The ORL₁ Receptor: Molecular Pharmacology and Signalling Mechanisms

David C. New Yung H. Wong

Department of Biochemistry, Molecular Neuroscience Center, and Biotechnology Research Institute, Hong Kong University of Science and Technology, Hong Kong, China

Key Words

ORL₁ · Nociceptin · Pharmacology, ORL₁ receptor · Receptor structure · G proteins · Signalling pathways

Abstract

The cloning of the opioid-receptor-like 1 (ORL₁) receptor and the identification of nociceptin as its endogenous agonist have revealed a new G-protein-coupled receptor signalling system. The structural and functional homology of ORL₁ to the opioid receptor systems has posed a number of challenges in understanding the often competing physiological responses elicited by these G-protein-coupled receptors. Thus, this review will attempt to summarize recent research by many groups that has revealed numerous subtleties of the ORL₁ receptor and its signalling pathways, as well as document the efforts to produce high-affinity selective ligands for the ORL₁ receptor that may be of value as research and therapeutic tools.

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Introduction

Following the isolation of DNAs encoding the δ -, κ -, and μ -opioid receptors, low stringency homology based screening of cDNA libraries identified a receptor that

appeared to be a fourth member of this receptor family [1–9]. The human, rat, and mouse clones had greater than 90% sequence identity with each other and over 60% homology with the three previously cloned opioid receptors. This similarity increases up to 80% within some of the transmembrane regions, suggesting that this receptor belongs to the opioid receptor family of G-protein-coupled receptors (GPCRs). Initially each group gave their independently isolated clones a different name, but the term ORL₁ (opioid-receptor-like 1) has become widely accepted. However, the International Union of Pharmacology has proposed that opioid receptors be termed OP₁, OP₂, OP₃, and OP₄, with the ORL₁ receptor designated as OP₄ [10, 11]. More recently, the terms DOP-R, KOP-R, MOP-R, and NOP-R have also been proposed, but this new nomenclature has yet to gain common currency, and the four opioid receptors are more often referred to as δ -, κ -, μ -, and ORL₁.

Early ligand-binding studies found very low levels of binding of all known opioid agonists and antagonists to the ORL₁ receptor [4, 9], and so it was considered to be an orphan. Two groups quickly identified the endogenous peptide for the ORL₁ receptor from rat brain and porcine hypothalamus [12, 13]. The new ligand was determined to be a heptadecapeptide closely related to the opioid dynorphin A. The peptide, originally termed nociceptin or orphanin FQ (OFQ), bound to the ORL₁ receptor in a saturable manner with a high affinity, but did not compete

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Dr. Y.H. Wong
Department of Biochemistry
Hong Kong University of Science and Technology
Clear Water Bay/Hong Kong (China)
Tel. +852 2358 7328, Fax +852 2358 1552, E-Mail boyung@ust.hk

Fig. 1. Serpentine representation of the primary sequence of the human ORL₁ receptor. The putative sites of glycosylation (◆), cysteine bridge formation (S-S), phosphorylation (P), and C-terminal tail acylation are indicated. Amino acid residues that have been directly implicated in ligand binding and signal transduction are also marked.

for binding to δ -, κ -, or μ -opioid receptors. Both groups also confirmed that receptor activation causes potent inhibition of the adenylyl cyclase activity.

In vivo use of this newly isolated peptide revealed that, in contrast to the analgesic effects of opioid receptor ligands, hyperalgesic effects were observed. Further detailed studies have revealed that nociceptin acts as a hyperalgesic or analgesic either acting alone or by modulating the effects of endogenous opioid compounds and other analgesics. The observed effect of nociceptin varies, depending on the method of application, concentration of peptide administered, the length of administration, and the method of determining the effect [for a review see 14]. Regardless of the contradictory results that have been obtained, it is clear that nociceptin plays a significant role in a range of physiological processes, including a number of phenomena related to pain perception.

A considerable body of work has also been accumulated describing the tissue distribution of nociceptin and the ORL₁ receptor. The ORL₁ receptor is widely expressed in the nervous system as well as in peripheral

organs and the immune system. Of particular interest is the wide variety of brain structures that express the receptor and the diversity of processes with which these areas are associated. These include pain and sensory perception, hormonal regulation, stress, memory, and the modulation of autonomic functions [for reviews see 15, 16]. Such a wide pattern of expression explains why the observed effect of administration of nociceptin varies so greatly. This review will outline the recent efforts that have led to our understanding of the receptor-ligand interactions and the signalling mechanisms that define the ORL₁ receptor system.

Receptor Structure

The 370 amino acid protein consists of seven hydrophobic regions of 23–25 amino acids, each linked with intra- and extracellular regions of varying length. In these loop regions, there are a number of readily identifiable features (fig. 1). There are three potential asparagine-linked glycosylation sites in the extracellular N-terminal region (Asn²¹, Asn²⁸, and Asn³⁹), conserved cysteine residues in extracellular loops (EL) 2 and 3 (Cys¹²³ and Cys²⁰⁰), and a potential acylation site in the intracellular C-terminal region (Cys³²⁴). A number of putative serine and threonine phosphorylation sites are present in the C-terminal region and the intracellular loop (IL) regions.

The highest regions of homology with the opioid receptors are within the transmembrane (TM) domains and intracellular loops. When those residues that are retained in the ORL₁ receptor and all three opioid receptors are considered, the conservation in TMs 2, 3, and 7 and in ILs 1 and 3 is over 70%. If conservative substitutions and conservation between the ORL₁ receptor and individual members of the opioid receptor family are considered, the level of homology is even greater. In contrast, in TM-4 only six amino acids are conserved across all four receptors, and EL-2 and EL-3 have two and one amino acids conserved, respectively. Both the N-terminal and C-terminal regions are also bereft of extensive regions of homology.

These dramatic contrasts of regions of high and low homology may provide a structural explanation for the apparent differences in ORL₁ and opioid receptor pharmacology and the physiological responses that they induce. In this regard a number of fascinating studies provide considerable insight.

Analysis of Receptor-Ligand-Binding Sites

Opioid peptides have a common motif at their N-terminus (Tyr-Gly-Gly-Phe), and the tyrosine residue is essential for binding to opioid receptors [17, 18]. The N-terminal sequence of nociceptin (Phe-Gly-Gly-Phe) differs from this common opioid sequence by the absence of a hydroxyl group. Changing the Phe¹ of nociceptin to a Tyr residue creates a ligand (Tyr¹-OFQ) that retains its ability to bind and activate the ORL₁ receptor [19]. Tyr¹-OFQ also binds to κ -opioid receptors with increased affinity ($K_i = 3.3$ nM) [20], but only acts as a very weak partial agonist with an EC₅₀ value > 100 μ M.

Further work revealed that residues 10–15 of nociceptin are critical for its activation of the ORL₁ receptor, but also provide it with its selectivity. This was shown by exchanging these residues for the cognate residues of dynorphin A. This new peptide lost activity at the ORL₁ receptor, but was able to activate δ - and κ -opioid receptors [20]. This indicates that despite the high degree of conservation between ORL₁ and opioid receptors, a common 'opioid-binding pocket' may not be present in the ORL₁ receptor and that determinants of ligand binding and activation in ORL₁ and opioid receptors may not be at topologically equivalent sites. This notion was reinforced when ORL₁ receptor mutants were created and tested for their ligand-binding ability. Mutation of single or multiple nonconserved residues in TMs 5–7 of the ORL₁ receptor to the residues conserved in opioid receptors (fig. 1) resulted in a receptor that could bind both nociceptin and dynorphin A with high affinity [21].

By taking advantage of the ligand selectivity of ORL₁ and κ -opioid receptors, a series of ORL₁/ κ -opioid receptor chimeras was constructed to determine which regions of the ORL₁ receptor are required for ligand binding and activation. Eleven chimeras were constructed and analyzed for their ability to bind nociceptin or dynorphin A [22]. It is clear from comparisons of the binding affinities of the two ligands for these chimeras that the regions of the receptors required for high-affinity binding do not overlap. Introduction of the top half of TM-2, EL-1, and the top half of TM-3 into the κ -opioid receptor produces a chimera that binds both nociceptin and dynorphin A with good affinity. Further addition of TM-1/IL-1 or EL-2 to this chimera results in subnanomolar binding affinities for both ligands [22]. In contrast, only those mutants with κ -opioid TMs 5–7 bound dynorphin A, confirming previous observations that point mutants of the ORL₁ receptor in these domains enabled both ligands to bind the receptor [21].

When the same chimeras were tested for their ability to inhibit forskolin-stimulated cAMP accumulation follow-

ing challenge with nociceptin and dynorphin A, it became apparent that regions beyond those required for ligand binding are necessary for ORL₁ function. The N-terminal region and EL-2 of ORL₁ are required for potent activation by nociceptin, but for the potencies of the chimeric receptors to equal those of wild-type ORL₁, TM-1 and IL-1 must also be included [22]. However, residues within other regions of the receptor have also been implicated in functional activation of the receptor. A single point mutation at Gln²⁸⁶ in TM-6 (Q286A; fig. 1) resulted in a receptor that bound nociceptin with wild-type affinities, but was incapable of inhibiting forskolin-stimulated cAMP synthesis [23]. In all of the ORL₁/ κ -opioid chimeras tested that were able to signal in response to nociceptin, TM-6 was derived from the κ -opioid receptor which does not share this Gln residue. This would suggest that certain regions of the two receptors are interchangeable, but the selectivity of the receptors is determined at distinct sites.

Topham et al. [24] have used computer simulations to model the interactions between the ORL₁ receptor and nociceptin. They predicted that the N-terminal FGGF tetrapeptide binds in a conserved region formed by two distinct hydrophobic cavities bound by TMs 3, 5, 6, and 7. The postulated binding of the N-terminal tetrapeptide of nociceptin within a region of the TM core structure that is highly conserved across all the opioid receptors explains why Tyr¹-OFQ binds to both ORL₁ and κ -opioid receptors.

Since the publication of this computer simulation, several lines of evidence have emerged that support the identification of these TM cavities as important binding sites for nociceptin. Amino acid residues Asp¹³⁰, Tyr¹³¹, Phe²²⁰, Phe²²⁴, and Trp²⁷⁶ (fig. 1) are all predicted to lie within 5 Å of the N-terminal tetrapeptide of nociceptin. Mutation of any one of these residues has been observed to affect the ability of nociceptin to bind to and activate the ORL₁ receptor [23; unpubl. observations from this laboratory]. In addition, we have recently produced a constitutively active mutant (CAM) of the ORL₁ receptor by replacing Asn¹³³ with a tryptophan residue (fig. 1). Mutant N133W exhibited increases in the basal inhibition of forskolin-stimulated activity, and the basal activity of signalling through G α_{14} and G α_{16} subunits increased by two- and threefold, respectively [manuscript in preparation]. A mechanistic model has been proposed to explain the creation of CAM receptors by mutation of this conserved Asn residue in TM-3. Molecular modelling of the angiotensin II type 1 (AT₁) receptor [25] predicted that in the nonactivated receptor this Asn residue (Asn¹¹¹ in AT₁) interacts with Tyr²⁹² in TM-7 (Tyr³¹⁹ in ORL₁). Occupation by the

agonist was postulated to disrupt this interaction and to result in the interaction of Tyr²⁹² with Asp⁷⁴ in TM-2 (conserved as Asp⁹⁷ in ORL₁). Disruption of this stabilizing interaction between TM-3 and TM-7 by mutation of the Asn residue to Ala in both AT_{1A} and bradykinin B₂ receptors [26, 27] resulted in CAMs. We postulate that binding of nociceptin in the TM cavities of the ORL₁ receptor causes disruption of similar stabilizing interactions, promoting the formation of an active form of the receptor.

Access to the ligand-binding site is regulated by EL-2 which caps the cavities formed by the TM helices. The computer simulation details a number of receptor-ligand contacts that provide an explanation as to why ORL₁ and opioid receptors show such high selectivity for ligands. Chimeric ORL₁/κ-opioid receptors identified EL-2 as a key region required for the activation of the receptor. The model places this highly negatively charged loop in close proximity to the highly positively charged region of nociceptin (between residues 8 and 13) and predicts that four positively charged residues of nociceptin form ion pairs with this acidic loop region. Experimental evidence has confirmed that charged side chains at Arg⁸ and Arg¹² of nociceptin are critical for binding and activity [28, 19]. Furthermore, introducing an Arg residue into position 8 of dynorphin A allows it to activate the ORL₁ receptor [20]. The model also describes a putative interaction between Thr⁵ of nociceptin and Gln²⁸⁶ of the ORL₁ receptor which has been shown to be essential, as mutant Q286A is inactive [23].

In a striking example of how selectivity between nociceptin and dynorphin A is achieved, the model predicts that Gly⁶ of nociceptin is positioned within a hydrophobic cleft between TM-4 (Gly¹⁸²-Gly¹⁸⁹) and TM-5 (Gly²¹²-Ile²¹⁹) regions. Position 6 of dynorphin A is occupied by a charged Arg residue which would be unable to form stable contacts within this region. Finally, irreversible cross-linking of the ORL₁ receptor using a derivative of nociceptin with a photolabile group introduced at position 10 identified the region of interaction as occurring in a section spanning the interface of EL-3 and TM-7 [29]. Mapping of residue 10 of nociceptin to this position is entirely consistent with the computer-simulated model.

Pharmacological Properties

Due to the similarities of the ORL₁ receptor sequence and those of opioid receptors, several of the groups that cloned ORL₁ attempted to use opioid ligands to pharmacologically characterize the new receptor. It was quickly

realized that the ORL₁ receptor had pharmacological characteristics distinct from those of the opioid receptors as up to 10 nM of radiolabelled diprenorphine, etorphine, or β-endorphin failed to bind to CHO or COS-7 cells expressing the receptor [4].

Following the isolation of nociceptin and the production of radiolabelled analogues, competition binding studies confirmed the selectivity of the ORL₁ receptor for its endogenous ligand. Pan et al. [30] used the iodinated analogue of nociceptin, [¹²⁵I]Tyr¹⁴-OFQ/N, to show that the opioid ligands DPDPE, U50,488, morphine, and diprenorphine all displaced nociceptin with K_i values of approximately 1 μM. In contrast, nociceptin was able to displace its iodinated derivative with K_i values <0.1 nM. Nociceptin also displayed very low potency at δ-, κ-, or μ-opioid receptors and was unable to inhibit the adenylyl cyclase activity in cells expressing these receptors [12]. These and subsequent studies have shown that of all the opioid peptides tested, dynorphin A has the highest affinity (IC₅₀ = 76 nM) [31] for ORL₁. Even though 6 of the 17 amino acids that constitute nociceptin and dynorphin A are conserved, the difference in affinities measured for the two peptides is several hundredfold and underlines the point that the ORL₁ receptor is pharmacologically distinct from the opioid receptors.

Studies by numerous authors using radiolabelled ORL₁ receptor ligands have identified high-affinity binding sites in brain tissues from various species [for a review see 32]. The majority of these studies identify a single high-affinity saturable binding site, although a number of exceptions have occurred. Saturation-binding studies in the mouse brain using [¹²⁵I]Tyr¹⁴-OFQ/N as the tracer ligand produced nonlinear Scatchard plots from which two binding sites, with K_d values of 4 pM and 0.9 nM, were identified [31]. Two sites were also observed in CHO and HEK-293 cells transfected with the ORL₁ receptor [33, 34]. It has been suggested that the existence of two binding sites, with high but distinct affinities for nociceptin, provides evidence for multiple forms of the ORL₁ receptor [31]. However, the preponderance of evidence suggests that tissues of the brain and transfected cells express a single binding site. Consideration should also be given to the possibility that the measurement of two binding sites represents binding to alternatively spliced forms of the ORL₁ receptor or perhaps to G-protein-coupled and G-protein-uncoupled states. The contribution of these two complicating factors has yet to be fully accounted for in vivo, but in cells transfected with the ORL₁ receptor the affinity of radiolabelled nociceptin and analogues has been shown to be sensitive to the presence of

Na²⁺ ions and nonhydrolyzable analogues of GTP [34, 35]. This indicates functional interaction between the ORL₁ receptor and heterotrimeric G proteins.

Two recent studies and our own observations sound a note of caution for the analysis of binding data using nociceptin or an iodinated derivative as a tracer ligand. It is apparent that nociceptin exhibits high-affinity binding to the filters often used to separate bound and free ligands following radioligand-binding assays. This occurs even when filters are pretreated with polyethyleneimine to prevent nonspecific binding. Dooley and Houghten [32] have observed affinities for filters of between 10 and 100 nM, and we have measured IC₅₀ values in the low nanomolar range. The addition of bovine serum albumin to the binding assay buffer effectively eliminates the problem; however, its use in published studies varies and may provide a partial explanation as to why there is such variability in reported affinities. Values have been reported that range from the low picomolar to a greater than nanomolar range [32]. A further level of uncertainty has been introduced by the discovery that tritiated nociceptin is degraded during storage and that its integrity is significantly compromised during incubation with cell membranes [36]. Incubation of tritiated nociceptin with COS-7 cell membranes for 15 min resulted in 85% degradation of the ligand. The degradation products identified have all been reported to have low affinities for the ORL₁ receptor [28], but the potential depletion of the tracer ligand to such an extent could lead to erroneous interpretation of equilibrium binding data.

The use of nociceptin and a number of its derivatives has proven that the cloned ORL₁ receptor and the binding sites identified in brain tissues are in fact the same. Nociceptin is degraded to a number of shorter peptides by the actions of aminopeptidases and endopeptidases in blood, hippocampus, and spinal cord [37–39]. These metabolites bind to the ORL₁ receptor with low affinities and have much reduced potencies in functional assays [28, 38]. However, amidation of the C-terminus of two of these peptides, NC(1–13) and NC(1–9) [40, 41], is sufficient to restore some degree of potency and efficacy. A number of groups have used these ligands to show that the order of binding affinities and potencies is the same regardless of whether the data are obtained from brain cell membranes or cell lines transfected with the cloned human receptor [41, 42]. Similar data from mouse, guinea pig, and rat tissues find that the order of affinity and potency for a number of derivatives of nociceptin is largely consistent [for comparative data see 43]. This suggests that the cloned receptor and the binding site identified in mammalian tissues are the same pharmacological entity.

ORL₁ Receptor Ligands

In order to fully characterize and ultimately exert a degree of control over a GPCR signalling system, it is vital to have a number of pharmacological tools available. A comprehensive characterization of receptor binding and expression requires agonists and antagonists of different character (i.e., peptidic and nonpeptidic). Functional characterization is facilitated by the introduction of partial and inverse agonists, but perhaps the greatest challenge is to exert *in vivo* control over a signalling system. Ideally, ligands must be resistant to degradation, highly specific, and must be able to cross the blood-brain barrier. Development of ligands with diverse properties poses considerable problems; nevertheless, great progress has been made since the isolation of nociceptin and its identification as the endogenous ligand of the ORL₁ receptor [12, 13].

A number of agonists of nociceptin have been produced with increased binding affinities and potencies at the ORL₁ receptor [for example, see 44], with the most effective ones incorporating further positively charged residues into the already basic central region of the peptide. [Arg¹⁴, Lys¹⁵] nociceptin is up to 17-fold more potent than nociceptin in functional assays and 30-fold more potent when the *in vivo* pronociceptive effects are analyzed. This is thought to result not only from improving the receptor-ligand interaction characteristics, but also from stabilizing the ligand against degradation by endopeptidases [45]. However, perhaps the most significant step in the development of potent nociceptin analogues was the discovery that although the nociceptin fragment NC(1–13) has greatly reduced binding affinities and potencies, amidation of the C-terminal end to produce NC(1–13)NH₂ created a stable ligand with binding affinities and agonist potencies just as high as those of nociceptin [40]. This ligand has been used as a template from which a large number of ligands have been developed. The most active of these compounds all contain modifications of the phenyl ring at position 4 [46]. Introduction of a fluoride, NO₂, or CN group at the para position of the phenyl ring increased the activity of NC(1–13)NH₂ by up to fivefold.

The agonist NC(1–13)NH₂ has also been used as a template from which ORL₁ receptor antagonists have been developed. A series of structure-activity relationship studies identified the ligand [Phe¹ψ(CH₂-NH)Gly²]-NC(1–13)NH₂ [47]. This ligand retained its ability to bind to the ORL₁ receptor with high affinity (K_i = 10 nM) [43] and was able to inhibit the electrically invoked con-

traction of mouse vas deferens, as opposed to the stimulatory effect caused by nociceptin and NC(1–13)NH₂. Subsequent *in vitro* studies have identified partial and full agonistic effects invoked by [Phe¹ψ(CH₂-NH)Gly²]-NC(1–13)NH₂ [42, 48, 49]. Unfortunately, when the peptide was injected into the mouse, it inhibited morphine-induced analgesia and reduced the latency in tail withdrawal tests [50], suggesting that *in vivo* [Phe¹ψ(CH₂-NH)Gly²]-NC(1–13)NH₂ acts as an agonist.

The mixed responses elicited by [Phe¹ψ(CH₂-NH)Gly²]-NC(1–13)NH₂ limit its usefulness; however, a new peptide has recently been described that behaves as a pure antagonist. [Nphe¹]-NC(1–13)NH₂ inhibits the binding of a tritiated nociceptin analogue to mouse brain membranes with a K_i = 100 nM [51]. Despite this low affinity, [Nphe¹]-NC(1–13)NH₂ was able to antagonize forskolin-stimulated cAMP accumulation in CHO cells transfected with human ORL₁ with a pK_b of 6.1 and to antagonize the nociceptin-induced contraction in electrically stimulated mouse vas deferens with a pK_b of 6.4 [51]. Most promisingly, the ligand did not show any residual agonist activity and was able to prevent the effects of nociceptin in the mouse tail withdrawal assay and to potentiate morphine-induced analgesia [52]. It also displayed a high degree of selectivity for the ORL₁ receptor over opioid receptors [52, 53]. In rat models of neuropathic pain and in response to thermal and mechanical stimuli, [Nphe¹]-NC(1–13)NH₂ was able to reduce the effect of nociceptin in a dose-dependent manner [54, 55]. [Nphe¹]-NC(1–13)NH₂ is also able to antagonize nociceptin-induced hypotension, bradycardia, and vasodilation in the rat [56]. These effects were not observed in ORL₁ receptor knockout mice and, unlike the μ-opioid agonist, DAMGO, did not cause drug tolerance [57]. This is particularly promising, as tolerance is a significant problem in the use of opioids and opiates to manage pain, and the availability of a drug that has a longer effective time frame would be of significant benefit in a clinical setting.

There is an increasingly large library of selective ORL₁ peptidic receptor agonists [58, 59]. However, the number of nonpeptide ligands is still relatively small. In recent years, the techniques of combinatorial chemistry and high-throughput screening have been combined to identify compounds that act as potent agonists and antagonists of the ORL₁ receptor. Several small molecule agonists have been described for ORL₁ receptors that mimic the *in vivo* effects of nociceptin and are able to penetrate the blood-brain barrier [60–62]. The most selective of these appears to be compound Ro 64-6198. It has been reported to have a pK_i for HEK-293 cells expressing human ORL₁

receptor of 9.4, with 100-fold selectivity over κ- and μ-opioid receptors and greater than 1,000-fold selectivity over δ-opioid receptors, 40 other GPCRs, and channel-binding sites [63]. *In vitro* GTPγS-binding assays suggest that Ro 64-6198 acts as a full agonist with potencies equal to those of nociceptin. *In vivo*, Ro 64-6198 exhibited dose-dependent anxiolytic effects [63, 64], with no signs of drug tolerance following 15 days of daily drug exposure [65].

Two recent studies in rats and mice suggest that the clinical use of this compound may have limitations. Low doses of Ro 64-6198 administered to mice affect balance and motor coordination, while higher doses in both rats and mice cause hypothermia [66]. A comparison of the effects of Ro 64-6198 on isolated vas deferens tissue showed that the apparent agonistic effects of the ligand, in mice but not rats, were not antagonized by J-113397 (see below) or [Nphe¹]-NC(1–13)NH₂ [67]. This suggests that despite the apparent *in vitro* selectivity of Ro 64-6198, it is able to activate unidentified signalling pathways in a species-dependent manner.

J-113397 is a derivative of a compound isolated from a chemical library. The lead compound bound to ORL₁ receptors with an IC₅₀ of 200 nM and acted as an agonist in GTPγS-binding assays. A series of derivations resulted in compounds with nanomolar affinities and increasing selectivity, until compound J-113397 was produced which bound to CHO cells expressing human ORL₁ receptors with an IC₅₀ of 2.3 nM and antagonized nociceptin-induced GTPγS binding with an IC₅₀ of 5.6 nM. Furthermore, the IC₅₀ values for δ-, κ-, and μ-receptors were all at least 600-fold higher than for ORL₁ receptors [68]. When administered subcutaneously, J-113397 dose-dependently inhibited hyperalgesia elicited by administration of nociceptin in a mouse tail-flick test [69]. J-113397 has also been shown to potently antagonize the effects of nociceptin in human and rat neocortical slices [70], membranes of the external plexiform layer, and granule cell layer of the rat main olfactory bulb [71]. It also inhibits the effects of nociceptin on neuronal action potential firing within the rat rostral ventromedial medulla neurons [72] and potently inhibits the proinflammatory effects of nociceptin [73]. In summary, J-113397 is a small-molecule ORL₁ receptor ligand that is potent and selective in all the *in vitro* and *in vivo* assays so far examined and holds tremendous potential as a pharmacological and therapeutic tool.

Another nonpeptide antagonist, JTC-801, has recently been reported that has potent antinociceptive effects in acute-pain animal models when applied by intravenous

Fig. 2. Intracellular signalling pathways activated by the ORL₁ receptor. The diagram identifies the heterotrimeric G protein subunits that have been shown to couple with the ORL₁ receptor, as well as the signalling pathways that these interactions activate. Dashed lines indicate that the role of intermediate proteins has not yet been elucidated. The experimental evidence identifying individual pathways and the interactions between their intermediates is described in the text.

injection or by oral administration [74]. The increasing number of small-molecule agonists and antagonists being identified and characterized give hope that new therapeutic agents will be generated to treat a number of conditions where current therapies have lost efficacy, due to the development of tolerance, or become counterproductive because of the development of dependence and side effects.

ORL₁ Receptor Signalling Pathways

The high homology between the ORL₁ receptor and the three opioid receptor subtypes immediately alerted researchers that the ORL₁ receptor is also likely to be a G_{i/o}-coupled receptor that regulates the activity of adenylyl cyclase, inwardly rectifying K⁺ channels and voltage-gated Ca²⁺ channels. (The reader is referred to figure 2 for an overview of the ORL₁ signalling pathways.)

When comparing opioid and nociceptin signalling, there is a particular paradox, as nociceptin is often observed to counteract the effects of opioids while apparently acting via the same signal transduction pathways. This may in part be due to the distinct localization of ORL₁ and opioid receptors in the neuronal networks responsible for pain transmission [75] and the inhibition of two distinct groups of neurons by these receptor agonists [76–78]. However, there remains a significant gap in our understanding of the molecular mechanisms underlying these differences and the role that activation of individual effectors plays in mediating them.

Adenylyl Cyclase

ORL₁ receptor coupling to the adenylyl cyclase second-messenger system was confirmed following the isolation of nociceptin. In CHO and HEK-293 cells transfected with the ORL₁ receptor, nociceptin inhibits forskolin-stimulated cAMP accumulation with ED₅₀ values of ap-

proximately 1 nM [12, 13, 19]. In these systems, pretreating the cells with pertussis toxin (PTX) abolishes the response [for example, see 79, 80], identifying $G_{i/o}$ heterotrimeric G proteins as the intermediates between receptor and effector. To exclude the possibility that these effects are an artifact of heterologous expression systems, inhibition of cAMP accumulation by nociceptin has also been observed in neuroblastoma cells endogenously expressing ORL₁ receptors [79, 81] as well as in mouse brain homogenates with an EC_{50} value < 1 nM [31].

The ORL₁ receptor is able to functionally interact with PTX-insensitive G_z proteins. In HEK-293 cells transiently expressing the ORL₁, dopamine D₁ receptors, and the α -subunit of G_z , nociceptin dose-dependently inhibited dopamine-stimulated cAMP accumulation in a PTX-insensitive manner. A similar result was obtained with retinoic-acid-differentiated SH-SY5Y cells, which endogenously express both the ORL₁ receptor and G_z , where a component of the response to nociceptin was insensitive to PTX [80]. Although G_z is a close relative of $G_{i/o}$ proteins, it is more than a PTX-insensitive version of $G_{i/o}$, because it can regulate unique effectors not shared by the $G_{i/o}$ proteins [82].

The phenomenon of supersensitization of adenylyl cyclase is a hallmark of cellular tolerance induced by chronic activation of opioid receptors. Chronic activation of the ORL₁ receptor also leads to a PTX-sensitive increase in the sensitivity to forskolin of transfected HEK-293 cells as well as neuroblastoma SK-N-SH cells which endogenously express the ORL₁ receptor [83]. This suggests that nociceptin may induce drug tolerance, but it should be remembered that the small-molecule agonist Ro 64-6198 caused no signs of tolerance in the rat following 15 days of exposure [65]. It has not been established whether Ro 64-6198 causes adenylyl cyclase supersensitivity.

Phospholipase C/ Ca^{2+}

Several studies have suggested that activation of the ORL₁ receptor results in activation of a phospholipase C/ Ca^{2+} /protein kinase C (PKC) pathway. Activation of PKC was observed in CHO cells stably expressing the ORL₁ receptor with an EC_{50} of 0.2 nM. This response was PTX sensitive and blocked by PKC inhibitors, Ca^{2+} chelators, and a phospholipase C (PLC) inhibitor [84]. This pattern of activation resembles that observed for the opioid receptor activation of PLC β -coupled pathways by $G\beta\gamma$ subunits [85, 86], although the involvement of $G\beta\gamma$ subunits in the nociceptin induction of PLC β pathways has yet to be proven. The physiological relevance of nociceptin-induced stimulation of PLC β is not obvious; however, a

recent report suggested that PKC activation by nociceptin generates superoxide in pig pial arteries which impairs hypotensive cerebrovasodilation after hypoxia/ischemia [87].

$G\alpha_{16}$ and $G\alpha_{14}$ subunits have been shown to interact with numerous G_i -linked GPCRs, enabling them to activate PLC β -coupled pathways [88, 89]. We have investigated whether the promiscuity of these two G_q family member subunits extends to the ORL₁ receptor. When the ORL₁ receptor and either $G\alpha_{16}$ or $G\alpha_{14}$ subunits were transiently expressed in COS-7 cells, nociceptin stimulated the accumulation of inositol phosphates by six- and fourfold, respectively [80, 89]. $G\alpha_{14}$ and $G\alpha_{16}$ subunits are expressed in hematopoietic cells, including monocytes and T cells ($G\alpha_{16}$) [90] and B cells ($G\alpha_{14}$) [91]. The ORL₁ receptor transcript has also been identified in these cell lineages [92, 93], but a functional coupling between receptor and effector in these tissues has yet to be demonstrated. Functional association with $G\alpha_{14}$ and $G\alpha_{16}$ confers the ORL₁ receptors with the ability to regulate the mitogen-activated protein kinases (MAPKs) and hence gene transcription. Moreover, we envisage the ability of ORL₁ receptors and these $G\alpha$ subunits to functionally interact in heterologous transfection systems to have a practical use by enabling the coupling of ORL₁ receptors to signal transduction systems that are amenable to the development of high-throughput screening platforms.

MAPK Pathways

GPCRs regulate cell proliferation and differentiation through a family of MAPKs. These serine/threonine protein kinases are capable of phosphorylating several transcription factors [94], thereby regulating subsequent transcriptional events. There are at least three subtypes of MAPK: the extracellular-signal-regulated kinases (ERKs) are mainly stimulated by growth factors [95], whereas c-Jun NH₂-terminal kinases (JNKs; also referred to as the stress-activated protein kinases) and p38 MAPK are more responsive to cellular stress [96, 97].

Nociceptin stimulation of CHO cells expressing the ORL₁ receptor induces ERK activation via a PTX-sensitive mechanism, resulting in activation of the transcription factors Elk-1 and Sap1a [98]. The activation was partially blocked by inhibition of PKC or phospholipase C [99, 100], suggesting that a p21^{ras}-independent pathway may partially mediate the response. The nociceptin-induced MAPK activity was affected by wortmannin and genistein, phosphatidylinositol-3-kinase (PI3K) and tyrosine protein kinase inhibitors, respectively, indicating that the induction is probably mediated via $G\beta\gamma$ -subunit

activation of a PI3K/Src/SOS/p21^{ras} pathway. The nociceptin-induced MAPK activation was also inhibited by expression of the carboxy-terminal domain of β -adrenergic receptor kinase, which eliminates signalling via G $\beta\gamma$, or by expression of the proline-rich domain of SOS which inhibits signalling via SOS/p21^{ras} pathways [101].

Nociceptin induces the activation of p38 MAPK via ORL₁ receptors endogenously expressed in NG108-15 neuroblastoma $\times$ glioma hybrid cells in a PTX-sensitive manner. Activation of p38 MAPK was diminished by inhibition of protein kinase A (PKA), but potentiated by PKC inhibitors [102]. Furthermore, we have demonstrated that agonist stimulation of the ORL₁ receptor leads to stimulation of the JNK subgroup of MAPKs in a Ras/Rac-dependent manner. This effect was observed in both transfected COS-7 cells and NG108-15 cells endogenously expressing the ORL₁ receptor [103]. Transfection of the cells with either dominant-negative mutants of Ras (RasS17N) or Rac (RacT17N) significantly inhibited the activation of JNK. On the other hand, the specific PI3K inhibitor wortmannin had no effect on the induced JNK activity. The ORL₁-induced JNK activation was primarily mediated via PTX-sensitive proteins, although there was also a small contribution by a PTX-insensitive component. The G α subunit responsible for this PTX-insensitive signalling was not identified, but cotransfection experiments showed that the ORL₁ receptor was able to induce JNK activation through PTX-insensitive G α_z , G α_{12} , G α_{14} , and G α_{16} .

It is particularly interesting that although the opioid receptors also activate MAPK pathways, there seem to be some clear differences in the regulation of this activation. All three opioid receptors activate MAPK pathways through G $\beta\gamma$ -dependent pathways [104], but ERK activation by nociceptin shows a partial dependence on PLC activation [99, 100]. The divergence in signalling patterns of G i/o -coupled receptors may be of importance in understanding the different *in vivo* effects of nociceptin and opioids. It is also interesting to note that pretreatment of cells expressing both ORL₁ and μ -opioid receptors with nociceptin decreased subsequent μ -opioid agonist stimulation of ERK activation [101]. While it is not yet possible to extrapolate these findings to physiological responses, it is a clear example of nociceptin inhibiting the effects of opioids at a molecular level.

The ability of nociceptin to activate all three classes of the MAPK pathway and the observations that JNK pathways mediate the phosphorylation of transcription factors c-Jun and ATF-2 [103], and ERK pathways the phosphorylation of Elk-1 and Sap1a [98], indicate the importance

of nociceptin signalling at the nuclear level. We have recently identified the signal transducer and activator of transcription (STAT3) as a target of ORL₁ pathways. In heterologous transfection systems expressing the ORL₁ receptor and G α_{16} , STAT3 is tyrosine phosphorylated (Tyr⁷⁰⁵) following challenge with nociceptin. Using a STAT3-responsive promoter-driven luciferase gene reporter system, we have shown that inhibitors of Janus kinase 2 (JAK2) and Raf-1 reduce the nociceptin-induced stimulation of the transcriptional activity of STAT3. Using cells expressing a constitutively active form of G α_{16} , we have delineated a complex system of regulation of STAT3 phosphorylation by a calmodulin kinase II/c-Src/Ras/Raf-1/MEK/ERK pathway as well as a JAK2 pathway. In addition, we observed inhibition of STAT3 transcriptional activity by a Cdc42/JNK1-coupled pathway. However, the relative contributions of these pathways to the transduction of ORL₁ signalling have not yet been fully established.

The STAT proteins have been implicated as mediators for numerous cytokines, growth factors, and hormones that regulate immune responses as well as cell proliferation, apoptosis, and differentiation [105]. Nociceptin has been demonstrated to have effects on neuronal differentiation [106], but the G protein subtypes and signalling pathways involved have not been investigated, although G α_{12} and G α_o subunits are known to activate STAT3 in NIH-3T3 cells [107–109]. It is clear that further refinement of our understanding of the nociceptin activation of MAPK pathways and the cooperativity of MAPK subtypes that results in transcriptional activation will be required before we can fully understand the relationship between nociceptin-induced MAPK activation and the resulting physiological consequences. Such a task is further complicated by the ability of GPCRs to regulate the signalling pathways that are typically under the control of growth factors and cytokines [110].

Voltage-Gated Ca²⁺ Channels

In SH-SY5Y human neuroblastoma cells that endogenously express the ORL₁ receptor, nociceptin partially inhibits N-type Ca²⁺ channels with an IC₅₀ value of 42 nM [111]. This effect was PTX sensitive and was mirrored by findings in human NG108-15 neuroblastoma cells transfected with the ORL₁ receptor [112].

In rat dorsal root ganglion (DRG) neurons and mouse small nociceptive trigeminal ganglion neurons, it has been observed that nociceptin suppressed high-voltage-activated N-type Ca²⁺ channels [113, 114]. In DRG neurons, this effect was attenuated by GTP γ S, GDP β S, and alu-

minum fluoride, suggesting the involvement of G-protein-coupled signalling mechanisms. Opioid agonists had much the same effects, but a difference was noted when low-voltage T-type Ca^{2+} channels were investigated. Morphine was unable to suppress the low-voltage channels, but nociceptin could, although in a manner that did not seem to depend on G protein involvement [113]. As these T-type channels contribute to the development of convulsive activity [115], treatment with nociceptin may yet prove to have benefits in anticonvulsive therapy.

In DRG neurons isolated from nerve-injured rats, it was shown that the effectiveness of morphine in inhibiting N-type channels was functionally downregulated, whereas the effectiveness of nociceptin was increased [116]. These findings are in line with observations that morphine has limited uses in treating neuropathic pain [117], but that nociceptin has analgesic properties in animal models of nerve injury [118, 119].

Inhibition of Ca^{2+} channels by nociceptin has also been observed in other areas of the nervous system implicated in transmitting and processing painful stimuli. In dissociated rat periaqueductal gray matter (PAG) neurons, nociceptin inhibited N-type and P/Q-type channels via a $\text{G}\beta\gamma$ -subunit-mediated pathway [120]. In addition, nociceptin inhibits rat rostral ventromedial medulla (RVM) neurons by inhibiting calcium conductances. Nociceptin also inhibits GABA release within the RVM via a presynaptic Ca^{2+} -dependent mechanism. Therefore, nociceptin is able to exert both disinhibitory and inhibitory effects on neuronal action potential firing within the RVM [121]. Whole-cell patch-clamp techniques on rat locus ceruleus neurons have also detected inhibitory calcium channel activity [122].

Nociceptin has been shown to inhibit N-type Ca^{2+} channels in rat sympathetic ganglion neurons in a PTX-sensitive manner [123]. These authors have suggested that this maybe a mechanism by which nociceptin exerts its depressive effects on the cardiovascular system [43, 56]. In dissociated rat hippocampal neurons nociceptin partially inhibits L-, N-, and P/Q-type Ca^{2+} channels in a PTX- and $\text{GTP}\gamma\text{S}$ -sensitive manner [124]. These observations correlate with evidence that nociceptin inhibits synaptic transmission and long-term potentiation in the hippocampus [125] and that ORL_1 knockout mice show enhanced long-term potentiation in the hippocampus which facilitates learning and memory [126].

When the inhibition of Ca^{2+} channels is investigated, a number of examples are apparent, where the effects of ORL_1 signalling correlate with observed physiological effects of nociceptin. Clear differences also emerge be-

tween the effects of nociceptin and opioids that may explain the differential effects of these compounds.

Inwardly Rectifying K^+ Channels

At the postsynaptic membrane, many GPCRs produce hyperpolarization by activating K^+ channels, preventing excitation or the propagation of action potentials. Studies in transfected *Xenopus* oocytes have confirmed that the ORL_1 receptor can activate K^+ channels. Coexpression of ORL_1 and potassium channel subunits Kir3.1 (GIRK1) either alone or with Kir3.4 (CIR, reKATP) allowed nociceptin to evoke potassium currents with 50% of the maximal effect elicited by 1 nM of agonist [127, 128].

Functional coupling of ORL_1 receptors and potassium channels has been observed in neurons endogenously expressing ORL_1 receptors. Nociceptin activates inwardly rectifying K^+ channels in all PAG neurons and inhibited evoked fast GABAergic and glutamatergic postsynaptic currents in a significant subpopulation of PAG neurons [121]. These findings suggest that nociceptin, via its presynaptic inhibitory effect on transmitter release and via its postsynaptic actions, has the potential to modulate the analgesic, behavioral, and autonomic functions of the PAG. Similarly, nociceptin activates inwardly rectifying K^+ channels in dorsal raphe, rat trigeminal nucleus caudalis neurons, and locus ceruleus neurons [129–131]. A similar picture emerges in the hippocampus where nociceptin activates K^+ channels, thereby modulating the excitability of hippocampal cells [128, 132–134] which are implicated in learning and memory and the generation of seizures [126]. Observations that stimulation of the ORL_1 receptor activates inwardly rectifying K^+ channels are consistent with the inhibitory actions of nociceptin in neuronal cells. However, considerable progress needs to be made before these cellular actions can explain the different *in vivo* effects of nociceptin. As was shown for the involvement of $\text{PLC}\beta$ and Ca^{2+} channel signalling mechanisms in the modulation of cardiovascular activity, nociceptin activation of inwardly rectifying K^+ channels has also been implicated in these processes [135].

ORL_1 Receptor Regulation

Desensitization of an ORL_1 receptor induced response was first observed when the effect of continuous application of nociceptin to the rat locus coeruleus neurons was investigated [129]. Activation of K^+ channels decreased to 70% of the maximal response following treatment of the neurons with 3 μM nociceptin. Desensitization occurred

with a half-time of 2 min and was homologous, as the response of the neurons to Met-enkephalin was largely unchanged by continuous application of nociceptin [129]. However, desensitization of the cells with Met-enkephalin reduced the maximal response produced by nociceptin by 22%, implying a degree of heterologous desensitization induced by opioid receptors.

Pei et al. [136] used a microphysiometer to examine the extracellular acidification induced by nociceptin stimulation of CHO cells stably transfected with the ORL₁ receptor. These authors observed that the extracellular acidification rate stimulated by nociceptin was dose dependent and PTX sensitive, but that exposure to 10 nM nociceptin for 6 min reduced the responsiveness of the cells by approximately 50%. This attenuation of the nociceptin-induced response recovered with a half-life of 12 min. This desensitization was not inhibited by PKA inhibitors but was almost completely abolished by PKC inhibitors. Analogy with other receptor systems would suggest that the desensitization of the ORL₁-receptor-induced response is mediated by PKC phosphorylation of serine and threonine residues in the C-terminal tail and intracellular loops of the receptor.

Homologous desensitization of the endogenously expressed ORL₁ receptor has similarly been demonstrated in neuroblastoma × glioma NG108-15 cells and SK-N-SH cells. In both cell types the ability of nociceptin to inhibit the forskolin-induced accumulation of cAMP was almost completely abolished after prechallenging the cells with 10 μM agonist for 10 min [79, 81]. Treatment of NG108-15 cells with the δ-opioid agonist DPDPE was unable to heterologously desensitize the ORL₁ receptor. In both NG108-15 and SK-N-SH cells, G protein activation and inhibition of adenylyl cyclase by nociceptin were significantly attenuated by prior stimulation of the NMDA receptor. Interestingly, desensitization of opioid receptor signalling was observed in NG108-15 cells, but not in SK-N-SH cells, suggesting differential modulation of ORL₁ and opioid responses by NMDA receptors [137].

Nociceptin also induced desensitization of the nociceptin-induced N-type Ca²⁺ channel inhibition in NG108-15 cells and rat hippocampal pyramidal cells [112, 138]. Rat hippocampal cells recovered from the desensitization within 20 min and were heterologously desensitized by the GABA_B receptor agonist baclofen. The response of hippocampal cells to nociceptin was attenuated by treatment with the PKC activator, phorbol myristate acetate, once again implying a role for PKC-mediated desensitization of the ORL₁ receptor [138].

The effect on desensitization and internalization of ORL₁ receptors by different agonists has been investigated [65]. Pretreatment of HEK-293 cells expressing ORL₁ receptors with either nociceptin or Ro 64-6198 reduced the ability of both agonists to inhibit adenylyl cyclase by almost 50%. Ligand-binding studies of pretreated cells revealed a surprising difference. Following stringent washing of the cells treated with nociceptin, the number of binding sites and the affinity of the cells for nociceptin were unchanged, but cells pretreated with Ro 64-6198 had a reduced binding capacity (~50%) and affinity (~4-fold) [65]. This suggests that either the desensitization induced by nociceptin is not accompanied by receptor internalization or that the nociceptin-desensitized receptors are recycled much more rapidly than those desensitized by Ro 64-6198. In vivo, acute and chronic treatment of rats with Ro 64-6198 also reduced the number of brain ORL₁-binding sites with a half-time for recovery of approximately 5.5 h [65].

A similar set of studies on SK-N-BE human neuroblastoma cells identified nociceptin-induced internalization of the ORL₁ receptor that was dependent on the formation of clathrin-coated vesicles and ATP. Overexpression of β-arrestin 2 in these cells increased the internalization of ORL₁ receptors, and the recovery of cell surface receptor number was dependent on acid phosphatases [139]. This set of data confirms that, in this cell type at least, ORL₁ receptors do follow a pattern of desensitization, sequestration, internalization, and recycling. Although much work remains to be done to identify which amino acid residues are phosphorylated and how they contribute to the desensitization and internalization processes. In addition, little is known about the fate of internalized receptors; it has yet to be established whether receptors are recycled back to the cell surface following internalization or whether they are targeted for lysosomal degradation.

Receptor-Receptor Interactions

Homo- and heterodimerization of GPCRs is an increasingly reported phenomenon. Direct physical interactions between numerous receptor subtypes have been measured as well as the resulting alterations in receptor expression, ligand binding, agonist-mediated endocytosis, G protein activation, and second-messenger coupling [140].

We have recently completed a study [141] that involved measuring the effects of coexpression of ORL₁ and

opioid receptors. ORL₁ receptor signalling via Gα₁₆-coupled pathways was reduced by approximately 50% by coexpression with δ-, but not κ- or μ-opioid receptors. Furthermore, upon coexpression of ORL₁ and individual opioid receptors, signalling via κ- and μ-receptors was almost completely abolished [141]. We have not demonstrated a physical interaction between ORL₁ and opioid receptors, but our data certainly suggest that, given colocalization of these receptors, a modulation of their signalling properties is possible. It has not yet been determined whether such a pattern of modulation occurs in cells endogenously expressing these receptors or whether the abolition of κ- and μ-opioid signalling by ORL₁ receptors provides an explanation for the ability of nociceptin to inhibit some of the effects of opioids.

Alteration of the binding and signalling properties of opioid receptors occurs when different opioid receptor subtypes are coexpressed. Cells coexpressing μ- and δ-opioid receptors exhibited altered potency and rank order of affinities for opioid ligands [142]. The ability of the receptor complex to inhibit forskolin-stimulated cAMP production was retained, but was insensitive to PTX treatment, suggesting an altered pattern of G protein interactions. This accumulated data suggest that the interaction of opioid and ORL₁ receptors has implications for the pharmacology, signalling, and physiology of these signal transduction systems.

Concluding Remarks

Great progress has been made since the first descriptions of the orphan ORL₁ receptor. The isolation of nociceptin and the pharmacological characterization of the ORL₁ receptor have enabled researchers to describe a multitude of physiological processes that are modulated by ORL₁ receptor activation. This has in turn suggested that controlling the ORL₁ signalling activity may be beneficial to the treatment of numerous pathophysiological states. In many of these cases, *in vivo* studies have confirmed that nociceptin and its analogues do have therapeutic potential [43].

Our understanding of the interactions that occur between nociceptin and ORL₁ receptors are such that it has been possible to design ligands with enhanced binding and specificity characteristics. The peptidic analogues of nociceptin are both useful pharmacological tools and potential clinical useful drugs, but perhaps more excitingly, a number of small-molecule agonists and antagonists have now been described that appear to have real poten-

tial. Antagonists J-113997 and JTC-801 inhibit hyperalgesic responses, and JTC-801 has been shown to be orally active [69, 74]. The increasing use of the ORL₁ receptor in high-throughput drug screening protocols and the observations that ORL₁ couples to the universal signalling adaptors, Gα₁₆ and Gα_{16z44} [80, 143], will certainly facilitate the discovery of new ligands.

Agonist binding to ORL₁ receptors has been shown to activate signalling via several intracellular pathways. However, comparison with the complexity of responses triggered by opioid receptors [104] would suggest that much more detail about the regulation of these pathways and the interplay between them remains to be uncovered. For example, no attempt has yet been made to determine the contribution made to the ORL₁ modulation of cAMP levels by the many isoforms of adenylyl cyclase. Similarly, we have only just begun to recognize that GPCRs form interactions with each other. The selectivity of these complex formations is little studied, and our understanding of the regulation of their formation and the consequences for signalling and physiological processes is in its infancy. It may prove to be the case that regulation of signalling at the receptor level is an important mode of regulation of opioid signalling by nociceptin.

Perhaps the biggest challenge will be the correlation of the *in vitro* signalling responses measured with the *in vivo* actions of nociceptin. This is undoubtedly a daunting task, but one which will result in a much fuller understanding of how we may be able to regulate many physiological processes.

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