

RESEARCH ARTICLE



Comprehensive transduceromic profiling of NOP receptor ligands at different G α subunits

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Abstract

Background and Purpose: The nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) is an opioid receptor family member with distinct pharmacology and broad therapeutic potential. NOP receptors regulate functions such as nociception, stress reactivity, reward, mood, autonomic tone, immunity and sleep/wake cycle. Biased signalling has been proposed to enhance efficacy and minimise adverse effects at many G protein-coupled receptors (GPCRs), yet the transducer coupling preferences of NOP remain poorly defined.

Experimental Approach: We applied the (TRansdUcer PATHways) BRET-based G protein profiling platform (TRUPATH) biosensor suite to resolve NOP receptor coupling. Human NOP receptor was expressed in HEK293 cells and challenged with 20 chemically diverse ligands, including the endogenous neuropeptide, as well as peptide and non-peptide agonists. We compared TRUPATH data with those obtained in validated assays, that is, calcium mobilisation (via G α q β 5), NOP-G protein and NOP- β -Arrestin 2 interaction.

Key Results: N/OFQ activated six inhibitory G proteins (Gi1, Gi2, Gi3, GoA, GoB, Gz) with high potency (pEC₅₀ 9.08–10.49) but modest signal amplitude (15–30% of basal). Ligands UFP-113 and Comp 26 showed the most pronounced deviations across G protein isoforms.

Conclusions and Implications: This study provides a comprehensive fingerprint of NOP receptor pharmacology at the single-transducer level. The lack of marked G protein bias at the NOP receptor, more pronounced than for the classical opioid receptors, may reflect a combination of intrinsic receptor properties and assay limitations. These findings underscore the need for innovative chemotypes and complementary experimental strategies to fully resolve potential transducer bias at the NOP receptor.

Abbreviations: BRET, bioluminescence resonance energy transfer; CHO, Chinese hamster ovary; CL_{95%}, 95% confidence limits; G α , G protein alpha subunit; G $\beta\gamma$, G protein beta-gamma complex; HEK293, human embryonic kidney 293; N/OFQ, nociceptin/orphanin FQ; NOP, nociceptin/orphanin FQ peptide receptor; PTX, pertussis toxin; TRUPATH, (TRansdUcer PATHways) BRET-based G protein profiling platform.

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KEYWORDS

G proteins, GPCR, N/OFQ, NOP receptor, Transduceromics, TRUPATH

1 | INTRODUCTION

The nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP) was cloned in 1994 by Mollereau and colleagues (Mollereau et al., 1994) and was orphanised the following year by two independent groups (Meunier et al., 1995; Reinscheid et al., 1995). Unlike classical opioid receptors, NOP is activated exclusively by its endogenous ligand N/OFQ and shows negligible affinity for classical opioid receptor ligands. NOP transcripts and protein are widely distributed throughout the central nervous system—brainstem, limbic structures, cortex—and in peripheral tissues, including dorsal root ganglia, immune cells, heart, lung and gut (Calo & Lambert, 2018; Toll et al., 2016). Consequently, the N/OFQ-NOP receptor system modulates several different functions, including nociception, stress reactivity, reward, mood, autonomic tone, immunity (Lambert, 2008) and wake/sleep cycle control (Morairty et al., 2023; Whiteside et al., 2024); these pleiotropic actions make the NOP receptor an intriguing but challenging drug target.

Medicinal chemistry programmes have yielded a broad array of peptide and non-peptide NOP ligands with diverse efficacy and selectivity profiles (Pacifico et al., 2020; Zaveri, 2016). Clinical development has explored compounds such as the NOP agonists (i) SCH-486757 for cough (McLeod et al., 2010), (ii) UFP-112 for overactive bladder (Angelico et al., 2019), (iii) SER100 for hypertension (Kantola et al., 2017; Villar et al., 2016) and (iv) sunobinop for insomnia (Cipriano, Kapil, Zhou, Shet, Whiteside, et al., 2024; Whiteside et al., 2024, 2025); and the NOP antagonist LY2940094/BTRX-246040 for depression, alcohol use disorder and Parkinson's disease (Post, Smart, Jackson, et al., 2016; Post, Smart, Krikke-Workel, et al., 2016). Moreover, cibranoladol, a mixed NOP/opioid agonist is currently in Phase III studies for acute pain (Christoph et al., 2017; Scholz et al., 2018). These efforts suggest the translational promise of targeting NOP.

Because NOP activation can generate multiple, sometimes opposing, biological outputs, several complementary strategies have been proposed to improve the therapeutic potential of NOP ligands, including (i) compartmentalisation—confining activity to specific anatomical sites, such as UFP-112 acting as spinal analgesic after intrathecal administration (Hu et al., 2010; Rizzi et al., 2007) or as overactive bladder treatment after intravesicular administration (Angelico et al., 2019); (ii) polypharmacology—balancing NOP and μ opioid receptor agonism—as in cibranoladol and AT-121 to synergise analgesia while mitigating side effects (Ding et al., 2018; Linz et al., 2014); (iii) biased agonism—ligands that preferentially engage G proteins over β -arrestins or vice versa—with numerous G protein-biased NOP agonists reported (Camilotto et al., 2025; Chang et al., 2015; Cipriano, Kapil, Zhou, Shet, Harris, et al., 2024; Faouzi et al., 2020; Ferrari et al., 2016, 2017; Malfacini et al., 2015; Pacifico et al., 2020; Parker & Bruchas, 2019), although the in vivo consequences of NOP receptor signalling selectivity remain to be explored (Azevedo Neto

What is already known?

- The NOP receptor couples mainly to pertussis toxin (PTX)-sensitive G proteins.
- TRUPATH enables systematic mapping of GPCR transducer profiles.

What does this study add?

- This study provides a complete NOP receptor coupling profile across G α subfamilies.
- This study compares NOP-TRUPATH signalling to established pharmacological readouts on six key transducers.

What is the clinical significance?

- This study e-establishes a reliable NOP signalling reference useful for interpreting clinical candidates.
- This study offers a forward-looking signalling benchmark to apply in future NOP drug development.

et al., 2021). Unlike classical opioid receptors, where allosteric modulation is already object of intense research (Griebel et al., 2025; Kaneko et al., 2024; Król et al., 2025; Ramos-Gonzalez et al., 2025), no NOP receptor allosteric modulators have been reported to date.

NOP receptor couples predominantly to pertussis-toxin (PTX)-sensitive Gi/o proteins (Malfacini et al., 2018). As with many G protein-coupled receptors (GPCRs), NOP receptor signalling strictly depends on functional G proteins (Grundmann et al., 2018), and dissecting which biological effects are mediated by individual G α subunits is of significant relevance.

Different technologies allow the investigation of GPCR transducer coupling at single-G protein resolution: the bioluminescence Resonance Energy Transfer (BRET)-based heterotrimer dissociation sensors measure real-time G α -G $\beta\gamma$ separation across multiple G protein families (Avet et al., 2022; Galés et al., 2005); the open-source (TRAnsDUCer PATHways) BRET-based G protein profiling platform (TRUPATH) biosensor suite comprises 14 BRET probes covering all human heterotrimeric G proteins (Olsen et al., 2020); and the TGF- α shedding assay uses chimeric G α subunits to convert GPCR activation into ectodomain release of alkaline-phosphatase-tagged TGF- α for high-throughput profiling (Inoue et al., 2019).

To investigate the complement of $G\alpha$ subunits that underpin the intracellular signalling of the activated NOP receptor and its G protein bias, we applied the TRUPATH system in cells overexpressing human NOP receptors. Experiments with N/OFQ demonstrated, aligning with previous indications, that NOP couples primarily with $Gi1$, $Gi2$, $Gi3$, GoA , GoB and Gz —all G proteins that decrease cAMP (although Gz is not blocked by PTX). Then, 20 NOP receptor ligands encompassing peptide-based and non-peptide ligands possessing full or partial agonist properties were assayed. Notably, all ligands were generated in previous structure–activity relationship (SAR) studies and were chosen to span the widest possible chemical diversity among known NOP receptor agonists to assess whether distinct chemotypes could differentially engage specific G protein subtypes (i.e. transducer bias). Peptide ligands employed were synthesised at the University of Ferrara (Italy) and included N/OFQ, UFP-112 and UFP-113 (Arduin et al., 2007), PWT2-N/OFQ (Guerrini et al., 2014), the Dooley hexapeptides Ac-RYYRIK-NH₂ and Ac-RYYRWK-NH₂ (Dooley et al., 1997), and a selected panel of N/OFQ(1-13)-NH₂ derivatives described by Pacifico and colleagues (Pacifico et al., 2020): Comp 3 ([Ala³]N/OFQ(1-13)-NH₂), Comp 22 ([Cha¹]N/OFQ(1-13)-NH₂), Comp 26 ([D-Ala²]N/OFQ(1-13)-NH₂), Comp 29 ([pPhe⁴]N/OFQ(1-13)-NH₂), including the strongly G protein (vs. arrestin) biased Comp 40 ([Cys¹⁴-Palmitoyl]N/OFQ(1-14)-NH₂) and Comp 43 ([Cys¹⁴-(2,5-dioxopyrrolidine-1-yl-(AlaLys)₆NH₂)]N/OFQ(1-14)-NH₂). Reference small-molecule agonists Ro 65-6570 (Wichmann et al., 1999), SCH-486757 (McLeod et al., 2010) and MCOPPB (Hirao et al., 2008) were synthesised by us, and other small molecules AT-090 (Ferrari et al., 2016), AT-202/SR16835 (Toll et al., 2016), AT-312 (Zaveri et al., 2018), AT-390 (Arcuri et al., 2018) and AT-403 (Ferrari et al., 2017) were from Astraera Therapeutics. All ligands' effects were either experimentally, or based on the available literature, evaluated in calcium mobilisation (NOP- $G\alpha_{q15}$ -Ca²⁺), NOP-G protein, and NOP- β -arrestin 2 couplings experiments. This thorough characterisation provided a comprehensive in vitro fingerprint of NOP pharmacology at a single-transducer resolution via TRUPATH, therefore representing an important milestone in understanding how NOP receptor intracellular signalling works.

2 | METHODS

2.1 | Drugs and reagents

N/OFQ, UFP-113 (Arduin et al., 2007), UFP-112 (Arduin et al., 2007), Ac-RYYRIK-NH₂, Ac-RYYRWK-NH₂, PWT2-N/OFQ (Guerrini et al., 2014), Comp 3 ([Ala³]N/OFQ(1-13)-NH₂), Comp 26 ([D-Ala²]N/OFQ(1-13)-NH₂), Comp 40 ([Cys¹⁴-Palmitoyl]N/OFQ(1-14)-NH₂), Comp 43 ([Cys¹⁴-(2,5-dioxopyrrolidine-1-yl-(AlaLys)₆-NH₂)]N/OFQ(1-14)-NH₂), Comp 22 ([Cha¹]N/OFQ(1-13)-NH₂), Comp 29 ([pPhe⁴]N/OFQ(1-13)-NH₂) (Pacifico et al., 2020), Ro 65-6570 and SCH-486757 were synthesised by us at the University of Ferrara (IT) as described in the references. AT-390 (Arcuri et al., 2018), AT-312 (Zaveri et al., 2018), AT-090 (Ferrari et al., 2016), AT-202/SR16835 (Toll et al., 2009) and AT-403 (Ferrari et al., 2017) were synthesised in the lab of Dr. Nurulain Zaveri at the Astraera Therapeutics (Mountain

View, CA, USA). Chemical structures of all ligands tested are provided in the Figure S1. We prepared concentrated solutions (10 mM) of ligands in ultrapure water or dimethyl sulfoxide (DMSO) to dilute peptides or small molecules, respectively, and stored them in aliquots at −20°C until use. The maximum ligand concentration used was 10 μ M, corresponding to a final DMSO content of 0.1%, which did not influence any of the assay readouts described in this study. We employed chemicals and reagents from Merck/Millipore Sigma (Burlington, MA, USA), chemiluminescent dyes from NanoLight Technology (Norman, OK, USA), cell culture reagents from Euroclone (Pero, Italy), plasticwares from Sarstedt (Nümbrecht, Germany) and Corning (Corning, NY, USA).

2.2 | BRET $G\alpha$ - $G\beta\gamma$ dissociation assay (TRUPATH)

We adapted this assay from that reported by Olsen and co-workers (Olsen et al., 2020), and the kit for establishing TRUPATH was a gift from Bryan Roth (Addgene kit #1000000163, RRIDs available via Addgene). Here, we used all RLuc8-tethered $G\alpha$ proteins and co-transfected them with the indicated $G\beta$ and the GFP2-linked $G\gamma$ in Human Embryonic Kidney (HEK293, RRID:CVCL_0045) cells stably expressing the (human) NOP receptor (provided by Prof. DG Lambert). HEK293_{NOP} culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g ml^{−1} Hygromycin B, 100 IU ml^{−1} penicillin and 100 IU ml^{−1} streptomycin. We carried out the episomal transfection routines in HEK293 cells stably expressing the NOP receptor with a mixture of DNA and polyethyleneimine (1:3 μ g) 48 h prior to assessing drugs' effects on $G\alpha$ - $G\beta\gamma$ dissociation. About 20 h before the experiments, in white opaque 96-well microplates (PerkinElmer, Waltham, MA, USA), we plated about 75,000 cells previously transfected with a 1:1:1 mixture of $G\alpha$, $G\beta$ and $G\gamma$ (40 ng cm^{−2} each). On the day of the experiment, we washed the cells, added 2 μ M Prolume Purple coelenterazine (NanoLight Technology, Norman, OK, USA) to the cells, and subsequently, serial dilutions of the ligands all in HBSS/HEPES (10 mM) solution. We then recorded the occurring light emission at 405(10) and 510(30) nm with the Victor Nivo (PerkinElmer, Waltham, MA, USA) and then averaged three BRET ratios (computed by dividing light at 510 for 405) measured at 5, 10 and 15 min after agonist addition. Additionally, to assess expression levels of the different $G\alpha$ proteins, we compared the light emitted at 405 within all transducers (Figure S1).

2.3 | Calcium mobilisation assay

Chinese Hamster Ovary (CHO, RRID:CVCL_0214) cells stably co-expressing the human NOP receptor and the $G\alpha_{q15}$ chimeric G protein (to force the NOP receptor triggering Ca²⁺ flux) were used in this assay. Cells were cultured in DMEM/Ham's F12 Nutrient Mix (F12) (1:1) medium supplemented with 10% FBS, 2 mM L-glutamine, 200 μ g ml^{−1} G418, 100 μ g ml^{−1} Hygromycin B, 100 IU ml^{−1} penicillin and 100 IU ml^{−1} streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were seeded at 12,000 cells

per well into 384-well black, clear-bottom plates 24 h prior to the test. On the day of the experiment, we loaded plated cells for 60 min with a solution consisting of HBSS/HEPES (20 mM) supplemented with 2.5 mM probenecid, 3 μ M Fluo-4 AM and 0.01% pluronic acid, and then exchanged with HBSS/HEPES (20 mM), 2.5 mM probenecid and 500 μ M Brilliant Black (which decreases fluorescence background). Serial dilutions of ligands were prepared in HBSS/HEPES (20 mM) buffer with 0.02% bovine serum albumin (BSA). The automated microplate reader FlexStation III (Molecular Device, CA, USA) was set to 37°C and changes in fluorescence intensity detected. Maximum change in percentage over the baseline fluorescence of the vehicle was computed to express the effects of all compounds.

2.4 | BRET NOP-G protein and β -arrestin 2 interaction assay

A BRET interaction assay was used to study ligand-induced NOP receptor-G protein and β -arrestin 2 interactions. In brief, HEK293 cells stably co-expressing two pairs of fusion proteins were employed (i) Renilla Luciferase (RLuc)-tethered human NOP receptor, (ii) the bovine G β 1 tagged with Renilla Green Fluorescent Protein (RGFP) or (iii) the human β -arrestin 2 tagged with RGFP. To measure NOP receptor-G protein interaction, membranes taken from HEK293 cells stably co-expressing the fusoproteins NOP-RLuc and G β 1-RGFP were used, while NOP receptor- β -arrestin 2 coupling was assayed on living cells. Such cells were grown in DMEM medium supplemented with 10% FBS, 2 mM L-Glutamine, 200 μ g ml⁻¹ G418, 100 μ g ml⁻¹ Hygromycin B, 100 IU ml⁻¹ penicillin and 100 IU ml⁻¹ streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. Experiments were carried out in white opaque 96 wells microplates (PerkinElmer, CT, USA); NOP- β -arrestin 2 coupling was assessed on cells seeded at a density of 100,000 cells per well 24 h prior to measure BRET. The day of the experiment, medium was discarded, and cells were incubated in phosphate-buffered saline (PBS) (Mg²⁺ 0.5 mM, Ca²⁺ 0.9 mM) and 2 μ M Prolume Purple coelenterazine (NanoLight Technology, CA, USA) before agonist injections and bioluminescence reading. NOP-G protein coupling was recorded on cell membranes resuspended in PBS, corresponding to an amount of 3 μ g of total protein per each of the 96 well in the presence of 2 μ M Prolume Purple coelenterazine. Ligands were added, and averaged BRET ratios from 15 min measurements were computed. BRET ratios were computed as counts per second (CPS) obtained on a Victor Nivo (PerkinElmer, CT, USA) with 405- (10) and 510 (30)-nm bandpass filters. BRET ratios were derived from light passed through 510 divided by that passed through 405 filters. Vehicle BRET values were subtracted from all computations and datasets normalised as a fraction of N/OFQ maximal effects.

2.5 | Nomenclature of targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived

in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023). The NOP receptor (OPRL1) is indexed in the Guide to Pharmacology under target ID 320. In the same database, following ligands are described with indicated ID: N/OFQ ID 1681, UFP-112 ID 3917, PWT2-N/OFQ ID 8865, Ac-RYYRIK-NH₂ ID 1679, Ac-RYYRWK-NH₂ ID 1680, SCH-486757 ID 8869, Ro 65-6570 ID 8864, MCOPPB ID 12419, AT-403 ID 9747, AT-202 ID 8867. Ligands not currently listed in the Guide to Pharmacology are reported using the nomenclature defined in the original medicinal chemistry references, and structures are reported in Table S1.

2.6 | Data analysis and terminology

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2025). In this study, we employed terminology and computations consistent with the recommendations of the International Union of Basic and Clinical Pharmacology (IUPHAR) (Kolb et al., 2022; Neubig et al., 2003). We performed statistical evaluation of our datasets with Excel for Microsoft 365 (Microsoft, Redmond, Washington, USA) and Prism 10 (GraphPad Software Inc., San Diego, CA, USA). We expressed experimental points as mean $\pm$ SEM of n experiments, where n was always greater or equal to five. We fitted all concentration-response curves to agonists to the four-parameter logistic nonlinear regression model as follows: $\text{effect} = \text{basal} + (E_{\text{max}} - \text{basal}) / (1 + 10^{[(\text{LogEC}_{50} - \text{Log}[\text{ligand}]) \times \text{Slope}]})$. We expressed agonists' potency (pEC₅₀) values as the means with computed confidence limits (CL_{95%}). N/OFQ maximal effects (E_{max}) were expressed as percentage $\pm$ SEM of inhibition of the basal BRET value (TRUPATH). E_{max} of ligands were then normalised to N/OFQ E_{max} and expressed as percentage $\pm$ SEM of N/OFQ E_{max} . All ligands with E_{max} CL_{95%} not including N/OFQ value were considered as partial agonists. Differences in terms of potency were represented in the heatmap as Log (CR), where CR is the concentration ratio between EC₅₀ of a given ligand and that of N/OFQ.

3 | RESULTS

3.1 | N/OFQ effects on NOP-G protein activation

First, we successfully expressed 14 different G α proteins as indicated by luminescence values detected (Figure S1A). To identify the physiologically relevant couplers, we examined N/OFQ responses across all sensors and selected those showing nanomolar potency and measurable efficacy. Specifically, only transducers yielding pEC₅₀ > 7 and E_{max} > 10% of basal BRET were considered engaged (Figures S1B,C and Table S2). These thresholds served as empirical criteria to identify reproducible, receptor-mediated responses. Based on this analysis, N/OFQ activated six inhibitory G proteins—Gi1, Gi2, Gi3, GoA, GoB and Gz. N/OFQ evoked measurable E_{max} (~ 15–30%) and high pEC₅₀ (9.08–10.49) on Gi1, Gi2, Gi3, GoA, GoB and Gz that were selected for further studies (Figure 1).

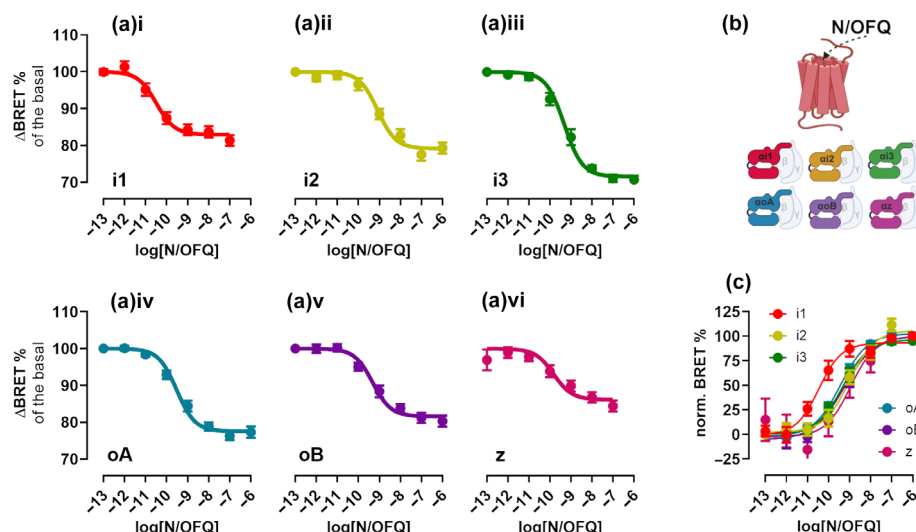


FIGURE 1 Effects of N/OFQ on NOP coupling with Gi1, Gi2, Gi3, GoA, GoB and Gz. Increasing concentrations of N/OFQ diminished the BRET ratio as depicted in the curves on the indicated transducers (Gi1 ●, Gi2 ●, Gi3 ●, GoA ●, GoB ●, and Gz ●) (a)–(vi). Data are mean $\pm$ SEM of >15 experiments performed in duplicate and are expressed as % inhibition of basal BRET. Note that the y-axes were truncated between 70 and 110 to enhance visualisation of concentration–response profiles. Schematic depiction of the partners of the $G\alpha$ - $G\beta\gamma$ dissociation (TRUPATH) assay (b). Luciferase ($G\alpha$ -linked) and fluorescent proteins ($G\gamma$ -tethered) have been omitted for simplicity (designed with BioRender). (c) Concentration–response curves depict normalised concentration–response curves to N/OFQ on all transducers.

3.2 | Standard peptide agonist effects on NOP-G protein activation

UFP-112 (known potent NOP receptor agonist) evoked a consistent concentration-dependent stimulation of all six G protein dissociation with very high potency (8.79–10.74) and maximal effects like those of N/OFQ, although diminished at Gi1 and GoA (Figure 2, UFP-112). UFP-113 (described as potent NOP receptor partial agonist) maximal effects were diminished in four out of six transducers (Gi2, Gi3, GoA, GoB) and its potency varied across transducers (6.99–10.45) being the highest at Gi1 and the lowest at GoA (Figure 2, UFP-113). The tetrameric PWT2-N/OFQ—consisting of a cyclam core fused to four N/OFQ sequences—displayed consistent high $E_{\max}$, not significantly different from that of the endogenous peptide, and high pEC_{50} (8.52–9.75) irrespective of the transducer (Figure 2, PWT-N/OFQ). Lastly, Ac-RYYRIK-NH₂ and Ac-RYYRWK-NH₂ hexapeptides—well-known NOP partial agonists—displayed moderate potency (7.25–8.98 and 7.51–9.01, respectively) and relatively low maximal effects, with Gi2, Gi3 and GoA being the less activated G proteins (Figure 2, Ac-RYYRIK-NH₂ and Ac-RYYRWK-NH₂).

3.3 | Standard non peptide agonist effects on NOP-G protein activation

SCH-486757, Ro 65-6570 and MCOPPB (well-known NOP-receptor agonists) all displayed high maximal effects, in terms of potency, MCOPPB (9.31–11.24) > Ro 65-6570 (7.04–8.39) > SCH-486757 (6.06–7.18), across all G proteins (Figure 3, SCH-486757, Ro 65-6570, and MCOPPB).

3.4 | Astraea Therapeutics (AT) non peptide agonist effects on NOP-G proteins activation

AT-403 (among the most potent nonpeptide NOP receptor agonists discovered) mimicked the stimulatory effects of N/OFQ with no difference in $E_{\max}$ and very high pEC_{50} (8.47–11.47) throughout our transduceromics analysis (Figure 4, AT-403). AT-090 displayed high $E_{\max}$ with no difference from N/OFQ and moderate potency (7.46–9.18) (Figure 4, AT-090). AT-390 $E_{\max}$ was always the same as N/OFQ apart from Gi3; as for potency, it was moderate (7.53–9.51) (Figure 4, AT-390). AT-312 $E_{\max}$ was the same as the standard across all transducers, while potency was slightly lower (7.03–9.08) (Figure 4, AT-312). AT-202 also displayed high $E_{\max}$ but with relatively low pEC_{50} (7.31–8.56) (Figure 4, AT-202).

3.5 | N/OFQ peptide derivative effects on NOP-G proteins activation

Compound 3 (Figure 5, Compound 3) and Compound 26 (Figure 5, Compound 26), slightly less potent and efficacious than N/OFQ (1-13)-NH₂ in previous studies, here showed diminished potency (7.81–9.51 and 8.27–9.21, respectively) and $E_{\max}$ being significantly lower for Comp 3 in Gi3 and for Comp 26 in Gi3, GoA, GoB and Gz. Comp 40 (Figure 5, Comp 40) and Comp 43 (Figure 5, Comp 43)—displayed strong bias towards G protein vs. arrestin in another BRET assay—were very potent across all transducers (8.76–9.84 and 9.01–10.69, respectively) with maximal effects lower for Comp 40 at Gi3, GoA, GoB, and Gz and at Gz for Comp 43. Comp 22 (Figure 5) and Comp 29 (Figure 5)—developed as highly potent unbiased full

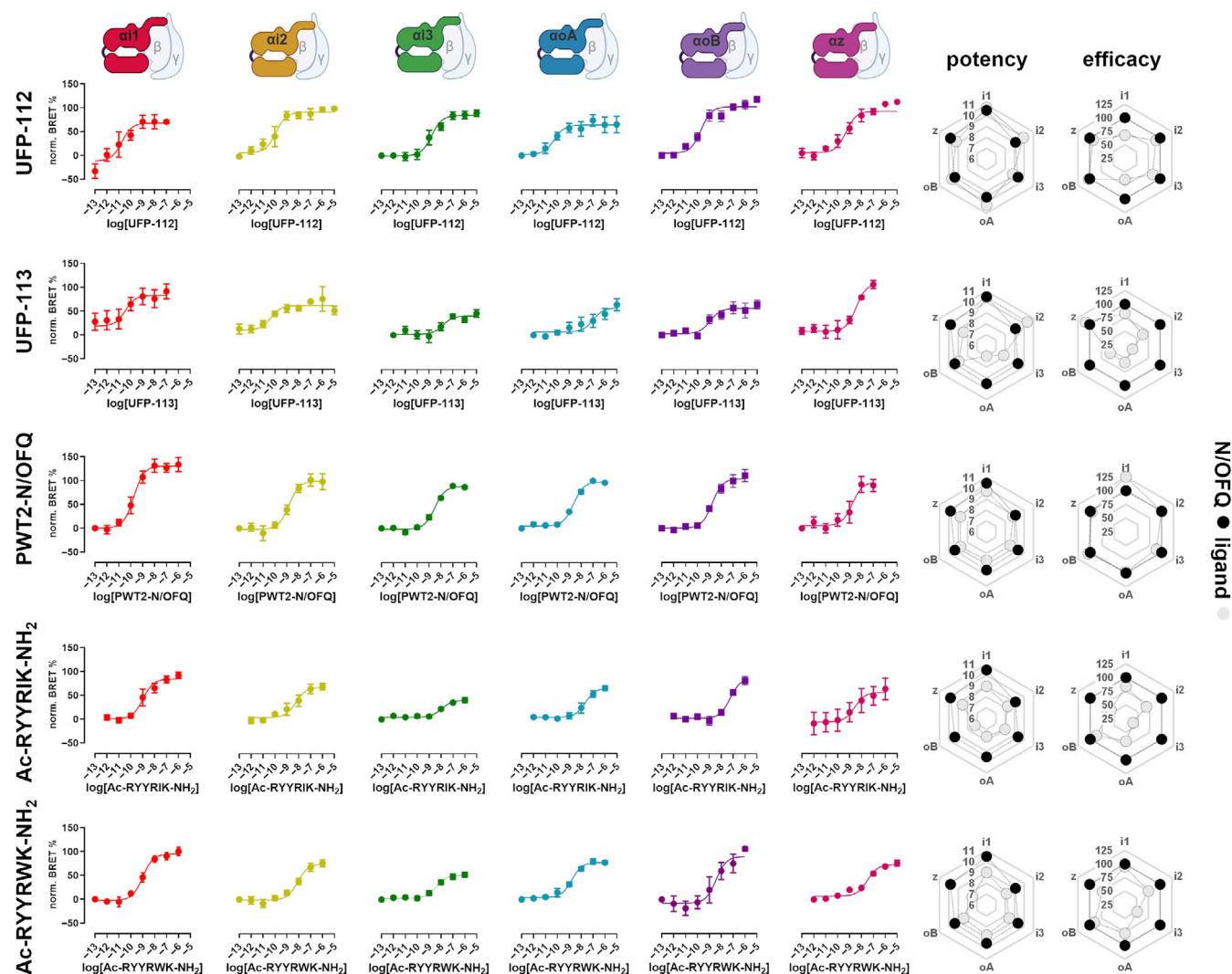


FIGURE 2 UFP-112, UFP-113, PWT2-N/OFQ, Ac-RYRIK-NH₂ and Ac-RYRWK-NH₂ activities on NOP stimulation. Concentration–response curves to UFP-112, UFP-113, PWT2-N/OFQ, Ac-RYRIK-NH₂ and Ac-RYRWK-NH₂ for α -G β dissociation (left–right, Gi1, Gi2, Gi3, GoA, GoB, Gz). Spider plots for potency and efficacy are displayed on the right side. Data are mean $\pm$ SEM of five experiments performed in duplicate and are expressed as the percentage of N/OFQ $E_{\max}$.

agonists—behaved as very high potency agonists (9.13–10.57 and 9.25–10.50, respectively) with $E_{\max}$ slightly although significantly lower than N/OFQ: at Gi2, GoA, GoB, Gz for Comp 22 and at GoB and Gz for Comp 29.

A comprehensive overview of the pharmacological parameters is provided in Tables 1 and S3.

3.6 | Pharmacological profile of ligands in orthogonal assays

In order to compare the results obtained with TRUPATH with those of three different assays, that is, calcium mobilisation via chimeric G protein and BRET assays measuring NOP–G β ₁ and NOP– β -arrestin 2 interaction, we prepared Table 2 in which we include published data and the results of novel experiments.

Specifically, in the calcium mobilisation assay, N/OFQ triggered NOP–G α q5–Ca²⁺ fluxes in a concentration-dependent manner with high potency (pEC_{50} 9.63) and maximal effects (230%) (Figure S2 and Table S4). Results for UFP-112, UFP-113, PWT2-N/OFQ, Ac-RYRIK-NH₂, Ac-RYRWK-NH₂, SCH-486757, Ro 65-6570, MCOPPB, AT-403 and AT-090 were already available in the literature (Table 2); therefore, we performed additional experiments with AT-390, AT-312, AT-202, Comp 3, Comp 26, Comp 40, Comp 43, Comp 22 and Comp 29. All compounds mimicked the stimulatory effect of N/OFQ, showing high $E_{\max}$ values comparable to that of N/OFQ, while their potency ranged from 8.25 to 9.72 (Figure S2 and Table S4). Overall, regarding maximal effects, only UFP-113, Ac-RYRIK-NH₂ and Ac-RYRWK-NH₂ behaved as partial agonists in the calcium mobilisation assay (Table 2). In terms of potency, the rank order normalised to the corresponding standard (N/OFQ or N/OFQ(1–13)-NH₂), including both published and new data, was as follows: MCOPPB $\geq$ Comp



FIGURE 3 SCH-486757, Ro 65-6570 and MCOPPB effects on NOP activation (i.e. $G\alpha 1$, $G\alpha 2$, $G\alpha 3$, $G\alpha O$ and $G\alpha B$). Concentration–response curves to SCH-486757, Ro 65-6570 and MCOPPB for $G\alpha$ – $G\beta$ dissociation (left–right, $G\alpha 1$, $G\alpha 2$, $G\alpha 3$, $G\alpha O$, $G\alpha B$, $G\alpha Z$). Spider plots for potency and efficacy are displayed on the right side. Data are mean $\pm$ SEM of five experiments performed in duplicate and are expressed as the percentage of N/O FQ E_{max} .

43 = Comp 29 $\geq$ Comp 22 = N/O FQ = N/O FQ(1-13)-NH₂ $\geq$ UFP-112 = Comp 3 = Comp 26 = PWT2-N/O FQ = Comp 40 = AT-403 $\geq$ Ac-RYYRWK-NH₂ = AT-312 = AT-090 $\geq$ Ro 65-6570 $\geq$ AT-390 $\gg$ AT-202 = Ac-RYYRIK-NH₂ > UFP-113 $\gg$ SCH-486757 (Table 2).

We then evaluated NOP– G -protein interaction; here, N/O FQ concentration-dependently stimulated the proximity between the receptor and $G\beta$ with high potency (two separate sets of experiments pEC₅₀ 8.04 and 8.61) and maximal effects set to 1 (Figure S3). We assayed Ac-RYYRIK-NH₂, Ac-RYYRWK-NH₂, AT-390, AT-312 and AT-202 (Figure S3 and Table S5), because all remaining ligands were already described in the literature (Table S5). Ligands with efficacy lower than 80% of the standard were ranked as follows: AT-202 $\geq$ Ac-RYYRWK-NH₂ > Ac-RYYRIK-NH₂ > AT-312 = AT-090 = AT-390 $\geq$ UFP-113. As far as potency is concerned, the rank order, including new and previously published data, was Comp 40 $\geq$ MCOPPB $\geq$ Comp 43 $\geq$ UFP-112 = UFP-113 $\geq$ PWT2-N/O FQ $\geq$ Ac-RYYRIK-NH₂ = Ac-RYYRWK-NH₂ = Comp 29 > Comp 22 $\geq$ N/O FQ = N/O FQ(1-13)-NH₂ $\geq$ AT-403 = AT-390 $\geq$ Ro 65-6570 $\geq$ Comp 3 $\geq$ AT-312 $\geq$ Comp 26 $\geq$ AT-090 > SCH-486757 > AT-202 (Table 2).

Finally, we used a NOP– β -arrestin 2 interaction assay, where N/O FQ showed high potency (two separate sets of experiments pEC₅₀ 7.78 and 9.07) and maximal effects normalised to 1 (Figure S4). We studied Ac-RYYRIK-NH₂, Ac-RYYRWK-NH₂, AT-390, AT-312 and AT-202 (Figure S4 and Table S5), while the remaining ligands were available from the literature (Table 2). Ligands with efficacy lower than

80% of the standard followed this order: AT-403 > Comp 3 $\geq$ AT-202 $\geq$ AT-090 $\geq$ AT-312 $\geq$ AT-390 $\geq$ Ac-RYYRWK-NH₂ $\geq$ SCH-486757 $\geq$ Ac-RYYRIK-NH₂ $\gg$ UFP-113. In terms of potency (with UFP-113 reported for its antagonist potency), the rank order, including new and literature data, was UFP-113 $\gg$ UFP-112 > Ac-RYYRIK-NH₂ = Ac-RYYRWK-NH₂ > MCOPPB $\geq$ Comp 29 $\geq$ Comp 22 = N/O FQ = N/O FQ(1-13)-NH₂ $\geq$ Comp 43 = PWT2-N/O FQ = AT-403 = Comp 40 = Comp 3 = AT-390 > AT-090 > Comp 26 > AT-312 > Ro 65-6570 $\gg$ SCH-486757 > AT-202 (Table 2).

4 | DISCUSSION

The present work expands the classic view that NOP couples mainly to $G\alpha i/o$ by resolving the individual α -subunits engaged by 20 chemically diverse agonists. Using the TRUPATH BRET sensor suite, we confirm N/O FQ-mediated activation of $G\alpha 1$, $G\alpha 2$, $G\alpha 3$, $G\alpha O$, $G\alpha B$ and $G\alpha Z$, whereas coupling to $G\alpha 15$ and Gustducin was detectable with higher concentrations. These findings align with earlier PTX-sensitivity data and single-readout studies, yet provide sub-family resolution that was not yet shown. Although the absolute responses were modest (15–30% BRET change for N/O FQ, a point that must be considered for further comparison and speculation), N/O FQ stimulated the six main G -protein subtypes with very high potency (pEC₅₀ 9.08–10.49). Of high importance, the pharmacological profile of all ligands, with very few exceptions, was largely superimposable to that obtained by other in vitro assays as demonstrated by calcium mobilisation (NOP–

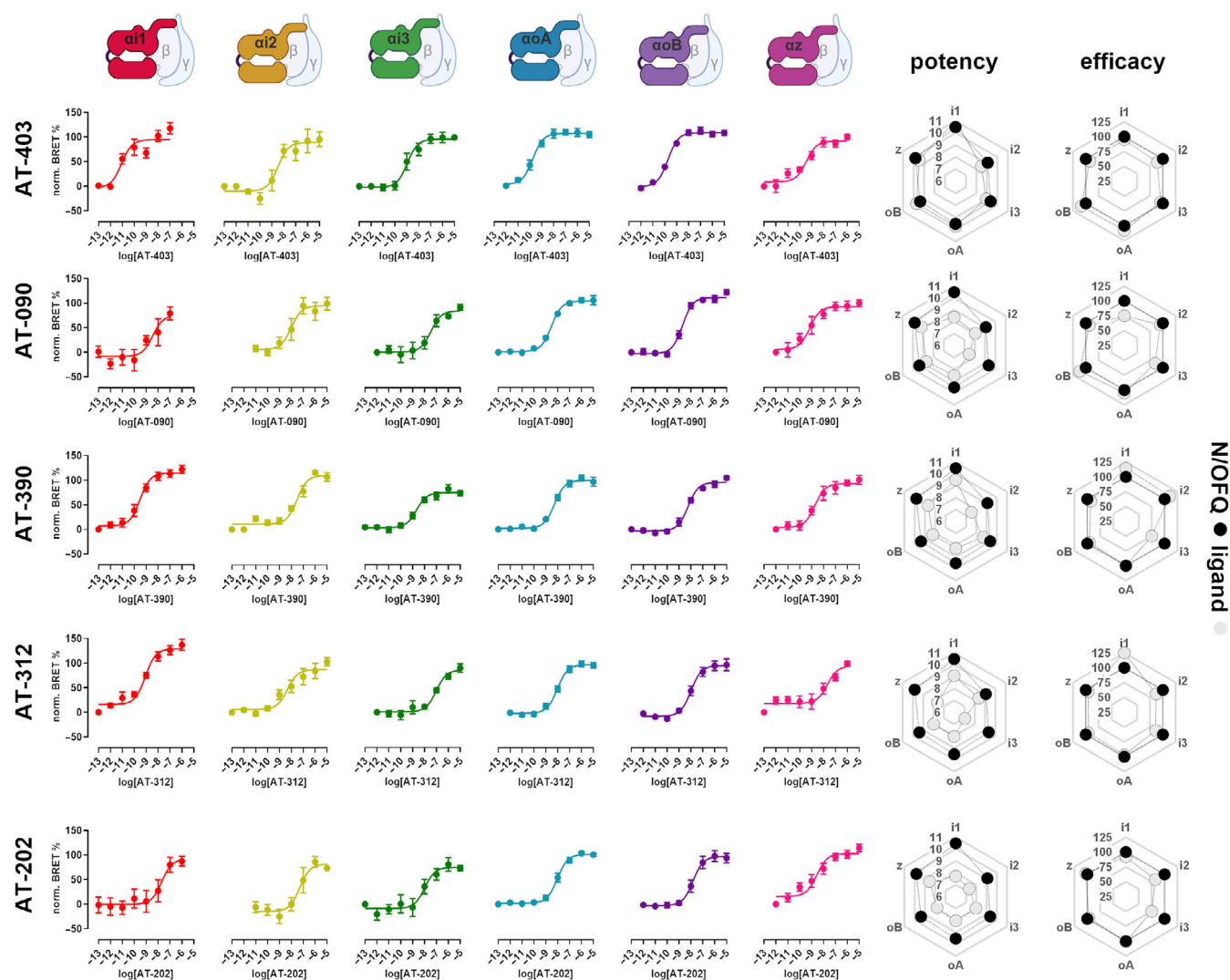


FIGURE 4 Effects of AT-403, AT-090, AT-390, AT-312 and AT-202 on NOP activation (i.e. Gi1, Gi2, Gi3, GoA and GoB). Concentration–response curves to AT-403, AT-090, AT-390, AT-312 and AT-202 for $G\alpha$ – $G\beta\gamma$ dissociation (left–right, Gi1, Gi2, Gi3, GoA, GoB, Gz). Spider plots for potency and efficacy are displayed on the right side. Data are mean $\pm$ SEM of five experiments performed in duplicate and are expressed as the percentage of N/OFQ E_{max} .

$G\alpha q5$ – Ca^{2+}), NOP–G protein and NOP– β -arrestin 2 couplings experiments. Nonetheless, some ligands may represent exceptions to the trendline.

Here, applying the TRUPATH suite, N/OFQ fostered a highly potent activation of the six cAMP-inhibiting transducers with following rank order of potency: $Gi1 > Gz > GoA \geq GoB = Gi3 \geq Gi2$. Remaining tested G proteins displayed much lower agonist potency values: $G15 = Gust \gg G12 = G13 = GsS = GsL$. N/OFQ potency (pEC_{50} 9.08–10.49) was very high, in line with approaches such as forskolin-induced cAMP inhibition (Meunier et al., 1995; Reinscheid et al., 1995) and other BRET-based approaches (Chang et al., 2015; Malfacini et al., 2015) focussed on G protein activation. These values of potencies align well with the affinity of the neuropeptide to the NOP receptor (McDonald, Calo, et al., 2003). As for maximal effects, the following rank order of efficacy— $Gi3 \geq G15 \geq GoA \geq Gi2 \geq GoB \geq Gi1 \geq Gust \geq Gz$ was observed—with transducers G12, G13, GsS

and GsL being totally inactive when the NOP receptor was stimulated by N/OFQ. This trend aligns with what was recently reported by Avet and colleagues (Avet et al., 2022) using a similar approach. Quite remarkably, Margas and co-workers (Margas et al., 2008) showed that rat sympathetic stellate ganglion expresses Gi1, Gi3 and Go, and that siRNA down-regulation highlights only Gi1 as pivotal in NOP-mediated effects on Ca^{2+} currents. Unfortunately, limited information is available on the role of each $G\alpha$ subunits triggered by the NOP receptor. The physiological role of each of these $G\alpha$ subunit is yet to be fully understood. Genetic alteration of $G\alpha$ expression in mice showed how (i) depletion of $G\alpha i1$ and $G\alpha i3$ can elicit depressive-like phenotype, fully rescued by viral re-expression of $G\alpha i3$ in the hippocampus (Marshall et al., 2018); (ii) RGS-insensitive $G\alpha i2$ (G184S) allele exhibits constitutively enhanced 5-HT_{1A} receptor signalling accompanied by anxiolytic and antidepressant-like behaviour (Talbot et al., 2010); (iii) genetic depletion of $G\alpha o$ impairs cerebellar cortical

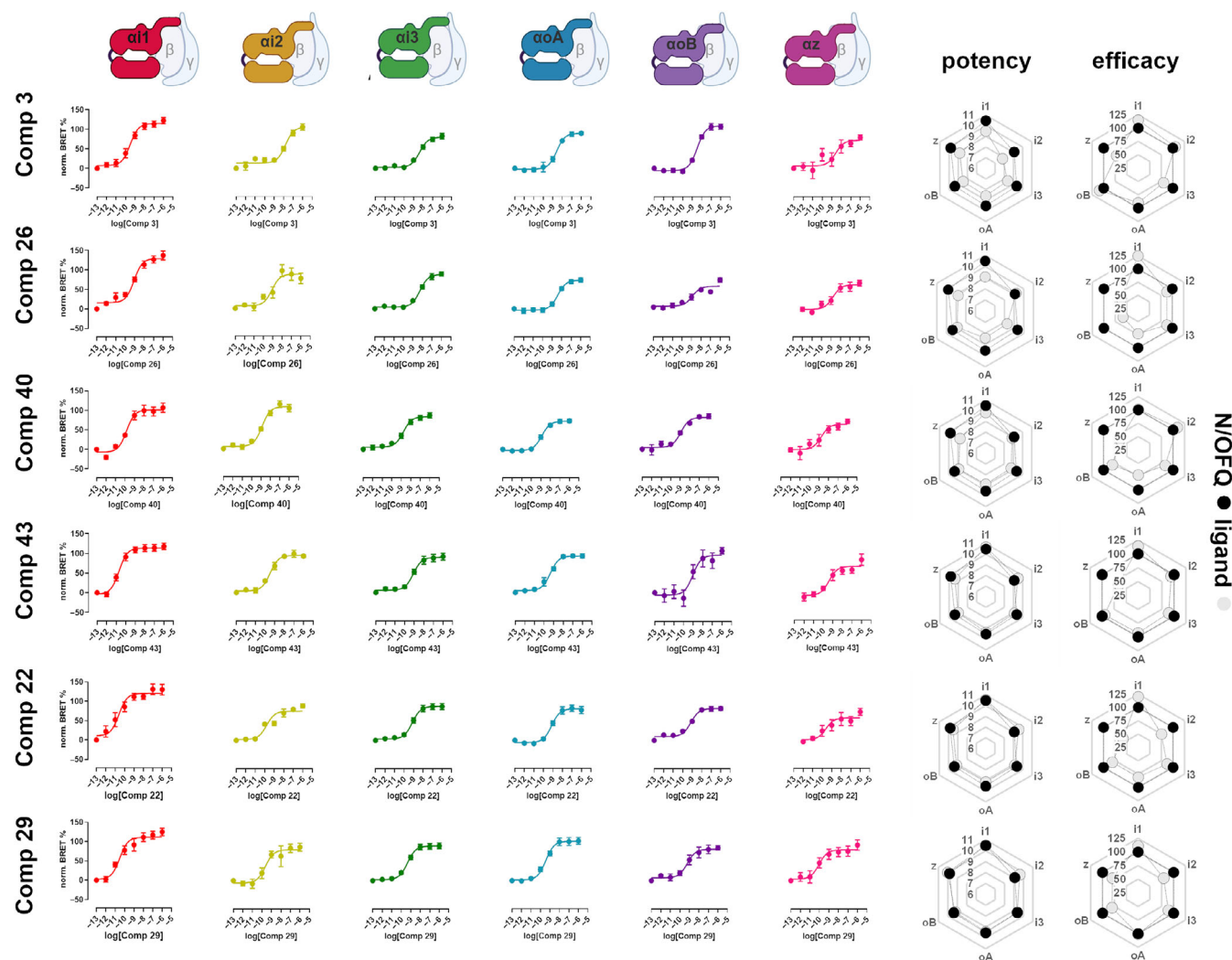


FIGURE 5 Effects of compounds (Comp) 3, 26, 40, 43, 22, and 29 on NOP activation (i.e. Gi1, Gi2, Gi3, GoA, and GoB). Concentration-response curves to Comp 3 ([Ala³]N/OFQ(1-13)-NH₂), Comp 26 ([D-Ala²]N/OFQ(1-13)-NH₂), Comp 40 ([Cys¹⁴Palmitoyl]N/OFQ(1-14)-NH₂), Comp 43 ([Cys¹⁴(2,5-dioxopyrrolidine-1-yl-(AlaLys)₆-NH₂)]N/OFQ(1-14)-NH₂), Comp 22 ([Cha¹]N/OFQ(1-13)-NH₂) Comp 29 ([pPhe⁴]N/OFQ(1-13)-NH₂) for Gα-Gβγ dissociation (left-right, Gi1, Gi2, Gi3, GoA, GoB, Gz). Spider plots for potency and efficacy are displayed on the right side. Data are mean ± SEM of five experiments performed in duplicate and are expressed as the percentage of N/OFQ E_{max}.

development, reduces synapse formation and disrupts synaptic plasticity (Cha et al., 2019; Silachev et al., 2022); Gαo is also described as a core brake on dopaminergic signalling (Muntean et al., 2021); (iv) Gαz-null animals show heightened cocaine-induced locomotor activity and diminished morphine analgesia (Yang et al., 2000). Altogether, it is not yet fully clear the output of biased agonists towards each of the G protein subunits; the availability of approaches such as TRUPATH will open up the search for biased agonists capable of solving this important question, possibly bypassing the issue of system compensation in knock-out studies.

Besides the evaluation of N/OFQ effects on all 14 transducers offered by the TRUPATH suite, the characterisation of 19 molecules of different chemical nature unveiled interesting insights. Of standard peptides, that is, UFP-112, UFP-113, PWT2-N/OFQ, Ac-RYYRIK-NH₂ and Ac-RYYRWK-NH₂, all molecules' pharmacological parameters aligned to that from the literature both in terms of potency and

efficacy (Toll et al., 2016). UFP-112 and PWT2-N/OFQ behaved as potent full agonists, while UFP-113, Ac-RYYRIK-NH₂ and Ac-RYYRWK-NH₂ displayed a more variable array of potencies and efficacies. UFP-113 showed a clear reversal of potency being very potent on Gi2 and of only weak-moderate potency on Gi3, GoA and Gz. This molecule's affinity towards the NOP receptor is described to be similar to that of N/OFQ, with functional potency values described to be variable; for instance, the potency of UFP-113 in the calcium mobilisation assay that uses chimeric G proteins (Gαqi5) was more than 30-fold less than that of N/OFQ. Thus, altogether, these results suggest that the NOP/UFP-113 complex has different ability to interact with the different G proteins compared to the complex NOP/N/OFQ. In terms of efficacy, UFP-113 displayed variable results on the different G proteins investigated in TRUPATH experiments, from 0.41 to 1.09. Similar results were obtained in previous studies where UFP-113 behaved as a NOP antagonist (mouse and rat vas deferens

TABLE 1 Summary of pharmacological parameters.

	GI1		GI2		GI3		GoA		GoB		Gz	
	pEC50	E _{max}	pEC50	E _{max}	pEC50	E _{max}	pEC50	E _{max}	pEC50	E _{max}	pEC50	E _{max}
N/OFQ	10.49 (10.19–10.80)	100	9.08 (8.84–9.32)	100	9.35 (9.18–9.53)	100	9.53 (9.35–9.71)	100	9.38 (9.12–9.64)	100	9.83 (9.48–10.17)	100
UFP-112	10.74 (10.03–11.46)	68 ± 8	9.92 (9.46–10.40)	91 ± 5	8.79 (8.30–9.20)	84 ± 5	10.26 (9.47–11.04)	64 ± 5	9.70 (9.31–10.05)	102 ± 4	9.23 (8.79–9.88)	93 ± 6
UFP-113	10.45 (9.32–11.77)	83 ± 10	10.34 (9.60–11.07)	63 ± 4	7.83 (7.05–8.55)	41 ± 5	6.99 (5.64–9.09)	58 ± 9	8.95 (8.07–9.59)	56 ± 5	8.44 (7.98–8.91)	109 ± 10
PWT2-N/OFQ	9.75 (9.42–10.06)	130 ± 6	8.82 (8.38–9.24)	100 ± 7	8.52 (8.35–8.67)	89 ± 3	8.63 (8.49–8.77)	98 ± 2	8.76 (8.43–9.06)	104 ± 5	8.77 (8.16–9.42)	97 ± 12
Ac-RYYRIK-NH ₂	8.98 (8.43–9.45)	84 ± 6	8.25 (7.61–9.09)	68 ± 7	7.96 (7.59–8.32)	40 ± 3	7.65 (7.21–8.06)	66 ± 5	7.25 (6.92–7.59)	87 ± 7	8.56 (6.52–10.43)	57 ± 15
Ac-RYYRWK-NH ₂	9.01 (8.72–9.30)	95 ± 4	8.12 (7.71–8.52)	75 ± 5	8.40 (8.03–8.77)	51 ± 3	8.78 (8.50–9.06)	78 ± 3	8.43 (7.62–9.24)	90 ± 13	7.51 (7.21–7.81)	73 ± 3
SCH-486757	7.11 (6.05–8.05)	97 ± 10	7.18 (6.47–7.95)	100 ± 14	6.06 (5.60–6.59)	99 ± 12	6.24 (5.67–6.90)	108 ± 15	6.43 (5.82–7.07)	99 ± 13	6.89 (6.09–7.60)	77 ± 10
Ro 65-6570	8.16 (7.75–8.63)	130 ± 8	8.12 (7.73–8.56)	113 ± 7	7.04 (6.66–7.43)	88 ± 7	7.60 (7.12–8.06)	89 ± 6	8.14 (7.65–8.73)	91 ± 7	8.39 (8.04–8.75)	114 ± 6
MCOPPB	11.24 (10.16–12.10)	94 ± 9	10.03 (9.27–10.64)	86 ± 8	9.78 (9.30–10.13)	84 ± 3	9.31 (8.92–9.69)	78 ± 4	9.47 (8.95–9.96)	82 ± 7	10.17 (9.69–10.74)	99 ± 7
AT-403	11.04 (10.56–11.51)	95 ± 6	8.47 (7.95–9.00)	89 ± 8	8.94 (8.57–9.31)	96 ± 5	9.74 (9.45–10.03)	108 ± 3	9.72 (9.54–9.91)	109 ± 2	9.47 (8.94–10.01)	92 ± 5
AT-090	8.38 (7.21–9.56)	75 ± 17	8.05 (7.37–8.72)	95 ± 9	7.46 (6.93–7.99)	85 ± 8	8.53 (8.41–8.65)	105 ± 2	8.70 (8.51–8.89)	112 ± 3	9.18 (8.55–9.81)	93 ± 6
AT-390	9.51 (9.18–9.84)	115 ± 5	7.53 (7.18–7.88)	110 ± 5	8.67 (8.33–9.00)	75 ± 3	8.28 (8.08–8.48)	100 ± 3	8.24 (8.03–8.45)	96 ± 3	8.69 (8.23–9.15)	93 ± 5
AT-312	9.08 (8.74–9.42)	129 ± 5	8.38 (7.56–9.19)	87 ± 7	7.03 (6.63–7.42)	86 ± 6	8.03 (7.83–8.24)	97 ± 3	7.99 (7.70–8.27)	95 ± 5	7.62 (6.93–8.32)	95 ± 9
AT-202	7.72 (6.94–8.51)	92 ± 16	7.31 (6.74–7.88)	82 ± 11	7.96 (7.35–8.58)	75 ± 8	8.03 (7.87–8.20)	101 ± 3	7.82 (7.50–8.13)	97 ± 5	8.56 (7.91–9.21)	103 ± 6
Comp 3	9.51 (9.18–9.84)	115 ± 5	7.81 (7.48–8.14)	105 ± 6	8.37 (8.13–8.62)	80 ± 3	8.59 (8.34–8.84)	90 ± 4	8.48 (8.31–8.65)	109 ± 4	8.85 (7.61–10.09)	72 ± 9
Comp 26	9.08 (8.74–9.42)	129 ± 5	9.21 (8.52–9.90)	88 ± 7	8.27 (8.06–8.47)	88 ± 3	8.41 (8.16–8.66)	73 ± 3	8.90 (8.36–9.43)	58 ± 4	8.80 (8.21–9.39)	62 ± 5
Comp 40	9.84 (9.50–10.17)	101 ± 5	8.88 (8.63–9.14)	109 ± 4	8.76 (8.48–9.05)	83 ± 3	8.89 (8.71–9.08)	72 ± 2	8.94 (8.57–9.30)	81 ± 4	8.79 (7.92–9.66)	64 ± 7
Comp 43	10.69 (10.42–10.96)	114 ± 4	9.48 (9.22–9.75)	94 ± 3	9.10 (8.80–9.40)	89 ± 4	9.36 (9.09–9.62)	93 ± 3	9.01 (8.41–9.61)	95 ± 10	9.39 (8.72–10.06)	65 ± 6

TABLE 1 (Continued)

	Gi1		Gi2		Gi3		GoA		GoB		Gz	
	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}
Comp 22	10.57 (9.95–11.20)	120 ± 6	9.55 (8.83–10.27)	75 ± 4	9.14 (8.88–9.40)	87 ± 3	9.13 (8.87–9.40)	81 ± 4	9.15 (8.94–9.37)	81 ± 2	9.53 (8.33–10.74)	58 ± 7
Comp 29	10.50 (9.99–11.00)	111 ± 5	9.61 (9.02–10.20)	79 ± 7	9.34 (9.10–9.59)	89 ± 3	9.44 (9.15–9.73)	101 ± 4	9.25 (8.75–9.74)	80 ± 5	10.01 (9.33–10.70)	79 ± 6

Note: Values are the mean pEC₅₀ with CL_{95%} and the mean E_{max} with SEM. Ligands whose E_{max} CL_{95%} (reported in Table S3) do not include the N/OFQ E_{max} reference value are highlighted in green. Comp. compound.

bioassays, NOP-β-arrestin 2 interaction) or as low-medium efficacy partial agonists (NOP-G protein interaction and [³⁵S]GTPγS) (Toll et al., 2016) (Table 2), up to almost full agonist in label-free experiments (Camilotto et al., 2025). Thus, these results confirm that in different preparations a partial agonist may behave as partial or full agonist, when the stimulus-response coupling is particularly moderate to high, or even as an antagonist, when the efficiency of the stimulus-response coupling is particularly low (Kenakin, 2014). Interestingly, a study with NOP inducible system experimentally demonstrated this by altering the efficiency of the stimulus-response coupling with the manipulation of the receptor density (McDonald, Barnes, et al., 2003). The Dooley hexapeptides, Ac-RYYRIK-NH₂, consistently displayed higher potency and maximal effects than Ac-RYYRWK-NH₂; this perfectly matches what was reported in the literature (Dooley et al., 1997). In terms of E_{max}, Gi2, Gi3 and GoA displayed the weakest efficacy compared to N/OFQ for all standard peptide partial agonists. Nonetheless, pharmacological parameters of standard peptide agonists align to that obtained by other approaches (Table 2).

From the several NOP receptor non-peptide ligands we characterised in this study, standard small molecules agonists SCH-486757, Ro 65-6570, and MCOPPB showed E_{max} similar to that of the endogenous neuropeptide N/OFQ, thus acting as NOP full agonists. MCOPPB was the most potent agonist, followed by Ro 65-6570 and by SCH-486757. Variations across the transducers were not significant. Of note, MCOPPB, was found to be approximately threefold more potent than N/OFQ across different assays (Ferrari et al., 2017; Hirao et al., 2008). Ro 65-6570 was found to be at least 10-fold less potent than N/OFQ (Ferrari et al., 2017; Wichmann et al., 1999). SCH-486757 is described to be at least 100-fold less potent than the standard (Ferrari et al., 2017; McLeod et al., 2010) (Table 2). On the one hand, these results corroborate the reliability of this assay to fingerprint NOP receptor pharmacology; on the other hand, the different chemotypes did not appear to produce any differential activation across G proteins. Therefore, in order to increase the variety of chemotypes under study, we pharmacologically characterised a series of five different *Astraea* Therapeutics NOP receptor agonists. AT-403 produced the most potent and efficacious profile in the AT series on all six G proteins. This NOP ligand behaves as a highly potent, unbiased full agonist in G protein vs. β-arrestin 2 interaction, as previously reported (Azevedo Neto et al., 2021; Ferrari et al., 2017) (Table 2). AT-090 displayed moderate potency and reveals efficacy nearly the same as N/OFQ. Of interest, AT-090 showed the weakest potency and efficacy at fostering NOP-Gi1 compared to other transducers. Of note, this molecule was found to be a NOP receptor partial agonist (E_{max} approximately 60%) with an unbiased profile when comparing receptor-arrestin and Gβ protein interaction (Asth et al., 2016; Ferrari et al., 2016). In the stimulation of [³⁵S]GTPγS binding (low amplification), its efficacy is low (approximately 20%) but higher (approximately 80%) in calcium mobilisation (with chimeric G proteins, high amplification) (Ferrari et al., 2016) (Table 2). Thus, compared to the other approaches, TRUPATH, seems to emphasise the efficacy of AT-090. A chemically distinct NOP agonist, AT-390, displayed moderate potency and E_{max} similar to that of N/OFQ excepted for the coupling of Gi3,

TABLE 2 Effects of ligands on orthogonal assays.

	Ca ²⁺ (Gα _{q15})		NOP-Gβ		NOP-β arrestin 2	
	CR	E _{max}	CR	E _{max}	CR	E _{max}
N/OFQ	1 ^{a, c, d, f}	100	1 ^{b, c, d}	100	1 ^{b, c, d}	100
N/OFQ(1-13)-NH ₂	2 ^a	96	1 ^e	100	1 ^e	100
UFP-112	3 ^a	103	0.12 ^b	98	0.45 ^b	89
UFP-113	37 ^a	62	0.12 ^b	45	0.04 ^{b, g}	
PWT2-N/OFQ	4 ^f	100	0.19 ^b	110	3 ^b	130
Ac-RYYRIK-NH ₂	24 ^a	59	0.2 ^g	58	0.2 ^g	26
Ac-RYYRWK-NH ₂	7 ^a	58	0.2 ^g	73	0.2 ^g	39
SCH-486757	1047 ^d	102	72 ^d	86	151 ^d	36
Ro 65-6570	10 ^c	95	5 ^b	96	45 ^b	84
MCOPPB	0.5 ^d	102	0.08 ^d	104	0.6 ^d	99
AT-403	4 ^d	100	2 ^d	99	4 ^d	77
AT-090	9 ^c	80	18 ^c	49	11 ^c	57
AT-390	11	100	2	49	5	42
AT-312	8	102	10	50	32	47
AT-202	24	102	251	77	200	63
Comp 3	3	95	8 ^e	87	5 ^e	65
Comp 26	3	96	13 ^e	103	13 ^e	95
Comp 40	4	100	0.06 ^e	103	5 ^e	93
Comp 43	0.8	104	0.1 ^e	93	2 ^e	93
Comp 22	1	103	0.6 ^e	108	1 ^e	103
Comp 29	0.9	105	0.2 ^e	108	0.8 ^e	93

Note: CR, EC₅₀ of a given ligand divided by EC₅₀ of corresponding standard (N/OFQ or N/OFQ(1-13)-NH₂); E_{max} are presented setting standards to 100%.

^a(Camarda et al., 2009).

^b(Malfacini et al., 2015).

^c(Ferrari et al., 2016).

^d(Ferrari et al., 2017).

^e(Pacífico et al., 2020).

^f(Rizzi et al., 2014).

^gUFP-113 CR was computed comparing the molar antagonist's potency to the standard's EC₅₀.

where AT-390 efficacy was significantly lower than that of the standard. Previous reports indicate that AT-390 behaves as full agonist with approximately three-fold lower potency than AT-403 (Arcuri et al., 2018), in partial agreement with these datasets. NOP receptor agonist AT-312 preserved full efficacy across every G protein, with moderate values of potency being lower on Gi3 and Gz. Literature data showed full agonist activity and approximately 10-fold lower potency than N/OFQ in the stimulation of [³⁵S] GTPγS binding; this molecule also behave as a fairly potent partial agonist at the μ opioid receptor (Zaveri et al., 2018) (Table 2). AT-202 (SR16835) displayed high E_{max} yet the weakest potency of the group, in line with previous datasets (Ferrari et al., 2017) (Table 2). Across these five non-peptide agonists, a clear potency–efficacy continuum emerges across transducers activated by the NOP receptor. Ligands, such as AT-312, whose activity is proposed to be similar at the NOP and μ opioid receptor, may be instrumental to answer questions on the nature of G protein bias within members of the same GPCR subfamily.

Unfortunately, concerning G protein discrimination, none of the small molecules tested exhibited any notable bias among the various Gα subtypes (Figure 6). N/OFQ(1-13)-NH₂ is the smaller fragment of N/OFQ preserving all endogenous peptide's features (Guerrini et al., 1997); therefore, Pacífico and colleagues (Pacífico et al., 2020) reported a rather large collection of N/OFQ(1-13)-NH₂ derivatives with enhanced/reduced potency towards NOP-G protein interaction and biased/unbiased behaviour versus NOP-β-arrestin 2 recruitment. We shortlisted six derivatives, Compound 3 in which Gly³ was substituted by Ala, displayed a homogenous loss of about 10-fold potency compared to N/OFQ, with E_{max} lower on Gi3. A slightly higher loss in potency in FSK-induced cAMP was detected by Reinscheid and colleagues (Reinscheid et al., 1996). Pacífico and colleagues showed eightfold loss of potency with E_{max} near that of the standard. Compound 26 instead of Gly² had the D-Ala non-natural amino acid; in the manuscript by Reinscheid, this modification would mimic the conformation induced by the natural Gly residue having similar functional and binding features as N/OFQ. This is in line with the

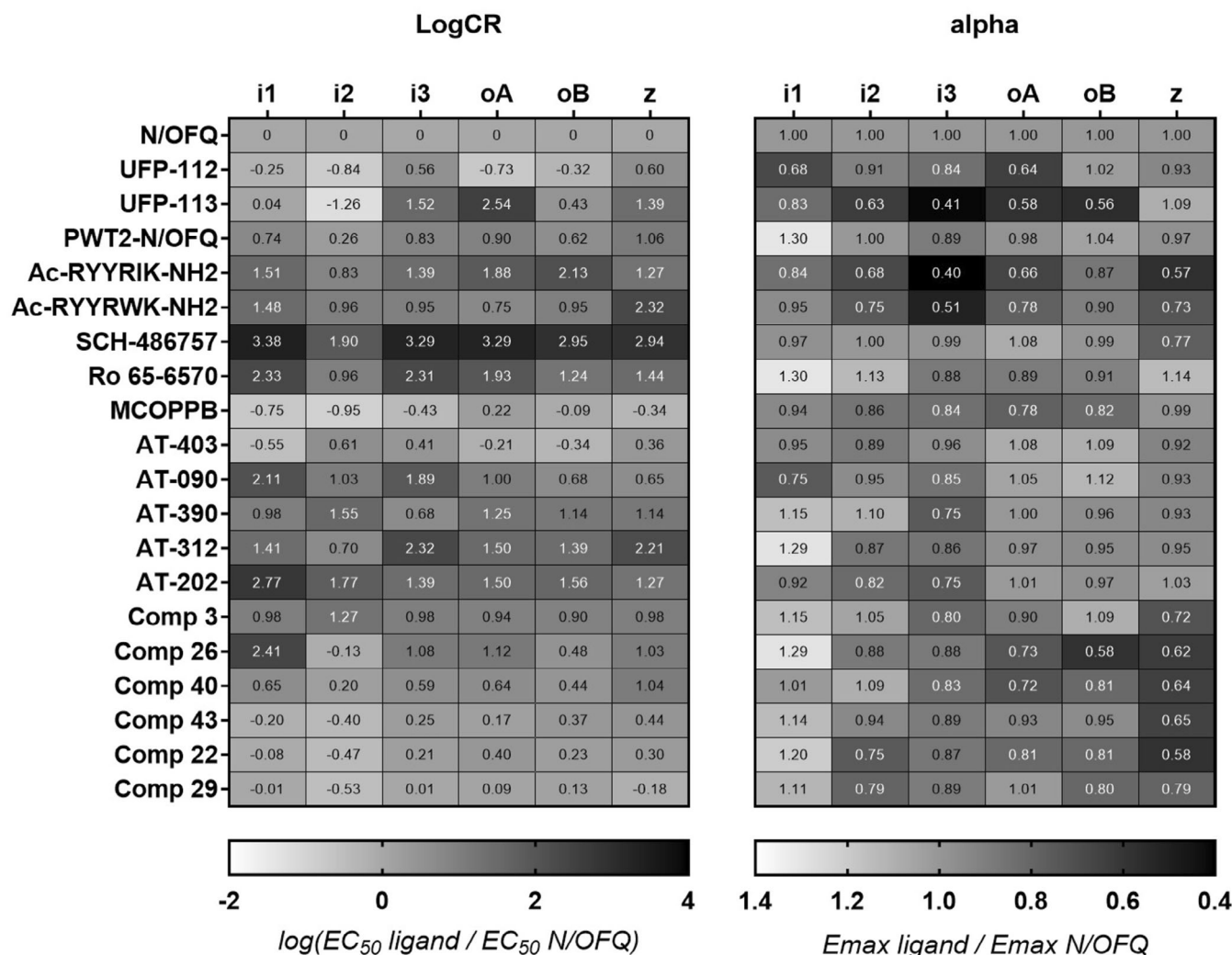


FIGURE 6 Heat maps summarising NOP receptor coupling to individual inhibitory $G\alpha$ subunits, showing ligand-specific potency and efficacy profiles. (a) Potency, expressed as $\log(EC_{50}$ ligand / EC_{50} N/OFQ) (log CR). (b) Efficacy, expressed as E_{max} ligand / E_{max} N/OFQ ($\alpha = 1$ for N/OFQ). White/light grey shades denote higher potency or efficacy than N/OFQ; black/dark grey shades denote lower values. Data are mean from five independent experiments carried out in duplicate. Comp, compound.

approximately 13-fold loss of pEC_{50} reported by Pacifico and co-workers. In the TRUPATH assay, the potency was indeed lower on all transducers; while the E_{max} was significantly diminished on some of the transducers (Gi3, GoA, GoB and Gz). All remaining peptide derivatives were described as capable of increasing their functional potency unbiasedly (Compound 22 and Compound 29, carrying single aa alteration in the message domain) or with a certain bias towards G protein (Compound 40 and Compound 43, both harbouring modification of the address domain). Compound 40, for instance, was very potent on all six transducers, while displayed a slight although significant diminishment of E_{max} on Gi3, GoA, GoB and Gz. In the paper of Pacifico, the compound showed that E_{max} always equal to that of the standard, and the bias was caused by variations in terms of potency. As for Compound 43, it was very potent across all transducers (9.01–10.69) with E_{max} lower only at Gz. High potency was measured also in the original manuscript. Finally, Compound 22 and Compound 29 behaved as very high potency agonists with E_{max} slightly although significantly

lower than N/OFQ (on Gi2, GoA, GoB, Gz for Compound 22 and on GoB and Gz for Compound 29). Such molecules were highly potent unbiased full agonists in the original paper (Pacifico et al., 2020). Altogether, the present results suggest that, at least for the NOP receptor G protein vs. arrestin, bias does not predict functional selectivity within G proteins (Tables 1 and 2).

In Figure 6, UFP-113 and Compound 26 showed the most pronounced divergence across transducers. These outcomes align with modifications in the N-terminal message domain (Phe¹-Gly²), which is critical for initiating activation, as demonstrated by structure–activity relationship (SAR) studies (Guerrini et al., 1997; Pacifico et al., 2020) and visualised in inactive NOP structures bound to antagonists (Miller et al., 2015; Thompson et al., 2012). Cryo-EM analyses of μ , δ , κ opioid and NOP receptors in complex with their endogenous peptides and Gi proteins (Wang et al., 2023) highlight the conserved features of GPCR activation, including inward movement of TM5, outward displacement of TM6, rotation of TM7 and the rearrangement of the

conserved toggle switch (W6.48), of DRY and NPxxY motifs. Within this conserved framework, ligands, such as UFP-113, Compound 26, and to a lesser extent AT-312 may fail to efficiently stabilise TM7 rearrangements required for selective engagement of some transducers, thereby contributing to the relatively uniform coupling profile observed here.

Taken together, the apparent absence of robust bias at NOP may arise from both receptor conformational features, lack of chemical diversity of ligands and assay sensitivity. Yet the contrasting signatures of UFP-113 and Compound 26 highlight that deviations can manifest either as uneven potency (UFP-113) or as broad potency loss with selective efficacy deficits (Comp 26). At present, it is not possible to disentangle whether these differences reflect intrinsic receptor properties or limitations of the system, and complementary approaches will be required to determine whether NOP is inherently less selective across G proteins or whether subtle differences are simply masked by methodological constraints.

Interestingly, μ receptor ligands can display marked, structurally validated divergence across transducers (Qu et al., 2023). In comparison, the discrepancies observed for NOP ligands were modest. κ Receptor profiling revealed a degree of potency bias of similar magnitude to NOP (El Daibani et al., 2023; Olsen et al., 2020). For the δ receptor, uniform efficacy was reported with only mild potency divergence (Varga et al., 2025). These comparisons suggest that the lack of $G\alpha$ subtype preference or selectivity observed at NOP is not unique but part of a broader continuum across opioid receptors. Nevertheless, the engineered nature of the TRUPATH sensors and the relatively low signal amplitude warrant caution, as these may limit sensitivity to subtle differences. In addition, the recombinant expression system reduces direct translational relevance. The use of orthogonal assays such as One-Go (Janicot & Garcia-Marcos, 2024) and studies in native systems will be valuable to refine these observations.

There are caveats of the present study we need to underline. First, the TRUPATH assay was performed in heterologous systems, where receptor density and G protein level may not fully reproduce the native environment. Moreover, the TRUPATH platform itself is characterised by a relatively modest signal-to-noise ratio, a recognised limitation inherent to this BRET-based setup (Olsen et al., 2020). Nevertheless, we attempted to optimise experimental parameters, such as receptor and sensor expression, and emission integration time, achieving reproducible curves and consistent agonist rank orders of potency across replicates and transducers, and in line with the results obtained in different assays. Second, our data do not demonstrate that all the effects of the ligands are exclusively because of the activation of the NOP receptor; however, based on the following considerations, we are confident that this is the case: (i) most of the ligand tested have been shown in previous studies to be highly selective for the NOP receptor, and for some of them, this has been confirmed in NOP receptor knockout studies, (ii) the potency values of most of the compounds tested with TRUPATH are high or very high, thus strongly suggesting on-target action; (iii) the signal to noise ratio of the TRUPATH assay is at best modest, this makes the possibility that receptors endogenously expressed by HEK293 may interfere with the

signal coming from the overexpressed NOP receptor unlikely. Together, these points provide strong confidence that the pharmacological effects we report solely reflect selective NOP receptor activation.

In conclusion, although strong NOP receptor biased ligands across G protein isoforms still need to be detected, this study aligns NOP to the other opioid receptors and implements the pharmacology of this class of GPCR with novel tools to begin the dissection of NOP signalling outputs at the single transducer level.

AUTHOR CONTRIBUTIONS

Davide Malfacini: Conceptualization; methodology; supervision; formal analysis; data curation; writing—original draft; writing—review and editing; funding acquisition. **Riccardo Camilotto:** Investigation; data curation; formal analysis; writing—review and editing. **Michele Maritan:** Investigation; writing—review and editing; formal analysis; data curation. **Erika Morrone:** Investigation; writing—review and editing; formal analysis; data curation. **Stephen Afriyie Boateng:** Data curation; formal analysis; investigation; writing—review and editing. **Anna Vacilotto:** Investigation; data curation; formal analysis; writing—review and editing. **Salvatore Pacifico:** Resources; investigation; validation; writing—review and editing. **Remo Guerrini:** Investigation; validation; writing—review and editing; resources; funding acquisition. **Nurulain T. Zaveri:** Resources; validation; writing—review and editing. **Girolamo Calo:** Conceptualization; funding acquisition; data curation; supervision; writing—review and editing; resources.

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CONFLICT OF INTEREST STATEMENT

Dr Nurulain T. Zaveri is an employee and shareholder of Astraea Therapeutics (Mountain View, CA, USA), which provided several of the compounds used in this study. All other authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are included in this article and its Supporting Information files. Additional datasets are available from the corresponding author upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This study was conducted and reported in accordance with the *British Journal of Pharmacology* guidelines on transparency and scientific rigour. Experimental procedures, materials and analytical methods are described in sufficient detail to allow replication, and key resources

(including cell lines and reagents) are identified where applicable. Biological replicates, statistical approaches and data handling procedures are reported in this section.

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