP receptor and a strong G-protein bias at the NOP receptor. Further β-arrestin2 recruitment assays done by Grünenthal (T. Koch, unpublished data) confirm the strong G-protein bias profile of cebranopadol at the NOP receptor observed by Rizzi et al. (2016) (Table 1).

Human MOP, DOP, KOP, and NOP receptor binding assays were performed using cell membrane preparations of CHO-K1 cells transfected with the human MOP or DOP receptor and HEK293 cells transfected with the human NOP or KOP receptor (Linz et al. 2014). Rat MOP, KOP, and NOP receptor binding assays were run using membrane suspensions from rat brain, using [^{3}H]DAMGO (MOP receptor assay), [^{3}H]N/OFQ (NOP receptor assay), and [^{3}H]Ci-977 (KOP receptor assay) as ligands (see Linz et al. 2014). The agonist-stimulated [^{35}S] guanosine-5′-[γ-thio]triphosphate (GTPγS) binding assay was carried out as a homogeneous scintillation proximity assay (as described by Linz et al. 2014). The β-arrestin2 recruitment assays in the present publication were performed using the DiscoveRx PathHunter enzyme complementation assay (PathHunter CHO-K1 NOP β-Arrestin Cell Line and PathHunter CHO-K1 MOP β-Arrestin Cell Line) according to the manufacturer's instruction.

Table 1 Summary of available in vitro receptor binding and functional efficacy data on cebranopadol

	Radioligand binding		GTPγS binding (human)		β-arrestin2 recruitment (human)		Calcium mobilization (human)		Bret assay (human)							
									G-protein				β-arrestin2			
	K_i [nM]		EC_{50} [nM]	Relative efficacy [%]	EC_{50} [nM]	Relative efficacy [%]	EC_{50} [nM]	Relative efficacy [%]	EC_{50} [nM]		Relative efficacy [%]		EC_{50} [nM]		Relative efficacy [%]	
	Mean ± SD						Mean (CL95%)	Mean ± SEM	Mean (CL95%)		Mean ± SEM		Mean (CL95%)		Mean ± SEM	
	Rat	Human							5′	60′	5′	60′	5′	60′	5′	60′
NOP	1.0 ± 0.5	0.9 ± 0.2	13.0 ± 2.0	88.9 ± 3.9	2,200 ± 530	36.0 ± 13	52 (15–182)	89 ± 6	13 (6–30)	3 (2–4)	83 ± 1	86 ± 1	Inactive up to 1 μM			
MOP	2.4 ± 1.2	0.7 ± 0.3	1.2 ± 0.4	103.5 ± 4.7	50 ± 19	97 ± 13	63 (13–309)	99 ± 5	1 (0.7–3)	0.2 (0.1–0.7)	114 ± 13	100 ± 4	50 (30–83)	4 (2–9)	121 ± 2	86 ± 4
KOP	64.0 ± 11.0	2.6 ± 1.4	17.0 ± 5.0	67.2 ± 5.3	–	–	1,047 (537–2,042)	55 ± 3	–	–	–	–	–	–	–	–
DOP	N.D.	18.0 ± 20.0	110.0 ± 28.0	105.0 ± 8.5	–	–	490 (126–1,950)	81 ± 6	–	–	–	–	–	–	–	–

Data from Linz et al. (2014) (radioligand binding and GTPγS binding) and from Rizzi et al. (2016), reproduced in Fantinati et al. (2017) (calcium mobilization and BRET assay). EC_{50} values for the calcium mobilization and BRET assays were recalculated from the pEC_{50} values published in Rizzi et al. (2016). Note that the shown values are rounded. The β-arrestin2 recruitment data presented here were performed by Grünenthal (T. Koch, unpublished data)
SD standard deviation, *CL* confidence limits, *SEM* standard error of the mean

Cebranopadol bound with high affinity to NOP and opioid receptors (Table 1). The highest binding affinities were observed at human NOP and MOP receptors with subnanomolar inhibitory constants. Binding affinity for the human KOP receptor was approx. 3–4-fold lower, and binding affinity for the human DOP receptor was approx. 20–26-fold lower. A comparable binding profile was observed for rat NOP, MOP, and KOP receptors. In [^{35}S]GTPγS binding assays with membranes from cells expressing the respective recombinant human receptors, cebranopadol showed full agonistic efficacy at the human MOP and DOP receptors, almost full efficacy at the human NOP receptor, and partial efficacy at the human KOP receptor (Table 1). In the β-arrestin2 recruitment assay, cebranopadol revealed an efficacy of 97% and an EC_{50} of 50 nM at the MOP receptor but only partial efficacy (36%) and a very low potency (2,200 nM) at the NOP receptor (Table 1). Notably, affinities to more than 100 neuronal and safety-relevant receptors, ion channels, and enzymes tested in an extensive off-target profile were at least 100–1,000 times lower than opioid receptor affinities and can thus be considered as biologically irrelevant. The only exception was the serotonin 5A (5-HT5A) receptor for which a Ki of 8.7 nM was found. However, in a functional [^{35}S]GTPγS binding assay, cebranopadol did not show agonistic or antagonistic effects at this receptor at concentrations up to 10.0 μM; thus the affinity for this receptor is also considered to be biologically irrelevant.

Rizzi et al. (2016) studied the intrinsic efficacy of cebranopadol in a calcium mobilization assay using CHO cells stably co-expressing the human NOP, MOP, or KOP receptor with the C-terminally modified $G\alpha_{qi5}$ and CHO cells co-expressing the DOP receptor with the $G\alpha_{qG66Di5}$ protein. Bioluminescence resonance energy transfer (BRET) assay was used for studying receptor interaction with G-proteins and β-arrestin2 for MOP (in SH-SY5Y neuroblastoma cells) and NOP receptors (in HEK293 cells), using permanently expressing lines. Agonist responses were quantified as stimulated BRET ratio obtained by subtracting the vehicle value to that measured in the presence of ligand.

In the calcium mobilization studies, cebranopadol elicited concentration-dependent stimulation of calcium release in the four cell lines with maximal effects similar to those of reference ligands (N/OFQ, fentanyl, DPDPE, and dynorphin A for NOP, MOP, DOP, and KOP receptors, respectively), except in KOP receptor-expressing cells, where it behaved as a partial agonist. Cebranopadol was equipotent in activating NOP and MOP receptors but tenfold less potent at DOP and KOP receptors (Table 1). In the BRET studies, in membrane preparations from NOP receptor/G-protein-expressing cells, the nonpeptide NOP receptor agonist Ro 65-6570 and cebranopadol were approx. tenfold less potent than N/OFQ to enhance receptor–G-protein interaction. However, prolonging the incubation time from 5 to 60 min virtually abolished this difference (Table 1). In membranes expressing MOP receptor/G-protein, cebranopadol displayed similar efficacy but tenfold greater potency than the reference agonists dermorphin and fentanyl.

With regard to β-arrestin2–receptor interactions, cebranopadol was virtually inactive in cells expressing the NOP receptor but maintained full agonist activity in cells expressing the MOP receptor. Prolonging incubation time only slightly increased cebranopadol potency at the MOP receptor, but did not recover the lack of efficacy at the NOP receptor (Table 1).

Taken together, the findings of Rizzi et al. (2016) confirm and extend previous findings (Linz et al. 2014) by showing that cebranopadol is a highly potent mixed NOP/opioid receptor agonist. In addition, their findings suggest that cebranopadol is a G-protein-biased agonist at MOP and particularly at NOP receptors. This observation of Rizzi et al. (2016) was confirmed by further β-arresstin2 recruitment studies performed by Grünenthal with cebranopadol at the MOP and NOP receptor and presented here for the first time (Table 1). The rank order of potencies of cebranopadol for activating Gq/i-mediated calcium signaling through the NOP and opioid receptors (NOP > MOP > DOP ≥ KOP) agrees with the profile of affinities measured in receptor binding assays (Linz et al. 2014). Likewise, full agonism at NOP, MOP, and DOP receptors and partial agonism at the KOP receptor are consistent with the data of Linz et al. (2014) from [^{35}S]GTPγS binding assays. On the other hand, data from a BRET assay suggested an incubation time-dependent ~tenfold greater potency for MOP compared to NOP receptors. Incubation time-dependency was more pronounced for cebranopadol than for other agonists, and the authors speculated that cebranopadol may have unusually slow kinetics of receptor activation, also in light of the observation that the absolute potency values measured in the calcium assay were lower than those determined in their BRET assay and in [^{35}S]GTPγS binding assays reported by Linz et al. (2014). Based on their own previous studies, Rizzi et al. (2016) suggest that the calcium assay tends to underestimate the potency of agonists with slow activation kinetics (Rizzi et al. 2014; Ruzza et al. 2014; Camarda et al. 2009) and that the relatively low potency displayed by cebranopadol in the calcium assay may result from a non-equilibrium between slow receptor activation and the transient nature of the calcium response (Charlton and Vauquelin 2010).

G-protein-biased agonism is clearly an advantageous feature for a MOP receptor agonist. Analgesic properties of morphine are strongly associated with G-protein-dependent signaling (Bohn et al. 1999), whereas tolerance development, respiratory depression, and constipation are more dependent on β-arrestin2 signaling (Bohn et al. 2000; Raehal et al. 2005). On the other hand, the possible functional implications of the strong G-protein bias displayed by cebranopadol at NOP receptors are currently not known. Data on functional selectivity in the NOP receptor field with respect to different intracellular signaling pathways are just beginning to emerge (Malfacini et al. 2015; Chang et al. 2015b). Asth et al. (2016) provided the probably first demonstration of the functional consequence at the behavioral level of a divergent G-protein and β-arrestin2 signaling by showing that the presence or absence of anxiolytic or antidepressant effects was linked to the degree of β-arrestin2 recruitment rather than to the degree of G-protein recruitment. NOP receptor-selective ligands need to be evaluated in β-arrestin2 knockout mice, and novel selective NOP receptor ligands with a large bias toward G-protein or toward β-arrestin2 signaling need to be developed and tested. This will further broaden the understanding of the possible role of functional G-protein vs. β-arrestin2 selectivity in the development of NOP receptor agonists as novel innovative analgesics.

3 In Vivo Pain Pharmacology

Several preclinical studies have been published demonstrating consistent analgesic efficacy of cebranopadol across a broad range of models of acute and chronic pain in rat (Table 2) and mouse (Table 3).

In rats, cebranopadol was tested in models of acute nociception, neuropathic allodynia and hyperalgesia, visceral allodynia, inflammatory hypersensitivity, and bone cancer-induced allodynia. Cebranopadol shows full efficacy and high potency (ED_{50} 5.6 μg/kg i.v.) in the tail-flick model of acute nociception with a high stimulus intensity (baseline latency times in the range of 4 s, cutoff time 12 s). The duration of

Table 2 Effect of cebranopadol in rat models of pain

Indication	Model	ED_{50} (95% CI)	Reference
Acute pain	Tail flick	5.6 (4.4–7.0) μg/kg i.v.	Linz et al. (2014)
Acute pain	Tail flick	25.1 (20.7–30.4) μg/kg p.o.	Linz et al. (2014)
Acute pain	Low-intensity tail flick	7.4 (6.6–8.2) μg/kg i.v.	Linz et al. (2017)
Neuropathic pain	STZ PPT	0.5 (0.2–0.8) μg/kg i.v.	Linz et al. (2014)
Neuropathic pain	STZ PPT	0.8 μg (single dose)/paw i.pl. selective anti-HA	Tzschentke et al. (2017b)
Neuropathic pain	CCI CP	0.8 μg (single dose)/paw i.pl. selective anti-AD	Tzschentke et al. (2017b)
Neuropathic pain	CCI CP	0.25–0.8 μg/kg i.p.	Linz et al. (2014)
Neuropathic pain	SNL vFH	3.3 (2.7–4.0), 3.6 (2.8–4.6) μg/kg i.p. + J-113397 14.1 (10.3–17.7) μg/kg i.p. + naloxone 16.9 (12.5–21.4) μg/kg i.p. + naltrindole 17.3 (14.2–20.5) μg/kg i.p. + nor-BNI 15.0 (12.7–17.5) μg/kg i.p. + naloxone/naltrindole/nor-BNI 65.5 (52.3–81.1) μg/kg i.p.	Christoph et al. (2018)
Neuropathic pain	SNL vFH	0.8 (0.5–1.1) μg/kg i.v.	Linz et al. (2014)
Visceral pain	Pancreatitis vFH	0.13 (0.03–0.49) μg/kg i.v. (rAD)	Schiene et al. (2018a)
Inflammatory pain	CFA WB	5.5 (3.2–21.0) μg/kg i.v.	Linz et al. (2014)
Inflammatory pain	CFA WB	ED_{25} 1.6 (0.8–1.6) μg/kg i.v. + J-113397 3.2 (2.4–4.0) μg/kg i.v. + naloxone 18.3 (9.6–146) μg/kg i.v.	Schiene et al. (2018b)
Bone cancer pain	BCP vFH	3.6 (1.6–7.0) μg/kg i.v.	Linz et al. (2014)

ED effective dose, *CI* confidence interval, *MPE* maximal possible effect, *i.v.* intravenous, *i.p.* intraperitoneal, *i.pl.* intraplantar, *p.o.* per os, *vFH* von Frey hair, *STZ* streptozotocin, *CCI* chronic constriction injury, *SNL* spinal nerve ligation, *CFA* complete Freund's adjuvans, *WB* weight-bearing, *PPT* paw pressure test, *CP* cold plate

Table 3 Effect of cebranopadol in mouse models of pain

Indication	Model	ED50 (95%CI)	Reference
Acute pain	Tail flick	200 μg/kg i.v.	Rizzi et al. (2016)
Acute pain	Tail flick	40.1 (30.4–48.5) nmol/kg (= 15.2 (11.5–19.1) μg/kg) i.v.	Schunk et al. (2014)
Acute pain	Tail flick	77.0 (57.8–98.1) nmol/kg (= 29.1 (21.9–37.1) μg/kg) p.o.	Schunk et al. (2014)
Persistent pain	Formalin test	40 μg/kg i.v. (1st phase) 30 μg/kg i.v. (2nd phase)	Rizzi et al. (2016)
Persistent pain	Orofacial formalin test	20 μg/kg i.v. (1st phase) 20 μg/kg i.v. (2nd phase)	Rizzi et al. (2017)
Neuropathic pain	STZ heat HA	54.1% MPE at 2.1 nmol/kg (= 0.8 μg/kg) i.v.	Schunk et al. (2014)
Neuropathic pain	STZ heat HA	0.037 (0.026–0.050) nmol/animal (= 0.018 (0.012–0.024) μg/animal i.th. (0.01 selective anti-HA)) 0.037 (0.029–0.040) nmol/animal (= 0.017 (0.014–0.019) μg/animal i.c.v. (0.01 selective anti-HA))	Tzschentke et al. (2017b)
Visceral pain	MO-VP	4.6 (2.9–7.9) μg/kg i.v. (SP) 2.2 (1.3–3.49) μg/kg i.v. (rAD) 2.4 (1.4–3.6) μg/kg i.v. (rHA)	Schiene et al. (2018a)
Others	HP/WR/ CT/FT/ OXA-CP	Efficacy at 10 mg/kg s.c.	Salat et al. (2018)

ED effective dose, *CI* confidence interval, *MPE* maximal possible effect, *i.v.* intravenous, *i.p.* intraperitoneal, *i.th.* intrathecal, *i.c.v.* intracerebroventricular, *s.c.* subcutaneous, *p.o.* per os, *HA* hyperalgesia, *MO* mustard oil, *VP* visceral pain, *STZ* streptozotocin, *SP* spontaneous pain, *rAD* referred allodynia, *rHA* referred hyperalgesia, *HP* hot plate, *WR* writhing, *CT* capsaicin test, *FT* formalin test, *OXA* oxaliplatin, *CP* cold plate

action of more than 7 h corresponds well with the pharmacokinetic characteristics in the rat, a long half-life (4.52 h) and rapid absorption. Oral bioavailability was reported to be between 13 and 23%, which again is reflected in the potency in the same tail-flick paradigm after oral administration (ED_{50} 25.1 μg/kg p.o.) (Linz et al. 2014). A cutoff time of 12 s predominantly assesses a spinal nocifensive reflex. Lowering of the stimulus intensity gradually includes contribution of supraspinal regulation. In a tail-flick test with baseline latencies in the range of 8 s and a cutoff of 30 s, cebranopadol shows efficacy of similar magnitude and duration of action and a slightly reduced potency (ED_{50} 7.4 μg/kg i.v.; Linz et al. 2017). The confidence intervals of both ED_{50} values (high- and low-intensity stimulus) do overlap; thus these values are not significantly different from each other. This suggests that cebranopadol acts on all levels of the neuraxis with similar potency.

While the antinociceptive potency of cebranopadol in rat models of acute heat nociception is around 200-fold higher as compared to morphine (ED_{50} 1.1 mg/kg i.v.) (Tzschentke et al. 2009) and in the same range as fentanyl (Linz et al. 2014), there is a remarkable increase in potency in models of neuropathic pain. Mechanical

hypersensitivity in the rat spinal nerve ligation (SNL) model was measured by means of an electronic von Frey transducer, and cebranopadol completely abolished hypersensitivity with an ED_{50} of 0.8 μg/kg i.v., which is sevenfold more potent as the inhibition of heat nociception (Linz et al. 2014). Morphine showed a potency of 3.7 mg/kg i.v. in the same model (Christoph et al. 2007), i.e., the anti-hypersensitive potency of cebranopadol in this model is more than 1,000-fold higher than that of morphine. Cold allodynia was measured in rats with unilateral chronic constriction injury (CCI) by analyzing the nocifensive behavior on a cold metal plate for a period of 2 min. Cebranopadol was effective at doses below 1 μg/kg i.p., although an ED_{50} value was not determined in this experiment (Linz et al. 2014). Again, a more than 100-fold difference in potency compared to morphine can be assumed. Morphine was tested in the same setting and showed inhibition of cold allodynia with an ED_{50} value of 13.8 mg/kg i.p. (Tzschentke et al. 2006).

Pre-treatment of rodents with a single dose of streptozotocin (STZ) induces depletion of pancreatic ß-cells resulting in diabetes. Mechanical hyperalgesia can be assessed in this model by determination of the withdrawal threshold induced by continuously increased paw pressure test. Cebranopadol inhibits diabetic mechanical hyperalgesia with an ED_{50} of 0.5 μg/kg i.v. (Linz et al. 2014). While full inhibition of hyperalgesia reflected by 100% MPE is reached in diabetic rats, the same doses of cebranopadol did not change the mechanical paw withdrawal thresholds in nondiabetic control rats demonstrating selective anti-hyperalgesia at these low doses. An important aspect in the interpretation of an increased potency in neuropathic pain conditions is the potential site of action. Opioid receptors as well as the NOP receptor are expressed in all major parts of the ascending and descending pain pathways and therefore offer multiple target sites for analgesic interaction (Schröder et al. 2014). Local peripheral administration of cebranopadol results in efficacy in a mononeuropathic (CCI) and a polyneuropathic (STZ) pain model suggesting a substantial contribution of target receptors in distal primary afferent neurons. Intraplantar administration of cebranopadol results in selective ipsilateral efficacy without "leakage" toward the contralateral hind paw (Tzschentke et al. 2017b). In fact, a local dose that significantly inhibited cold allodynia upon ipsilateral administration was without effect on the ipsilateral paw when injected in the contralateral paw. Likewise, a dose which was efficacious on the ipsilateral side was without effect on the contralateral side in STZ-induced bilateral mechanical hyperalgesia. Beside the localization of potential analgesic contribution, the mechanistic type of analgesic efficacy is worthwhile to be considered. Studies combining anti-hypersensitive doses of cebranopadol with antagonists of the different opioid receptors as well as the NOP receptor reveal a synergistic interaction in the SNL model (Christoph et al. 2018). Pre-treatment with antagonists for NOP (J-113397), MOP (naloxone), DOP (naltrindole), and KOP (norbinaltorphimine, nor-BNI) receptors at dosages which have previously been shown to be selective for the respective receptor in the SNL model (Rutten et al. 2018) were able to inhibit cebranopadol's efficacy in a comparable range. In addition, analysis of NOP receptor antagonism and combined MOP/KOP/DOP receptor antagonism revealed synergistic interaction of the NOP component with the effect on the classical opioid receptors.

Beside acute and neuropathic pain models, cebranopadol was tested in rat models of visceral, inflammatory, and bone cancer-induced pain conditions. Treatment with dibutyltin dichloride induces subacute inflammation of the pancreas and leads to referred mechanical allodynia which can be measured at the corresponding dermatome of the abdominal wall by using calibrated von Frey filaments. Cebranopadol shows dose-dependent inhibition of referred visceral pain originating from pancreatitis with an ED_{50} of 0.13 μg/kg i.v. (Schiene et al. 2018a). As in models of neuropathic pain, the pathology of subacute visceral pain seems to be very sensitive for a drug targeting NOP and classical opioid receptors. Subacute local inflammation of the knee joint induced by intra-articular administration of complete Freund's adjuvants (CFA) models osteoarthritis pain and can be measured in a weight-bearing paradigm comparing the use of both hind paws. Cebranopadol normalizes CFA-induced impairment of weight-bearing with an ED_{50} of 5.5 μg/kg i.v. (Linz et al. 2014). In comparison, morphine is efficacious with an ED_{50} of 1.0 mg/kg i.v. (Schiene et al. 2011) suggesting a similar relative potency difference between cebranopadol and morphine as for acute nociceptive pain. However, heat nocifensive reflex reaction of the rat tail might well differ from pressure distribution on the hind paws with an ipsilateral knee joint inflammation. Experiments using selective doses of the NOP receptor antagonist J-113397 and the MOP receptor antagonist naloxone demonstrated contribution of both receptor types to the anti-hypersensitive efficacy of cebranopadol in the same rat model (Schiene et al. 2018b). The ED_{25} value of 1.6 μg/kg i.v. was shifted to 3.2 μg/kg i.v. by J-113397 and to a larger extent of 18.3 μg/kg i.v. by naloxone. In contrast to the situation in neuropathic pain (SNL model), these data suggest a relatively larger MOP receptor contribution in this model of osteoarthritis pain.

Ipsilateral tibial injection of mammary gland carcinoma cells results in local development of bone cancer which induces mechanical hypersensitivity on the ipsi- but not on the contralateral side. Treatment of the rats with cebranopadol results in dose-dependent anti-hypersensitive efficacy with an ED_{50} of 3.6 μg/kg i.v. (Linz et al. 2014) measured by von Frey filaments. Similar to the findings in neuropathic pain models, there was a selective inhibition of mechanical hypersensitivity on the ipsilateral hind paw, while mechanical antinociception on the contralateral hind paw was detected only at a higher dose.

In mice, cebranopadol was tested in models of acute and chronic pain covering a number of different pain etiologies and stimuli. Robust dose-dependent antinociception was demonstrated in different labs. In a tail-flick setting using NMRI mice with a baseline latency of 3–5 s and a cutoff latency of 12 s, cebranopadol showed full efficacy with potencies of 15.2 μg/kg i.v. and 29.2 μg/kg p.o. reflecting an oral availability in the range of 50% (Schunk et al. 2014). An independent group demonstrated full efficacy with an ED_{50} of 200 μg/kg i.v. in CD-1 mice (Rizzi et al. 2016). Beside the different application regime comprising a cumulative design in the latter study, further differences such as mouse strain and stimulus intensity may well contribute to the tenfold difference in potency.

In contrast to the rat, data in mouse neuropathic pain models are sparse. Diabetic STZ-induced heat hyperalgesia was inhibited by 54% MPE at a dose of 0.8 μg/kg

i.v. (Schunk et al. 2014). While this single dose does not allow proper quantification of efficacy and potency, an estimated ED_{50} value in the single digit μg/kg range mirrors the situation in the rat with a potency shift from antinociceptive to anti-neuropathic potency. Notably, both antinociceptive and anti-hyperalgesic measures make use of the same stimulus (i.e., heat), allowing for a more direct comparison, unlike the situation in rats using heat nociception versus mechanical or cold hypersensitivity. Spinal and supraspinal efficacy and potency were analyzed in STZ-induced heat hyperalgesia. This model allows for parallel assessment of anti-hyperalgesia (in diabetic STZ mice) and antinociception (in nondiabetic control mice) with the same readout in the same mouse strain. Spinal (i.th.) and supraspinal (i.c.v.) administration results in full anti-hyperalgesic efficacy with a potency of 0.018 μg/animal i.th. and 0.017 μg/animal i.c.v. (Tzschentke et al. 2017b). With both routes of administration, cebranopadol was more potent in diabetic as in nondiabetic conditions, reflected by a significant selective anti-hyperalgesic efficacy at a dose of 0.01 μg/animal. The virtually identical similar potency after spinal and supraspinal administration corroborates the conclusions drawn from the rat high- and -low-intensity tail-flick studies mentioned above. Assuming equal access of cebranopadol to the brain and spinal cord, in the central compartment, supraspinal and spinal sites/receptors are likely to contribute equally to the effects of cebranopadol after systemic administration. Interestingly, the identical minimal effective dose (0.0316 μg/animal) in both compartments for antinociceptive effects in nondiabetic animals further suggests that the purported anti-opioid effect of supraspinal NOP receptors under acute pain conditions was of no functional relevance when NOP receptors were activated concurrently with MOP receptors by cebranopadol.

Intraplantar injection of 20 μL of 1.5% formalin solution into the dorsal surface of the right hind paw (known as formalin test) induces a biphasic nociceptive behavior, thought to reflect acute (1st phase, 0–15 min) and persistent (2nd phase, 15–45 min) pain. Cebranopadol shows full efficacy in both phases of the formalin test with ED_{50} values of 40 μg/kg i.v. in 1st phase and 30 μg/kg i.v. in 2nd phase (Rizzi et al. 2016). A more sophisticated variant of the formalin test analyzes efficacy and potency of analgesics in the trigeminal territory upon orofacial administration of formalin. Again, cebranopadol shows full efficacy and similar potency in both phases (ED_{50} 20 μg/kg i.v. in 1st and 2nd phase) and no relevant potency shift as compared to the classical formalin test assessing the hind paw (Rizzi et al. 2017).

Acute colitis resulting in spontaneous and referred visceral pain symptoms can be induced by colorectal administration of mustard oil. Spontaneous visceral pain can be quantified by counting the occurrence of visceral pain reactions (licking of the abdomen, stretching, squashing, mounting, backward movement, or contraction of the flank muscles), and referred hypersensitivity can be measured by assessing the withdrawal reaction toward light touch (1 mN reflecting referred allodynia) or nociceptive stimulation (16 mN reflecting referred hyperalgesia) of the abdominal dermatomes with von Frey filaments. Cebranopadol shows full efficacy in all three parameters of visceral pain with ED_{50} values of 4.6 μg/kg i.v. for spontaneous visceral pain, 2.2 μg/kg i.v. for referred visceral allodynia, and 2.4 μg/kg i.v. for referred visceral hyperalgesia (Schiene et al. 2018a).

Overall, mouse data demonstrate a similar analgesic profile as in the rat, with a clear potency increase in models of neuropathic pain, potentially suggesting particularly beneficial effects of cebranopadol in clinical chronic neuropathic pain conditions. Taken together, there is very good agreement in terms of potency and efficacy between the findings in the Grünenthal studies and those of other labs (Rizzi et al. 2016, 2017). There is one study, however, that is completely at odds with all other studies in terms of potency (Salat et al. 2018). These authors reported that cebranopadol was efficacious in the hot plate test, the writhing test, the capsaicin test, the formalin test, and the oxaliplatin-induced neuropathic pain model without confounding effects in the rota-rod test of motor coordination at the extremely high dose of 10 mg/kg s.c. In fact, a no-observed-effect level (NOEL) of 0.142 mg/kg i.v. was reported for the mouse rota-rod (Schunk et al. 2014; see below), which appears to reflect a much more realistic therapeutic window when compared to the mouse efficacy data (summarized in Table 3). We are not aware of another study where cebranopadol was administered in mice via the subcutaneous route, but it is difficult to envision how this different route should result in a manyfold reduced potency.

Cebranopadol was also tested in the rhesus monkey 50 °C tail-dip assay of acute thermal nociception (W. Schröder and M.-C. Ko, personal communication). Cebranopadol exerted potent (ED50, 2.2 μg/kg; CI, 1.9, 2.7) and fully efficacious (100% MPE) antinociception in a dose- and time-dependent manner. The peak effect was observed at 30 min after i.v. administration of 4.5 μg/kg, and efficacy was sustained throughout the 2.5 h test session. Pre-treatment with a selective NOP receptor antagonist (J-113397, 0.1 mg/kg s.c.) or a selective MOP receptor antagonist (naltrexone, 0.3 mg/kg s.c.) inhibited the antinociceptive effects. Up to the highest dose tested, no sedative effects occurred that could have confounded the antinociceptive readout. Scratching responses were observed at all doses. In conclusion, systemic cebranopadol produced efficacious and potent antinociception in nonhuman primates that was mediated by activation of NOP and MOP receptors.

Taken together, rodent efficacy data demonstrate a broad analgesic profile of cebranopadol including inhibition of responses to thermal, mechanical, or chemical stimulation in a number of pain etiologies, including acute nociceptive as well as (sub)chronic inflammatory, visceral, and chronic neuropathic pain. There is a potency shift from acute toward chronic pain models which might be based on involvement of multiple potential sites of action (peripheral to central) as well as synergistic interaction between NOP and classical opioid receptor agonism. Efficacy of cebranopadol in nonhuman primates has also been demonstrated.

4 Preclinical Safety and Tolerability

Preclinical safety and tolerability of cebranopadol have been evaluated extensively in rodent models. These investigations focused on the CNS, the respiratory system, and the gastrointestinal system as typical target organs for opioid-type side effects. The outcome of these studies is summarized in Table 4.

Table 4 Effect of cebranopadol in rodent models on CNS, respiratory, and gastrointestinal function

Organ system	Species	Test parameter/ test system	Observation	Reference
CNS	Rat	Locomotor activity/video tracking	No effect at 25 μg/kg p.o.	De Guglielmo et al. (2017)
	Rat	Motor coordination/ rota-rod test	NOEL ≥16 μg/kg i.v.	Linz et al. (2014)
	Mouse	Motor coordination/ rota-rod test	NOEL = 376 nmol/kg (~142 μg/kg) i.v.; transient impairment of motor coordination (30–60 min post admin.) at 454 nmol/kg (~172 μg/kg) i.v.	Schunk et al. (2014)
	Mouse	Motor coordination/ rota-rod test	No effect at 1 mg/kg i.v.	Rizzi et al. (2016)
	Mouse	Motor coordination/ rota-rod test	No effect at 10 mg/kg s.c.	Salat et al. (2018)
Respiratory system	Rat	Respiratory function/whole-body plethysmography	NOAEL ≥16 μg/kg i.v. Transient but nonsignificant increase in respiratory rate and tidal volume at 4, 8, and 16 μg/kg i.v.; no effects on minute volume, peak inspiratory and expiratory flows, inspiration and expiration times, and calculated airway resistance index	Linz et al. (2014)
	Rat	Arterial blood gas tensions/ blood gas analysis	No significant changes of pCO_2 and pO_2 up to 17.1 μg/kg i.v.; co-administration of the selective NOP receptor antagonist J-113397 led to significant increase in pCO_2/decrease in pO_2 at 17.1 μg/kg i.v. cebranopadol which could be fully reversed by a MOP receptor antagonist (naloxone)	Linz et al. (2017)

(continued)

Table 4 (continued)

Organ system	Species	Test parameter/ test system	Observation	Reference
Gastrointestinal system	Rat	Intestinal transit time/charcoal test	Dose-dependent inhibition of intestinal transit NOEL: 4 μg/kg i.v.; 25% and 63% reduction of intestinal transit rate at 8 and 16 μg/kg i.v., respectively	Grünenthal GmbH, unpublished data
	Mouse	Intestinal transit time/charcoal test	Dose-dependent inhibition of intestinal transit NOEL: 21 nmol/kg (= 8 μg/kg) i.v. ED_{50} = 87 nmol/kg (= 33 μg/kg) i.v.	Schunk et al. (2014)

NOEL no-observed-effect level, *NOAEL* no-observed-adverse-effect level, ED_{50} half-maximum effective dose, *i.v.* intravenous, *s.c.* subcutaneous, *p.o.* per os

4.1 Central Nervous System

In contrast to pure MOP receptor agonists like morphine or oxycodone (Winter et al. 2003), cebranopadol is devoid of CNS side effects like sedation or disruption of motor coordination up to doses significantly higher than those required to produce antinociception in models of acute pain (Table 4).

In particular, cebranopadol did not influence general locomotor activity when administered orally to rats at an antinociceptive dose of 25 μg/kg (de Guglielmo et al. 2017). Likewise it was demonstrated that cebranopadol up to a dose of 16 μg/kg i.v. did not affect performance of rats in the rota-rod test (Linz et al. 2014). This dose is approximately 3 times higher than the ED_{50} in rat acute pain models and up to 30 times higher than ED_{50} values obtained in models of neuropathic pain (compare Table 2). In the same study, morphine induced dose-dependent impairment of motor coordination starting already at analgesic ED_{50} and leading to complete loss of motor coordination at a fully analgesic dose. Comparable results were also published for morphine and other MOP receptor agonists by Meert and Vermeirsch (2005). In mice, performance on the rota-rod was investigated after intravenous administration of cebranopadol (Schunk et al. 2014). In this study, the no-observed-effect level (NOEL) for effects on motor coordination was determined to be approximately 10-fold higher than the ED_{50} for antinociceptive activity in the tail-flick test and more than 175-fold higher than a half-maximum effective dose in a STZ model of diabetic neuropathic pain. In two recent studies, the low potential of cebranopadol to affect motor coordination in rats was confirmed (Rizzi et al. 2016; Salat et al. 2018).

4.2 Respiratory System

Respiratory depression is a potentially life-threatening side effect induced by classic opioid pain medications. It is therefore a main factor determining the therapeutic window of opioids.

The effect of cebranopadol on the respiratory system was investigated in two models of respiratory function in rats. In a whole-body plethysmography model, up to the highest test dose of 16 μg/kg i.v., cebranopadol did not induce significant changes in respiratory function (Linz et al. 2014). Although a transient increase in respiratory rate and tidal volume was observed, the effects were small, and minute volume was consequently not significantly changed during the 4 h investigational period following administration of cebranopadol. Other respiratory parameters, including peak inspiratory and expiratory flows, inspiration and expiration times, and airway resistance, were also not significantly affected by cebranopadol. In another study, arterial blood gas tensions were monitored to assess respiratory depressant effects in conscious rats (Linz et al. 2017). Likewise, cebranopadol up to 17.1 μg/kg i.v. had no statistically significant effects on arterial blood gas tensions. At this supra-analgesic dose, only a moderate decrease in arterial oxygen tension (pO_2) was observed, accompanied by a slight increase in arterial carbon dioxide tension (pCO_2). The absence of clear respiratory depressant activity even at doses being more than 3 times or 30 times higher than ED_{50} values in models of nociceptive or neuropathic pain is in clear contrast to classical opioids. Starting at analgesic doses, subcutaneous morphine induced a significant decrease in tidal volume that despite an increase in respiratory rate resulted in a significant reduction of minute volume in rat whole-body plethysmography (Linz et al. 2014). In addition, respiratory depression induced by morphine was apparent from dose-dependent decreases in peak inspiratory flow and expiration time, as well as significant increases in airway resistance index and a general disturbance of respiratory rhythm, none of which was observed even with very high doses of cebranopadol. Correspondingly, equianalgesic doses of intravenous fentanyl produced rapid and pronounced changes in blood gas tensions in rats, with statistically significant decreases in mean pO_2 and increases in mean pCO_2 (Linz et al. 2017). Taken together, these findings suggest that cebranopadol may be more likely than classic MOP receptor agonists to produce clinically meaningful levels of analgesia without potentially dangerous levels of respiratory depression. First evidence for a potential translation of the improved therapeutic window from animals to the clinical situation comes from results of a phase 1 trial in healthy human volunteers (Dahan et al. 2017; see below, Sect. 6).

Using the selective NOP receptor antagonist J-113397 and the MOP receptor antagonist naloxone, Linz and coworkers provided evidence that the intrinsic NOP receptor agonist activity of cebranopadol is responsible for limiting the respiratory depressant effect of its MOP receptor agonist activity in rats (Linz et al. 2017). Co-administration of J-113397 significantly increased the effects of cebranopadol on arterial blood gas tensions leading to changes in pO_2 and pCO_2 similar to those induced by equianalgesic doses of fentanyl. The respiratory depressant effects of the combination of cebranopadol and J-113397 could be fully reversed by additional

administration of naloxone. From these observations, the authors conclude that simultaneous activation of the NOP receptor by cebranopadol counterbalances the MOP receptor-dependent respiratory depression in rats.

4.3 Gastrointestinal System

Another adverse effect that is characteristic for opioids is the inhibition of gastrointestinal function. While standard opioids inhibit gastrointestinal transit at doses below or within the half-maximum effective analgesic dose range (Meert and Vermeirsch 2005), cebranopadol was shown to have better margins between the analgesic dose range and doses that inhibit gastrointestinal function. Schunk et al. (2014) observed a dose-dependent inhibition of gastrointestinal transit with intravenous cebranopadol in a charcoal test in mice. The ED_{50} was determined to be 33 μg/kg, which corresponds to approximately 2 times or 40 times the ED_{50} in mouse models of nociceptive or neuropathic pain, respectively. Similar results were obtained in rats, in which intravenous cebranopadol was found to reduce the gastrointestinal transit of a charcoal meal only at high analgesic and supra-analgesic doses (Grünenthal GmbH, unpublished data). The NOEL was determined to be 4 μg/kg, while 8 μg/kg and 16 μg/kg reduced the transit rate by 25% and 63%. Hence, about 50% inhibition of gut propulsion is reached at a dose of 2–3 times the ED_{50} in a rat model of nociceptive pain or 10–12 times the ED_{50} in a rat model of neuropathic pain.

In summary, several investigations on the potential of cebranopadol to induce opioid-type side effects in the CNS, the respiratory system, and the gastrointestinal system in rodents point toward a markedly broader therapeutic window than seen for classical opioids. Although this needs to be evaluated in further clinical trials, the animal data as well as the first clinical data (see below) suggest that cebranopadol may offer an advantage beyond classical opioid analgesics by reducing the potential for severe or even life-threatening adverse events.

5 Abuse Potential

There is good evidence that NOP receptor agonists can attenuate some of the MOP receptor-related (side) effects, such as reward, reinforcement, tolerance development, and physical dependence (Lutfy et al. 2001; Ciccocioppo et al. 2000; Sukhtankar et al. 2014; Murphy et al. 1999; Rutten et al. 2010, 2011, and references therein). The best evidence for "anti-abuse" effects of NOP receptor agonism comes from conditioned place preference (CPP) studies. The early prototypical small molecule NOP receptor agonists (Ro64-6198 and Ro65-6570) that have been used in many studies have very prominent sedative effects that can interfere with the behavioral readout in operant tasks. Thus, whereas the readout in CPP is not affected since the test is conducted in a drug-free state, interpreting the effects of NOP

receptor agonists on operant intravenous opioid self-administration has been difficult (Podlesnik et al. 2011; Sukhtankar et al. 2014).

In models of abuse liability, cebranopadol behaved as an opioid, but the magnitude of effects also clearly differentiated from morphine. Physical dependency was reduced in terms of spontaneous and precipitated withdrawal (Tzschentke et al. 2017a, b), tolerance development to the antiallodynic effect in a neuropathic pain model was much slower (Linz et al. 2014), and in a drug discrimination assay, cebranopadol fully generalized to a morphine cue only at clearly supranalgesic doses (Tzschentke and Rutten 2018). The intravenous self-administration (IVSA) of cebranopadol has not been evaluated; however, cebranopadol was reported to reduce cocaine self-administration under both fixed-ratio and progressive-ratio schedules (Shen et al. 2017) and to block the escalation of cocaine intake and conditioned reinstatement of cocaine seeking in rats (de Guglielmo et al. 2017). Published data on rewarding effects in the CPP paradigm are equivocal, as in one study (Shen et al. 2017) no effects were seen, while another study (de Guglielmo et al. 2017) demonstrated CPP at a single test dose. Whether the anti-reinforcement effects of cebranopadol in IVSA and the benign CPP effects are related to the drug's (potentially aversive) activity at the KOP receptor remains to be established. What is clear, however, is that the activity at the KOP receptor does not confer an overall aversive effect to cebranopadol, as signified by the lack of contribution of the KOP receptor to the cebranopadol cue (Tzschentke and Rutten 2018) and, more directly, by the absence of a conditioned place aversion (Shen et al. 2017; de Guglielmo et al. 2017).

5.1 Intravenous Self-Administration (IVSA)

The intravenous self-administration (IVSA) of cebranopadol has not yet been evaluated directly. However, two studies have evaluated the impact of cebranopadol on several aspects of cocaine IVSA in rats.

De Guglielmo et al. (2017) reported that cebranopadol reversed the escalation of cocaine IVSA and reduced total intake in rats that were given extended (6 h) access to cocaine, whereas it did not affect the self-administration of sweetened condensed milk or responding on the inactive lever, suggesting that the effects on cocaine self-administration were not attributable to nonspecific motor effects. Furthermore, the lack of effect of cebranopadol on sweetened condensed milk self-administration did not likely result from different levels of responding for each reward because responses during milk self-administration were analyzed at two different time points to match both the number of rewards and length of self-administration sessions. Interestingly, cebranopadol also blocked the cue-induced reinstatement of cocaine seeking, without affecting responding on the inactive lever. As cebranopadol decreased the reinstatement of cocaine seeking in the absence of cocaine under extinction conditions, it may decrease the motivation to seek and take cocaine, independent of changes in blood levels of cocaine. As such, cebranopadol may not only be effective to reduce actual cocaine intake, but it may also be able to prevent relapse to cocaine-taking behavior during abstinence. The potential utility of

selective NOP receptor agonists and combined NOP-MOP receptor agonists for the treatment of drug abuse and dependence has been discussed previously (Zaveri 2011).

Shen et al. (2017) confirmed and extended these findings by showing that under a fixed-ratio-5 schedule of reinforcement, cebranopadol decreased cocaine but not saccharin self-administration, again indicating a specific inhibition of psychostimulant consumption that is not due to sedation or general disruption of motor activity. In addition, cebranopadol decreased the motivation for cocaine as evidenced by reduction of the break point measured in a progressive-ratio paradigm. Cebranopadol retained its effect on cocaine consumption throughout a 7-day chronic treatment, suggesting a lack of tolerance development. Only simultaneous blockade of NOP and MOP receptors by concomitant administration of the NOP receptor antagonist SB-612111 and naltrexone reversed cebranopadol-induced decrease of cocaine IVSA, suggesting that cebranopadol activates both NOP and classical opioid receptors to exert its effect.

Theoretically, the ability of cebranopadol to act as KOP receptor agonist might also contribute to the effect seen in cocaine IVSA. However, several lines of evidence argue against this possibility. First, the effect on cocaine IVSA was fully blocked by co-administration of a MOP and a NOP receptor antagonist (Shen et al. 2017). Second, the KOP receptor agonistic effect of cebranopadol did not contribute to cebranopadol's stimulus properties (Tzschentke and Rutten 2018). Third, cebranopadol did not produce a conditioned place aversion in place conditioning paradigms (Shen et al. 2017; de Guglielmo et al. 2017). These findings indicate that the KOP receptor agonistic activity of cebranopadol does not convey an overall net aversive effect of the drug. On the other hand, one cannot exclude the possibility that the KOP receptor agonistic component contributes an element to the overall effect, which acts, like the NOP receptor agonistic activity, to reduce or limit the overall rewarding and reinforcing effects of cebranopadol.

5.2 Conditioned Place Preference (CPP)

Published data on rewarding effects in the conditioned place preference (CPP) paradigm are equivocal, as Shen et al. (2017) have found only a nonsignificant trend (at 10 and 50 μg/kg i.p.), while de Guglielmo et al. (2017) demonstrated CPP at the single dose tested (25 μg/kg p.o.) (in the absence of effects on locomotor activity during the conditioning sessions). This difference cannot easily be accounted for. In fact, due to a higher C_{max} and an earlier t_{max}, if anything, i.p. administration would have been expected to produce CPP, not oral administration. Beside different routes of administration, the two studies differ in a number of other variables, such as duration and number of conditioning sessions. Potentially the most relevant difference is that Shen et al. (2017) used an unbiased conditioning design, whereas de Guglielmo et al. (2017) used a biased design, pairing cebranopadol with the non-preferred compartment of each animal. As conditioning to the non-preferred compartment increases the effect window, it may be easier to observe an increase in

time spent in the drug-paired compartment. Furthermore, nonspecific effects, such as anxiolytic effects, may contribute to an increase in time spent in the initially non-preferred compartment. As such, overall, Shen et al. (2017) may have used the more appropriate, yet more stringent, design, using i.p. administration and an unbiased design.

Despite the discrepancy regarding presence or absence of place preference, an important and consistent finding was that cebranopadol did not produce conditioned place aversion. Hence, unlike selective KOP receptor agonists, cebranopadol appears to be devoid of negative affective properties. Its affective effects appear rather to be driven by its MOP receptor activity, held in check by its NOP receptor activity.

5.3 Drug Discrimination

In drug discrimination studies in the rat, Tzschentke and Rutten (2018) addressed the question of which of the pharmacological activities of cebranopadol (NOP, MOP, KOP, and DOP receptor agonism) contribute to its interoceptive stimulus properties. To this end, cebranopadol was first tested in generalization tests against a morphine cue, including receptor-specific antagonism. Cebranopadol generalized to the morphine cue; however, full generalization was only seen at clearly supra-analgesic doses. The effect of cebranopadol was reduced by the MOP receptor antagonist naloxone but, notably, was enhanced by the NOP receptor antagonist J-113397. In a second step, cebranopadol was established as a cue, and MOP, NOP, KOP, and DOP receptor-selective agonists were tested in generalization tests. In cebranopadol-trained rats, cebranopadol as well as morphine produced dose-dependent generalization. A NOP receptor agonist (Ro 65-6570) did not, while a DOP receptor agonist (SNC-80) and a KOP receptor agonist (U50488) weakly generalized to the cebranopadol cue. Finally, cebranopadol in combination with receptor-selective antagonists was tested against the cebranopadol cue. Generalization of cebranopadol was reduced by naloxone and J-113397, but not by a DOP (naltrindole) or a KOP (norbinaltorphimine) receptor antagonist. Taken together, these results suggest a clear contribution of MOP receptor activity and a relative lack of contribution of DOP and KOP receptor activity to cebranopadol's stimulus properties. Despite the lack of generalization of Ro 65-6570 to the cebranopadol cue, the observed reduction of the cue by J-113397, and the fact that J-113397 increased generalization of cebranopadol to a morphine cue, suggests that the NOP receptor activity of cebranopadol not only contributes to its discriminative stimulus properties but also attenuates the morphine-like stimulus property of cebranopadol through an intrinsic interaction with MOP receptor activity. This view is in line with data showing that NOP receptor activity of cebranopadol attenuates respiratory depression due to cebranopadol's MOP receptor activity (Linz et al. 2017; see below) and with reports demonstrating that NOP receptor agonists reduce certain (side-) effects of MOP receptor agonists (Rutten et al. 2010; Sukhtankar et al. 2014). It is also perfectly in line with the findings of Walentiny et al. (2018) who showed that the NOP receptor

agonist Ro 64-6198 produced a rightward shift in the dose-response curve for oxycodone generalization to an established oxycodone cue. Overall, these results are consistent with a unique profile and pharmacology of cebranopadol relative to classical opioids (i.e., only moderate morphine-like stimulus properties in the analgesic dose range and contribution of NOP activity to the overall cue).

The reason for the somewhat equivocal findings regarding NOP receptor contribution was not clear, but Tzschentke and Rutten (2018) argued that it may be related to shortcomings of the tool compounds used. The use of Ro 65-6570 was hampered by dose-limiting side effects that may have precluded the testing of doses necessary to observe generalization to the cebranopadol cue. Also, the possibility that higher doses of Ro 65-6570 would have shown a greater degree of generalization to cebranopadol cannot be excluded.

Another issue of potential relevance in the present context is the concept of "biased agonism." Many G-protein-coupled receptors (GPCRs) signal not only via G-proteins but also via other intracellular signaling pathways, which can mediate different and independent effects (Benredjem et al. 2017; Bologna et al. 2017). Rizzi et al. (2016) have demonstrated that cebranopadol is a biased agonist at the NOP receptor and as such has a very strong bias for G-protein over β-arrestin2 signaling subsequent to NOP receptor activation. Unfortunately, the functional relevance of biased signaling at the NOP receptor has not been characterized yet. However, cebranopadol lacks the typical side effects of N/OFQ and the early non-peptidergic NOP receptor agonists (such as Ro 64-6198 and Ro 65-6570), such as sedation, motor incoordination, or hypnotic-like state at higher doses (Linz et al. 2014; Kotlinska et al. 2003; Higgins et al. 2001) despite its high potency and efficacy at the NOP receptor. N/OFQ shows no and Ro 65-6570 has only a weak bias for G-protein signaling (Rizzi et al. 2016). Thus the lack of β-arrestin2 signaling is an attractive hypothesis to explain better tolerability of cebranopadol as compared to other NOP receptor agonists. It may also give first hints regarding a possible functional relevance of biased signaling at the NOP receptor with respect to discriminative stimulus properties related to NOP receptor activation. Ro65-6570 did not generalize to the cebranopadol cue, although J-113397, by reducing the cebranopadol cue and by increasing the morphine-like property, clearly demonstrated a functional role of cebranopadol's NOP receptor activity. If the degree or direction of biased signaling at the NOP receptor has an influence on the interoceptive cue produced by an agonist, then Ro65-6570 may produce a cue different from that produced by the NOP receptor activity of cebranopadol, and this may explain the lack of generalization between the two. Clearly, more work is needed to elucidate the functional significance of biased signaling at the NOP receptor.

5.4 Physical Dependency

Tzschentke et al. (2017a) evaluated opioid-type physical dependence produced by cebranopadol in mice and rats. In a naloxone-precipitated withdrawal assay in mice, a regimen of seven escalating doses of cebranopadol over 2 days produced only very

limited physical dependence as evidenced by very little withdrawal symptoms (jumping) even at cebranopadol doses clearly exceeding the analgesic dose range. In contrast, mice showed clear withdrawal symptoms when treated with morphine within the analgesic dose range. In the rat, spontaneous withdrawal (by cessation of drug treatment; in terms of weight loss and behavioral score) was studied after 4-week subacute administration. Naloxone-precipitated withdrawal (in terms of weight loss and behavioral score) was studied in the same groups of rats after 1 week re-administration following the spontaneous withdrawal period. In both tests, cebranopadol-treated rats showed only few signs of withdrawal, while withdrawal effects in rats treated with morphine were clearly evident. The findings suggest a low potential of cebranopadol to produce opioid-type physical dependence in rodents.

These studies did not include mechanistic investigations. However, Tzschentke et al. (2017a) discuss the possibility that the limited potential of cebranopadol to produce physical dependence may be related to its NOP receptor agonistic activity. As mentioned above, NOP receptor agonists can reduce a number of typical (side-) effects associated with classical opioids. Although these findings do not bear direct relevance for physical dependence, they nevertheless show that cebranopadol behaves differently from pure MOP receptor agonists. Kotlińska et al. (2000) have shown that the NOP receptor agonist N/OFQ can inhibit the expression of naloxone-precipitated withdrawal after morphine treatment in the rat. On the other hand, administration of the NOP receptor agonist Ro 64-6198 during the dependence induction phase did not prevent the development of morphine dependence in mice (Kotlinska et al. 2003). The latter finding appears to be at odds with the findings of Tzschentke et al. (2017a). But as in the case of drug discrimination, biased signaling at the NOP receptor might help to resolve this issue. Cebranopadol has a strong NOP G-protein bias, whereas the early non-peptidergic NOP receptor agonists (such as Ro 64-6198 and Ro 65-6570) have no or only a weak bias for G-protein signaling (Chang et al. 2015a; Rizzi et al. 2016). Again, the functional relevance for biased signaling at the NOP receptor is currently not known, but it could be hypothesized that the lack of G-protein-biased signaling of the NOP receptor agonist might be responsible for the lack of effect of this compound on the development of morphine dependence. Regarding the mild effects in the spontaneous withdrawal situation, an additional possibility can be considered. Given the long duration of action of cebranopadol (>6 h after intravenous administration [Linz et al. 2014], >8 h after p.o. administration [Schunk et al. 2014]), the slow elimination of cebranopadol could mimic a tapering-like effect, thus reducing the occurrence of spontaneous withdrawal.

In a very recent study, Ruzza et al. (2018) have tested the hypothesis that the reduced physical dependence liability of cebranopadol is due to its NOP receptor agonistic activity by conducting a naloxone-precipitated withdrawal experiment in NOP receptor knockout mice. It was found that in wild-type mice the degree of naloxone-precipitated withdrawal did not differ between cebranopadol- and morphine-treated animals. However, in mice lacking the NOP receptor, naloxone precipitated a larger withdrawal in cebranopadol as compared to morphine-treated

animals. On the one hand, these findings show that the intrinsic NOP receptor activity reduces dependence development conveyed by its MOP receptor activity, analogous to the reduction of morphine-like discriminative stimulus properties described above (Tzschentke and Rutten 2018). On the other hand, the findings are at odds with those of Tzschentke et al. (2017a), who found very little naloxone-precipitated withdrawal in mice treated with cebranopadol compared to morphine-treated animals. This difference may be due to a number of methodological aspects, such as different mouse strains (CD-1 versus NMRI), different treatment schedules (nine doses over 5 days versus seven doses over 2 days), naloxone dose (10 versus 30 mg/kg), and observation period (30 versus 15 min). The most striking and probably most relevant difference, however, appears to be to the relative drug doses that were administered. Ruzza et al. (2018) based their dose selection on ED_{50} values from a previous mouse orofacial formalin test. Total doses that were administered in their withdrawal study were 255 mg/kg for morphine and 10.2 mg/kg for cebranopadol, i.e., there was a factor of approx. 25 between the two doses. In the study of Tzschentke et al. (2017a), total doses were 567 mg/kg for morphine and 0.453 mg/kg for cebranopadol, i.e., a factor of approx. 1,250. This means that in relative terms, animals received a 50-fold higher cebranopadol dose (relative to morphine) in the Ruzza et al. study as compared to the Tzschentke et al. study. The dose selection in the latter study was based on the equianalgesic potency ratio of morphine and cebranopadol in the mouse known to the authors at the time. In a recently published study (Schiene et al. 2018a), using a mouse colitis model of visceral pain, ED50 values were 0.8–1.0 mg/kg i.v. (depending on the readout) for morphine and 2.2–4.6 μg/kg i.v. for cebranopadol, resulting in a dose ratio of approx. 300. From a translational point of view, it is difficult to foresee which of these two outcomes is more predictive for the clinical situation. However, it is of interest to note that emerging clinical data appear to confirm the low potential of cebranopadol to produce physical dependence (Christoph et al. 2017; see below).

Taken together, these findings demonstrated that the mild withdrawal effects of cebranopadol are not limited to acute precipitated withdrawal in the mouse but extend to a subacute situation and to spontaneous withdrawal in the rat. First evidence from clinical trials with cebranopadol suggests that the low potential to produce physical dependence in rodents may translate to humans.

5.5 Tolerance Development

Linz et al. (2014) reported data on the development of analgesic tolerance to cebranopadol (anti-allodynic effect in a chronic constriction injury (CCI) model of mononeuropathic pain). Complete tolerance to cebranopadol had developed by day 26. For an (initially) equi-effective dose of morphine, complete tolerance had already developed by day 11. The morphine data were in accordance with a previous report on the development of tolerance to morphine (Tzschentke et al. 2007). Thus, tolerance to the antiallodynic effect of cebranopadol in the CCI model in the rat developed slowly and was significantly delayed compared to morphine. Although no

mechanistic study was done by Linz et al. (2014), it is tempting to speculate that the reduced rate of tolerance development seen for cebranopadol is due to its NOP component. The existing literature on the role of the NOP receptor in the development of morphine tolerance is conflicting. Lutfy et al. (2001) have shown that development of analgesic tolerance is reduced in rats if a NOP receptor agonist was co-administered with a selective MOP receptor agonist. On the other hand, a number of studies have reported reduced or absent morphine tolerance in animals lacking the NOP receptor or after administration of a NOP receptor antagonist (Ueda et al. 1997, 2000; Chung et al. 2006; Micheli et al. 2018). These studies differ widely in their methodologies (morphine regimen to induce tolerance, route of drug administration, pain model to assess tolerance, etc.) such that no definite conclusions about the role of the NOP receptor and its ligands in the development of morphine analgesic tolerance can be drawn at present.

6 Clinical Trials

The clinical pharmacokinetic (PK) properties of cebranopadol were described by Kleideiter et al. (2018). The analysis was based on noncompartmental methods in six phase I clinical trials in healthy subjects and patients and population PK analysis in two further phase I and six phase II clinical trials. After oral administration of the immediate-release formulation, cebranopadol showed a late time to reach maximum plasma concentration [C_{max}] (4–6 h), a long half-value duration (14–15 h), and a terminal phase half-life in the range of 62–96 h. After multiple once-daily dosing in patients, an operational half-life (the dosing interval resulting in an accumulation factor [AF] of 2) of 24 h was found to be the relevant factor to describe the multiple-dose PK of cebranopadol. The time to reach steady state was approximately 2 weeks, the AF was approximately 2, and peak-trough fluctuation was low (70–80%). Dose proportionality at steady state was shown for a broad range of cebranopadol doses (200–1,600 μg). A two-compartment disposition model with two lagged transition compartments and a first-order elimination process best described cebranopadol data in healthy subjects and patients after single- and multiple-dose administration. This PK profile suggests that cebranopadol is suitable for once-daily administration even without an extended release (ER) formulation. It reaches C_{max} only after 4–6 h, which may be a relevant factor contributing to a low abuse potential (see above and below). Furthermore, the abovementioned PK characteristics of cebranopadol were observed for a variety of different formulations (tablet, liquid-filled capsule, and oral solution), suggesting that the impact of tampering with the cebranopadol tablet formulation would be very limited. The apparent ER-like profile observed for cebranopadol from an IR formulation is considered to result from the physicochemical properties of cebranopadol, which is a poorly soluble Biopharmaceutics Classification System class 2 compound. Equilibrium solubility was determined to be 0.14, 1.23, 0.05, and <0.04 μg/mL at pH value 1.2, 4.8, 6.8, and 7.4, respectively (Grünenthal, unpublished data).

From the clinical phase II program of cebranopadol, three trials have been published in full and one further trial in abstract form.

Scholz et al. (2018) reported results of a phase IIa trial in postoperative pain. Patients who underwent primary bunionectomy were included in a randomized, multicenter, double-blind, double-dummy, placebo- and active-controlled, parallel group clinical trial. Cebranopadol at a single oral dose of 200, 400, or 600 μg was given and compared with 60 mg controlled release morphine and placebo. The primary efficacy endpoint was the sum of pain intensity assessed between 2 and 10 h after the first intake time point. While no difference between 200 μg cebranopadol and placebo was detected, 400 and 600 μg resulted in a reduction of postoperative pain which was more effective than placebo 2–22 h after first intake. Morphine as positive control was effective, although less as compared to cebranopadol when analyzing the primary endpoint. When assessing the global impression per subject, patients receiving cebranopadol 400 or 600 μg were more satisfied than patients receiving morphine. Notably, morphine efficacy was detected later than the efficacy of cebranopadol. In summary, doses of 400 and 600 μg cebranopadol induced more effective postoperative analgesia compared to the classical opioid morphine. Cebranopadol 400 and 600 μg ensured adequate 24-h pain relief while being safe, with 400 μg single-dose treatment being better tolerated than morphine. The relative frequency of patients with at least one treatment-emergent adverse event increased dose-dependently in those treated with cebranopadol but was highest in the group treated with morphine.

The efficacy of cebranopadol in chronic low back pain patients after a treatment period of 14 weeks was reported by Christoph et al. (2017). The trial was randomized, double-blind, and placebo- and active-controlled and evaluated analgesic efficacy, safety, and tolerability in patients with moderate to severe chronic low back pain. Patients with and without a component of neuropathic pain were assessed. Cebranopadol was administered once daily at assigned doses of 200, 400, or 600 μg. Tapentadol administered at 200 mg twice daily and placebo served as controls. Change from baseline pain to the weekly average 24-h pain during the entire 12 weeks and during week 12 of the maintenance phase was used as primary efficacy endpoint. Treatment with cebranopadol resulted in analgesic efficacy with statistically significant and clinically relevant improvements over placebo at all doses. Likewise, the positive control tapentadol showed efficacy. Analysis of the responder groups with $\geq$30% and $\geq$50% pain reduction confirmed these results. Sleep and functionality were also positively modulated by cebranopadol and tapentadol. Overall, positive effects of treatment with cebranopadol and tapentadol were observed, irrespective of the presence or absence of neuropathic pain components. The treatment with cebranopadol was safe, with higher doses leading to higher treatment discontinuations because of treatment-emergent adverse effect which occurred mostly during the titration phase of 14 days. An acceptable tolerability profile was described for patients who reached the target doses. During maintenance phase, the incidence rate of most frequently reported treatment-emergent adverse events was $\leq$10%.

The analgesic efficacy of cebranopadol compared with morphine prolonged release (PR) was examined by Eerdekens et al. (2018) in patients with moderate to severe cancer-related pain in a double-blind, parallel group, multiple-dose trial that was designed as a non-inferiority trial for efficacy of cebranopadol versus morphine PR. One hundred twenty-six patients were treated for up to 7 weeks. For the primary efficacy endpoint (average amount of daily rescue medication intake (morphine immediate release) over the last 2 weeks of treatment), non-inferiority of cebranopadol with and superiority over morphine PR was demonstrated. Cebranopadol also showed positive results on several additional efficacy endpoints. The vast majority of patients ($\geq$75% of either treatment) had clinically relevant pain reduction. Most frequently used doses were $\leq$800 μg cebranopadol or $\leq$120 mg morphine PR daily. A switch from previous opioid medication to cebranopadol was safe, generally well tolerated, and successful in terms of analgesia. A total of 83.1% of patients on cebranopadol and 82.0% on morphine PR experienced treatment-emergent adverse events. Taken together, this clinical trial showed that cebranopadol was effective, safe, and well tolerated in the dose range tested (200–1,000 μg) in patients suffering from chronic moderate to severe cancer-related pain and was superior to morphine PR on the primary endpoint.

A further trial on the efficacy, safety, and tolerability of cebranopadol in patients with pain due to diabetic peripheral neuropathy (DPN) was reported by Eerdekens et al. (2016). A randomized, multicenter, double-blind, double-dummy, placebo- and active-controlled, parallel group, multiple-dose, exploratory trial was conducted in patients with moderate to severe chronic pain due to DPN. Rapid titration (2 weeks) to the allocated dose was followed by 6 weeks of maintenance treatment. The primary endpoint was pain assessed on an 11-point numerical rating scale (NRS). Patients received placebo, pregabalin 300 mg BID, or cebranopadol 100 μg, 300 μg, or 600 μg QD. Mean (SD) baseline pain score was 6.83 (1.26) on the NRS. A clinically relevant difference of at least −0.7-point NRS compared to placebo on change from baseline was shown with all cebranopadol doses, with higher doses showing a larger difference. Cebranopadol 600 μg also significantly reduced pain according to MMRM analysis. All doses of cebranopadol were safe without systematic effects on ECG, vital signs, or laboratory parameters. 73.4%, 82.0%, and 85.5% of patients taking 100 μg, 300 μg, or 600 μg, respectively, experienced treatment-emergent adverse events, compared with 75.4% and 69.4% on pregabalin and placebo, respectively. The most common treatment-emergent adverse events across all cebranopadol groups were nausea, dizziness, vomiting, fatigue, and somnolence. In conclusion, in this trial, cebranopadol was effective, safe, and well tolerated in a population with pain due to DPN.

Dahan et al. (2017) performed a phase I pharmacokinetic-pharmacodynamic trial to quantify the effects of cebranopadol on respiration. Healthy male volunteers received a single dose of 600 μg cebranopadol orally. Ventilation at an elevated clamped end-tidal pressure of carbon dioxide, pain threshold to transcutaneous electrical stimulation, and plasma cebranopadol concentration were measured at regular time intervals for up to 11 h after drug administration. Cebranopadol produced typical opioid-like effects including miosis and analgesia. The blood-effect-site equilibration half-life for respiratory depression and analgesia was

1.2 ± 0.4 h and 8.1 ± 2.5 h, respectively. The effect-site concentration causing 50% respiratory depression was 62 ± 4 pg/mL; the effect-site concentration causing 25% increase in currents to obtain pain threshold was 97 ± 29 pg/mL. Thus, in terms of concentration-effect relationship, cebranopadol was relatively more potent to produce analgesia than respiratory depression. Under the current experimental conditions of a carbon dioxide clamp, this clinical trial modeled a ceiling of respiratory depression induced by cebranopadol at a minimum ventilation of 5 L/min. No such ceiling for respiratory depression is known for classic opioids, including fentanyl and morphine, which have a minimum ventilation value statistically indistinguishable from zero, meaning the complete absence of respiration (Dahan et al. 2005; Yassen et al. 2007). The relevance of this finding in healthy volunteers under experimental conditions for pain patient populations and at higher doses has to be further investigated.

6.1 Abuse Liability in Humans

The phase II trial of efficacy and safety of cebranopadol in patients with chronic low back pain (Christoph et al. 2017) included the assessment of physical dependence by means of the Clinical Opiate Withdrawal Scale (COWS). All tested doses of cebranopadol (200 μg, 400 μg, 600 μg once daily) produced significant analgesia. Following abrupt cessation of treatment at the end of the 14-week treatment period, only 4.6–6.5% of patients across the three assigned dose arms reported mild withdrawal symptoms. Moderate withdrawal symptoms were reported by one patient (0.9%) each in the 200 μg and 600 μg arms. In the placebo arm, a single case of moderately severe withdrawal was reported (0.9%). These findings are in line with the very weak withdrawal effects in the preclinical studies and suggest that even after long-term treatment with moderate to high doses of cebranopadol, a slow tapering off is not required.

Cebranopadol was further evaluated in a single-dose, nested-randomized, double-blind crossover trial in 42 non-dependent recreational opioid users, which assessed the abuse potential of single doses of cebranopadol relative to hydromorphone immediate release (IR) and placebo (Göhler et al. 2018). The trial consisted of a qualification phase and a seven-period treatment phase. Treatments were cebranopadol 200 μg, 400 μg, and 800 μg, hydromorphone 8 mg and 16 mg, and two placebos. Primary endpoint was the peak effect of drug liking at this moment, measured by visual analog scale (VAS). Secondary endpoints included VAS rating for good drug effects, high, bad drug effects, take drug again, drug similarity, and pupillometry. Cebranopadol 200 μg and 400 μg did not differentiate from placebo on the abuse potential assessments and generated smaller responses than hydromorphone. The magnitude of the responses observed with cebranopadol 800 μg was similar to hydromorphone 8 mg and smaller than hydromorphone 16 mg. The maximum effect for VAS drug liking at this moment was delayed compared to hydromorphone (3 h and 1.5 h, respectively). These results confirmed the hypothesis

that cebranopadol has lower abuse potential than the MOP receptor agonist hydromorphone.

There are three potential (and by no means mutually exclusive) explanations for why cebranopadol only produced a limited degree of liking in this trial. First, the long time to peak effect, consistent with a late maximum plasma concentration (t_{max} approx. 5 h), may be an important factor. It has been shown that a high speed of onset of action and an early t_{max} for plasma (and brain) concentrations is an important determinant for the reinforcing efficacy of a drug (Comer et al. 2009; Winger et al. 2002). From a behaviorist point of view, the long delay between action (drug taking) and effect would weaken the reinforcement derived from the consumption of the drug. Pharmacometric simulations showed that a higher dose of cebranopadol than used in this trial would be needed to reach the same maximum probability of having a drug liking (at this moment) VAS score higher than 60, as observed for hydromorphone IR 8 mg. Furthermore, this effect would be reached approx. 5 h later for cebranopadol than for hydromorphone IR (Piana et al. 2016). Second, Göhler et al. (2018) described that the highest dose of cebranopadol tested in the trial (800 μg) produced higher incidences of nausea and vomiting as well as negative effect measures than 8 mg hydromorphone. Thus, the increased experience of negative effects may have curbed the likability of high-dose cebranopadol. Third, the intrinsic potent NOP receptor agonistic activity of cebranopadol may limit its MOP receptor-mediated rewarding effects, as has been repeatedly shown in animal models of reward and reinforcement (Ciccocioppo et al. 2000; Sukhtankar et al. 2014; Murphy et al. 1999; Rutten et al. 2010, 2011).

7 Conclusions

Cebranopadol appears to bear out the expectations grounded in a large volume of preclinical, neurobiological, and pharmacological work on the beneficial interaction between MOP and NOP receptor activation. This benefit is twofold: an additive or even synergistic interaction with respect to analgesic efficacy of both components and a "protective" effect of NOP receptor activation with respect to typical opioid side effects, such as respiratory depression, and abuse and dependence liability. First clinical findings are promising regarding efficacy as well as tolerability. These findings now need to be extended and corroborated in further clinical trials. In light of the current opioid crises, a highly potent and efficacious novel opioid drug with an improved safety and dependence liability profile may be an important addition to the armamentarium for the management of severe and chronic pain.

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Therapeutic Approaches for NOP Receptor Antagonists in Neurobehavioral Disorders: Clinical Studies in Major Depressive Disorder and Alcohol Use Disorder with BTRX-246040 (LY2940094)

Jeffrey M. Witkin, Tanya L. Wallace, and William J. Martin

Contents

Abstract

Conventional antidepressants increase the efflux of biogenic amine neurotransmitters (the monoamine hypothesis of depression) in the central nervous system (CNS) and are the principle drugs used to treat major depressive disorder (MDD). However, the lack of efficacy in some patients, the slow onset of action, and the side effect profiles of existing antidepressants necessitate the exploration of additional treatment options. The discovery of the nociceptin/orphanin FQ peptide NOP receptor (N/OFQ-NOP receptor) system and its characterization in preclinical biological and pharmacological stress-related conditions supports the potential antidepressant and anti-stress properties of a NOP receptor antagonist for the treatment of neurobehavioral disorders. BTRX-246040 (formerly LY2940094) was designed to test this hypothesis in the clinic. A small clinical proof of concept study demonstrated efficacy of BTRX-246040 in MDD patients. In this study, BTRX-246040 (40 mg, p.o.) significantly reduced negative bias as assessed by the facial recognition test within 1 week of treatment and decreased

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depression symptoms after 8 weeks. BTRX-246040 also reduced depression symptoms in a second trial with heavy alcohol drinkers. Given the comorbidity of MDD and alcohol use disorder, a compound with such effects in patients could be a valuable addition to the medications available. A proof of concept study showed efficacy of BTRX-246040 in reducing heavy drinking and increasing the probability of abstinence in individuals diagnosed with alcohol dependence. In addition, plasma levels of gamma-glutamyl transferase were decreased by BTRX-246040 compared to placebo control implying improvement in liver function. Collectively, the clinical data reviewed within this chapter suggest that BTRX-264040 functions to normalize dysfunction in reward circuits. The overall efficacy and safety of this compound with a novel mechanism of action are encouraging of further clinical development. BTRX-246040 is currently under development for MDD by BlackThorn Therapeutics.

Keywords

Alcohol use disorder · Antidepressant · BTRX-246040 · LY2940094 · Major depressive disorder · N/OFQ · NOP receptor antagonist · Reward processing

Abbreviations

AUD	Alcohol use disorder
BTRX-246040 = LY2940094	[2-[4-[(2-chloro-4,4-difluoro-spiro[5Hthieno [2,3-c]pyran-7,4′-piperidine]-1′-yl)methyl]-3-methyl-pyrazol-1-yl]-3-pyridyl]methanol
CGI-I	Clinical Global Impression of Improvement
CGI-S	Clinical Global Impression of Illness Severity
GRID-HAMD-17	Grid format of the Hamilton Depression Rating Scale, 17 items
HADS	Hospital Anxiety and Depression Scale
HAMA	Hamilton Anxiety Rating Scale
LY2940094	BTRX-246040
MADRS	Montgomery-Asberg Depression Rating Scale
MDD	Major depressive disorder
MPS	Maier-Philipp subscale of the GRID-HAMD-17
N/OFQ	Nociceptin/orphanin FQ

1 From Hypotheses to Clinical Testing

The localization of the nociceptin/orphanin FQ peptide NOP receptor (N/OFQ-NOP receptor) system along with the activity of compounds in preclinical models served as an initial guide for clinical development of NOP receptor modulators. NOP

receptors are broadly expressed in cortical regions, including the prefrontal and cingulate cortices, as well in the hippocampus and striatum (Berthele et al. 2003). This expression pattern positions N/OFQ to interact with multiple neural circuits that regulate mood, learning, and motor control (Zaveri 2016; Witkin et al. 2014). Indeed, preclinical data derived primarily from rodent models consistently suggested that blockade of NOP receptors would yield antidepressant activity (see Gavioli and Calo' 2013; Witkin et al. 2014). Figure 1 provides a simplified summary of biological responses to nociception and to NOP receptor antagonists showing the dynamic interplay of the N/OFQ-NOP receptor system on stress and mood regulation (see Gavioli and Calo' 2013; Witkin et al. 2014 for further discussion).

By contrast, understanding of the N/OFQ-NOP receptor system within the context of ethanol consumption had been confounded by the use of a variety of distinct rodent-based models (e.g., genetically selected alcohol-preferring rats vs. heterogeneous rats with no alcohol preference), pharmacological interventions (e.g., agonists or antagonists), treatment paradigms (e.g., alcohol consumption vs. relapse prevention), and treatment durations (e.g., acute vs. chronic). Initially, NOP receptor agonists were shown to reduce ethanol-driven behaviors (see Ciccocioppo et al. 2003; Witkin et al. 2014). However, the finding that NOP receptors desensitize rapidly following exposure to agonists (Spampinato et al. 2007) suggested that actions attributed to "agonist" activity could reflect inactivation of the NOP receptor. The possibility that enhanced NOP function may increase vulnerability to developing alcohol abuse (Ulbaldi et al. 2016) and that selective

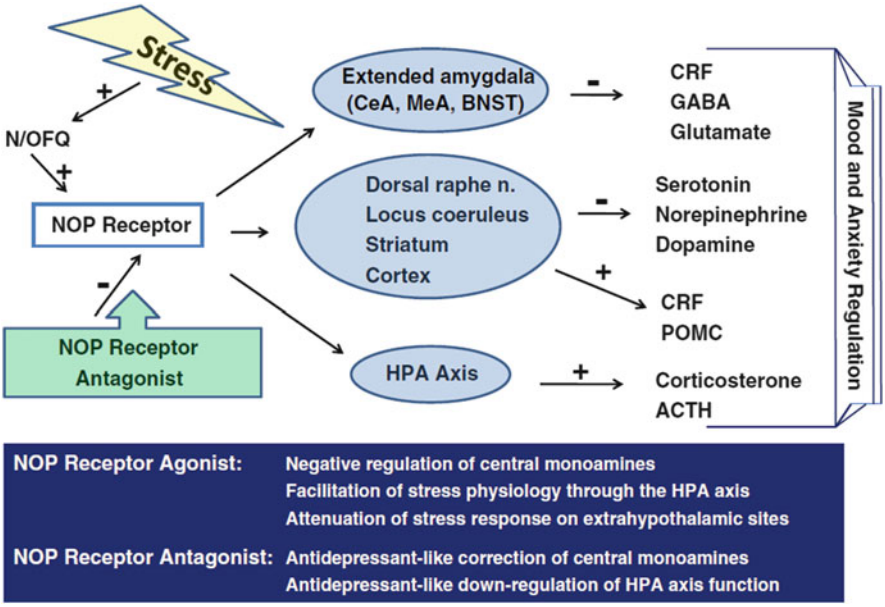

Fig. 1 Role of nociception pathways in stress-induced modulation of neurotransmission. From Witkin et al. (2014) with permission

NOP receptor antagonists produced anti-alcohol effects (Rorick-Kehn et al. 2016; Kallupi et al. 2017) created a unique rationale and opportunity to evaluate the clinical utility of NOP receptor antagonists in patients.

Disorders associated with depression or alcohol use (dependence or abuse) are among the most prevalent psychiatric conditions (Grant et al. 2004). Although each can develop independently of the other, the co-occurrence of major depressive disorder (MDD) and alcohol use disorder (AUD) is high (Cranford et al. 2011). Given the shared biological substrates for MDD and AUD and the supporting preclinical data on the N/OFQ-NOP receptor system, one challenge in developing a novel, first-in-class NOP receptor antagonist is the segmentation between the "learning" and "confirming" phases of clinical development (Sheiner 1997) as they relate to a new mechanism of action drug. One possibility to "confirm" the preclinical efficacy is enabled by studying the drug in homogeneous patient populations. A possibility to "learn" about the drug's mechanism can occur by studying it in heterogeneous clinical populations. Below, we review aspects of each approach as it relates to the initial and ongoing development of BTRX-246040.

2 Major Depressive Disorder

Major depressive disorder (MDD) is a debilitating neuropsychiatric disorder affecting millions of people worldwide (Chiu et al. 2018; de la Vega et al. 2018), that inflicts damage to individuals, families, society, and to our world economy (Jansen et al. 2018). For the most part, antidepressant drugs all function by the same putative mechanism limiting the diversity of medicinal options for MDD patients. Their primary mechanism of action is thought to be initiated through the increase in extracellular monoamines, the subsequent impact of these monoamines on neurotransmission, and the ultimate long-term impact of these mechanics on synaptic integrity in mood-regulating brain areas (Duman and Duman 2015). The primary monoamines that are hypothesized to be relevant to antidepressant action are 5-hydroxytryptamine (5-HT) or serotonin and norepinephrine (NE) (Iversen 2005).

The vast majority of all antidepressant drugs used worldwide have this basic mechanism of action. They are classified as to the protein they primarily impact to initiate the increase in CNS monoamine bioavailability. Antidepressants selectively block the reuptake of 5-HT (serotonin reuptake inhibitors or SRIs), NE (norepinephrine uptake inhibitors or NRIs), or both 5-HT and NE (dual-acting inhibitors or serotonin/norepinephrine reuptake inhibitors or SNRIs). Although these antidepressants have demonstrated efficacy, tolerability, and safety, antidepressant response (diminution of some symptoms) is produced generally in only one third of patients. Remission (full arrest of symptoms) generally is observed in another one third of patients. The remaining one third of MDD patients are treatment resistant (Rush et al. 2006). Furthermore, these antidepressants generally require weeks of daily dosing to achieve full-treatment response (Katz et al. 1996, 2004).

If augmentation of monoamine neurotransmission is not a panacea for MDD patients for the reasons outlined above, then it follows that the engagement of other

biological mechanisms is needed. This approach has been used to engineer multiple compounds with SRI, NRI, or SNRI mechanisms plus additional pharmacological properties that might enhance antidepressant response. Recently, for example, vortioxetine was introduced into clinical practice as such a multimodal antidepressant, the additional pharmacology of which is hypothesized to increase its ability to impact cognitive symptoms of depression which often go untreated by conventional antidepressants (Pan et al. 2017). In addition, alternative medicinal strategies to treat MDD exist that do not depend, at least initially, on monoamine augmentation as their primary mechanism of action and indeed have unique antidepressant properties (Witkin et al. 2018); however, to date, these mechanisms are not in general clinical use (e.g., ketamine, psilocybin, and other putative rapid-acting antidepressants). For other compounds with completely novel mechanisms of action, that is those that produce their biological effects primarily or initially through non-monoaminergic process, the list of alternative antidepressants drops to near zero.

One reason for the paucity of novel mechanism antidepressant drugs is the difficulty in generating mechanistic insight in human clinical patient populations. For the N/OFQ-NOP receptor system, three small clinical studies had previously shown elevated plasma levels of N/OFQ across different patient populations with depression (MDD, bipolar depression, and postpartum depression) and that peptide levels decreased after successful antidepressant treatment in one study (Gu et al. 2003; Wang et al. 2009; Zhang et al. 2009). Unfortunately, this clinical work comes from one research group and the manuscripts are published only in Chinese. Translation verified the statements made in the English language abstracts, but, to our knowledge, these results have not been replicated by another group. Based upon the existing data, a NOP receptor antagonist was conceived and synthesized that could be tested in MDD patients. BTRX-246040 (formerly LY2940094), a dihydrospiro(piperidine-4,7′-thieno[2,3-c]pyran) (Fig. 2), binds to hNOP receptors with a K_i of 0.10 nM and functionally blocks hNOP receptors with a K_b of 0.17 nM; no agonist activity at hNOP was observed at 10 μM (Toledo et al. 2014; Statnick et al. 2016). BTRX-246040 is selective for hNOP receptors over other opioid receptors (K_i at mu >451 nM, kappa >430 nM, delta >471 nM) (Toledo et al. 2014; Statnick et al. 2016). In contrast to monoamine-based antidepressants,

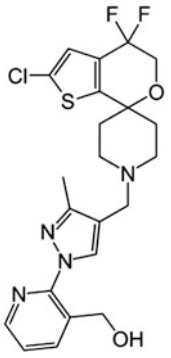

Fig. 2 Structure of the NOP receptor antagonist BTRX-246040 (LY2940094) in Phase 2 clinical development for the treatment of major depressive disorder

BTRX-246040 marginally increased cortical efflux of 5-HT in rats without significantly increasing cortical efflux of NE or dopamine (Post et al. 2016a). BTRX-246040 produced anti-stress and antidepressant-like activity in rodent models (Witkin et al. 2016; Post et al. 2016a). BTRX-246040 produced antidepressant-like effects in the forced swim test in rats (Post et al. 2016a) and mice and augmented this antidepressant-like effect of fluoxetine (Witkin et al. 2016). This effect was deleted in NOP−/− mice (Witkin et al. 2016). In contrast, anxiolytic-like effects were not observed in some animal models (conditioned suppression, four-plate test, novelty-suppressed feeding), but BTRX-246040 was active against fear-conditioned freezing, stress-induced increases in cerebellar cGMP, and stress-induced hyperthermia in rodents (Witkin et al. 2016). Importantly, BTRX-246040 was not disruptive of motor or cognitive performances in rodents (Witkin et al. 2016).

Preclinical data with BTRX-246040 and the demonstration of safety margins encouraged human research studies. BTRX-246040 was well tolerated and safe in human volunteers when given orally. Occupancy of NOP receptors was assessed using [^{11}C]NOP-1A as a tracer (Raddad et al. 2016). Brain occupancy of BTRX-246040 increased in prefrontal cortex, occipital cortex, putamen, and thalamus and increased as a function of plasma exposure reaching 90% at the highest plasma exposure levels (Raddad et al. 2016). Figure 3 shows the dose- and time-effects for the occupancy of BTRX-246040 in the prefrontal cortex of human volunteers. The values for the other brain areas measured were generally comparable. Thus, oral doses of BTRX-246040 in humans occupied up to a mean of 83% of brain NOP receptors when assessed at 2.5 h post dosing and a mean of 74% at 26.5 h post dosing with 40 mg. Given these values, it was expected that BTRX-246040 (40 mg) would achieve and maintain NOP receptor occupancy >80% upon a single oral dose. The tolerability and safety of BTRX-246040 and the demonstration that once-daily dosing of 40 mg achieves a sustainable and high level of NOP receptor occupancy provided dosing guidelines for clinical investigation of MDD patients.

Exploration of the potential antidepressant activity of BTRX-246040 in MDD patients was evaluated in a multicenter 8-week, double-blind, placebo-controlled

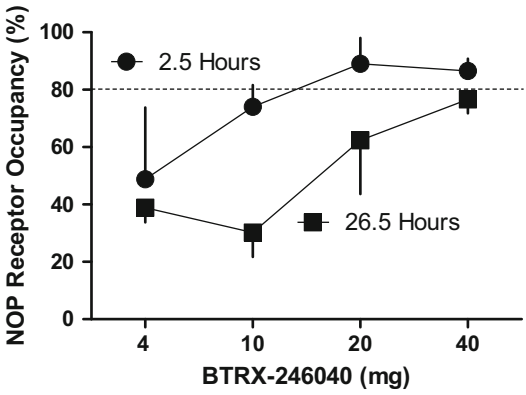

Fig. 3 Occupancy of NOP receptors in the prefrontal cortex by BTRX-246040 at 2.5 or 26.5 h after a single oral dose in healthy, human volunteers. Values are means ± S.E.M. $N = 2$ (20 mg), 4 (40 mg, 2.5 h), or 3 (all other data points). Data are plotted from those reported in Raddad et al. (2016)

trial (Post et al. 2016a). Male and female outpatients (18–65 years of age) were selected based upon meeting the following criteria for MDD: no psychotic features with total scores of >20 on the GRID-Hamilton Depression Rating Scale, >4 on the Clinical Global Impression of Severity, and >11 on the Hospital Anxiety and Depression Rating Scale depression subscale. Patients were not included in the study if they had any other prior or existing Axis I disorders, risk for suicide, or treatment-resistant depression (see Post et al. 2016a for complete criteria and patient demographics). The mean GRID-HAMD-17 total score was 25 which would categorize patients as having moderate to severe depression (Zimmerman et al. 2013). Selection and exclusion criteria resulted in a study design that consisted of 69 and 65 patients in the BTRX-246040 and placebo groups, respectively.

In addition to rating scales to assess antidepressant efficacy, this study included an emotional test battery to quantify emotional processing. Previous studies have shown that MDD patients interpret facial expressions with a negative bias and that antidepressant-induced modification of this emotional bias can precede changes in mood (Harmer et al. 2011). BTRX-246040 demonstrated a significant effect on the facial recognition test of the emotional test battery after 1 week of treatment. Accuracy in identifying positive faces was enhanced by BTRX-246040 with a probability of 92.4% compared to placebo-treated patients (Fig. 4). The least squares (LS) mean percentage accuracy of identifying positive faces in the BTRX-246040 group was 60.2% and was 56.9% in the placebo group. Further, the accuracy in discriminating positive from negative emotional faces was increased by BTRX-246040 with a probability of 88.6%. The onset of this effect was striking because it contrasted with the multiple weeks of dosing required to significantly impact MDD scores globally (Post et al. 2016a). Moreover, the reduction of negative emotional bias by BTRX-246040 was comparable to that observed with citalopram and reboxetine (Harmer et al. 2004; Tranter et al. 2009; Post et al. 2016a). Taken as a whole, the results with BTRX-246040 in the facial recognition test suggested that

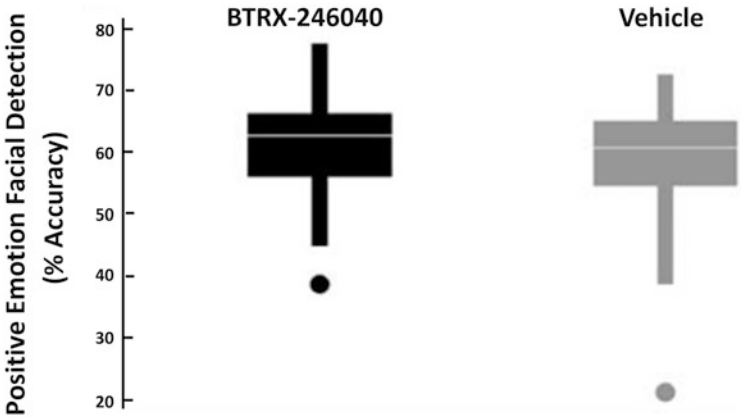

Fig. 4 Effects of BTRX-246040 (LY2940094) on a facial expression recognition task. Data are expressed as the least squares (LS) mean percentage accuracy for positive emotions at 7 days post daily oral dosing with 40 mg. Data are from Post et al. (2016a) with permission

antagonism of the NOP receptor could influence emotional processing in a manner consistent with monoamine-based antidepressants.

The effect of BTRX-246040 on depression symptoms was evaluated as change from baseline in the GRID-HAMD-17 total score. Depression symptoms improved to a greater extent in patients receiving drug than in patients given placebo (Fig. 5). The overall effects of BTRX-246040 were generally comparable to standard-of-care antidepressants as discussed below. The LS mean changes at week 8 on the GRID-HAMD-17 total scores were −11.4 and −9.8 for patients in the BTRX-246040 and placebo treatment groups, respectively. Statistical LS estimation that drug was superior to placebo was 82.9%. This probability was increased to 97.4% when the analysis included the follow-up at weeks 9–10, consistent with the plasma half-life of the compound (Raddad et al. 2016). Separate items analyzed from the GRID-HAMD-17 in the full analysis data set showed >90% probability of being better with drug on board including mood (99%), loss of appetite (98%), weight loss (90%), sexual interest (91%), and general somatic symptoms (91%). Consistent with these results, positive signals indicating an antidepressant response of BTRX-246040 versus placebo with >80% probability were observed on secondary endpoints including clinical impression measures (CGI-I, CGI-S) and the Maier-Philipp subscale (MPS) of the GRID-HAMD-17.

In contrast BTRX-246040 had a higher probability than placebo in disrupting sleep (increased insomnia). These findings are consistent with rat EEG data showing selective suppression by BTRX-246040 of NREM sleep (Post et al. 2016a), an effect distinct from that of conventional antidepressants that selectively suppress REM sleep.

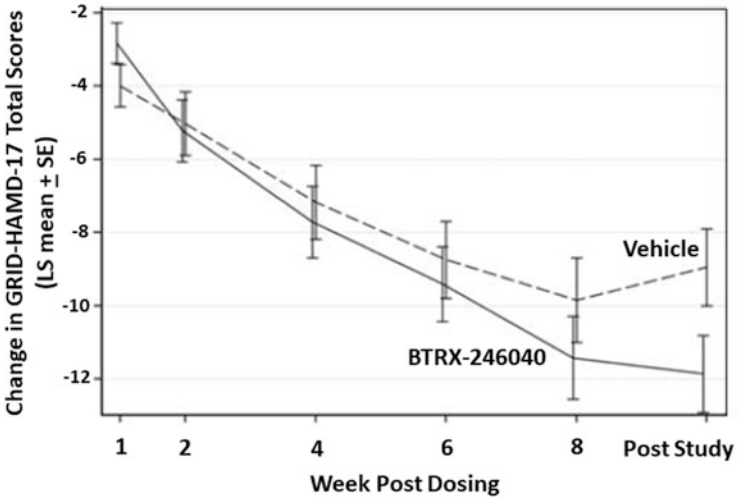

Fig. 5 Effects of BTRX-246040 (LY2940094) in MDD patients. Shown are changes from baseline in the total score from the GRID-Hamilton Depression Rating Scale, 17 items (GRID-HAMD-17) over weeks of daily dosing with 40 mg. Data are expressed as least-square (LS) mean change scores. Post study is from weeks 9–10. Data are from Post et al. (2016a) with permission

Conventional biogenic amine-based antidepressants have been shown to reduce anxiety in MDD patients and in generalized anxiety disorder (Gomez et al. 2018). BTRX-246040 did not show evidence of anxiolytic activity at week 4 as assessed by the HAMA or on the anxiety subscale items of the GRID-HAMD-17 or the patient-rated HADS anxiety subscale (Post et al. 2016a). However, this study was not specifically designed to evaluate antianxiety efficacy. Further, the anxiety levels of the MDD patients studied prior to dosing were low. Therefore, it is believed that a true test of an anxiolytic impact of BTRX-246040 in patients is required to make any firm conclusion on anxiolytic properties of the compound.

As observed in the acute treatment study of receptor occupancy in healthy human volunteers (Raddad et al. 2016), daily oral dosing of BTRX-246040 over 8 weeks was generally safe and well tolerated by MDD patients (Post et al. 2016a). Treatment-emergent adverse events were reported by both placebo (63.1%) and drug-treated patients (63.9%). Events with the greatest probability of occurrence were headache (23.2%), nausea (10.1%), insomnia (8.7%), upper respiratory tract infection (7.2%), diarrhea (7.2%), dizziness (7.2%), constipation (5.8%), and anxiety (5.8%). For most of the reported adverse events, they were considered mild to moderate in intensity and for the most part did not significantly differ from placebo. The only statistical differences between adverse event reporting in drug vs. placebo groups were observed with insomnia and dizziness (only reported by drug-treated patients). No clinically significant findings in laboratory assessments, vital signs, ECGs, or suicidality were observed during treatment with BTRX-246040. The clinical findings represent the first human evidence that the blockade of NOP receptors might have antidepressant properties, consistent with a large database of preclinical science (c.f., Witkin et al. 2014, 2016).

The use of different clinical trial instruments and study designs makes it difficult to compare the effects of BTRX-246040 in this MDD study (Post et al. 2016a) with those of conventional antidepressants (Jain et al. 2013; Jacobsen et al. 2015; Mathews et al. 2015). Comparing across studies that used the HAMD-17 suggests that the effect size of BTRX-246040 on mood scales in the MDD patients was generally comparable to effects demonstrated by SSRI antidepressants (Hieronymus et al. 2016). Since the multidimensional nature of the HAMD-17 can mask improvement on specific behavioral domains (Hieronymus et al. 2016), it is worth noting that the effects of BTRX-246040 on specific items of the MDD inventory were greater than on the total scores on the inventory. This finding highlighted the potential that BTRX-246040 could influence behavioral domains that are distinct from those modulated by SRI antidepressants.

3 Alcohol Use Disorder

Medicines to treat AUD are severely needed. AUD (DSM-5) is highly prevalent (29%) (Grant et al. 2015) and is associated with severe individual consequences both medical (including MDD) and otherwise and has a large impact on society and economy (Grant et al. 2004; Peacock et al. 2018). AUD also has a major impact upon

morbidity and mortality (Grant et al. 2015). Fortunately, there are some medicines approved for AUD. These include disulfiram (a deterrent treatment), acamprosate, and naloxone (reducing craving and relapse). Some medications are used for treatment off-label such as the opioid nalmefene and the antiepileptic topiramate (Soyka and Müller 2018). However, while generally safe, especially in non-liver compromised patients, definitive evidence for efficacy of these drugs is weak. Within the confines of the current literature, these drugs demonstrate only small to moderate effects on alcohol use and no significant impact on health outcomes (Palpacuer et al. 2018).

Preclinical data had shown that the NOP receptor antagonist BTRX-246040 reduced ethanol drinking in two lines of alcohol-preferring rats, attenuated responding maintained by alcohol, and reduced behavioral measures of motivation under the control of ethanol. Importantly, BTRX-246040 also blocked ethanol-seeking behaviors that had been suppressed by extinction and reinstated by stress. BTRX-246040 also attenuated cortical efflux of dopamine induced by ethanol injection (Rorick-Kehn et al. 2016). These preclinical studies were the first to suggest that NOP receptor antagonism might be beneficial in AUD and encouraged clinical investigation of this possibility.

The first clinical trial of a NOP receptor antagonist against alcohol-drinking was conducted using BTRX-246040 (Post et al. 2016b). In this study, data from 44 (drug) and 42 (placebo) subjects (males and females, aged 21–66 years old) were analyzed from a double-blind, placebo-controlled trial of 8 weeks duration where BTRX-246040 was dosed orally (capsules) at 40 mg/day as in the MDD study (Post et al. 2016a) described above. The individuals in the study were diagnosed with alcohol dependence and were additionally selected on the basis of exhibiting 3–6 heavy drinking days per week. BTRX-246040 did not significantly decrease the number of nondrinking days compared to placebo after 8 weeks of single daily doses. However, BTRX-246040 did produce a significant reduction in heavy drinking days and in the percent days abstinent (Table 1). Although complete abstinence is a goal in drug and alcohol abuse therapeutics, reduction in heavy drinking has significant health benefits and is considered by the US FDA to be an acceptable goal in AUD drug therapy (USDHHS 2015).

BTRX-246040 also decreased the plasma levels of gamma-glutamyl transferase with significant decreases from placebo beginning at week 4. The adverse events reported with BTRX-246040 dosing were insomnia, vomiting, and anxiety, but no

Table 1 Effects of BTRX-246040 on alcohol drinking in alcohol-dependent patients

Measure	Mean drug vs. placebo difference (95% CL)	Probability of drug/placebo difference <0 as percent
Mean drinks/day – raw	0.07 (−0.93 to 1.08)	44.3
Mean drinks/day – percent	−5.8 (−22 to 10)	76.0
Percent heavy drinking days	−8.8 (−21 to 3.2)	92.7
Percent abstinent days	7.1 (−3.4 to 17.6)	91.0

Data are from Post et al. (2016b)

serious adverse events or significant changes in laboratory chemistries or vital signs were reported. The decrease in a liver enzyme marker, gamma-glutamyl transferase, is significant as it suggests a potential biomarker for future investigations of BTRX-246040 in AUD studies. Importantly, these data also suggest that BTRX-246040 might be valuable for AUD patients in helping to protect liver function, an effect that is associated with a reduction in alcohol consumption.

Secondary endpoints of anxiety and depression were also evaluated in this study. BTRX-246040 did not significantly alter anxiety symptoms suggesting that decreases in anxiety were not causal in driving the suppression of alcohol consumption. However, despite the explicit exclusion of MDD from the patient subject pool, BTRX-246040 decreased HADS depression subscale scores compared to placebo ($p = 88.9\%$). These data in alcohol use disorder patients thus provide a systematic replication and an extension of the generality of an antidepressant response to BTRX-246040. Furthermore, since MDD diagnosis patients were excluded from this study, the impact of BTRX-246040 on MDD symptoms was also not a likely cause of its effects on alcohol drinking.

Overall, the effects observed in alcohol dependent subjects with BTRX-246040 are promising for further investigation of this NOP receptor mechanism. Significant decreases in the number of heavy drinking days, increases in the number of days abstinent from drinking, and the corresponding decreases in plasma levels of gamma-glutamyl transferase converge to suggest potential health benefit in AUD. Longer duration clinical studies with larger groups of patients seems warranted. This conclusion is supported by the tolerability and safety exhibited by BTRX-246040 along with the great medical need for improved medicines for this highly prevalent disorder that produces large health, societal, and economic consequences.

4 Dimensional Psychiatry: Reward Processing and Ongoing Clinical Development

Each of the investigations of BTRX-246040 in MDD and AUD highlight the need for drugs with new mechanisms to address unmet clinical needs, and the potential to alter mood and behavior in patient populations by selectively blocking NOP receptors. The historical approach to psychiatric drug development focused on isolating patients by their diagnosis and pursuing approval for a categorical indication. Shifts toward dimensional psychiatry wherein one focuses on identifying core mechanisms of neurobehavioral disorders across diagnostic boundaries presented a unique clinical development opportunity for BTRX-246040. Could the potential confound of comorbidity of alcohol and depression be turned into an advantage and advance in understanding by focusing on common underlying neurobiological mechanisms? Dysregulation of reward systems transcends diagnostic categories and is clearly a recognized problem within the therapeutic domains of depression (Knowland and Lim 2018; Lambert et al. 2018) and alcohol dependence (You et al. 2018). N/OFQ has been implicated as a key biological mediator of this reward pathway pathophysiology for depression (Der-Avakian et al. 2017; Vitale et al.

2017) and for alcohol dependence (Koob 2015; Witkin et al. 2014). Figure 6 illustrates key neural pathways involved in the evaluation of reward value and the control of behavior by reinforcing stimuli like ethanol. Comparable and overlapping neural circuits have been identified for depression (Price and Drevets 2012; see also Fig. 1). These pathways are intercalated with opiate receptor systems and the hypothalamic-pituitary axis. NOP receptors are integrated into the neural network and have been shown to be critical modulators of reward and mood (see Witkin et al. 2014 for a more detailed overview). Importantly, reward dysfunction can be objectively measured behaviorally and/or through measurement of changes in brain network activity (Hägele et al. 2015) thus enabling the integration of this concept into clinical trial designs.

BlackThorn Therapeutics is currently conducting another double-blind, placebo-controlled Phase 2a study with BTRX-246040 in MDD patients. The study uses a multicenter design to evaluate the efficacy, safety, and tolerability of BTRX-246040 administered orally once daily at up to 80 mg for 8 weeks, with the Montgomery-Asberg Depression Rating Scale (MADRS) change in total score between BTRX-246040 and placebo as the primary endpoint (clinicaltrials.gov identifier: NCT03193398). The study aims to identify objectively defined subtypes of patients

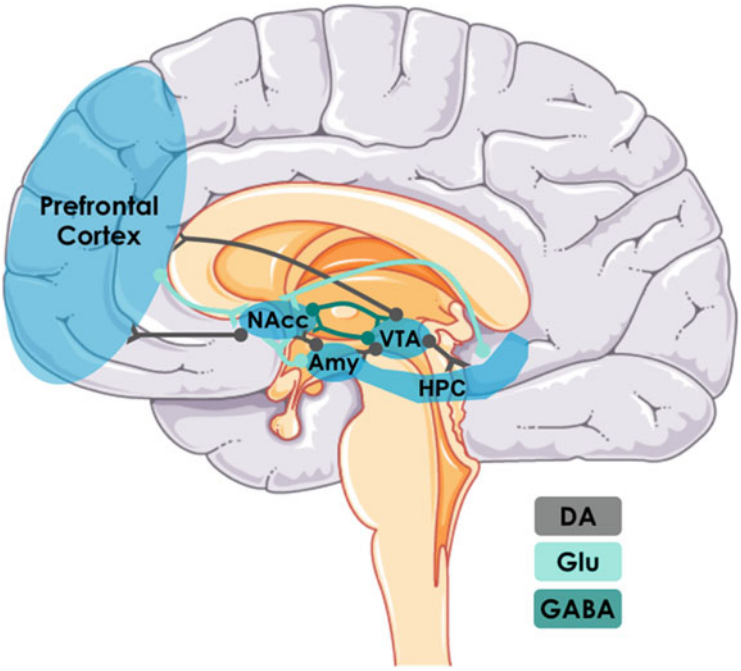

Fig. 6 A simplified overview of key neural pathways involved in the evaluation of reward value, mood, and the control of behavior by reinforcing stimuli like ethanol. Neural circuitry involved in alcohol addiction and the involvement of NOP receptors. *Amy* amygdala, *DA* dopamine, *Glu* glutamate, *HPC* hippocampus, *NAcc* nucleus accumbens, *VTA* ventral tegmental area

with MDD to determine which subtype may be most responsive to NOP receptor antagonism (Madrid et al. 2017). This study carries forward elements from the MDD study conducted by Post and colleagues (Post et al. 2016a; e.g., HADS and the emotional test battery) and adds clinical scales and quantitative behavioral assessments that measure anhedonia. Anhedonia is generally not well controlled by antidepressant treatment (Argyropoulos and Nutt 2013). Thus, this follow-on study presents an opportunity to evaluate BTRX-246040 against anhedonia in patients with MDD.

5 Conclusions

BTRX-246040 (formerly LY2940094), an orally bioavailable antagonist selective for NOP receptors (Toledo et al. 2014), has the potential to be a first-in-class treatment for neurobehavioral disorders. In three clinical studies, BTRX-246040 was safe and well tolerated (healthy volunteers, MDD patients, and AUD patients) (Raddad et al. 2016; Post et al. 2016a, b). BTRX-246040 decreased depression symptoms in patients with MDD as well as in the AUD study which was not explicitly designed to evaluate depression. BTRX-246040 occupies human brain NOP receptors at 80% for ~24 h post a single oral dose (40 mg) (Raddad et al. 2016). In one clinical trial with MDD patients, BTRX-246040 produced antidepressant efficacy after 8 weeks after a single daily oral dose (Post et al. 2016a). In the same study, antidepressant-like emotional face recognition bias was altered within 1 week. A second clinical study in heavy drinkers found significant decreases in heavy drinking and increases in abstinence periods (Post et al. 2016b). The need for medicines for AUD is clear and pressing (Palpacuer et al. 2018; Peacock et al. 2018). The comorbidity of alcohol use and depression is high (Beaulieu et al. 2012), thus making the conjunction of these two findings particularly intriguing. Given the tolerability and apparent safety of BTRX-246040, further development of this molecule is ongoing (BlackThorn Therapeutics). The additional pharmacology of NOP receptor antagonism demonstrated with BTRX-246040 includes decreases in excessive eating and body weight in rodents that suggests potential in the treatment of binge-eating disorder (Statnick et al. 2016). Additionally, given the comorbidity of obesity, overeating, and metabolic syndrome with MDD (Repousi et al. 2018) and the liability of some antidepressants to engender weight increases (Carvalho et al. 2016), BTRX-246040 has another potential advantage as an antidepressant should these findings translate to humans. In addition, preclinical data suggest that antagonism of NOP receptors would facilitate cognitive function (c.f., Kuzmin et al. 2009; Rekik et al. 2017). Given the failure of antidepressants to generally help with this depression symptom (Pan et al. 2017), BTRX-246040 might provide added efficacy along this MDD symptom dimension. Ongoing preclinical and clinical assessments of BTRX-246040 will provide insights into the underlying biology and potential therapeutic utility of NOP receptor antagonism.

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JMW dedicates this review to the memory of creative and energetic chemist, devoted humanitarian, and friend Conception Pedregal, who led the chemistry effort to discover BTRX-246040.

Conflict of Interest WJM and TLW are employees of and shareholders in BlackThorn Therapeutics which is actively evaluating BTRX-246040 for the treatment of neurobehavioral disorders.

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Correction to: Electrophysiological Actions of N/OFQ

Bryony L. Winters, Macdonald J. Christie, and Christopher W. Vaughan

Correction to:
Chapter "Electrophysiological Actions of N/OFQ" in:
B. L. Winters et al., Handbook of Experimental Pharmacology,
https://doi.org/10.1007/164_2019_205

In the last paragraph of Sect. 2.1.2 on line 3 the word 'off-cells' is misspelt. It should be 'on-cells'. The sentence "On the other hand, secondary neurons, which are presumed off-cells (although see Cleary et al. 2008), are insensitive to κ-opioids, but μ-opioids induce a K^+ conductance (Pan et al. 1990; Vaughan et al. 2001)." should read as "On the other hand, secondary neurons, which are presumed on-cells (although see Cleary et al. 2008), are insensitive to κ-opioids, but μ-opioids induce a K^+ conductance (Pan et al. 1990; Vaughan et al. 2001)."

The original chapter was corrected.

The updated online version of this chapter can be found at https://doi.org/10.1007/164_2019_205

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