ACS Chemical Neuroscience

Research Article

pubs.acs.org/chemneuro

Novel Synthesis and Pharmacological Characterization of NOP Receptor Agonist 8-[(1*S*,3a*S*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (Ro 64-6198)

Steven D. Chang,[†] Lawrence E. Brieaddy,[‡] Joseph D. Harvey,[†] Anita H. Lewin,[‡] S. Wayne Mascarella,[‡] Herbert H. Seltzman,[‡] P. Anantha Reddy,[‡] Ann M. Decker,[‡] Charles J. McElhinny, Jr.,[‡] Desong Zhong,[‡] Elisha E. Peterson,[†] Hernán A. Navarro,[‡] Michael R. Bruchas,*[,§] and F. Ivy Carroll*[,‡]

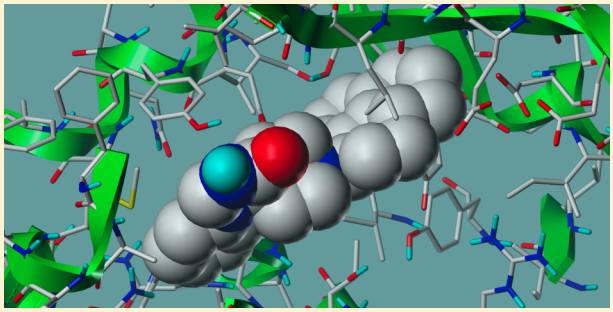
[†]Department of Anesthesiology, Basic Research Division, and Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110, United States

[‡]Research Triangle Institute, P.O. Box 12194, Research Triangle Park, North Carolina 27709-2194, United States

[§]Department of Anesthesiology, Basic Research Division, and Department of Anatomy and Neurobiology, Washington University Pain Center, Washington University School of Medicine, St. Louis, Missouri 63110, United States

Supporting Information

ABSTRACT: The nociceptin/orphanin FQ opioid peptide (NOP) receptor is a widely expressed GPCR involved in the modulation of pain, anxiety, and motor behaviors. Dissecting the functional properties of this receptor is limited by the lack of systemically active ligands that are brain permeant. The small molecule NOP receptor-selective, full agonist 8-[(1*S*,3a*S*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (Ro 64-6198) hydrochloride is an active, brain penetrant ligand, but its difficult and cost-prohibitive synthesis limits its widespread use and availability for animal studies. Here, we detail a more efficient and convenient method of synthesis, and use both in vitro and in vivo pharmacological assays to fully characterize this ligand. Specifically, we characterize the pharmacodynamics of Ro 64-6198 in cAMP and G-protein coupling in vitro and examine, for the first time, the effects of nociceptin/orphanin FQ and Ro 64-6198 in arrestin recruitment assays. Further, we examine the effects of Ro 64-6198 on analgesia, anxiety, and locomotor responses in vivo. This new synthesis and pharmacological characterization provide additional insights into the useful, systemically active, NOP receptor agonist Ro 64-6198.



KEYWORDS: *Ro 64-6198, nociceptin/orphanin FQ opioid peptide receptor, cAMP, locomotor activity, arrestin, GPCR*

After the reported cloning of the classical MOP, DOP, and KOP receptors, a highly homologous receptor, which was not activated by the classical opioid ligands, was cloned;[1−4] it was known as the ORL-1, or NOP receptor. The NOP receptor, which is activated by the endogenous peptide nociceptin/orphanin FQ (N/OFQ, **1**), (Figure 1) modulates Gα_i-mediated cAMP inhibition and voltage-gated Ca^{2+} and K^+ channels.[5] Administration of N/OFQ has been found to affect the levels of stress hormones[6−9] and to cause a decrease in locomotor activity.[10] Most importantly, studies have strongly suggested that neuropsychiatric disorders such as anxiety, depression, anorexia, obesity, as well as substance abuse and pain may be linked to the NOP receptor.[11,12] Ever since the discovery of the endogenous peptide N/OFQ,[10,13] efforts have been directed toward the development of small molecule NOP receptor agonists and antagonists for pharmacological manipulation of neuropsychiatric disorders potentially associated with NOP receptors.

Due to its brain permeability the small molecule NOP receptor agonist 8-[(1*S*,3a*S*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one Ro 64-6198 (**2**), has proven to be a highly useful ligand for studying this receptor system in vivo (Figure 1).[14] Similar to intracerebroventricular (i.c.v.) administration of N/OFQ, Ro 64-6198 administered intraperitoneally (i.p.) reduced acquisition and reinstatement of alcohol-induced place preference in place conditioning experiments in mice but, when administered alone, Ro 64-6198 produced neither place preference nor place aversion.[15,16] Ro 64-6198 significantly reduced alcohol self-administration in mice[16] and in rats[17] and did not induce conditioned place preference in rats.[18] In addition, Ro 64-6198 was shown to be active in some mouse[19] and monkey[20] pain models.[21]

To facilitate further investigations of the small molecule NOP agonist Ro 64-6198 we have developed a modified synthesis of Ro 64-6198 that avoids the high-pressure hydrogenation used in the reported synthesis[22] and also provides the (+)-enantiomer

Received: July 30, 2015
Revised: August 26, 2015
Published: September 14, 2015

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

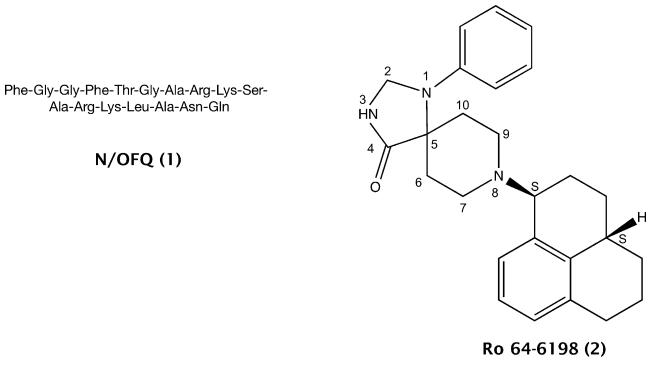


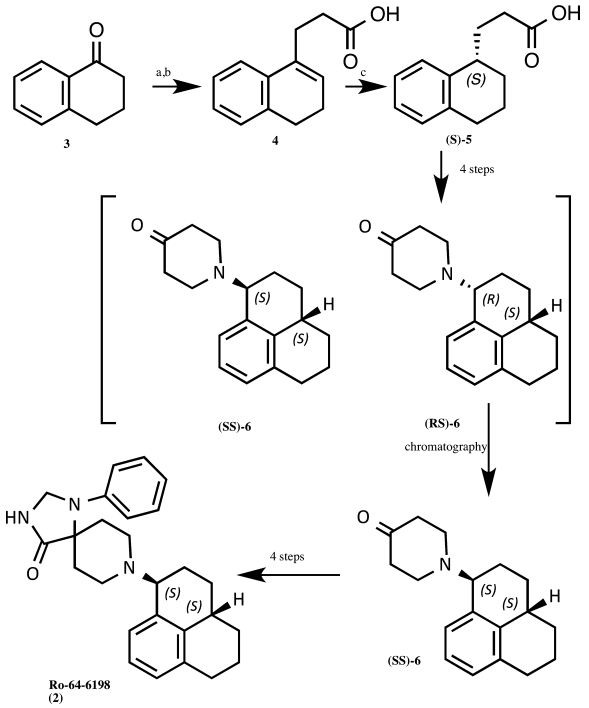
Figure 1. Structures of N/OFQ (**1**) and Ro 64-6198 (**2**).

of Ro 64-6198. In addition, we confirmed the chiral purity of both samples by developing analytical chiral HPLC conditions to provide excellent separation of Ro 64-6198 from its mirror image. Thus, synthesized Ro 64-6198 was used in a live-cell cAMP assay, as well as in calcium mobilization assays, in which Ro 64-6198 and its enantiomer were compared to N/OFQ. Additionally, we have investigated, and herein report, the first identification of arrestin recruitment by Ro 64-6198 using bioluminescence resonance energy transfer (BRET). Finally, we corroborated these in vitro results by using in vivo assays to measure the locomotor, motor coordination, and anxiolytic effects of i.p. administered Ro 64-6198.

CHEMISTRY

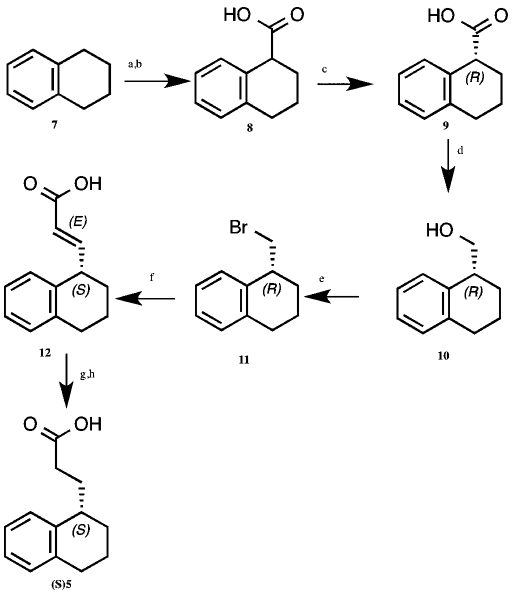
Scheme 1 shows the reported synthesis of Ro 64-6198 (**2**)[22] where the stereochemistry at C-3 of **2** is established by the stereoselective reduction of the acid **4** to give (*S*)-**5**. Since the equipment required for the high-pressure hydrogenation of **4** to (*S*)-**5** was not available at RTI, and because all attempts

Scheme 1[a]



[a]Reagents: (a) diethyl succinate, KOtBu, reflux; (b) AcOH, HCl, reflux; (c) (**5**)-MeOBIPHEP/Ru(OAc)$_2$; Pd/C, H_2; 1400 psi.

Scheme 2[a]



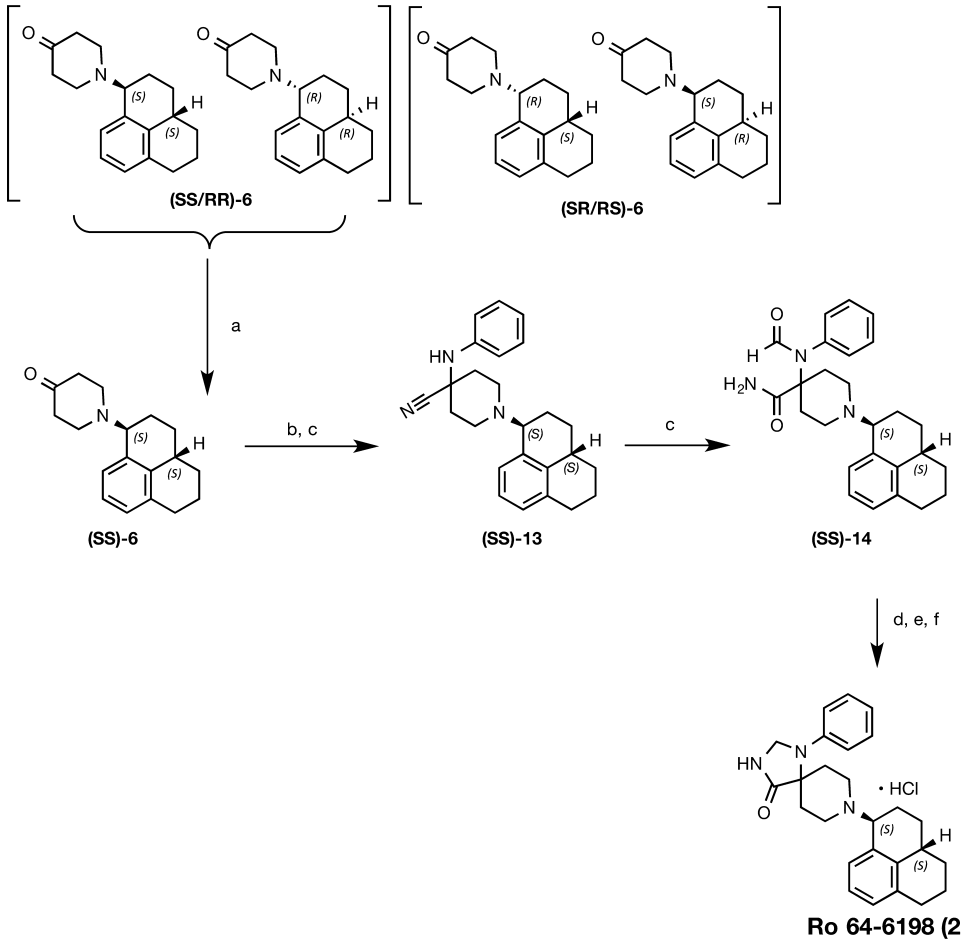
[a](a) BuLi; (b) CO_2; (c) resolve (quinine); (d) B_2H_6; (e) $(Ph)_3P$, CBr_4; (f) BuLi, $HC(O)CO_2H$; (g) H_2/Pd−C; (h) K_2CO_3.

to resolve racemic **5** were unsuccessful, an alternate synthesis of (*S*)-**5** was sought (Scheme 2). Initially, it was planned to establish the desired stereochemistry of **5** by preparing the known[23] (*S*)-1,2,3,4-tetrahydro-1-naphthoic acid (**9**) and elaborating this compound to (*S*)-3-(1,2,3,4-terahydronaphthalen-1-yl)propionic acid ((*S*)-**5**).[22] Although **9** is commercially available, the cost of the amount required to synthesize gram quantities of **2** was prohibitive. Therefore, the racemic acid **8** was prepared by carboxylation of tetrahydronaphthalene (**7**) and resolved via the quinine salt.[23] The resolved acid **9** was converted to tetrahydronaphthylmethyl bromide **11**, via the alcohol **10**, that was used in an attempted Wittig coupling with glyoxylic acid to prepare the required intermediate (*S*)-**5**.

Since this reaction sequence was unsuccessful the alternative procedure described in Scheme 3 was developed. With the knowledge that the diastereomers (1*S*,3a*S*)- and (1*R*,3a*S*)-**6** were readily separable chromatographically,[22] the diastereomeric 1-[(1*RS*,3a*RS*)- and 1-[(1*SR*,3a*RS*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]piperidin-4-one (*SS*/*RR*-**6** and *SR*/*RS*-**6**, respectively) were synthesized as a mixture, as reported,[22] and separated by column chromatography. Resolution of *SS*/*RR*-**6** via the di-*p*-toluoyl-D-tartarate salts gave both (+)-(*RR*)- and (−)-(*SS*)-**6**. The latter was treated with aniline, followed by trimethylsilyl cyanide, to give (+)-1-[(1*S*,3a*S*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]-4-(phenylamino)piperidine-4-carbonitrile ((+)-*SS*-**13**) that was formylated with concomitant nitrile hydrolysis to afford (+)-1-[(1*S*,3a*S*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]-4-(*N*-phenylformamido)piperidine-4-carboxamide (+)-(*SS*)-**14**. Treatment of (+)-(*SS*)-**14** with triethyl orthoformate, followed by sodium borohydride reduction, gave 8-[(1*S*,3a*S*)-2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, Ro 64-6198, that was converted to the hydrochloride salt (**2**·HCl). To confirm chiral purity, HPLC conditions that would give good separation of the enantiomers were sought. Using a Chiralpak IA column and eluting with common solvent combinations such as alkane/alcohol, with diethylamine as modifier, gave either no separation or minimal separation; strikingly, replacement of

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

Scheme 3[a]



[a]Reagents: (a) Resolution with di-*p*-toluoyl-D-tartaric acid; (b) aniline, TMSCN; (c) HCO_2H; (d) $(EtO)_3CH$; (e) $NaBH_4$; (f) HCl.

hexane by a 1:1 mixture of hexane/methyl *t*-butyl ether led to excellent separation (R_t 13.20 min for the (+)-isomer and 24.09 min for the (−)-isomer. Chiral HPLC analysis of **2**·HCl demonstrated that the resolution was incomplete (80% ee). Therefore, the free base **2** (from the HCl salt **2**·HCl) was treated with (+)-dibenzoyl-D-tartaric acid, converted back to **2**·HCl, which was recrystallized from methanol, to give **2**·HCl with 99.1% ee. The (−)-isomer, prepared likewise, had >99.1% ee.

IN VITRO PHARMACOLOGY

Calcium Mobilization Assay. An in vitro calcium mobilization assay was utilized to compare the agonist activity of N/OFQ, Ro 64-6198, and of the mirror image (*R*,*R*)-Ro 64-6198, at the human NOP receptor (Table 1). A stable NOP receptor cell line was created using RD-HGA16 cells (Molecular Devices), which overexpress the promiscuous $G\alpha_{16}$ protein and allow GPCRs not coupled to $G\alpha_{q/11}$ to mobilize internal calcium when activated.[24] In this assay, the endogenous agonist N/OFQ was observed to have an EC_{50} value of 0.17 nM whereas Ro 64-6198 had an, of EC_{50} = 25.6 nM. These observed EC_{50} values are similar to previously published potencies (N/OFQ EC_{50} = 0.29 nM; Ro 64-6198 EC_{50} = 10.5 nM).[25] The mirror image, (*R*,*R*)-Ro 64-6198, had an EC_{50} value of 311 nM and was 1829-fold and 12-fold less potent than N/OFQ and Ro 64-6198, respectively. Further, Ro 64-6198 was a full agonist at the NOP receptor (E_{MAX} = 106%) while (*R*,*R*)-Ro 64-6198 was slightly less efficacious with an E_{MAX} value of 88%.

Table 1. Calcium Mobilization Agonist Activity of N/OFQ (1), Ro 64-6198 (2), and (*R*,*R*)-Ro 64-6198 at the Human NOP Receptor

compd	EC_{50} (nM)[a]	% of N/OFQ E_{MAX}[a]	N/OFQ/(2) potency ratio
N/OFQ (**1**)	0.17 ± 0.04	100 ± 7	
Ro 64-6198 (**2**)	25.6 ± 2.0	106 ± 3	0.007 (0.03[b])
(*R*,*R*)-Ro 64-6198	311 ± 22	88 ± 3	

[a]Calcium mobilization EC_{50} and E_{MAX} values are reported as mean ± SEM and are the result of three independent experiments conducted in duplicate. [b]Ratio calculated from EC_{50} values reported in ref 25.

Signal Transduction. Characterization of G-protein activity and arrestin recruitment was performed in transfected HEK-293 cells expressing the NOP receptor. Using the GloSensor assay (Promega), we assessed $G\alpha_i$-mediated inhibition of cAMP accumulation in real time (Figure 2A). The observed EC_{50} (0.178 μM) is similar to previously published potencies (Table 2),[26] but it is important to note that Ro 64-6198 and N/OFQ have been shown to have broadly varying potencies in different tissues using different methodologies.[27] As expected, Ro 64-6198 displays a G-protein E_{MAX} consistent with that of a full agonist at the NOP receptor (Figure 2E).

Investigation of arrestin recruitment to the NOP receptor by Ro 64-6198, using a receptor-arrestin BRET assay recently established in our laboratories,[28] showed that Ro 64-6198 does

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

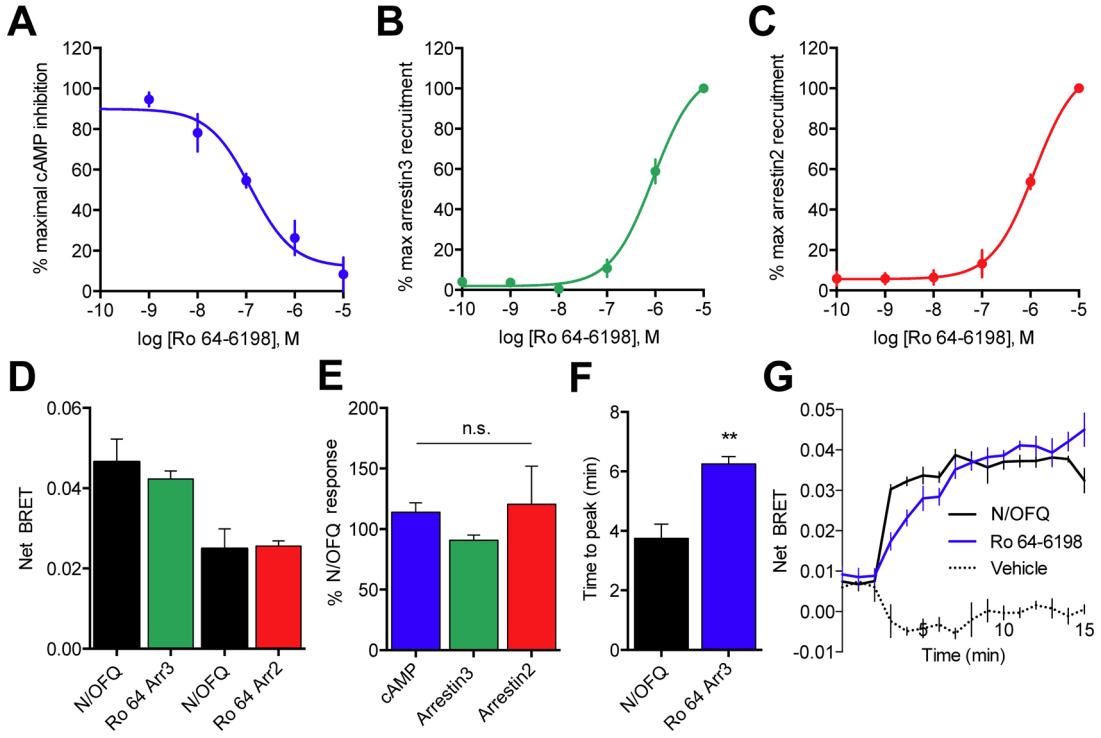


Figure 2. In vitro signaling profile of Ro 64-6198. (A) Inhibition of cAMP accumulation in stably transfected HEK293 cells after treatment with Ro 64-6198 in various concentrations. (B, C) Concentration−response curve for synthate-induced recruitment of arrestin3 (B) and arrestin2 (C) assessed by BRET. (D) Ro 64-6198 maximal arrestin3 and arrestin2 recruitment shown as raw NET BRET compared to N/OFQ's maximal recruitment. Ro 64-6198 shows expected full agonist efficacy in comparison to nociceptin. (E) Maximum efficacy in cAMP inhibition and arrestin3/2 recruitment shows no difference when compared to N/OFQ. (F) Average and representative time trace (G) of arrestin3 recruitment after treatment, showing a slower time-to-peak for Ro 64-6198. (**$p < 0.005$, $n = 3–4$, triplicate samples).

Table 2. cAMP and Arrestin Agonist Activity of N/OFQ (1) and Ro 64-6198 (2) at the Human NOP Receptor

	cAMP mobilization		arrestin3			
compd	EC_{50} (nM)[a]	% of N/OFQ E_{MAX}[a]	EC_{50} (nM)[b]	% of N/OFQ E_{MAX}[b]	cAMP potency ratio[d]	arrestin3 potency ratio[d]
N/OFQ (**1**)	4.5 ± 0.1[c]	100 ± 4	100 ± 13[c]	100 ± 12		
Ro 64-6198 (**2**)	178	113 ± 7	912 ± 107	91 ± 4	0.038	1.09

[a]cAMP, EC_{50}, and E_{MAX} values are reported as mean ± SEM and are the result of three to four independent experiments conducted in triplicate. [b]Arrestin recruitment EC_{50} and E_{MAX} values are reported as mean ± SEM and are the result of three to four independent experiments conducted in triplicate. [c]As previously reported in ref 28. [d]Ratio calculated vs N/OFQ from EC_{50} values given in ref 28.

recruit both arrestin3 (EC_{50} = 0.912 μM) and arrestin2 (EC_{50} = 1.20 μM) to the NOP receptor in a concentration-dependent manner comparably with N/OFQ (Figure 2B and C, Table 2). These results agree with previous findings showing arrestin2-recruitment E_{MAX} to the NOP receptor to be lower than that of arrestin3[28] and show that Ro 64-6198 recruits both arrestin3 and arrestin2 with full agonist activity, displaying arrestin3-recruitment E_{MAX} values similar to those for the endogenous ligand N/OFQ (Figure 2D and 2E, Table 2). Additionally, we observed that Ro 64-6198 recruits arrestin more slowly than N/OFQ (Figure 2F and 2G), thus resembling SCH 221,510 in time-to-peak recruitment of arrestin.[28] Taken together, these experiments suggest that Ro 64-6198 acts as a full NOP receptor agonist in vitro.

In Vivo Pharmacology. Functional determination of selected biological activity was performed in behavioral assessments for NOP receptor effects that had been previously reported for this receptor.[19] In the hot plate heat analgesia assay, mice dosed with Ro 64-6198 (3 mg/kg i.p.) displayed significant increase in latency to hindpaw withdrawal (Figure 3A). During the assay, we also observed a motor dysfunction behavior that may contribute to the latency to paw withdrawal, independent of an analgesic effect. To further characterize this sedation-like effect, we further assessed motor function in Ro 64-6198 treated mice.

To test for sedation-like effects of Ro 64-6198, we systemically treated mice with 3 different doses (0.3−3 mg/kg, i.p.) of Ro 64-6198 and assessed the locomotor and anxiolytic effects by comparison with vehicle control groups. In the open-field test, we observed a robust 2-fold decrease in the locomotor activity of mice treated with the highest dose (3 mg/kg) (Figure 4A) consistent over the 1 h testing period (Figure 4B) as well as a strong and statistically significant increase in time spent in the center of the open-field arena (Figure 3B). When looking more closely at the temporal locomotor traces, we noted that the increase in time spent in the center half (% of total area) of the arena was consistent over the course of the trial (Figure 3C). The lower dose groups showed no appreciable increase or decrease in center time or locomotor activity when compared with vehicle controls.

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

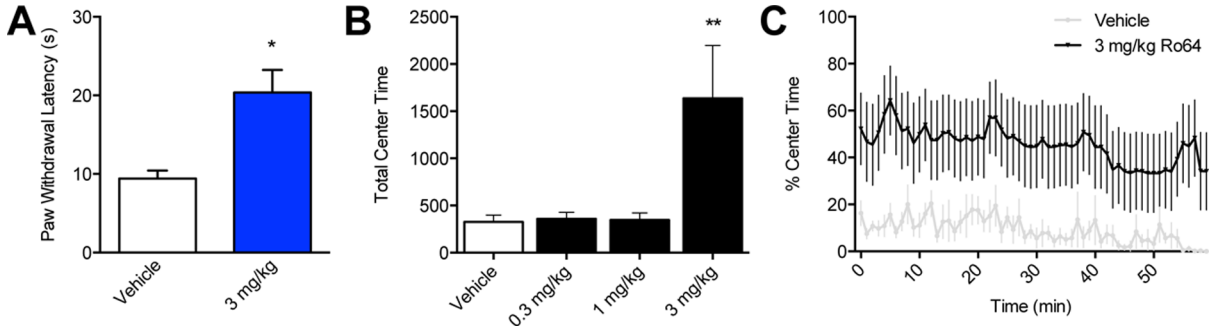


Figure 3. Analgesic and anxiolytic activity of Ro 64-6198. (A) Ro 64-6198 treatment group shows increased latency to paw withdrawal after thermal stimulus (n = 4/group). (B) Total time spent in the center of the testing arena depicts the anxiolytic effect of high dose (3 mg/kg) treatment. (C) Time trace data shows the observed trend of increased center time at the high dose are consistent throughout the duration of the testing period (*p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, n = 8−9/group).

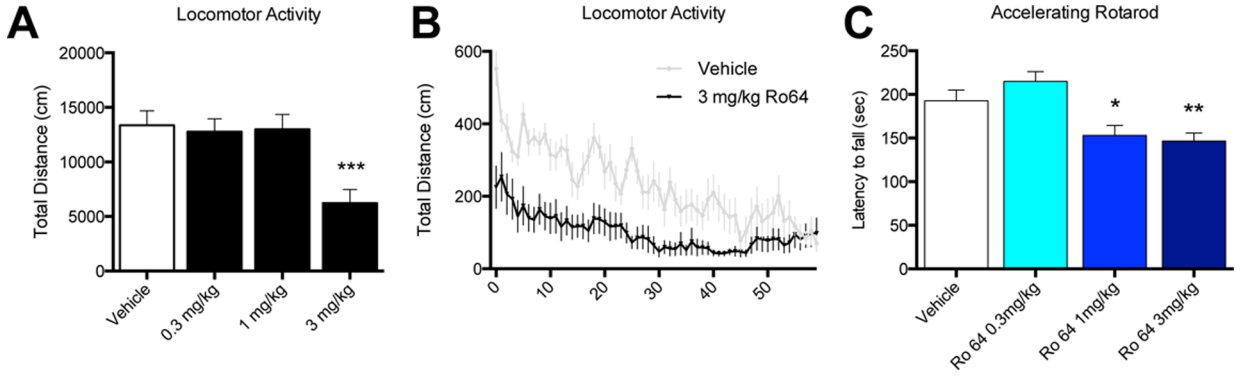


Figure 4. Motor deficits induced by Ro 64-6198 administration. (A) Locomotor activity of vehicle control and systemically (i.p.) treated animals in the open-field test. High dose (3 mg/kg) animals show significantly decreased locomotor activity over a 1 h testing period. (B) Time trace data shows the observed trend of reduced locomotor activity at the high dose are consistent throughout the duration of the testing period (n = 8−9/group). (C) Accelerating rotarod demonstrates a decrease in motor coordination beginning at a lower dose of 1 mg/kg (n = 8−10/group) (*$p \leq 0.05$, **p < 0.01, ***p < 0.001, one-way ANOVA).

To further characterize the potential motor deficits induced by Ro 64-6198, we employed the accelerating Rotarod test on naïve mice at the same treatment doses (0.3−3 mg/kg) as in the open field test. As has been shown in previous studies,[29,30] decreased motor coordination was observed at an effective dose of 3 mg/kg. Interestingly, an increase in dose sensitivity, showing a significant decrease in motor coordination beginning at the 1 mg/kg dose (Figure 4C), was also noted.

RESULTS AND DISCUSSION

The development of a relatively convenient synthesis for the NOP receptor agonist Ro 64-6198, as a complement to the Wichmann approach,[22] will allow for this important ligand to be more extensively explored and utilized in studies of NOP receptor function in vitro and in vivo. The two stereoisomers (Ro 64-6198 and its mirror image (*R*,*R*)-Ro 64-6198), prepared via the modified synthetic approach, have been characterized to be of high chemical and stereochemical purity. The latter was determined by developing chiral HPLC conditions that gave a large (>10 min) separation between enantiomers to determine enantiomeric excess (%ee). The %ee for our synthesized samples of Ro 64-6198, and of its mirror image, were 99.1% and >99.1%, respectively; the %ee reported for the Wichmann[22] sample is >98%, with the HPLC conditions not described. We also noted that the physical properties determined by us were slightly different from those reported in the literature (see experimental). In particular, the melting point of the sample of Ro 64-6198·HCl prepared by us was significantly lower than the reported value[22] as was the optical rotation, despite the fact that elemental analysis of our sample indicated that, just like the sample prepared by the original synthetic approach,[22] our sample was a monohydrochloride and was not hydrated.

The results of in vitro calcium mobilization assays showed Ro 64-6198 to be a full agonist at the NOP receptor, as expected, but to have reduced potency relative to N/OFQ; the "mirror image isomer" was found to be an order of magnitude less potent than Ro 64-6198 and also to be less efficacious. As expected, Ro 64-6198 inhibited cAMP accumulation in stably transfected HEK293 cells with maximum efficacy similar to that of N/OFQ, indicating full agonist activity at the NOP receptor, consistent with all previous studies.

Prior to our studies, it was unknown whether Ro 64-6198 would induce the recruitment of arrestins to the NOP receptor, which isoforms would be recruited, and in what proportion. Our data show, for the first time, that Ro 64-6198 effectively recruits arrestin2 and arrestin3 to the NOP receptor. Quantification demonstrated both N/OFQ and Ro 64-6198 to be effective in recruitment of arrestin3 and arrestin2 in a dose-dependent manner. Moreover, Ro 64-6198 exhibited full agonist efficacy for both arrestins in comparison to N/OFQ. The observed lower recruitment of arrestin2 relative to arrestin3 by N/OFQ, taken together with the differential regulation by arrestin2 and arrestin3 of both LPS-induced inflammatory responses and TLR4 inflammatory responses,[31] raises intriguing

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

questions regarding the role of arrestin recruitment by N/OFQ in the in vivo effects of N/OFQ agonists.

Further studies of Ro 64-6198 to assay NOP receptor agonist activity involved an array of in vivo pharmacological behavioral models and utilized mice dosed with Ro 64-6198 at 0.3−3 mg/kg i.p. Significant increases in latency of hindpaw withdrawal was observed in the hot plate heat analgesia assay at the 3 mg/kg dose, as had been previously shown.[19] Similarly, statistically significant effects on anxiety-like behavior and locomotor activity were observed using a high intraperitoneal dose (3 mg/kg) that had been shown[19,29] to decrease locomotor activity and coordination, and to exert anxiolytic effects in multiple measures of anxiety.[14,29,32] Moreover, we have identified a lower effective dose (1 mg/kg) for impairment of motor coordination using an accelerating Rotarod assay. This increased sensitivity is likely due to differences in protocol, namely, our use of an accelerating paradigm as opposed to fixed speeds in the aforementioned studies. However, these data are important in informing future studies using Ro 64-6198 in vivo, and caution against high doses, which may confound behavioral analysis due to the induction of hypolocomotor responses. The observed effects on latency to hindpaw withdrawal, anxiety-like behavior and locomotor activity, are consistent with, and may be due in part to, a sedative effect of Ro 64-6198. The results do not completely isolate a sedative effect, but they strongly suggest that sedation may play a role in the reported anxiolytic and analgesic effects of Ro 64-6198, as has been previously suggested.[15] Importantly, our in vivo data also suggest that it is also important to consider species differences in the therapeutic window of Ro 64-6198. Prior reports have shown that in mice the dose−response curve is very steep, whereas in rats there is a broader dose−response window in which one can use Ro 64-6198, in vivo.[33]

In summary, we have developed a novel, convenient approach to the synthesis of the important NOP receptor agonist Ro 64-6198 that also yields the mirror image (*R*,*R*)-Ro 64-6198. Functional results from the in vitro and in vivo studies of our sample of Ro 64-6198 confirm previously reported studies and show the expected modulations of analgesia, anxiety, and motor function as well as revealing a 3-fold lower effective dose for impairment of motor coordination. Noteworthy is the determination, for the first time, that Ro 64-6198 recruits both arrestin2 and 3 to the NOP receptor, in a profile consistent with a full agonist. This detailed pharmacological profile of Ro 64-6198 adds to the growing body of work describing the role of NOP receptors in vivo, and our new method of synthesis of Ro 64-6198 may provide a useful tool for further examination of the NOP receptor system for potential therapeutic intervention.

METHODS

Melting points were determined using a MEL-TEMP II capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were obtained on a Varian Avance DPX-500 MHz NMR spectrometer or a Bruker Unity Inova 300 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) with reference to TMS as internal standard. Mass spectra (MS) were determined using a PerkinElmer Sciex AP1 150 EX mass spectrometer equipped with an ESI (turbospray) source. Elemental analyses were performed by Atlantic Microlab Inc., Atlanta, GA. The purity of the compounds (>95%) was established by elemental analysis. Optical rotations were measured on a Rudolf Research AutoPol III polarimeter. Analytical HPLC was performed using a dual pump system (Varian Prostar 210 solvent system delivery system with 5 mL pump heads), a Rheodyne injector and a Varian ProStar 335 diode-array detector (DAD) controlled by Varian Star Workstation software. Unless otherwise stated, reagent-grade chemicals were obtained from commercial sources and were used without purification. All moisture- and air-sensitive reactions and reagent transfers were carried out under dry nitrogen.

Resolution of 1-[(1*RS*,3a*RS*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]piperidin-4-one (*RR*/*SS*-6). To a clear solution of (1*RS*,3a*RS*)-1-(2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl)piperidin-4-one (*RR*/*SS*-**6**)[22] (23.05 g, 0.0875 mol) in diethyl ether (500 mL) was added di-*p*-toluoyl-D-tartaric acid (34.9 g, 0.0875 mol, 97%). Solids formed within minutes. After standing at room temperature overnight, the white solids were collected, washed with ether, and dried under high vacuum overnight to afford 32.3 g (mp 155−156 °C) of the salt. The salt was recrystallized from MeOH to give 23.4 g of solids (mp 167−168 °C) that were converted to the free base (10% NaOH) to give 8.58 g (37%) of (−)-(*SS*)-**6** as a light yellow oil; $[\alpha]_D$ = −25° (c 1.05, MeOH).

(+)-1-[(1*S*,3a*S*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]-4-(phenylamino)piperidine-4-carbonitrile ((+)-(*S*,*S*)-13). To a solution of (−)-1-(2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-ylpiperidin-4-one [(−)-(*S*,*S*)-**6**] (8.00 g, 0.0304 mol) in acetic acid (30 mL) at 0 °C was added aniline (3.13 g, 0.0336 mol) followed by TMSCN (3.02 g, 0.0304 mol). The mixture was stirred at room temperature for 4 h and then added to ice water (450 mL)/NH_4OH (150 mL). The resulting solids were extracted with CH_2Cl_2 to give 10.9 g of a beige solid. The solids were purified by chromatography on SiO_2, using hexane/EtOAc, (3:1) as the eluent, to yield 9.38 g (83%) of (+)-**13** as a white solid; mp 145−146 °C (lit[22] 152 °C); $[\alpha]_D$ = +26.30 (c 0.460, $CHCl_3$) [lit[22] $[\alpha]_D$ = +28.40 (c 0.26, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 1.26−1.45 (m, 2H, H-2), 1.84−2.04 (m, 8H, H-4, H-5, H-2′a, H-6′a), 2.20−2.89 (m, 9H, H-3, H-6, H-3′, H-5′, H-1), 3.64 (bs, 1H, NH), 3.91 (dd, *J* = 9.5, 5.5 Hz, 1H, H-3a), 6.89−6.97 (m, 4H, ArH), 7.08 (t, *J* = 7.5 Hz, 1H, H-8), 7.22−7.27 (m, 2H, H-9, ArH), 7.47 (d, *J* = 7.5 Hz,1H, H-7). MS (EI) *m*/*z* calcd for $C_{25}H_{39}N_3$, 371; found, 372 (M + H).

(+)-1-[(1*S*,3a*S*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]-4-(*N*-phenylformamido)piperidine-4-carboxamide [(+)-(*S*,*S*)-14]. To an ice chilled solution of Ac_2O (100 mL) was added (+)-1-(2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl)-4-phenylaminopiperidine-4-carbonitrile [(+)-(*S*,*S*)-**13**] (9.23 g, 0.0248 mol) in formic acid (200 mL, 88%). After stirring at RT for 3 h, the mixture was concentrated, the residue was dissolved in formic acid (100 mL)/AcOH (10 mL), and the solution was allowed to stir at RT overnight. The solution was concentrated to 50 mL, and 10% $NaHCO_3$ was added very slowly. The mixture was extracted with CH_2Cl_2 to give a yellow oil (10.87 g). The oil was chromatographed on SiO_2, using CH_2Cl_2/MeOH, (9:1) as eluent, to give 6.75 g (65%) of [(+)-(*S*,*S*)-**14**] as an amorphous white solid. ^{1}H NMR ($CDCl_3$): δ: 1.20−1.30 (m, 2H, H-2), 1.71−2.11 (m, 8H, H-4, H-5, H-2′a, H-6′a), 2.27−2.66 (m, 7H, H-6, H-3′, H-5′, H-1), 2.78 (dd, *J* = 7.5, 5.5 Hz, 2H, H-3), 4.38 (dd, *J* = 9.5, 5.5 Hz, 1H, H-3a), 6.00 (bs, 2H, NH_2), 7.01 (d, *J* = 7.5 Hz, 1H, H-7), 7.03 (t, *J* = 7.5 Hz, 1H, H-8), 7.16−7.48 (m, 7H, ArH, H-9), 8.22 (S, 1H, CHO). MS (EI) *m*/*z* calcd for $C_{26}H_{31}N_3O_2$, 417; found, 418 (M + H).

8-[(1*S*,3a*S*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one hydrochloride (Ro 64-6198·HCl, 2·HCl). A mixture of (1*S*,3a*S*)-4-formylphenylamino)-1-(2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-yl)piperidine-4-carboxylic acid amide [(+)-(*S*,*S*)-**14**] (6.75 g, 0.0162 mol) in triethyl orthoformate (150 mL) was stirred at reflux for 4 days. The reaction mixture was concentrated, added to MeOH (180 mL)/THF (90 mL), and then treated with sodium borohydride (1.23 g, 0.0324 mol). The mixture was stirred at RT for 3 h, concentrated, added to 3 N NaOH (150 mL), and extracted with CH_2Cl_2. The organic extract was dried (Na_2SO_4) and concentrated to give 6.80 g of a yellow foam. Chromatographic purification on SiO_2, using CH_2Cl_2/MeOH/NH_4OH (300:20:2), as eluent, gave 5.60 g of Ro 64-6198 (**2**) as a foam. The free base Ro 64-6198 (**2**) was dissolved in CH_2Cl_2 and acidified with 2 M ethereal HCl. The precipitated solids were collected and recrystallized from MeOH/EtOAc to give Ro 64-6198·HCl (**2**·HCl) (2.73 g) as a white solid with mp 254−255 °C (lit[22] 262 °C). Chiral HPLC analysis

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

(Chiralpak, IA, 4.6 × 250 mm, 5 μM, 98% hexane/MTBE (1:1) containing 0.1% DEA; 2% EtOH containing 0.1% DEA, 1.0 mL/min, 254 nm) R_t 13.28 min, 80% ee. The HCl salt (**2**·HCl) was converted to the free base **2** (2.38 g), dissolved in EtOAc, and treated with (+)-dibenzoyl-D-tartaric acid (2.15 g) to give the corresponding salt (3.34 g). The salt was converted back to the free base **2** (1.81 g), which was treated with 2 M ethereal HCl to give the HCl salt (**2**·HCl). This salt was recrystallized from MeOH to yield 0.986 g (15%) of Ro 64-6198·HCl (**2**·HCl) (99.1% ee) as a white solid; mp 241−242 °C (lit[22] 262 °C); $[\alpha]_D$ = −97° (c 0.130, MeOH), [lit[22] $[\alpha]_D$ = −98.5°(c 0.11 MeOH)]. ^{1}H NMR (DMSO-d_6): δ:1.17−1.27 (m, 2H, H-2), 1.78−2.04 (m, 7H, H-4, H-5, H-6′a, H-10′a), 2.36−2.42 (m, 1H, H-3ax), 2.56−2.64 (m, 1H, H-3eq), 2.80−2.87 (m, 2H, H-7′ax, H-9′ax) 3.29−3.43 (m, 2H, 7′eq, H-9′eq), 3.65−3.95 (m, 2H, H-6), 4.58−4.63 (m, 1H, H-2′), 6.78 (t, J = 7.0 Hz, 1H, H-7), 7.10−7.27 (m, 6H, ArH, H-8), 7.80 (d, J = 7.0 Hz, 1H, H-9), 9.04 (s, 1H, NH), 10.43 (bs, 1H, NH). MS (EI) m/z calcd for $C_{26}H_{27}N_3O$, 401; found, 402 (M + H). Anal. Calcd for $C_{26}H_{27}N_3O$ HCl: C, 71.3; H, 7.36; Cl, 8.09; N, 9.59. Found: C, 71.27; H, 7.33; N, 9.60; Cl, 7.99.

Resolution of 1-[(1*RS*,3a*RS*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]piperidin-4-one ((*RR*/*SS*)-6). The mother liquor from the resolution of (*RR*/*SS*)-**6** was treated with di-*p*-toluoyl-L-tartaric acid to yield 8.0 g of (+)-1-(2,3,3a,4,5,6-hexahydro-1*H*-phenalen-1-ylpiperidin-4-one ((+)-(*R*,*R*)-**6**) as a light yellow oil; $[\alpha]_D$ = +24.5° (c 0.920, MeOH).

(−)-1-[(1*R*,3a*R*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]-4-(phenylamino)piperidine-4-carbonitrile [(−)-(*R*,*R*)-13]. Following the same procedure as for (+)-(*S*,*S*)-**13**, (−)-(*R*,*R*)-**13** was isolated to yield 9.38 g (83%); $[\alpha]_D$ = 25.9°(c 0.590, $CHCl_3$) (lit[22] + 29.2° (c 0.31, $CHCl_3$).

(−)-1-[(1*R*,3a*R*)-2,3,3a,4,5,6-Hexahydro-1H-phenalen-1-yl]-4-(*N*-phenylformamido)piperidine-4-carboxamide [(−)-(*R*,*R*)-14]. Following the same procedure as for (+)-(*S*,*S*)-**14**, (−)-(*R*,*R*)-**14** was isolated (6.75 g, 65%). MS (EI) m/z calcd for $C_{26}H_{31}N_3O_2$, 417; found, 418 (M + H).

8-[(1*R*,3a*R*)-2,3,3a,4,5,6-Hexahydro-1*H*-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one [(*R*,*R*)-Ro 64-6198]. Following the same procedure as for (*S*,*S*)-Ro 64-6198, (*R*,*R*)-Ro 64-6198 was isolated as the HCl salt (2.88 g, 24%); $[\alpha]_D$ = +100.5° (c 0.190, MeOH) (lit[22] + 94.6° (c 0.1, MeOH)); mp 251−252 °C (lit[22] 253 °C). Chiral HPLC gave 100% ee. Anal. Calcd for $C_{26}H_{27}N_3O$ HCl: C, 71.3; H, 7.36; Cl, 8.09; N, 9.59. Found: C, 71.01; H, 7.25; N, 9.43; Cl, 7.93.

In Vitro Pharmacology. *Calcium Mobilization.* CHO-RD-HGA16 (Molecular Devices) cells stably expressing the human NOP receptor were used. The day before the assay, cells were plated into 96-well black-walled assay plates at 30 000 cells/well (100 μL per well) in Ham's F12 supplemented with 10% fetal bovine serum and 100 units of penicillin and streptomycin. The cells were incubated overnight at 37 °C, 5% CO_2. Prior to the assay, Calcium 5 dye (Molecular Devices) was reconstituted according to the manufacturer instructions. The reconstituted dye was diluted 1:20 in prewarmed (37 °C) assay buffer (1× HBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4 at 37 °C), and 100 μL was added to each well. The cells were incubated for 45 min at 37 °C, 5% CO_2. During the incubation, serial dilutions of the test compounds were prepared in 0.25% BSA/1% DMSO/assay buffer, aliquoted into 96-well polypropylene plates, and warmed to 37 °C. After the dye-loading incubation period, the cells were pretreated with 25 μL of 2.25% BSA/9% DMSO/assay buffer and incubated for 15 min at 37 °C. After the pretreatment incubation period, the plate was read with a FlexStation II (Molecular Devices). Calcium-mediated changes in fluorescence were monitored every 1.52 s over a 60 s time period, with the FlexStation II adding 25 μL of test compound dilutions at the 19 s time point (excitation at 485 nm, detection at 525 nm). Peak kinetic reduction (SoftMax, Molecular Devices) relative fluorescent units (RFU) were plotted against the log of compound concentration. Data were fit to a three-parameter logistic curve to generate EC_{50} values (GraphPad Prism 6.0, GraphPad Software, Inc., San Diego, CA).

cAMP Accumulation Assay. HEK293 cells were stably transfected with both Promega's proprietary GloSensor plasmid (optimized for room temperature 25 °C experiments) and human NOPR. Cells were plated at ∼100 000 cell/well density on a 96-well opaque white plate. Cells were treated with 10 mM Forskolin and 1 mM IBMX for 10 min and treated with Ro 64-6198 concentrations ranging from 10 μM to 1 pM, and then luminescence was measured in a BioTek Synergy MX plate reader at 5 min increments for 45 min. A minimum of four independent experiments, consisting of three replicates each, were performed for each concentration form multiple passage variations of NOPR expressing pGlo cells.

Bioluminescence Resonance Energy Transfer Assay. HEK293 cells were transiently transfected with 125 ng of NOPR-Renilla Luciferase8 (NOPR-Rluc8) plasmids and 1 μg of Venus-Arrestin3 (or Venus-Arrestin2) using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, cells were plated into 96-well plates (Corning). And 24 h after plating, media were replaced with DMEM without phenol red (Cellgro). Fluorescence and luminescence signals were read using a Synergy H1 plate reader (Biotek). Initially, YFP expression was measured by fluorescence at 535 nm with 485 nm excitation. Immediately after, 5 μM coelenterazine-h (Biotium) was added to each well, and Rluc8 luminescence levels were measured. BRET between Rluc8 and Venus was measured sequentially with 460 and 528 nm filters 3 min before the addition of ligands for baseline, then every minute for 30 min after treatment. The BRET ratio was calculated as 528 nm emission divided by 460 nm emission. Ligand-induced BRET was calculated as the BRET ratio subtracted by the average BRET of the untreated baseline. All experiments were performed in triplicate.

In Vivo Pharmacology. *Paw withdrawal Test.* The heat analgesia test was performed in a polycarbonate chamber encompassing the hot plate (Model PE34 Series 8, IITC Life Science Inc.). Mice were acclimated to the chamber 1 day before testing for 15 min. Apparatus was set to 55 °C and mice were placed onto the plate until a hindpaw lick was observed, and scored as latency to paw withdrawal. A cutoff time of 20 s was used to prevent unnecessary pain or tissue damage.

Open-Field Test. The open-field testing was performed in polycarbonate open-top square boxes measuring 50 cm × 50 cm × 28 cm. Room lighting was maintained at 100 lx (±5), measured from the center of the box. Mice were injected with 10 μL/g volume (i.p.) 30 min prior to placement in the center of the arena, and then filmed via overhead camera for 1 h. Animals were tracked and analyzed by Ethovision 8.5 (Noldus Technologies, Leesburg, VA) for locomotor activity (distance traveled) and center time. Center time was used as our measure of anxiety. The center-time area was defined in Ethovision as the central 50% of the area within the box, equidistant from all sides. Naive adult (8−10 weeks) male mice were tested (n = 8−9).

Accelerating Rotarod Test. Accelerating Rotarod (Ugo Basile) testing was used to measure motor coordination. Mice were trained on the apparatus at 4 rpm until they were able to remain on the apparatus for 120 s. Subjects were given a maximum of five trials to complete training, or were excluded. At 30 min after training, the mice were injected (i.p.) with Ro 64-6198 (0.3−3 mg/kg) or vehicle control. At 1 h after training, the apparatus was set to accelerating (4−40 rpm), and latency to fall was measured. Five trials were performed at 5 min lengths, with 5 min in between in each trial. Naïve adult (8−10 weeks) male mice were tested (n = 8−10).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00208.

Chiral HPLC and determination of %ee of Ro 64-6198 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Telephone: 314-747-5754. E-mail: bruchasm@wustl.edu.
*Telephone: 919 541-6679. Fax: 919 541-8868. E-mail: fic@rti.org.

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

Author Contributions

L.E.B. performed synthesis of compounds, and C.J.M., Jr. and D.Z. developed and performed chiral HPLC analysis. S.D.C., J.D.H., E.E.P., and A.M.D. performed pharmacological studies. S.D.C., L.E.B., A.H.L., S.W.M., H.H.S., P.A.R., A.M.D., H.A.N., M.R.B., and F.I.C. designed the studies, performed analysis of the data, and wrote the manuscript.

Funding

This research was supported by the National Institute on Drug Abuse (NIDA), Projects N01DA-13-7784 (FIC), R21034929 (MRB), and T32DA007261 (SDC).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

NOP, nociceptin/orphanin FQ opioid peptide receptor; N/OFQ, nociceptin/orphanin FQ; DOP, delta opioid receptor; KOP, kappa opioid receptor; BRET, bioluminescence resonance energy transfer; RTI, Research Triangle Institute; GPCR, G protein coupled receptor; HEK293, human embryonic kidney 293; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's modified Eagle's medium; Rluc8, renilla luciferase 8

■ REFERENCES

(1) Bunzow, J. R., Saez, C., Mortrud, M., Bouvier, C., Williams, J. T., Low, M., and Grandy, D. K. (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a m, d, or k opioid receptor type. *FEBS Lett. 347*, 284−288.

(2) Chen, Y., Fan, Y., Liu, J., Mestek, A., Tian, M., Kozak, C. A., and Yu, L. (1994) Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. *FEBS Lett. 347*, 279−283.

(3) Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., Miyata, T., Houtani, T., and Sugimoto, T. (1994) cDNA cloning and regional distribution of a novel member of the opioid receptor family. *FEBS Lett. 343*, 42−46.

(4) Mollereau, C., Parmentier, M. L., Mailleux, P., Butour, J.-L., Moisand, C., Chalon, P., Caput, D., Vassart, G., and Meunier, J.-C. (1994) ORL1, a novel member of the opioid receptor family: Cloning functional expression and localization. *FEBS Lett. 341*, 33−38.

(5) Mogil, J. S., and Pasternak, G. W. (2001) The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol. Rev. 53*, 381−415.

(6) Devine, D. P., Watson, S. J., and Akil, H. (2001) Nociceptin/orphanin FQ regulates neuroendocrine function of the limbic-hypothalamic-pituitary-adrenal axis. *Neuroscience 102*, 541−553.

(7) Fernandez, F., Misilmeri, M. A., Felger, J. C., and Devine, D. P. (2004) Nociceptin/orphanin FQ increases anxiety-related behavior and circulating levels of corticosterone during neophobic tests of anxiety. *Neuropsychopharmacology 29*, 59−71.

(8) Leggett, J. D., Harbuz, M. S., Jessop, D. S., and Fulford, A. J. (2006) The nociceptin receptor antagonist [Nphe1,Arg14,Lys15]-nociceptin/orphanin FQ-NH2 blocks the stimulatory effects of nociceptin/orphanin FQ on the HPA axis in rats. *Neuroscience 141*, 2051−2057.

(9) Le Cudennec, C., Naudin, B., Do Rego, J. C., and Costentin, J. (2002) Nociceptin/orphanin FQ and related peptides reduce the increase in plasma corticosterone elicited in mice by an intracerebroventricular injection. *Life Sci. 72*, 163−171.

(10) Reinscheid, R. K., Nothacker, H.-P., Bourson, A., Ardati, A., Henningsen, R. A., Bunzow, J. R., Grandy, D. K., Langen, H., Monsma, F. J. J., and Civelli, O. (1995) Orphanin FQ: A neuropeptide that activates opioid-like G protein-coupled receptor. *Science 270*, 792−794.

(11) Lambert, D. G. (2008) The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat. Rev. Drug Discovery 7*, 694−710.

(12) Murphy, N. P. (2010) The nociceptin/orphanin FQ system as a target for treating alcoholism. *CNS Neurol. Disord.: Drug Targets 9*, 87−93.

(13) Meunier, J.-C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.-L., Guillemot, J.-C., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M., and Costentin, J. (1995) Isolation and structure of the endogenous agonist of the opioid receptor-like ORL_1 receptor. *Nature 377*, 532−535.

(14) Jenck, F., Wichmann, J., Dautzenberg, F. M., Moreau, J. L., Ouagazzal, A. M., Martin, J. R., Lundstrom, K., Cesura, A. M., Poli, S. M., Roever, S., Kolczewski, S., Adam, G., and Kilpatrick, G. (2000) A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat. *Proc. Natl. Acad. Sci. U. S. A. 97*, 4938−4943.

(15) Shoblock, J. R., Wichmann, J., and Maidment, N. T. (2005) The effect of a systemically active ORL-1 agonist, Ro 64-6198, on the acquisition, expression, extinction, and reinstatement of morphine conditioned place preference . *Neuropharmacology 49*, 439−446.

(16) Kuzmin, A., Sandin, J., Terenius, L., and Ogren, S. O. (2003) Acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice: effects of opioid receptor-like 1 receptor agonists and naloxone. *J. Pharmacol. Exp. Ther. 304*, 310−318.

(17) Kuzmin, A., Kreek, M. J., Bakalkin, G., and Liljequist, S. (2007) The nociceptin/orphanin FQ receptor agonist Ro 64-6198 reduces alcohol self-administration and prevents relapse-like alcohol drinking. *Neuropsychopharmacology 32*, 902−910.

(18) Le Pen, G., Wichmann, J., Moreau, J. C., and Jenck, F. (2002) The orphanin receptor agonist Ro 64-6198 does not induce place conditioning in rats. *NeuroReport 13*, 451−454.

(19) Reiss, D., Wichmann, J., Tekeshima, H., Kieffer, B. L., and Ouagazzal, A. M. (2008) Effects of nociceptin/orphanin FQ receptor (NOP) agonist, Ro 64-6198, on reactivity to acute pain in mice: comparison to morphine. *Eur. J. Pharmacol. 579*, 141−148.

(20) Ko, M. C., Woods, J. H., Fantegrossi, W. E., Galuska, C. M., Wichmann, J., and Prinssen, E. P. (2009) Behavioral effects of a synthetic agonist selective for nociceptin/orphanin FQ peptide receptors in monkeys. *Neuropsychopharmacology 34*, 2088−2096.

(21) Podlesnik, C. A., Ko, M. C., Winger, G., Wichmann, J., Prinssen, E. P., and Woods, J. H. (2011) The effects of nociceptin/orphanin FQ receptor agonist Ro 64-6198 and diazepam on antinociception and remifentanil self-administration in rhesus monkeys. *Psychopharmacology (Berl) 213*, 53−60.

(22) Wichmann, J., Adam, G., Rover, S., Hennig, M., Scalone, M., Cesura, A. M., Dautzenberg, F. M., and Jenck, F. (2000) Synthesis of (1S,3aS)-8-(2,3,3a,4,5, 6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4. 5]decan-4-one, a potent and selective orphanin FQ (OFQ) receptor agonist with anxiolytic-like properties. *Eur. J. Med. Chem. 35*, 839−851.

(23) Robinson, J. I., Rihloff, J., and Kowalczyk, B. (1996) *Process for Preparing 2-(1-azabicyclo[2.2.2]oct-3-yl)-2,3,3,4,5,6-hexahydro-1H-benz9(e)isoquinoli-1-one*, Syntex (USA) Inc., Palo Alto, CA.

(24) Milligan, G., Marshall, F., and Rees, S. (1996) G16 as a universal G protein adapter: implications for agonist screening strategies. *Trends Pharmacol. Sci. 17*, 235−237.

(25) Camarda, V., Fischetti, C., Anzellotti, N., Molinari, P., Ambrosio, C., Kostenis, E., Regoli, D., Trapella, C., Guerrini, R., Severo, S., and Calo, G. (2009) Pharmacological profile of NOP receptors coupled with calcium signaling via the chimeric protein G alpha qi5. *Naunyn-Schmiedeberg's Arch. Pharmacol. 379*, 599−607.

(26) Hashiba, E., Lambert, D. G., Jenck, F., Wichmann, J., and Smith, G. (2002) Characterisation of the non-peptide nociceptin receptor agonist, Ro 64-6198 in Chinese hamster ovary cells expressing recombinant human nociceptin receptors . *Life Sci. 70*, 1719−1725.

(27) Dooley, C. T., and Houghten, R. A. (2000) Orphanin FQ/nociceptin receptor binding studies. *Peptides 21*, 949−960.

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964

(28) Chang, S., Mascarella, S., Spangler, S., Gurevich, V., Navarro, H., Carroll, F., and Bruchas, M. (2015) Quantitative Signaling and Structure-Activity Analyses Demonstrate Functional Selectivity at the Nociceptin/Orphanin FQ Opioid Receptor. *Mol. Pharmacol. 88*, 502−511.
(29) Varty, G. B., Hyde, L. A., Hodgson, R. A., Lu, S. X., McCool, M. F., Kazdoba, T. M., Del Vecchio, R. A., Guthrie, D. H., Pond, A. J., Grzelak, M. E., Xu, X., Korfmacher, W. A., Tulshian, D., Parker, E. M., and Higgins, G. A. (2005) Characterization of the nociceptin receptor (ORL-1) agonist, Ro 64-6198, in tests of anxiety across multiple species. *Psychopharmacology (Berl) 182*, 132−143.
(30) Higgins, G. A., Grottick, A. J., Ballard, T. M., Richards, J. G., Messer, J., Takeshima, H., Pauly-Evers, M., Jenck, F., Adam, G., and Wichmann, J. (2001) Influence of the selective ORL1 receptor agonist, Ro 64-6198, on rodent neurological function. *Neuropharmacology 41*, 97−107.
(31) Fan, H., Luttrell, L. M., Tempel, G. E., Senn, J. J., Halushka, P. V., and Cook, J. A. (2007) Beta-arrestins 1 and 2 differentially regulate LPS-induced signaling and pro-inflammatory gene expression. *Mol. Immunol. 44*, 3092−3099.
(32) Goeldner, C., Spooren, W., Wichmann, J., and Prinssen, E. P. (2012) Further characterization of the prototypical nociceptin/orphanin FQ peptide receptor agonist Ro 64-6198 in rodent models of conflict anxiety and despair. *Psychopharmacology (Berlin, Ger.) 222*, 203−214.
(33) Shoblock, J. R. (2007) The pharmacology of Ro 64-6198, a systemically active, nonpeptide NOP receptor (opiate receptor-like 1, ORL-1) agonist with diverse preclinical therapeutic activity . *CNS Drug Rev. 13*, 107−136.

DOI: 10.1021/acschemneuro.5b00208
ACS Chem. Neurosci. 2015, 6, 1956−1964