Differential In Vitro Pharmacological Profiles of Structurally Diverse Nociceptin Receptor Agonists in Activating G Protein and Beta-Arrestin Signaling at the Human Nociceptin Opioid Receptor

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ABSTRACT

Agonists at the nociceptin opioid peptide receptor (NOP) are under investigation as therapeutics for nonaddicting analgesia, opioid use disorder, Parkinson’s disease, and other indications. NOP full and partial agonists have both been of interest, particularly since NOP partial agonists show a reduced propensity for behavioral disruption than NOP full agonists. Here, we investigated the in vitro pharmacological properties of chemically diverse NOP receptor agonists in assays measuring functional activation of the NOP receptor such as guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) binding, cAMP inhibition, G protein–coupled inwardly rectifying potassium (GIRK) channel activation, phosphorylation, beta-arrestin recruitment and receptor internalization. When normalized to the efficacy of the natural agonist nociceptin/orphanin FQ (N/OFQ), we found that different functional assays that measure intrinsic activity produce inconsistent levels of agonist efficacy, particularly for ligands that were partial agonists. Agonist efficacy obtained in the GTPγS assay tended to be lower than that in the cAMP and GIRK assays. These structurally diverse NOP agonists also showed differential receptor phosphorylation profiles at the phosphosites we examined and induced varying levels of receptor internalization. Interestingly, although the rank order for beta-arrestin recruitment by these NOP agonists was consistent with their ability to induce receptor internalization, their phosphorylation signatures at the time point we investigated were not indicative of the levels of beta-arrestin recruitment or internalization induced by these agonists. It is possible that other phosphorylation sites, yet to be identified, drive the recruitment of NOP receptor ensembles and subsequent receptor trafficking by some nonpeptide NOP agonists. These findings potentially help understand NOP agonist pharmacology in the context of ligand-activated receptor trafficking.

SIGNIFICANCE STATEMENT

Chemically diverse agonist ligands at the nociceptin opioid receptor G protein–coupled receptor showed differential efficacy for activating downstream events after receptor binding, in a suite of functional assays measuring guanosine 5'-O-[gamma-thio]triphosphate binding, cAMP inhibition, G protein–coupled inwardly rectifying potassium channel activation, beta-arrestin recruitment, receptor internalization and receptor phosphorylation. These analyses provide a context for understanding nociceptin opioid peptide receptor (NOP) agonist pharmacology driven by ligand-induced differential NOP receptor signaling.
decrease in the intracellular levels of cAMP. The Gβγ subunits couple to and activate GIRK channels, increasing potassium current (Connor et al., 1996a; Vaughan and Christie, 1996) and suppressing voltage-dependent calcium channels (Beedle et al., 2004; Connor et al., 1996b), resulting in cell hyperpolarization. This channel modulation upon agonist binding to the NOP receptor is consistent with the inhibitory actions of N/OFQ and agonists on neuronal activity and neurotransmitter release.

Like other GPCRs, NOP signaling and function are regulated by phosphorylation, internalization, and receptor desensitization (see Donica et al., 2013 for an excellent review). The Gβγ subunits that dissociate from the G protein–receptor complex recruit G protein receptor kinases (GRKs) for phosphorylation of GPCR intracellular domains. The agonist-bound phosphorylated GPCR undergoes a conformational change, allowing arrestin recruitment, receptor internalization, and desensitization, as shown for the MOR receptor (Williams et al., 2013). The NOP receptor contains several serine, threonine and tyrosine residues within its intracellular loops and C-terminal domain that can act as substrates for GRK-mediated NOP receptor phosphorylation. Using phosphospecific antibodies, Mann et al. (2019) recently showed that NOP C-terminal residues Ser346, Ser351, and Thr362/Ser363 were phosphorylated after treatment with diverse NOP agonists in a spatial and temporal manner, with Ser346 being the first site of phosphorylation. GRK2 and GRK3 were shown to be important for agonist-induced phosphorylation and receptor desensitization (Mandyam et al., 2002; Mandyam et al., 2003; Mann et al., 2019; Thakker and Standifer, 2002). Furthermore, alanine mutation of Ser363, which is phosphorylated by GRK3, reduced β-arrestin2 recruitment and NOP internalization (Zhang et al., 2012).

Arrestin recruitment by nonpeptide NOP ligands has been investigated in a limited number of studies, some of which included NOP full agonist AT-403 and NOP partial agonists AT-090 and AT-004 characterized here (Chang et al., 2015; Ferrari et al., 2016; Ferrari et al., 2017; Malfacini et al., 2015). NOP full agonist AT-403 showed significant arrestin recruitment but also activated G protein–mediated recruitment to the same extent, similar to the endogenous agonist N/OFQ (Ferrari et al., 2017). On the other hand, NOP partial agonist AT-090 [21% agonist efficacy in the GTPγS binding assay; Ferrari et al., 2016] showed significant efficacy for arrestin recruitment (Ferrari et al., 2016), unlike NOP partial agonist AT-004, which also showed ~20% agonist efficacy in GTPγS binding but was nearly inactive in recruiting β-arrestin (Chang et al., 2015; Ferrari et al., 2016; Malfacini et al., 2015). These NOP ligands have not been previously characterized for their effects on NOP receptor phosphorylation or internalization, but their differential efficacy of arrestin recruitment suggests that chemically diverse NOP agonists can have differential effects on NOP receptor signaling and trafficking. Here, we profiled phosphorylation signatures, arrestin recruitment, and receptor internalization induced by diverse NOP agonists. We also characterized the intrinsic activity of these ligands in mediating G protein–mediated downstream events. Commonly used assays to measure functional efficacy of opioid ligands in G protein–mediated signaling include simple proximal measurements of G protein recruitment like the GTPγS binding assay (McDonald and Lambert, 2010; Sim et al., 1996) as well as other assays of downstream signaling such as cAMP inhibition (Hill et al., 2010; Knapman et al., 2014), calcium release (Camarda et al., 2009; Coward et al., 1999), and GIRK activation (Parsons and Hirasa, 2011). These assays often provide inconsistent measures of intrinsic activity for the same ligand, and such inconsistencies become particularly significant when agonist efficacy obtained in nonamplified assay systems such as GTPγS assays are compared with that obtained in amplified assay systems such as the cAMP and GIRK assays. Levels of agonist efficacy seen in different G protein assays can also be confounded by receptor reserve, as agonists can produce a maximum response by occupying only a fraction of receptors (Selley et al., 1998; Traynor and Nahorski, 1995; Gillis et al., 2020; Kelly, 2013). Receptor reserve can also account for signal amplification and confound experimental measures of agonist efficacy, particularly when characterizing partial agonists in heterologous transfected cells (Adham et al., 1993; Baker et al., 2000; Selley et al., 1998; Traynor and Nahorski, 1995).

Here, we investigated the functional efficacy of several selective NOP agonists from our NOP ligand library, using multiple G protein assays and determined the range of intrinsic activity obtained among various assays for the same ligand. We also investigated the phosphorylation profiles, arrestin recruitment, and receptor internalization induced by these selective NOP agonists and found differential ligand-induced NOP receptor signaling and trafficking among the NOP ligands we examined.

**Materials and Methods**

**Chemical Compounds.** NOP-selective ligands AT-004 (Kamaklanu et al., 2020), AT-090 (Ferrari et al., 2016), AT-200 (Zaveeri et al., 2004), AT-312 (Zaveri et al., 2018b), AT-390 (Arcuri et al., 2018), and AT-403 (Arcuri et al., 2018) were synthesized at Astraea Therapeutics and have been previously reported. Stock solutions of N/OFQ at a 1 mM concentration and [D-Ala2, N-MePhe4, Gly-ol]–enkephalin (DAMGO) at a 10 mM concentration were prepared in deionized water, whereas those of the AT ligands were prepared in DMSO (10 mM) and diluted with water or assay medium to the desired concentration.

**Cell Culture and Transfection.** All cells were maintained in a 5% CO2 incubator under saturated humidity and 37°C. Human NOP, or human opioid receptor–like receptor (hORL), and human MOR (hMOR) opioid receptors were stably expressed individually in CHO cells as reported previously (Zaveri et al., 2001). The hORL CHO cells were cultured in 150 mm tissue culture dishes (Corning, New York City, NY) in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 400 µg/ml G418. The hMOR CHO cells were cultured in 50% F12/DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 400 µg/ml G418. The AtT-20/D16v-F2 (AtT-20) cells used in GIRK assays were a gift from Dr. Bryan Roth and cultured in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The HTLA cells [a human embryonic kidney 293 (HEK293) cell line stably expressing a tobacco etch virus protease fusion gene used for β-arrestin assays were a gift from Dr. Bryan Roth and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml hygromycin B, and 2 µg/ml puromycin. Then, the HTLA cells were transiently transfected with NOP or MOR receptors overnight before the β-arrestin assays were carried out.
out (see details below). For cAMP assays, the hORL and hMOR CHO cells were transiently transfected with a cAMP23F plasmid (Promega, Madison, WI) overnight before the cAMP assays were carried out (see details below).

Membrane Preparation. The hORL and hMOR CHO cells were grown to confluence and harvested for membrane preparation. The membrane was prepared in 50 mM Tris buffer (pH 7.4). Cells were scraped off 150-mm culture dishes and centrifuged at 500g for 15 minutes. The cell pellet was homogenized in 50 mM Tris with a Fisher Scientific PowerGen 125 rotor-stator type homogenizer, centrifuged at 20,000g for 25 minutes, washed and recentrifuged once more at 20,000g for 25 minutes, aliquoted at a concentration of 2 mg/ml protein per vial for hORL and 3 mg/ml protein per vial for hMOR, and stored in a −80°C freezer until use.

Receptor Binding Assay. AT compounds were dissolved in 100% DMSO to a concentration of 10 mM. The binding assays were performed in 96-well polystyrene plates using six concentrations of each test compound (final concentration of 10 μM to 0.1 nM in 10-fold dilutions) in triplicate, with each well containing 100 μl of compound and 100 μl of tritiated ligands [3H]DAMGO (48.0 Ci/mmol, Kd 0.69 nM for MOR) or [3H]N/OFQ (130 Ci/mmol, Kd 0.065 nM for NOP). Non-specific binding was determined using 1 μM of the unlabeled nociceptin for NOP and 1 μM of unlabeled DAMGO for MOR. Assays were initiated by the addition of 800 μl of membrane per well, after which the samples were incubated for 60 minutes at 25°C in a total volume of 1.0 ml. In NOP receptor experiments, 1 mg/ml bovine serum albumin was added to the compound dilution buffer. The incubation was terminated by rapid filtration through 0.05% polystyrene-imino-soaked glass fiber filters (GF/C Filtermat A, Perkin Elmer) on a Tomtec Mach III cell harvester and washed 5 times with 0.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4) buffer. The filters were dried overnight and soaked with scintillation cocktail before counting on a Wallac Beta plate 1205 liquid scintillation counter. Radioactivity was determined as counts per minute. IC50 values were determined using at least six concentrations of test compound and calculated using the variable slope curve-fitting method in GraphPad Prism 6.0 software (ISI, San Diego, CA). Kd values were determined by the method of Cheng and Prusoff (Cheng and Prusoff, 1973).

[35S] GTP-S Binding Assay. [35S] GTP-S binding assays were conduced as previously described (Khryonov et al., 2011; Toll et al., 2009). In brief, membranes (2 mg/ml protein for NOP and 3 mg/ml for MOR) were incubated for 60 minutes at 25°C with [35S] GTP-S (50 μM), GDP (10 μM) and the appropriate compound, in a total volume of 1.0 ml Buffer A containing 20 mM HEPES, 10 mM MgCl2, and 100 mM NaCl (pH 7.4). Samples were filtered over glass fiber filters and counted as described for the binding assays. Agonist efficacy (Emax) is reported as percentage of maximal stimulation relative to the standard full agonist N/OFQ or DAMGO and calculated as maximal stimulation by test compound divided by maximal stimulation by standard full agonist 100. The EC50 values were calculated using the variable slope dose-response curve fitting method in GraphPad Prism 6.0 software.

GloSensor cAMP Assay. The plasmid pGloSensor-23F-cAMP was transfected into hORL and hMOR CHO cells (1.5 × 10⁴ cells) using the Fugene 6 HD reagent (Promega, Madison, WI). Twenty-four hours after the transfection, the cells were harvested, suspended in the culture medium, and seeded (100 μl/well at a concentration of 1 × 10⁵ cells/ml) into 96-well white wall microtiter plates. After being incubated at 37°C for 24 hours in a humidified environment containing 5% CO₂, the medium was replaced with DMEM containing 6% GloSensor cAMP substrate (Promega, Madison, WI). After 2 hours of additional incubation at room temperature, different AT compounds at five concentrations (final concentration of 1 μM to 0.1 nM in 10-fold dilutions) were added into the wells for 15 minutes of incubation, followed by the addition of 10 μM of forskolin (final concentration) to stimulate cAMP synthesis. Then, the dynamic luminescence signal of the wells was detected immediately using a FluorStar Optima plate reader (BMG, Gütersloh, Germany) for 30 consecutive minutes at a rate of 1 read per well per second. Subsequently, to calculate Emax of the standard agonist, cAMP inhibition induced by 1 μM of the positive control N/OFQ or DAMGO in the presence of 10 μM of forskolin (i.e., the max level of cAMP signal in the presence of 1 μM N/OFQ or DAMGO plus 10 μM forskolin divided by the max level of cAMP signal in the presence of 10 μM forskolin only) was used as 100%, and the level of cAMP inhibition induced by test compounds was normalized to that of N/OFQ or DAMGO (net cAMP inhibition by 1 μM full agonist × 100). In addition, the IC50 of different compounds in inhibiting cAMP synthesis was calculated using the variable slope dose-response curve fitting method in GraphPad Prism 6.0 software. To minimize intra-assay variations caused by transient transfections carried out on different days, the key time points of the assay, including the time of transfection, the time of compound, forskolin, and reagent addition, and the time of signal readout on the plate reader, were kept the same among different days. Furthermore, only the experiments showing comparable baseline (negative control) and positive control (N/OFQ or DAMGO) signals were included in the comparative analyses to ensure intra-assay consistency.

GIRK Assay. AtT-20 cells stably expressing hNOP or hMOR receptors were plated in 96-well black, clear bottom plates covered with poly-l-lysine. Plates were kept for 48 hours at 37°C and 5% CO₂. Assay was performed as previously described (Günther et al., 2016) using Hanks' balanced salt solution, buffered with HEPES 20 mM (pH 7.4) as standard buffer. The membrane potential dye FMP (FLIPR Membrane Potential kit BLUE, Molecular Devices, Biberach, Germany) was reconstituted according to manufacturer's instructions. Test compounds were prepared right before assay measurements at the concentration indicated. The assay was performed in a FlexStation 3 microplate reader (Molecular Devices, Biberach, Germany).

Western Blot Analysis (Receptor Phosphorylation). Stably transfected HEK293 cells expressing human NOP receptors were plated onto poly-l-lysine–coated 60-mm dishes and grown for 2 days to 80% confluency. After incubating with 10 μM N/OFQ or agonists for 10 minutes at 37°C, the cells were lysed with a detergent buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 10 mM NaF; 10 mM disodium pyrophosphate; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS) in the presence of protease and phosphatase inhibitors. After 30 minutes of centrifugation at 4°C, human influenza hemagglutinin (HA)-tagged NOP receptors were enriched using anti-HA–agarose beads. Samples were then inverted for 1.5 hours at 4°C. After washing; proteins were eluted using an SDS sample buffer for 30 minutes at 50°C, and proteins were separated on 7.5% or 12% SDS-polyacrylamide gels. After electrophoretion, membranes were incubated with either 0.1 g/ml anti-human phosphorilated S346 (5034), anti–human phosphorilated S351 (4876), or anti–human phosphorilated T362/S363 (4874) antibodies overnight at 4°C, followed by detection of bound antibodies using an enhanced chemiluminescence buffer (Thermo Fisher Scientific, Schwerte, Germany). Blots were subsequently stripped and reprobed with the phosphorylation-independent anti–NOP receptor antibody (4871) or anti-HA antibody (0631) to ensure equal loading of the gels.

Analysis of NOP Receptor Internalization. Stably transfected cells expressing HA-tagged human NOP receptors were plated onto poly-l-lysine–coated coverslips and grown overnight. Cells were incubated with rabbit anti-HA antibody (0631) in serum-free medium for 2 hours at 4°C. After 60 minutes of agonist exposure at room temperature, cells were fixed with 4% paraformaldehyde and 0.2% picric acid in a phosphate buffer (pH 6.9) for 30 minutes at room temperature. Subsequently, cells were washed several times with a phosphate buffer (22.6 ml/L 1M NaH2PO4•H2O; 77.4 ml/L 1M Na2HPO4•H2O; 0.1% Triton X-100, pH 7.4), permeabilized, and then incubated with an Alexa488-coupled goat anti-rabbit antibody (Invitrogen, Darmstadt, Germany). Cells were mounted, and the internalization of receptors was examined using a Zeiss LSM510 META laser scanning confocal microscope (Jena, Germany).
For quantitative internalization assays, cells were plated onto 24-well plates and grown overnight. Then, the cells were preincubated with anti-HA antibody (0631) for 2 hours at 4°C and exposed to agonists for 60 minutes at 37°C. Subsequently, cells were fixed for 45 minutes at room temperature, washed three times with PBS, and incubated with a peroxidase-conjugated secondary antibody (Santa Cruz, Heidelberg, Germany). After washing, ABTS substrate [2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonaphthalene salt] was added, and optical density was measured at 405 nm using a FlexStation 3 microplate reader (Molecular Devices, Waltham, MA) and then seeded into white flat-bottom 96-well tissue culture plates (Corning, New York City, NY). On the following day when cells grew to 80% confluency (day 2), the cells were transfected with human NOP or MOR receptors using the FuGene 6 HD transfection reagent (Promega, Madison, WI) following the protocol provided by the manufacturer. The ratio of DNA plasmid to FuGene 6 HD transfection reagent in the transfection was 1 μg DNA:6 μl FuGene 6 HD. Thirty micrograms of DNA was used for the transfection of cells in each culture dish. After 24 hours of transfection (day 3), transfected cells were harvested by trypsinization (Trypsin-EDTA, Gibco, Thermo Fisher Scientific, Waltham, MA) and then seeded into white flat-bottom 96-well tissue culture plates (Corning, New York City, NY) at 5 × 10^4 cells per well. Twenty-four hours later (day 4), the cells were treated with AT compounds or positive controls (DAMGO or N/OFQ for MOR and NOP, respectively) for 18 hours at 37°C. On day 5, the luciferase signal in each well was detected by using a Bright Glo Luciferase Reporter Assay (Promega, Madison, WI) on a plate reader (BMG, FluoStar Optima, Giessen, Germany). The level of β-arrestin recruitment was determined by subtracting the baseline signal (medium only) from the signal detected in the presence of compound. Furthermore, to calculate percentage of β-arrestin recruitment induced by the positive control N/OFQ (1 μM) was used as 100%, and the amount of β-arrestin recruitment induced by AT compounds was normalized to that of N/OFQ and DAMGO. In addition, the EC50 values were calculated using the variable slope dose-response curve fitting method in GraphPad Prism 6.0 software. Each experiment was repeated at least three times, with each drug concentration in quintuplicate wells to obtain and report the average value ± S.D. Similar to our approach in the cAMP inhibition assay, we carried out the operations of the β-arrestin recruitment assay at fixed time points to minimize intra-assay variations. We also only included the experiments showing comparable baseline (negative control) and positive control (N/OFQ or DAMGO) signals for comparison.

Data Analysis. Experimental data in Tables 1–3 are expressed as means ± S.D. EC50 and EC50 values were calculated with GraphPad Prism 6.0 (GraphPad software, La Jolla, CA) using the respective curve-fitting methods indicated in the Materials and Methods. S.D. was derived using the S.D. function (stdev) in Excel software. For Western blot assays, the detected protein bands were quantified using ImageJ version 1.47 software (National Institutes of Health, Bethesda, MD). Statistical analysis for Fig. 3C was carried out with Student’s t test. A P value of <0.05 was considered statistically significant. Since this was an exploratory study, a graphical representation was used to compare ligand efficacies between β-arrestin recruitment and internalization, as shown in Fig. 5.

Results

Binding Affinity of AT Compounds for NOP and MOR Receptors. All tested NOP agonists (Fig. 1) showed high binding affinity with Kᵦ values ranging from 0.3 to 9.8 nM at the recombinant NOP receptor and from 6.0 to 375.5 nM at the recombinant MOR receptor expressed in CHO cells (Table 1). Ligands were grouped into highly selective NOP ligands with NOP selectivity >35-fold over the MOR receptor (AT-403, AT-390, and AT-004) and modestly selective NOP ligands with NOP selectivity ranging from 17- to 26-fold (AT-200, AT-312, and AT-090) over the MOR receptor.

Intrinsic Activity in the GTP·S Binding Assay at NOP and MOR Receptors. Agonist potency and efficacy were measured in the GTP·S binding assay using membranes from CHO cells expressing human NOP or MOR receptors. Percentage of stimulation was determined by normalization to maximum activity elicited by standard full agonists N/OFQ and DAMGO. At NOP, AT-312, AT-390, and AT-403 displayed full agonist efficacy (~100% of the standard N/OFQ), whereas AT-200 > AT-004 > AT-090 showed less than 50% efficacy compared with N/OFQ (Table 2; Supplemental Figure 1). Based on this, the compounds were grouped into NOP full agonists (AT-312, AT-390, and AT-403) and NOP partial agonists (AT-004, AT-200, and AT-090) (Table 2). With respect to potency, the EC50 values of all compounds at NOP were consistently greater than their Kᵦ values (Adapa and Toll, 1997). At MOR, all compounds showed significantly lower potency as well as lower efficacy compared with standard agonist DAMGO, consistent with their lower binding affinity at MOR.

cAMP Assay at NOP and MOR Receptors. In hORL or hMOR CHO cells transiently expressing the cAMP plasmid, both N/OFQ and DAMGO decreased forskolin-induced cAMP accumulation, yielding potencies (EC50) of 2.2 nM for N/OFQ and 9.1 nM for DAMGO, respectively (Table 2; see also graphs of cAMP experiments in Supplemental Fig. 2). All AT compounds displayed high efficacy (85%–106%) relative to N/OFQ at the NOP receptor, in rank order AT-403 > AT-090 > AT-312 > AT-004 > AT-200 > AT-090 (Table 2). Notably, the potencies for all the compounds in the NOP cAMP assay were consistently higher compared with those obtained in the GTP·S binding assay (Table 2). Furthermore, although the NOP full agonists in the GTP·S assay (AT-312, AT-390, and AT-403) also showed full agonist efficacy in the cAMP assay, the AT compounds that showed partial agonist efficacy at NOP in the GTP·S assay (AT-040, AT-090, and AT-200) all showed nearly full agonist efficacy, but lower potencies than N/OFQ (Table 2), with exception of AT-090, which showed significantly increased potency and full agonist efficacy in the cAMP assay compared with the GTP·S binding assay.

At the MOR receptor, efficacy of the compounds obtained in the cAMP assay was also higher than that obtained via the GTP·S binding assay (Table 2), although the differences were less pronounced since these compounds had lower potency and efficacy at the MOR overall.

GIRK Channel Activation by AT Compounds at NOP and MOR Receptors. In the GIRK assay in AtT-20 cells expressing NOP or MOR receptor, both N/OFQ and DAMGO induced agonist-dependent membrane hyperpolarization in a concentration-dependent manner, yielding EC50 values of 1.2 nM for N/OFQ and 1.0 nM for DAMGO (Table 2), respectively, consistent with potencies we previously reported for these agonists (Mann et al., 2019). The AT compounds tested in this assay all displayed full agonist efficacy (95%–105%) at NOP receptor compared with N/OFQ, in rank order AT-312 > AT-200 > AT-004 (see also graphs in Supplemental Fig. 3). Moreover, similar to the observations in the cAMP assay, the EC50 (potency) values from the GIRK assay at the NOP
receptor were consistently shifted to lower values (higher potency) compared with those obtained in the GTP\textsubscript{S} binding assay (i.e., the compounds appeared more potent in the GTP\textsubscript{S} assay than in the GTP\textsubscript{S} functional assay). Furthermore, the efficacies of the compounds obtained from the GIRK assay were also higher than those obtained in the GTP\textsubscript{S} binding assay. For example, AT-004 appears to be a low-ef
cacy partial agonist at NOP in the GTP\textsubscript{S} assay but showed nearly full agonist ef
cacy and higher potency in the NOP GIRK assay.

The differences in the levels of intrinsic activity at NOP obtained from the GTP\textsubscript{S} binding assay, GIRK assay, and cAMP assay for selected NOP ligands (AT-004, AT-200, and AT-312) are shown in Fig. 2. It can be seen that the intrinsic activity observed in the GIRK and cAMP assays are significantly higher than the stimulation achieved by these compounds in the GTP\textsubscript{S} binding assay, especially for compounds behaving as partial agonists in the GTP\textsubscript{S} assay, i.e., a stimulation level of 20\%–60\% (AT-004 and AT-200). AT-090 (Table 2) also showed partial agonist ef
cacy in the GTP\textsubscript{S} assay (21\% stimulation) but showed full agonist efficacy (104\% stimulation) and significantly higher potency in the cAMP assay. NOP partial agonists AT-004 and AT-200 also showed significantly higher po
tencies in the GIRK assay compared with that in the GTP\textsubscript{S} assay (Table 2).

On the other hand, the compounds displayed lower efficacy at the MOR receptor in the GIRK assay, in rank order AT-200 > AT-312 > AT-004, compared with DAMGO, and the efficacy and potency obtained from the GIRK assay appeared to be somewhat consistent with those observed in the GTP\textsubscript{S} assay. This is particularly true for the low-ef
cacy partial agonist AT-004, which showed low ef
cacy and poor potency at MOR in the GTP\textsubscript{S} assay and showed no GIRK channel activation (Table 2).

Phosphorylation of NOP Receptors Induced by AT Compounds. Using phosphosite-specific antibodies that we generated against residues in the NOP C-terminal tail, we previously showed that N/OFQ-induced NOP receptor phosphorylation occurs in a time-dependent manner, where S346 phospho
ylation occurred within 20 seconds, S351 phosphorylation occurred within 60 seconds, and T362/S363 phosphorylation

TABLE 1
Receptor binding affinities of NOP ligands at the human NOP and human MOR receptors expressed in CHO cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>hNOP (K_t) (nM)</th>
<th>hMOR (K_t) (nM)</th>
<th>Selectivity (hMOR (K_t)/hNOP (K_t))</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/OFQ</td>
<td>0.12 ± 0.01(^a)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DAMGO</td>
<td>—</td>
<td>0.59 ± 0.03(^a)</td>
<td>—</td>
</tr>
<tr>
<td>AT-004</td>
<td>9.8 ± 0.9 (4)</td>
<td>375.5 ± 36.5 (4)</td>
<td>38-fold</td>
</tr>
<tr>
<td>AT-090</td>
<td>5.6 ± 1.7 (3)</td>
<td>95.4 ± 3.5 (3)</td>
<td>17-fold</td>
</tr>
<tr>
<td>AT-200</td>
<td>4.1 ± 0.1 (4)</td>
<td>107.1 ± 9.9 (7)</td>
<td>26-fold</td>
</tr>
<tr>
<td>AT-312</td>
<td>0.3 ± 0.1 (6)</td>
<td>6.0 ± 1.0 (6)</td>
<td>20-fold</td>
</tr>
<tr>
<td>AT-390</td>
<td>0.9 ± 0.3 (5)</td>
<td>53.1 ± 16.5 (5)</td>
<td>59-fold</td>
</tr>
<tr>
<td>AT-403</td>
<td>1.1 ± 0.1 (4)</td>
<td>97.9 ± 15.0 (7)</td>
<td>91-fold</td>
</tr>
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</table>

\(^a\)The number of N/OFQ and DAMGO binding experiments was not labeled since each experiment of AT compounds was accompanied with N/OFQ or DAMGO as the standard.

TABLE 2
Functional assays of G protein signaling

<table>
<thead>
<tr>
<th>Compound</th>
<th>hNOP EC\textsubscript{50} (nM)</th>
<th>hNOP E\textsubscript{max} %Stim</th>
<th>hMOR EC\textsubscript{50} (nM)</th>
<th>hMOR E\textsubscript{max} %Stim</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNOP full agonists</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AT-004</td>
<td>6.3 ± 1.4</td>
<td>102.3 ± 0.8</td>
<td>14.9 ± 7.6</td>
<td>98.0 ± 12.9</td>
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<td>AT-090</td>
<td>15.2 ± 0.4</td>
<td>110.1 ± 11.4</td>
<td>12.6 ± 1.3</td>
<td>98.3 ± 5.8</td>
</tr>
<tr>
<td>AT-200</td>
<td>27.2 ± 0.8</td>
<td>104.6 ± 1.2</td>
<td>3.7 ± 4.5</td>
<td>105.9 ± 4.1</td>
</tr>
<tr>
<td>hNOP partial agonists</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AT-004</td>
<td>266.6 ± 73.7</td>
<td>39.7 ± 4.5</td>
<td>224.8 ± 123.4</td>
<td>84.8 ± 19.9</td>
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<tr>
<td>AT-090</td>
<td>50.1 ± 6.4</td>
<td>210.0 ± 6.5</td>
<td>4.5 ± 2.1</td>
<td>104.6 ± 11.6</td>
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<tr>
<td>AT-312</td>
<td>29.9 ± 1.4</td>
<td>102.3 ± 0.8</td>
<td>14.9 ± 7.6</td>
<td>98.0 ± 12.9</td>
</tr>
<tr>
<td>AT-390</td>
<td>15.2 ± 0.4</td>
<td>110.1 ± 11.4</td>
<td>12.6 ± 1.3</td>
<td>98.3 ± 5.8</td>
</tr>
<tr>
<td>AT-403</td>
<td>6.3 ± 1.4</td>
<td>104.6 ± 1.2</td>
<td>3.7 ± 4.5</td>
<td>105.9 ± 4.1</td>
</tr>
</tbody>
</table>

\(^%\text{Stim}\) indicates percentage of stimulation compared with that of the control N/OFQ or DAMGO, as described in Materials and Methods. N.A., not detectable; ND, not done. All results are expressed as means ± S.D. calculated from at least 3 independent experiments.
occurred after 3 minutes (Mann et al., 2019). Several known NOP agonists were also found to show varying profiles of phosphorylation after a 10-minute exposure (Mann et al., 2019). Using the same phosphosite-specific antibodies, we next investigated the phosphorylation signatures of the AT NOP full agonists and partial agonists at a single time point, i.e., after exposure for 10 minutes, and at different concentrations. Figure 3 shows that NOP full agonist AT-403 induced strong phosphorylation at S346, S351, and T362/S363 (Fig. 3A) at 10 μM concentration after a 10-minute exposure, similar to N/OFQ. NOP full agonist AT-312, however, showed phosphorylation only at S346 and S351 at 1 and 10 μM concentrations but no detectable phosphorylation at T362/S363 (Fig. 3A) when measured at this single time point (i.e., after 10 minutes of incubation). Interestingly, NOP full agonist AT-390 showed no detectable phosphorylation at any of the three phosphosites at any concentration after 10 minutes of incubation (Fig. 3A).

On the other hand, NOP partial agonists AT-004 and AT-200 showed no phosphorylation at 10 μM concentration at S351 and T362/S363 (Fig. 3A), but partial agonist AT-090 showed dose-dependent phosphorylation at all three sites, albeit more robust phosphorylation at S346 compared with the other two sites S351 and T362/S363 (Fig. 3A).

Among the NOP full agonists examined here, only AT-403 was able to induce a multisite phosphorylation profile similar to that of the endogenous agonist N/OFQ at all three sites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Phosphorylation intensity</th>
<th>Internalization %</th>
<th>EC50 nM</th>
<th>Recruitment %</th>
<th>EC50 nM</th>
<th>Recruitment %</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/OFQ</td>
<td>+</td>
<td>100</td>
<td>14.3 ± 9.0</td>
<td>100 ± 2.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DAMGO</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>46.5 ± 22.7</td>
<td>100 ± 1.3</td>
</tr>
<tr>
<td>AT-004</td>
<td>—</td>
<td>50</td>
<td>N.A.</td>
<td>26.7 ± 8.4</td>
<td>N.A.</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>AT-090</td>
<td>low</td>
<td>74</td>
<td>17.8 ± 12.3</td>
<td>146.3 ± 37.4</td>
<td>175.0 ± 80.3</td>
<td>84.4 ± 7.2</td>
</tr>
<tr>
<td>AT-200</td>
<td>low</td>
<td>25</td>
<td>241.0 ± 56.2</td>
<td>41.3 ± 4.7</td>
<td>29.6 ± 27.1</td>
<td>45.1 ± 2.9</td>
</tr>
<tr>
<td>AT-312</td>
<td>—</td>
<td>50</td>
<td>6.8 ± 3.5</td>
<td>97.3 ± 10.7</td>
<td>175.0 ± 28.2</td>
<td>84.5 ± 9.8</td>
</tr>
<tr>
<td>AT-390</td>
<td>—</td>
<td>122</td>
<td>6.3 ± 4.5</td>
<td>247.4 ± 64.8</td>
<td>165.0 ± 2.1</td>
<td>78.9 ± 4.1</td>
</tr>
<tr>
<td>AT-403</td>
<td>+</td>
<td>65</td>
<td>4.3 ± 3.2</td>
<td>77.8 ± 12.1</td>
<td>475.0 ± 196.0</td>
<td>92.1 ± 7.3</td>
</tr>
</tbody>
</table>

Recruitment: Percentage of β-arrestin recruitment compared with that induced by the control N/OFQ or DAMGO, as described in Materials and Methods. “−” indicates no phosphorylation, “+” indicates strong phosphorylation, “low” indicates a low level of phosphorylation between 10% and 30%. N.A., not available. The EC50 and Recruitment values of β-arrestin recruitment are expressed as means ± S.D. calculated from at least 3 independent experiments.

Table 3: Functional assays of phosphorylation, internalization, and β-arrestin recruitment.

Fig. 1. Chemical structures of NOP agonists investigated.
by NOP receptor internalization was visualized.

Compounds.

AT-390 show differential but distinct receptor phosphorylation probed within 10 minutes of incubation. In this regard, AT-403 appears to have a similar phosphorylation signature to other known NOP agonists that we have previously investigated (Ro64-6198, SCH221510, AT-202, and MCOPPB; Mann et al., 2019) at similar time points. It is further notable that, under these same conditions, NOP full agonist AT-390 induces no phosphorylation at the three phosphosites we probed, whereas full agonist AT-312 shows no receptor phosphorylation at T362/S363 but induces phosphorylation at the other two sites. These results indicate that at the 10-minute time point, diverse NOP full agonists AT-403, AT-312, and AT-390 show differential but distinct receptor phosphorylation signatures.

Internalization of NOP Receptors Induced by AT Compounds. NOP receptor internalization was visualized by fluorescence microscopy and quantified using a cell surface ELISA (Fig. 3B and 3C). In time-course experiments, we previously showed that NOP receptor internalization was first detectable after 5 minutes of agonist treatment and reached a maximum after 60 minutes (Mann et al., 2019). In initial experiments, internalization by N/OFQ, NOP full agonists AT-312, and partial agonists AT-004 and AT-200 was qualitatively characterized using fluorescence confocal microscopy (Fig. 3B) after 60 minutes of agonist treatment. In subsequent experiments, internalization by N/OFQ, NOP full agonists AT-403 and AT-390, and partial agonist AT-090 after a 60-minute treatment was characterized by a quantitative ELISA (Fig. 3C). Figure 3B shows that N/OFQ induces robust internalization of the NOP receptor after 60 minutes of agonist exposure, further quantified by ELISA (in Fig. 3C) as the percentage of internalized receptors in N/OFQ-treated cells. NOP full agonist AT-403, which showed a similar multisite phosphorylation profile as N/OFQ after a 10-minute exposure, showed a significantly lower level of receptor internalization compared with N/OFQ (Fig. 3C) after the 60-minute exposure. In Fig. 3B, NOP full agonist AT-312, which also showed robust phosphorylation, appeared to show lower internalization compared with N/OFQ. On the other hand, NOP full agonist AT-390, which showed no phosphorylation (Fig. 3A) after the 10-minute exposure, showed a level of receptor internalization not significantly different from that of N/OFQ itself (Fig. 3C).

NOP partial agonist AT-090, which showed robust phosphorylation at S346 (Fig. 3A), showed receptor internalization not significantly different than N/OFQ (Fig. 3C), whereas partial agonists AT-004 and AT-200 showed little internalization of the NOP receptor (Fig. 3B) after the 60-minute exposure but also did not show phosphorylation at S351 or T362/S363.

Taken together, these results suggest that NOP phosphorylation at S346, S351, and T362/S363 may be sufficient, but not the only phosphorylated residues, to induce receptor internalization and that phosphorylation at other as yet unidentified residues in the intracellular domains of the NOP receptor is likely involved in receptor internalization induced by compounds such as AT-390. The lower internalization observed with full agonist AT-403 compared with N/OFQ and AT-390, and the higher phosphorylation and internalization seen with partial agonist AT-090 compared with AT-004 and AT-200, further suggest that structurally diverse NOP ligands may induce differential receptor trafficking and receptor desensitization.

β-Arrestin Recruitment of AT Compounds via NOP and MOR Receptors. β-arrestin recruitment by selective NOP agonists is shown in Table 3 and in graphs shown in Supplemental Fig. 4. The potency (EC50) of the ligands and the percentage of stimulation of β-arrestin recruitment normalized to that by N/OFQ are shown in Fig. 4. NOP full agonist AT-403 showed significant β-arrestin recruitment with potency higher than that of N/OFQ, but with slightly lower efficacy (77% of N/OFQ recruitment). This lower efficacy for arrestin recruitment by AT-403 appears to be consistent with the lower percentage of internalization observed with AT-403 compared with that of N/OFQ. NOP full agonist AT-390, which shows comparable NOP internalization (but not phosphorylation), shows significant β-arrestin recruitment with potency higher than N/OFQ itself (Table 3). NOP full agonist AT-312 also shows nearly the same recruitment of β-arrestin with similar potency as N/OFQ. The rank order of β-arrestin recruitment by the synthetic NOP full agonists (AT-390 > AT-312 > AT-403) appears to be consistent with that for receptor internalization (AT-390 > AT-403), but not phosphorylation, particularly for AT-312 and AT-390. Among NOP partial agonists, AT-090 shows higher stimulation of arrestin recruitment than N/OFQ itself (Table 3; Supplemental Fig. 4). More importantly, even though AT-090 is a partial agonist of lower potency in the GTPγS assays, it shows the same potency as N/OFQ in the β-arrestin recruitment assay. Partial agonists AT-004 and AT-200 induced weak β-arrestin recruitment less than 50% of that induced by N/OFQ, and this appeared consistent with both their phosphorylation and internalization profiles.

At the MOR receptor, the selective NOP agonists AT-403, AT-390, and AT-312 had much lower potency for β-arrestin recruitment compared with the standard MOR agonist DAMGO (Table 3). Selective NOP partial agonist AT-004 showed undetectable recruitment of β-arrestin (Table 3). Modestly selective NOP partial agonist AT-200, which showed significant MOR partial agonist efficacy in the GTPγS assay and nearly full agonist efficacy in the GIRK assay (Table 2), had comparable potency for β-arrestin recruitment to MOR agonist DAMGO, albeit with lower efficacy (45% of DAMGO recruitment).

Overall, the efficacies of β-arrestin recruitment by the NOP agonists appeared to be associated with the levels of
Fig. 3. Phosphorylation and internalization induced by AT compounds at human NOP receptor. (A) HEK293 cells expressing human NOP receptors were treated with 10 μM of N/OFQ, AT-004, AT-200, AT-403, or vehicle (−) or with 1 nM to 10 μM of AT-090, AT-312, and AT-390, at 37°C for 10 minutes, and cell lysates were immunoblotted with antibodies to phosphorylated (p) S346, pS351, or pT362/S363. Blots were stripped and reprobed for human NOP receptor to ensure equal loading. All blots were representative of 3–5 experiments. (B) HEK293 cells stably expressing HA-tagged human NOP receptors were preincubated with HA antibody and then stimulated with 10 μM of N/OFQ, AT-390, AT-090, or AT-403 at 37°C for 60 minutes. Then, the cells were fixed and labeled with a peroxidase-conjugated secondary antibody. Receptor internalization was measured by ELISA and quantified as the percentage of internalized receptors in the cells treated with agonists. Data represent means ± S.D. from 12 independent experiments performed in quadruplicate. The *P < 0.05 versus N/OFQ.
OFQ binding (Mann et al., 2015). It is possible that residues higher further notable that NOP full agonist AT-390, which shows due to we previously identified OFQ, shows no phosphorylation at any of the phosphosite residues we labeled in the graph for each agonist. We used as being phosphorylated after N/OFQ binding (Mann et al., 2015). It is possible that residues other than the three phosphosites we probed with our available antibodies may be phosphorylated by NOP agonists such as AT-390 that lead to the robust β-arrestin recruitment and internalization observed with this nonpeptide NOP agonist.

**Discussion**

In this study, we characterized the intrinsic activity of chemically diverse NOP agonists in various G protein–mediated functional assays (GTPγS binding, cAMP inhibition, and GIRK activation) and characterized agonist-induced receptor trafficking by measuring ligand-induced NOP receptor phosphorylation of selected residues, arrestin recruitment, and receptor internalization. Targeting the NOP receptor has been proposed by us and others as an approach to reduce the side effects of MOR agonists (Bird and Lambert, 2015; Gunther et al., 2018; Kiguchi et al., 2020a; Kiguchi et al., 2020b; Toll et al., 2009). Using an array of ligands, we showed that NOP agonists with varying functional selectivity toward the MOR receptor, i.e., NOP-MOR bifunctional agonists, achieve antinociception comparable to that seen with MOR agonists but without the side effects of MOR such as opioid abuse liability, tolerance development, and respiratory depression (Ding et al., 2018; Sukhtankar et al., 2013; Toll et al., 2009; Vang et al., 2015). Selective NOP receptor full agonists such as AT-312 and AT-202 reduce rewarding effects of several abused substances such as alcohol, cocaine, and opioids (Toll et al., 2009; Zaveri et al., 2018a; Zaveri et al., 2018b). Other NOP full agonists such as AT-403 and AT-390 were found to attenuate the expression of levodopa-induced dyskinesia in animal models of Parkinson’s disease (Arcuri et al., 2018). On the other hand, NOP receptor partial agonists such as AT-004 reduce Parkinsonian motor symptoms in hemilesioned rats (Kamakolanu et al., 2020), whereas partial agonists like AT-090 were shown to have antidepressant-like properties in animal models (Asth et al., 2016).

NOP full agonist AT-403 and the well-characterized NOP agonist Ro 64-6198 have been found to disrupt motor and neurologic function at higher doses (Arcuri et al., 2018; Higginson et al., 2001), an effect not observed with NOP partial agonists such as AT-004 or AT-090 (Asth et al., 2016; Kamakolanu et al., 2020). Since NOP receptor full agonists and partial agonists showed varying levels of separation between therapeutically relevant effects and apparent motor suppressive activity, we undertook the detailed in vitro assessment of functional efficacies of these NOP agonists in assays of NOP receptor function and trafficking. It was hoped that these tools could elucidate the signaling profiles of NOP receptor agonists and provide information on differential effects of receptor regulation that may help explain the pharmacological effects of these NOP agonists.

Among the selective NOP agonists we examined, AT-312, AT-390, and AT-403 behaved as full agonists in the GTPγS assay, whereas AT-004, AT-090, and AT-200 showed lower efficacy and were partial agonists in this assay (Table 2). All these compounds showed even lower efficacy in the MOR GTPγS binding assay, confirming their selectivity for NOP agonist efficacy over MOR efficacy (Table 2).

Interestingly, however, we consistently found that ligands that appeared as partial agonists in the GTPγS assay showed significantly higher efficacy and potency as nearly full agonists in the cAMP and GIRK assays (Table 2). Comparison of intrinsic activities from the GTPγS, cAMP, and GIRK assays revealed a systematically leftward
shift of all dose-response events in cAMP and GIRK assays (Fig. 2). The overestimation of agonist efficacy in the cAMP and GIRK assays was seen at both NOP and MOR receptors (Table 2). For example, although AT-004 showed only low efficacy for GTP- \( \gamma \)S binding at NOP, it behaved as a full agonist in the cAMP and GIRK assays (Table 2). Similarly, although AT-312 induced less than 30% stimulation in GTP-\( \gamma \)S binding at the MOR, it induced about 60% and > 70% stimulation in the MOR cAMP and GIRK assays, respectively. Similar results were also observed with other AT compounds (Table 2). It is possible that higher efficacy seen in the cAMP and GIRK assays is due to signal amplification downstream in the signaling pathway. Intrinsic activity (percentage of stimulation) measured in G protein assays using heterologous transfected cell lines is also known to be affected by receptor reserve and signal amplification (Adham et al., 1993; Baker et al., 2000; Harrison and Traynor, 2003; McDonald et al., 2003; McDonald and Lambert, 2010). These factors may play a role in the different levels of agonist efficacy measured in these assays (Nickolls et al., 2011; Selley et al., 1997).

According to the classic model of agonist-induced GPCR activation, the agonist-bound receptor, complexed with G proteins, is phosphorylated by GRKs and recruits \( \beta \)-arrestin, after which the receptor complex is targeted for internalization, leading to desensitization of the primary receptor signal. This study is the first to characterize several NOP agonists in these three main signaling nodes involved in NOP receptor trafficking after agonist binding, i.e., NOP receptor phosphorylation, \( \beta \)-arrestin recruitment, and internalization were studied for each ligand. Our data presented interesting differences in the ability of various NOP agonists to induce these events. NOP full agonist AT-403 showed robust phosphorylation of all three examined phosphosites (S346, S351, and S362/S363) of NOP receptor after 10 minutes of incubation, comparable to the natural NOP full agonist N/OFQ (Fig. 3A). NOP full agonist AT-312 also showed comparable phosphorylation at S346 and S351 but not at T362/S363 under similar exposure. NOP full agonist AT-390, on the other hand, showed no phosphorylation at any of these residues at any concentration under the same conditions. NOP partial agonists AT-004 and AT-200 showed minimal phosphorylation of NOP receptor residues S351 and T362/S363 at 10 \( \mu \)M concentration (Fig. 3A), whereas NOP partial agonist AT-090 showed a robust dose-dependent phosphorylation at all three sites after 10 minutes of incubation. The human NOP receptor contains several residues in its intracellular loops and C-terminal tail that can potentially be phosphorylated after receptor activation. Mann et al. (2015) reported that N/OFQ agonist-induced NOP phosphorylation occurs primarily within the residues in the C-terminal tail. They generated phosphosite-specific antibodies to the C-terminal residues S346, S351, and T362/S363 and showed that N/OFQ and nonpeptide agonists showed a hierarchical and temporal phosphorylation of S346 followed by S351 and T363/S363. In their study, the phosphorylation induced by N/OFQ and nonpeptide agonists appeared to correlate with the NOP internalization observed after treatment with these agonists. However, in the current study, the level of NOP internalization induced by the NOP agonists studied here did not appear to be consistent with the phosphorylation signatures induced by these agonists at the three phosphosites S346, S351, and T362/S363. Particularly, full agonist AT-390 showed no phosphorylation at any of these sites but still showed robust internalization compared to N/OFQ, which showed significant phosphorylation at all three residues. On the other hand, full agonist AT-403 showed similar phosphorylation to N/OFQ but lower internalization than N/OFQ (Fig. 3C). Although ligand-induced receptor phosphorylation was probed after 10 minutes of incubation, whereas internalization was measured after 60-minute exposure with NOP agonists, these results still suggest that residues other than S346, S351, and T362/S363 are likely involved in ligand-induced NOP phosphorylation, leading to subsequent internalization particularly for NOP full agonist AT-390. Given that opioid receptor internalization leads to receptor desensitization, NOP full agonist AT-390 may induce more NOP desensitization compared to full agonist AT-403, which may lead to differences in the pharmacological responses between these two NOP full agonists. Indeed, we found that AT-403 and AT-390 showed different windows of separation between their antidyskinetic effects and disruption of locomotor activity (Arcuri et al., 2018).

The efficacy and potency of \( \beta \)-arrestin recruitment by these NOP full agonists appeared to be consistent with the internalization induced by these agonists (Figs. 4 and 5). NOP full agonist AT-390 showed significantly higher arrestin recruitment with higher potency than the standard agonist N/OFQ. Interestingly, AT-390 also induces greater internalization than most other NOP full agonists we tested, including N/OFQ (Fig. 3C). In contrast, the chemically distinct NOP full agonist AT-403 shows lower arrestin recruitment than N/OFQ (Table 3; Supplemental Fig. 4F) and also lower internalization than N/OFQ.

NOP receptor partial agonists AT-004 and AT-200 did not show significant arrestin recruitment, but partial agonist AT-090 showed a robust recruitment of \( \beta \)-arrestin, with potency and efficacy greater than N/OFQ itself (Table 3; Supplemental Fig. 4). AT-090 also showed significant receptor phosphorylation and robust internalization compared with N/OFQ and other full agonists. Thus, among NOP partial agonists characterized in this study, the internalization induced by AT-090 may suggest a greater propensity for receptor desensitization than NOP partial agonists AT-004 and AT-200.

In summary, the characterization of structurally diverse NOP full agonists and partial agonists presented here highlights the differential functional effects of chemically diverse NOP agonists on ligand-induced receptor signaling and trafficking. Furthermore, the results demonstrate that measurement of intrinsic activities of agonist ligands can vary with the type of functional assay used to measure intrinsic activity. These differences are particularly pronounced in the case of partial agonist ligands, which can show varying levels of agonist efficacies depending on the assay and system used. Therefore, characterizing GPCR agonist ligands as full agonists or partial agonists must take into account several factors discussed above to determine intrinsic activity of agonist ligands. The differential effects on receptor trafficking, particularly of NOP full agonist AT-390 and NOP partial agonist AT-090, compared with other NOP agonists may be important to understand the pharmacological profiles and actions of these NOP agonists.
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