Title page

NCP, a dual kappa and mu opioid receptor agonist, is a potent analgesic against inflammatory pain without reinforcing or aversive properties

Peng Huang¹, Conrad K. Ho¹, Danni Cao¹, Saadet Inan¹, Scott M. Rawls¹, Mengchu Li², Bo Shi Huang², Piyusha P. Pagare², E Andrew Townsend³, Justin L. Poklis³, Matthew S. Halquist⁴, Matthew Banks³, Yan Zhang²³⁵, and Lee-Yuan Liu-Chen¹

¹ Center for Substance Abuse Research, Temple University Lewis Katz School of Medicine, Philadelphia, PA, 19140, USA
² Department of Medicinal Chemistry, Virginia Commonwealth University School of Pharmacy, Richmond, VA, 23298 USA
³ Department of Pharmacology and Toxicology, Virginia Commonwealth University School of Medicine, Richmond, VA, 23298 USA
⁴ Department of Pharmaceutics, Virginia Commonwealth University School of Pharmacy, Richmond, VA, 23298 USA
⁵ Institute for Drug and Alcohol Studies, 203 East Cary Street, Virginia Commonwealth University, Richmond, VA 23298, USA

*Corresponding author:
Lee-Yuan Liu-Chen, Ph.D.
Center for Substance Abuse Research, Temple University Lewis Katz School of Medicine, 3500 North Broad Street, MERB 851, Philadelphia, PA 19140, USA
Tel.: +1 215 707 4188; fax: +1 215 707 7661.
lliuche@temple.edu
Running title: A non-addictive analgesic with KOR/MOR agonist activities

*Corresponding author:
Lee-Yuan Liu-Chen, Ph.D.
Center for Substance Abuse Research, Temple University Lewis Katz School of Medicine, 3500 North Broad Street, MERB 851, Philadelphia, PA 19140, USA
Tel.: +1 215 707 4188; fax: +1 215 707 7661.
lliuche@temple.edu

The number of text pages: 24 (from Abstract to Footnotes)
The number of tables: 1
The number of figures: 11 plus one supplemental figure
The number of references: 77
The number of words in the Abstract: 249
The number of words in Introduction: 746 including reference citations
The number of words in Discussion: 1954 including reference citations

A list of nonstandard abbreviations used in the paper (alphabetical order):

β-FNA, β-Funaltrexamine; CPA, conditioned place aversion; CPP, conditioned place preference; CR845, Difelikefalin; GPCRs, G protein-coupled receptors; KOR, kappa opioid receptor; MOR, mu opioid receptor; NCP, 17-Cyclopropymethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[[4′-(2′-cyanopyridyl)]carboxamido]morphinan; norBNI, Norbinaltorphimine.

A recommended section assignment to guide the listing in the table of contents:
Neuropharmacology
Abstract

While agonists of μ (MOR) and κ (KOR) opioid receptors have analgesic effects, they produce euphoria and dysphoria, respectively. Other side effects include respiratory depression and addiction for MOR agonists and sedation for KOR agonists. We reported that 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[[4′-(2′-cyanopyridyl)]carboxamido]cmorphinan (NCP) displayed potent KOR full agonist and MOR partial agonist activities (58%) with 6.5x KOR-over-MOR selectivity in vitro. Herein, we characterized pharmacological effects of NCP in rodents. In mice, NCP exerted analgesic effects against inflammatory pain in both the formalin test and the acetic acid writhing test, with A50 values of 47.6 and 14.4 μg/kg (s.c.), respectively. The analgesic effects in the acetic acid writhing test were mediated by the KOR. NCP at doses much higher than those effective in reducing inflammatory pain did not produce antinociception in the hot plate and tail flick tests, inhibit compound 48/80-induced scratching, cause conditioned place aversion (CPA) or preference, impair rotarod performance, inhibit locomotor activity, cause respiratory depression, or precipitate morphine withdrawal. However, NCP (10~100 μg/kg) inhibited gastrointestinal transit with a maximum of ~40% inhibition. In MOR knockout mice, NCP caused CPA, demonstrating that its lack of CPA is due to combined actions on the MOR and KOR. Following s.c. injection, NCP penetrated into the mouse brain. In rats trained to self-administer heroin, NCP (1~320 μg/kg/infusion) did not function as a reinforcer. Thus, NCP produces potent analgesic effects via KOR without side effects except constipation. Therefore, dual full KOR/partial MOR agonists with moderate KOR-over-MOR selectivity may be promising as non-addictive analgesics for inflammatory pain.
Significance Statement

Developing non-addictive analgesics is crucial for reducing opioid overdose deaths, minimizing drug misuse, and promoting safer pain management practices. Herein, pharmacology of a potential non-addictive analgesic, NCP, is reported. NCP has full KOR agonist / partial MOR agonist activities with a 6.5 x selectivity for KOR over MOR. Unlike MOR agonists, analgesic doses of NCP do not lead to self-administration or respiratory depression. Furthermore, NCP does not produce aversion, hypolocomotion, or motor incoordination, side effects typically associated with KOR activation.
Introduction

There are three opioid receptors: mu, delta, and kappa opioid receptors (MOR, DOR, and KOR, respectively), which are G<sub>i/o</sub> proteins-coupled receptors. Opioid receptors are distributed throughout the central and peripheral nervous system (Mansour et al., 1988) and activation of these receptors produces many effects, most notably analgesia, anti-pruritic effect, altered reward processing and mood regulation [reviewed in (Gaveriaux-Ruff and Kieffer, 2002; Darcq and Kieffer, 2018; Cahill et al., 2022)]. While the MOR is the main target for opioid analgesics used clinically, these drugs produced serious adverse effects, particularly addiction and respiratory depression (Darcq and Kieffer, 2018). KOR agonists produce analgesic and antipruritic effects (von Voigtlander et al., 1983; Cowan and Gmerek, 1986; Inan and Cowan, 2022); however, clinical development of these compounds has been limited by side effects such as psychotomimetic effect, dysphoria, and sedation (Rimoy et al., 1994; Pande et al., 1996), except for nalfurafine (formerly TRK820) [reviewed in (Miyamoto et al., 2022)] and difelikefalin (formerly CR845) (Fishbane et al., 2020; Lipman and Yosipovitch, 2021).

Recent trends in developing KOR agonists as analgesics and anti-pruritic agents with fewer side effects include peripherally acting agonists and G protein-biased agonists. The FDA recently granted approval for the treatment of systemic itch in hemodialysis patients using a peripherally acting KOR agonist, Difelikefalin (formerly CR845) (Fishbane et al., 2020; Lipman and Yosipovitch, 2021). It is desirable to develop G protein-biased agonists because it was postulated that following KOR activation, analgesic and anti-pruritic effects are mediated by G protein-mediated signaling, whereas aversion and sedation are attributed to β-arrestin signaling [reviewed in (Brust, 2022)]. Nalfurafine has been used in Japan for treatment of systemic itch in patients undergoing kidney dialysis or suffering from chronic liver diseases without producing aversion or sedation [reviewed in (Miyamoto et al., 2022)]. Whether nalfurafine is a G protein-biased KOR agonist is a matter of debate. It was reported to be G protein-biased, balanced, and β-arrestin-biased [reviewed in (Zhou et al., 2022)]. To date no newly developed G protein-biased KOR agonists have been approved for use in humans.

MOR agonists with agonist activities at other receptors have been postulated to be a valid avenue for developing non-addictive analgesics with reduced side effects (Gunther et al., 2018). For example, AT-121 and cebranopadol, both having partial agonist activity at MOR and nociceptin/orphanin FQ receptors, exerted morphine-like analgesic effects without causing side effects commonly associated with mu opioids (Linz et al., 2014; Ding et al., 2018). Both MOR agonists and KOR agonists produce analgesia, while they produce opposite hedonic states, euphoria and dysphoria, respectively (Pan, 1998; Darcq and Kieffer, 2018). Combined use of a KOR agonist and a MOR agonist has additive analgesic effects (Negus et al., 2008). Studies have shown that KOR agonists reduce the
rewarding effects of MOR opioids (Tsuji et al., 2001; Kaski et al., 2019). In self-administration studies in mice, rats and monkeys, treatment with KOR agonists, particularly non-conditioned place aversion (CPA)-producing nalfurafine, decreases reinforcing properties of and the choice for MOR agonists when mixed or co-administered with them (Kuzmin et al., 1997; Negus et al., 2008; Townsend et al., 2017; Zamarripa et al., 2020a; Zamarripa et al., 2020b; Zhang and Kreek, 2020). In addition, MOR agonists cause itch (Hales, 1980), whereas KOR agonists have anti-itch effects [reviewed in (Inan and Cowan, 2022)]. Thus, a compound with MOR agonist and KOR agonist activities is likely to be an effective analgesic with low likelihood of producing either dysphoria or addiction (Darcq and Kieffer, 2018).

Zhang’s lab has synthesized several series of 4,5-epoxymorphinan compounds. One of the compounds is 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[[4′-(2′-cyanopyridyl)]carboxamido]cmorphinan (NCP), which has substitutions at the 6b position (Huang et al., 2021; Wang et al., 2021) (Fig. 1). NCP displayed high affinities for the KOR and MOR with Ki values of 0.13 and 1.25 nM, respectively, and had much lower affinity for the DOR (Ki 75.3 nM). In in vitro [35S]GTPγS binding assay, NCP displayed potent KOR full agonist activity and MOR partial agonist activity (58% of the full agonist DAMGO) with EC50 values of 0.28 nM and 1.82 nM, respectively, showing a selectivity ratio of 6.5 for the KOR over MOR (Huang et al., 2021; Wang et al., 2021). NCP had a much lower potency at the DOR with high KOR/DOR selectivity (107x). We hypothesized that NCP may have favorable pharmacological properties in vivo due to its dual KOR and MOR agonist activities. In this study, we investigated its in vivo pharmacological effects. The prototypic KOR agonist U50,488H or MOR agonist morphine was included for comparison.
Materials and Methods

Drugs and Materials

U50,488H (U50,488 methanesulfonate), morphine sulfate, β-Funaltrexamine (β-FNA), Norbinaltorphimine (norBNI) and heroin HCl was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). U50,488H and morphine sulfate were dissolved in water. NCP was synthesized as described previously (Wang et al., 2021) and dissolved in saline or deionized water at 0.3-0.5 mg/ml as a stock solution, aliquoted and frozen and before use diluted with saline or deionized water, respectively. In mice, compounds were injected s.c. or i.p. at 10 μl/g body weight. Solutions for rat IV administration (heroin HCl and NCP) were passed through a 0.22-micron sterile filter (Millex GV, Millipore Sigma, Burlington, MA) before injection. Naloxone-d5, a reference standard, was purchased from Cerilliant Corp (Round Rock, TX). Paraformaldehyde and compound 48/80 were purchased from Sigma-Aldrich (St. Louis, MO). Formalin (5%) were prepared with paraformaldehyde (1.85%). Other commonly used chemicals were obtained from Sigma-Aldrich or ThermoFisher Scientific.

Animals

Adult male CD-1 mice, 30–45 g, purchased from Charles River Laboratories (Wilmington, MA), were used for most experiments. Male Swiss Webster mice (5-8 week, 25-35 g) from Envigo Laboratories (Frederick, MD) were used in the tail-flick assay and for drug distribution studies. Male C57BL/6J mice (20-25 g) from The Jackson Lab (Bar Harbor, ME) were used in the morphine withdrawal experiments. Male MOR knockout (MOR-/-) and wild type (WT) mice (22-35 g) were obtained from Animal Core of P30 Center of Excellence in our Center, which were bred by homozygous/homozygous pairing (C57BL/6 background) at Temple University. MOR-/- mice were originally developed in the lab of Dr. John Pintar by deletion of exon-1 of the oprm1 gene through homologous recombination (Schuller et al., 1999). All the mice were housed in a temperature- and humidity-controlled room on a light-dark cycle (22°C, 50–60 % humidity, 12:12 h light / dark cycle, lights off at 6 pm). All experiments were conducted when lights were on. The animals received food and water ad libitum, except during the experimental sessions. All procedures were approved by Institutional Animal Care and Use Committee of Temple University Lewis Katz School of Medicine.

For intravenous (IV) self-administration studies, female (240-260 g) and male (290-310 g) Sprague-Dawley rats were acquired (Envigo Laboratories, Frederick, MD, USA) and surgically implanted with custom-made jugular catheters and vascular access ports (Instech, Plymouth Meeting, PA, USA) as described previously (Townsend et al., 2021). Rats were singly housed in a temperature and humidity-controlled vivarium that was maintained on a 12-h light/dark cycle (lights off at 6:00 PM). Water and food (Teklad Rat Diet, Envigo) were provided ad libitum in the home cage. Behavioral
sessions were conducted 5 days per week at approximately the same time every day. Both research and enrichment protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Animal maintenance and research were conducted in accordance with the 2011 National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Behavioral tests in mice**

**Formalin test** was performed as we described (Liu et al., 2019a), which was based on the procedures of Murray et al. (1988). Briefly, after acclimation, mice were pretreated with saline (10 ml/kg, s.c.), U50,488H (2.5 mg/kg, s.c.) or NCP (20, 40, 80 μg/kg, s.c.) followed by intraplantar injection of 5% formalin (20 μl) 5 minutes later. The time each animal licked / groomed the formalin-injected paw was recorded from 15 to 35 min post-injection of formalin (phase II reaction).

**Acetic Acid Writhing Test** was carried out according to our published procedures (Huang et al., 2001; Suzuki et al., 2004) with some modifications. Male CD-1 mice were individually habituated in the observation chambers for at least 1 h, and then pretreated s.c. with either vehicle or NCP (10, 20, 40, 80 μg/kg, s.c.). Twenty min later, acetic acid (0.6%) was injected intraperitoneally (10 μl/g, i.p.). A further 5 min later, the number of writhes (abdominal stretches) was recorded for 15 min. The numbers of writhes in each group were then normalized to the mean of the vehicle-treated group. For investigation of the roles of KOR and MOR in the antinociceptive effects of NCP, mice were pretreated with the selective KOR antagonist norBNI (32 mg/kg, i.p.) or the selective MOR antagonist β-FNA (32 mg/kg, s.c.) for 24 h or 48 h, respectively, before the acetic acid writhing test. The dosing regimens were modified from those in previous pharmacological studies of opioids with the acetic acid writhing test as an endpoint (Ward et al., 1982; Broadbear et al., 1994). In addition, MOR-/- mice and WT mice were used to further examine the antinociceptive effects of NCP.

**Hot plate test** was conducted following our published procedures (Zhang et al., 2005) with some modifications. Mice were acclimated on a hot plate (Ugo Basile, Varese, Italy) first at room temperature about 1 min on the day of testing. Mice were tested on the hot plate at 52.5 ± 0.1°C according to the method of Dewey et al. (1970). Utilizing jumping, hind paw, or front paw licking as the nociceptive endpoint, two baseline response latency values were recorded for each animal following two conditioning runs with a minimum interval of 5 minutes between them. Mice (~80-90%) with the baseline average (8-20 s) were used. Then mice were pretreated with saline (10 ml/kg, s.c.), morphine (10 mg/kg, s.c.) or NCP (0.08, 0.80 or 10 mg/kg, s.c.). Twenty min later, each mouse underwent retesting on the hot plate at 15-minute intervals up to 60 minutes, using 30 s as the cutoff point (100%
of maximum possible antinociception). The latency of hind paw licking, front paw licking or jumping was measured and then normalized to the baseline latency. Antinociception was assessed as the percentage of maximal possible antinociception, determined by the formula: % of maximal possible antinociception = [(test latency − control latency)/(30 − control latency)] × 100.

**Warm water tail-flick test** was performed using a water bath with the temperature maintained at 56 ± 0.1°C as described previously (Li et al., 2022). Each mouse was gently wrapped in a cloth with only tail exposed. Baseline latency was measured before s.c. injections of saline or a selected compound. The distal one-third of the tail was immersed perpendicularly in water, and the mouse rapidly flicked his tail from the bath at the first sign of discomfort. The duration of time the tail remained in the water bath was counted as the baseline latency. Untreated mice with baseline latency reaction times ranging from 2 to 4 seconds were used. Test latency was obtained 20 minutes later after each subcutaneous injection. A 10-second maximum cutoff latency was used to prevent any tissue damage. Antinociception was quantified as the percentage of maximal possible antinociception, which was calculated as % of maximal possible antinociception = [(test latency − control latency)/(10 − control latency)] × 100.

**Compound 48/80 scratching test** was performed as we described (Liu et al., 2019a), which was based on the procedures of Wang et al. (2005). Briefly, after acclimation, mice were injected with saline (10 ml/kg, s.c.), U50,488H (2.5 mg/kg, s.c.) or NCP (80 μg/kg, s.c.) 20 min before administration of compound 48/80 (50 μg, s.c.), then the bouts of scratching were counted for 30 min.

**Rotarod test** was performed as we described (Liu et al., 2019a; Cao et al., 2020), which was adapted from the procedures of White et al. (2015). Male CD-1 mice (35-45 g) were used. Briefly, on the day preceding the test, mice underwent training on the rotarod, engaging in a 5-minute session with rotational speeds varying from 5 to 50 revolutions per minute. Each mouse underwent 2 to 3 training trials, with a 1-minute interval between each trial. After training, mice that demonstrated an ability to remain on the rotarod for over 240 seconds (equivalent to 80% of the 5-minute period), constituting approximately 50% of the mice, were selected. On the test day, the mice were initially assessed on the rotarod to establish baseline performance. Mice with baselines exceeding 240 seconds (comprising over 90% of the selected mice) were then injected with NCP (80 μg/kg, s.c.), U50,488H (2, 5 mg/kg, s.c.) or saline (10 ml/kg, s.c.) before the test, then the time each mouse stayed on the rotarod was recorded for 5 minutes at 10, 20, 30, and 40 min after injection.

**Conditioned place aversion (CPA)** was performed as we described (Liu et al., 2019a; Huang et al., 2022), which was adapted from our procedures of conditioned place preference (Xu et al., 2013),
using 2-chamber boxes. On day 1, mice were subject to pre-test for 15 min, in which the time each mouse spent in either chamber was recorded. On days 2-4, mice were pretreated with (morning/afternoon) saline / saline (10 ml/kg, s.c.), saline / U50,488H (2 or 5 mg/kg, s.c.) or saline / NCP (40, 80 or 800 μg/kg, s.c.) 10 min before each conditioning session, in which mouse was confined to one chamber for 30 min. Thus, mice were conditioned with U50,488H or NCP three times. On day 5, the time each mouse spent in either chamber was recorded in the 15-min post-test. For CD-1 mice, an unbiased design was adopted, and only mice with pre-test scores between 5.5 min and 9.5 min (~80-90% of mice) were used. For MOR(-/-) and WT C57BL/6 mice, a biased design was used due to the limited number of mice available.

**Measurement of locomotor activities** was performed as we described (Xu et al., 2013; Liu et al., 2019a). Mice were treated with saline (10 ml/kg, s.c.), U50,488H (5 mg/kg, s.c.) or NCP (80 μg/kg, s.c.) and put into locomotor chambers immediately. Activities were continuously monitored over a 60-min period.

**Measurement of gastrointestinal transit in mice** was performed as described previously (Raffa et al., 1982; Feng et al., 2006). Food was removed 18 h prior to the experiment, but animals had free access to water until 20–30 min before the start of the experiment. In order to prevent coprophagy during fasting each cage bedding was refreshed at the time of food removal. On the day of the experiment, mice were injected (s.c.) with either saline, morphine (1 mg/kg), or NCP (3-100 μg/kg) (n=7-8). Morphine was used as positive control. Thirty min following injections, animals received a charcoal meal (charcoal, wheat flour and water in a 1:2:6 weight–volume ratio) intragastrically through an 18-gauge feeding needle in a dosing volume of 0.2 ml per 10 g body weight. Then 20 min later, all animals were sacrificed, and the small intestine was excised from the pylorus to the ileocecal junction. The intestine was positioned on a ruled template, and measurements were taken for both the distance travelled by the charcoal and the total length of the intestine. Charcoal transit was calculated as a percent of the total intestinal length.

**Measurement of respiration in mice:** Experiments were performed as described by Inan et al. (2021). On the day of the experiment, mice were brought to the room and acclimated for 45–60 min in the observation boxes. Respiration rate (RR) and oxygen saturation (SpO2) were measured using MouseOx Plus Rat and Mouse Pulse Oximeter (Starr Life Sciences Corp, Oakmont, PA, USA) in conscious, freely moving animals. Animals were exposed to 4% isoflurane for 30 s to connect collar sensor to the neck of mice. They were injected (s.c.) with either saline, morphine (10 mg/kg), or NCP (20-160 μg/kg) (n=6-7). Mice were then placed into observation boxes and recording was started 5 min later to eliminate any anesthesia effect. Respiration and SpO2 were recorded every second and
averaged over 1-min periods for 60 min. Morphine (10 mg/kg) was used as a positive control (Hill et al., 2016). Area under the curve (AUC) was calculated for each group.

**Abuse liability experiments in rats**

*Apparatus and Catheter Maintenance*

Modular operant chambers located in sound-attenuating cubicles (Med Associates, St. Albans, VT) were equipped with two retractable levers, a set of three LED lights (red, yellow, green) mounted above each lever. Intravenous (IV) heroin or NCP was delivered by activation of a syringe pump (PHM-100, Med Associates) located inside the sound-attenuating cubicle as described previously (Townsend et al., 2019; Townsend et al., 2021). Following each behavioral session, catheters underwent flushing with gentamicin (0.4 mg) succeeded by 0.1 ml of heparinized saline (10 U/ml). To ensure catheter patency, verification was conducted at the conclusion of each experiment, involving the observation of instantaneous muscle tone loss following intravenous methohexital (0.5 mg) administration.

Rats were trained to self-administer heroin under a fixed-ratio (FR) schedule. After cannula implantation and recovery, seven rats (3 males and 4 females) were initially trained to respond for IV heroin (32 µg/kg/infusion) under a FR 5 / time out 20 s schedule of reinforcement during daily 2-h sessions. Each session commenced with a non-contingent administration of the available heroin dose, followed by a 60-second timeout. The onset of the response period was indicated by the extension of the right lever and the illumination of the right green stimulus light. Upon the completion of each response requirement, the lever retracted, the green light extinguished, and intravenous heroin was administered. This schedule remained in effect until the number of earned heroin infusions per session stabilized within 20% of the running mean for three consecutive sessions, displaying no consistent upward or downward trends. Following this stabilization, saline was substituted for heroin in every other session (i.e., SDSDS; S, saline; D, drug) until the earned saline infusions were at least 75% lower than the number of heroin infusions obtained during the preceding heroin session for two consecutive alternations. The same experimental program was utilized during the saline substitution sessions, using the same infusion duration as a 32 µg/kg/infusion of heroin of 5 s per 300g of rat weight. Once training criteria were met, test sessions were inserted into the sequence (i.e., DTSTD or STDTS; T, test) to evaluate responding maintained by a range of heroin unit doses (i.e., saline, 10, 32, or 100 µg/kg/infusion) and NCP doses (1, 3.2, 10, 32, 100, or 320 µg/kg/infusion). Saline and each unit heroin dose or NCP dose were tested once in each rat using a counterbalanced dosing order.

**In vivo blood-brain barrier penetration studies on NCP in mice.**
The experiment was performed per our previously reported protocol (Pagare et al., 2022). Male Swiss Webster mice (three mice / time point) were given NCP (10 mg/kg, s.c.) or the vehicle. At 5-, 10-, and 30-min post administration, mice were decapitated, and brains and blood were collected. Blood samples were centrifuged for 10 min at 15,000g at 4°C and plasma was collected. Brain and plasma samples were stored at −80°C until analysis.

The plasma and brain homogenate samples were then analyzed to determine the amount of NCP using Ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS), and the brain-to-plasma ratios were calculated. The experiment was performed using a modification of a previously described method and naloxone-d5 was used as the internal standard (Schwienteck et al., 2019). Chromatographic separation of NCP and naltrexone-d5 was achieved using a Shimadzu Nexera X2 liquid chromatography system with a Zorbax XDB-C18 4.6 × 75 mm, 3.5 μm column (Agilent Technologies, Santa Clara, CA). Mobile phase A consisted of water with 1 g/L ammonium formate and 0.1% formic acid, and mobile Phase B consisted of methanol. The flow rate was 1 mL/min. The detector was a Sciex 6500 QTRAP system with an IonDrive Turbo V source for TurbolonSpray (Sciex, Ontario, Canada). The following quantification and qualifying transition ions were monitored in a positive multiple reaction monitoring mode with collisions energies in parentheses: NCP, 473 > 455 (30), 473 > 308 (35), and 473 > 211 (51); naloxone-d5, 333 > 212 (45), 333 > 315 (25), and 333 > 273. Retention times for NCP and naloxone-d5 were 1.62 and 0.89 min, respectively, and the total run time was 5 min. Concentrations of NCP in blood and brain were determined by a linear regression plot based on peak area ratios of the calibrators.

**Morphine withdrawal** was precipitated following a protocol used by Kieffer and colleagues (Contet et al., 2008) with some modifications. Male mice (C57BL/6J) underwent twice daily (10:00 and 18:00) treatment for 5 days with escalating doses of morphine via s.c. injections (day 1: 20 mg/kg, day 2: 40 mg/kg, day 3: 60 mg/kg, day 4: 80 mg/kg, day 5: 100 mg/kg) or saline. On the morning of day 6, mice received another injection of 100 mg/kg morphine or saline. Two hours later, withdrawal was induced by a subcutaneous (s.c.) injection of 10 mg/kg naloxone or NCP at different doses. Subsequently, mice were placed inside a 50 cm high, 12 cm diameter transparent cylinder, positioned on white paper, under normal room lighting, and observed and videotaped for 30 minutes. Total numbers of jumps were recorded, with the majority of jumping occurring primarily during the initial 5-10 minutes. Other withdrawal signs usually observed in rats were minimal if any, such as wet dog shakes, paw tremor, sniffs, teeth chattering, ptosis, and piloerection.
Statistical analyses were performed using GraphPad Prism version 8.2.1 or 9.5.0 (GraphPad Software Inc., La Jolla, CA). All data are expressed as the mean ± standard error of the mean (SEM). Analysis of variance (one way or two-way ANOVA) and unpaired t-tests were used. Individual group comparisons were performed with Dunnett, Bonferroni, or Šídák’s multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference.
Results

NCP produced analgesic effects against inflammatory pain in the formalin test and the acetic acid writhing test

We examined activities of NCP in inhibiting pain-like behaviors in the formalin test (Murray and Cowan, 1991; Liu et al., 2019a) and in the acetic acid writhing test, and in attenuating compound 48/80-induced scratching (Wang et al., 2005; Liu et al., 2019a; Inan and Cowan, 2022).

In mice NCP (20, 40, 80 µg/kg) reduced licking time in phase II of the formalin test in a dose-dependent manner with an A$_{50}$ value of 47.6 µg/kg (Fig. 2A), indicating antinociceptive activities. We previously reported that in the same test U50,488H had an A$_{50}$ value of 0.58 mg/kg (Liu et al., 2019a), indicating that NCP is more potent than U50,488H.

KOR agonists have been reported to inhibit acetic acid-induced writhing in mice (Broadbear et al., 1994; Patrick et al., 1999; Huang et al., 2001). NCP (10, 20, 40, 80 µg/kg, s.c.) dose-dependently decreased the number of writhes induced by an i.p. injection of 0.6% acetic acid and the A$_{50}$ value was determined to be 14.4 µg/kg (Fig. 2B).

Surprisingly, NCP had no analgesic effects against thermal pain in the hot plate test and the tail-flick test up to a dose of 10 mg/kg (~700xA$_{50}$ of the writhing test) (supplemental Fig. 1).

Analgesic effects of NCP in the acetic acid writhing test are mediated by the KOR rather than the MOR

As shown in Fig. 3A, in male CD-1 mice pretreated with both norBNI (a KOR antagonist) and β-FNA (an irreversible MOR antagonist) (column 3) the antinociceptive effects of NCP (40 µg/mg, s.c.) in the acetic acid writhing test were blocked. In contrast, pretreatment of β-FNA alone (column 5) did not affect the antinociceptive effects of NCP (Fig. 3A). Pretreatment with norBNI alone (column 4) significantly reversed the effects of NCP (column 2). Although the norBNI + NCP group was not significantly different from the vehicle group (column 1), it almost reached statistical significance (p=0.056, by t-test), suggesting that norBNI may produce partial blockade.

NCP (80 µg/mg, s.c.) decreased writhing similarly in both MOR knockout and WT mice (males with C57BL/6 background) (Fig. 3B), consistent with the results of β-FNA pretreatment vs control groups of CD-1 mice (Fig. 3A). MOR deletion in MOR-/- mice was confirmed by Straub tail responses following injection of morphine (10 mg/kg, s.c.). Within about 15 min post-morphine, WT mice displayed hyperlocomotion with Straub tail responses for more than one hour, but MOR knockout mice did not.

These results suggest that it is KOR rather than MOR that mediates the antinociceptive effects of NCP in the acetic acid writhing test. The data on KOR-/- mice are not presented because the
wildtype littermates had very few acetic acid-induced writhes, which made it difficult to detect antinociceptive effect of any compound.

**NCP does not induce the adverse behaviors typically associated with KOR activation**

*a. Motor incoordination*

KOR activation results in impaired performance in the rotarod test and inhibition of novelty-induced hyperlocomotion (White et al., 2015; Liu et al., 2019a), measures of motor incoordination and sedation in rodents, respectively. We tested if NCP produces these effects in mice.

NCP (80 μg/kg) did not cause impaired performance in the rotarod test (Fig. 4A), indicating no motor incoordination. U50,488H (5 mg/kg) significantly decreased mouse rod-stay time at 10, 20, 30, and 40 min post-administration, with a sustained effect at 40 min (Liu et al., 2019a). U50,488H at 2 mg/kg, only reduced the time mice stayed on the rod at 40 min. Thus, NCP did not cause motor incoordination in the dose producing antinociceptive effects.

*b. CPA*

KOR agonists have been shown to cause dysphoria and psychotomimetic effects in humans (Pfeiffer et al., 1986; Rimoy et al., 1994; Pande et al., 1996) and CPA in rodents (Mucha and Herz, 1985; Shippenberg and Herz, 1986). We examined whether NCP caused CPA. NCP did not cause CPA in mice at the doses (40 or 80 μg/kg, s.c.) producing antinociceptive effects (Fig. 4B). Even at a very high dose of 800 μg/kg (s.c.), NCP did not induce CPA (Fig. 4B). We reported previously that U50,488H (2 or 5 mg/kg) induced significant CPA, compared to the saline group (Fig. 4B) (Liu et al., 2019a).

*c. Sedation or hypolocomotion*

NCP (80 μg/kg) did not cause significant decrease in novelty-induced hyperlocomotion in mice (Fig. 5), while U50,488H at 5 mg/kg showed significant inhibition (Liu et al., 2019a). Even at a super-high dose of 800 μg/kg, NCP had no effect. Thus, NCP did not cause sedation or hypolocomotion in the dose range producing maximal antinociceptive effects.

**NCP was not self-administered in rats trained to self-administer heroin**

Sprague-Dawley rats that were trained to consistently self-administer heroin did not self-administer NCP at 0.001, 0.01 and 0.1 mg/kg/injection. Fig. 6 shows heroin self-administration under an FR5 schedule of reinforcement. Under these conditions, heroin functioned as a reinforcer and displayed the typical inverted U-shaped dose-effect function \[F(1.5,7.5) = 5.1, \ p < 0.05\] with a heroin dose.
of 32 µg/kg/infusion maintaining greater rates of responding than saline. NCP failed to maintain rates of responding greater than saline control at all the dose examined (1.0, 3.3, 10, 32, 100, 320 µg/kg/infusion), indicating that NCP had no reinforcing effects in the dose range producing antinociceptive effects.

**Effects of NCP on gastrointestinal (GI) transit**

As shown in Fig. 7, NCP significantly slowed gastrointestinal transit of charcoal meal at doses of 10, 30, 80 and 100 µg/kg compared to saline, but not at 3 µg/kg. As expected, morphine at 1 mg/kg significantly reduced transit as well. Thus, like morphine, NCP induced significant decrease in GI transit in the dose range producing antinociceptive effects. It is noteworthy that the effects of NCP reached a plateau, producing about 40% maximal inhibition, compared to the control. In contrast, morphine dose-dependently inhibited GI transit with 100% maximal inhibition at 10 mg/kg, as did methadone and pethidine (Green, 1959).

**Effects of NCP on respiration**

NCP at 20, 40, 80, and 160 µg/kg did not have any effect on either respiratory rate or SpO2. Both respiratory rate and SpO2 were found similar to saline over the 60 min post injection as seen in Fig. 8. AUC calculations for NCP were not different from AUC of saline. However, morphine significantly reduced respiratory rate (****p < 0.0001) compared to saline and had no effect on SpO2 as it was observed previously (Hill et al., 2016; Inan et al., 2018). These results indicate that unlike morphine, NCP did not cause respiratory depression in the dose range producing antinociceptive effects.

**NCP induced CPA in mice in the absence of MOR**

To test whether lack of CPA following conditioning with NCP was due to actions on both the MOR and KOR, we examined if NCP induced CPA in MOR-/- and WT C57BL/6 mice. Fig. 9A shows that NCP (80 µg/kg, s.c.) induces strong CPA in MOR-/- mice. In contrast, NCP (80 or 800 µg/kg, s.c.) did not cause CPA in WT C57BL/6 mice in Fig. 9B, which is similar to the findings using CD-1 mice (Fig. 4B), suggesting that lack of CPA by NCP is due to its combined actions on the MOR and KOR.

**Distribution of NCP in the brain following injection into mice**
NCP doses effective in the formalin test and acetic acid writhing test did not cause CPA, hypolocomotion, impaired rotarod performance or respiratory depression, and it did not substitute for heroin in self-administration. One possibility for the observations is that NCP did not get into the brain. We thus examined if NCP crossed the blood brain barrier into the brain.

The plasma and brain homogenate samples of mice injected with NCP (10 mg/kg, s.c.) were analyzed to determine the amount of NCP using UPLC−MS/MS, and the brain-to-plasma ratios were calculated (Table 1). NCP appeared in plasma with the highest concentration (2.45 μg/mL) as early as 5 min after s.c. administration and declined at 10 min and 30 min. Brain concentrations of NCP after 5, 10, and 30 min were 0.93, 0.52, and 0.39 μg/g, respectively, indicating that NCP penetrated into the CNS after s.c. administration. Moreover, the amount of NCP in the brain was the highest 5 min post administration suggesting a fast onset of action. Although its concentration declined at 10 min, the highest brain-to-plasma concentration ratio of NCP was reached at 10 min post administration and remained constant over the 30 min period of the study (Table 1). The reason why we injected a high dose NCP, relative to its potency in the antinociception tests, is to be able to detect NCP in the samples.

**NCP did not precipitate morphine withdrawal-associated jumps**

In mice repeatedly treated with escalating doses of morphine, naloxone (10 mg/kg s.c.) induced 43 ± 4 jumps within 30 min, whereas NCP at 80 μg/kg and 800 μg/kg or even at 10 mg/kg s.c. (~700xA50 of writhing test) failed to induce jumps (Fig. 10).

**NCP had no anti-scratch effect**

We previously showed that U50.488 displayed significant anti-scratch effect in mice with an A50 value of 2.07 mg/kg (Liu et al., 2019a). In CD-1 mice, NCP at 80 or 300 μg/kg did not inhibit compound 48/80-induced scratching (Fig. 11A). As MOR activation induces itch (Hales, 1980) and KOR activation inhibits itch [reviewed in (Inan and Cowan, 2022)], the lack of anti-scratch effect of NCP may be due its combined actions on the MOR and KOR. We thus treated mice with β-FNA to block the MOR. In mice pretreated with β-FNA, 300 μg/kg NCP still did not inhibit compound 48/80-induced scratching (Fig. 11A), whereas U50,488H at 2.5 mg/kg significantly inhibited scratching. In MOR(-/-) mice (Fig. 11B), NCP up to 5 mg/kg did not inhibit compound 48/80-induced scratching, while U50,488H at 5 mg/kg significantly inhibited scratching. The results demonstrate that NCP has no anti-scratch effect at all.
Discussion

In the present study, we report the in vivo pharmacological profile of the dual KOR and MOR agonist NCP. NCP produces potent analgesic effects against inflammatory pain via the KOR without causing aversion, sedation, motor incoordination, reinforcing effects or respiratory depression. These findings suggest that NCP has potential to be an effective analgesic for management of inflammatory pain. In addition, dual full KOR/partial MOR agonists with moderate KOR-over-MOR selectivity may be generally promising as an avenue for developing non-addictive analgesics, which warrant further study.

NCP produced analgesic effects via the KOR with higher potencies than U50,488H

We found that NCP exerted antinociceptive effects in both the acetic acid-induced writhing test and the second phase of formalin test in mice, which are models of visceral pain and inflammatory pain, respectively.

Compared with our previous results, in the second phase of the mouse formalin test, the $A_{50}$ value of NCP (47.6 μg/kg) was approximately 8.2-fold higher than that of nalfurafine (5.8 μg/kg), but 12.2-fold lower than that of U50,488H (0.58 mg/kg) (Liu et al., 2019a). In addition, it is about 44-fold more potent than morphine (2.1 mg/kg) in this test (Murray et al., 1988). In the mouse acetic acid-induced writhing test, Endoh et al. (1999) reported $A_{50}$ values of 1.16 mg/kg, 0.58 mg/kg and 3.3 μg/kg for U50,488H, morphine and nalfurafine, respectively. Thus, NCP, with an $A_{50}$ value of 14.4 μg/kg in the acetic acid writhing test has a higher potency than U50,488H and morphine, but lower than nalfurafine.

NCP shows 6.5x selectivity for KOR over MOR in vitro and it is a full agonist at the KOR with partial agonist activity at the MOR (Huang et al., 2021; Wang et al., 2021). We thus investigated whether NCP acts on KOR or MOR to produce analgesic effects. While blockade of MOR by β-FNA or deletion of the MOR in mice did not affect NCP-induced antinociception, blockade of both KOR and MOR completely abolished the analgesic effects of NCP in the acetic acid-induced writhing test, suggesting that it is KOR, but not MOR, activation that mediates the analgesic effects of NCP. Although pretreatment with norBNI alone for 24 h significantly blocked the effect of NCP in the acetic acid-induced writhing test, the blockade appears to be partial. This observation may be due to additive or synergistic interaction between MOR and KOR activation in this behavioral end point. We also examined whether KOR deletion affected antinociceptive effects of NCP. Unexpectedly, the wildtype littermates had few writhes in response to acetic acid, therefore, we were not able to obtain meaningful data.

NCP produced analgesic effects against inflammatory, but not thermal pain
We used the second phase of formalin test and the acetic acid-induced abdominal writhing test in mice to test analgesic effects of NCP for two reasons. KOR agonists do not produce efficacious analgesic effects in tests using heat as the noxious stimuli, such as tail flick and hot plate tests, whereas they produce effective analgesia in the mechanical and inflammatory pain tests, including paw pressure test, tail pinch test, abdominal constriction test and the second phase of formalin test (Tyers, 1980; Hayes et al., 1987; Endoh et al., 1999; Wang et al., 2021) [reviewed in (Liu-Chen and Huang, 2022)]. The doses of KOR agonists considered "analgesic" in thermal pain tests exceed those in mechanical and inflammatory pain tests, and notably result in hypo-locomotion and motor incoordination. Thus, the uncertainty has persisted surrounding whether the observed reduction in latency of tail-flick or paw licking in the thermal tests induced by KOR agonists truly reflects their analgesic effects.

It is noteworthy that the dual KOR/MOR agonist NCP demonstrated no impact on locomotor activities and lacked analgesic effects in the two thermal pain tests. However, it exhibited robust analgesic effects in the two inflammatory pain tests. The absence of analgesic effects against thermal pain despite the MOR agonistic properties of NCP is surprising and intriguing, and may be attributed to the following factors. NCP has only partial MOR agonist activity, and in addition, studies have shown that KOR opioids, such as U50,488H or dynorphin, at doses that did not demonstrate analgesic effects in rodent tail flick or hot plate tests, reduce the analgesic effects of MOR opioids like morphine or DAMGO [reviewed in (Pan, 1998)].

Significantly, MOR opioids failed to alleviate the chronic pancreatitis-associated pain in up to 50% of patients (Schneider and Hirth, 2021). A peripherally acting KOR agonist, ADL 10-0101, demonstrated significant reduction in pain scores compared to a placebo in individuals with chronic pancreatitis who continued to experience abdominal pain despite receiving MOR opioid agonist therapy (Eisenach et al., 2003). Acetic acid triggers an inflammatory response in the abdominal cavity, leading to the subsequent activation of nociceptors in mice (Collier et al., 1968). Thus, it is intriguing to hypothesize that NCP, as a likely non-addictive opioid, might produce analgesia with chronic visceral pain in patients.

**NCP does not produce CPA, likely because of its actions on both the MOR and KOR**

KOR agonists, such as U50,488, have been shown to cause CPA in rodents (Mucha and Herz, 1985; Shippenberg and Herz, 1986; Liu et al., 2019a; Liu et al., 2020). However, NCP at doses producing effective analgesia (40, 80 or 800 μg/kg, s.c.) did not cause CPA in male CD-1 mice. In contrast U50,488H at 2 or 5 mg/kg produced robust CPA. The results on U50,488H are consistent with our previous findings (Liu et al., 2019a; Liu et al., 2020) and those of Kaski et al. (2019) that U50,488H at 0.25 to 10 mg/kg cause CPA independent of doses. In contrast, Robles et al. (2014) and Liu et al.
(2019b) showed that U50,488H induced CPA in male mice only at 10 mg/kg but not at 1, 2.5, or 5 mg/kg. The differences may be attributed to differences in CPA apparatus, experimental designs, and mouse strains.

In wildtype C57BL/6 mice NCP did not produce CPA or CPP, similar to the finding in CD-1 mice. In contrast, in MOR-/- mice in C57BL/6 genetic background NCP produced significant CPA, suggesting that lack of CPA by NCP is due to its dual actions on the MOR and KOR. Actions on the MOR cause CPP, but activation of the KOR produces CPA. Its actions on the MOR and KOR appear to counteract each other. As partial agonists at the MOR may not induce consistent CPP, we did not examine if NCP caused CPP in KOR-/- mice.

**NCP does not have anti-itch effect**

KOR agonists have been demonstrated to have anti-pruritic effects [reviewed in (Inan and Cowan, 2022)]. NCP acts as a potent KOR full agonist in [35S]GTPγS binding assay and its antinociceptive activity in the mouse acetic acid writhing test is mediated via the KOR; however, NCP did not inhibit compound 48/80-induced scratching even at 300 μg/kg (~20xA50 of writhing test) in CD-1 and wildtype C57BL/6 mice. We initially hypothesized that this might be due to its opposing actions on the MOR vs. the KOR as while MOR activation induces itch sensation (Hales, 1980), KOR activation inhibits itch [reviewed in (Inan and Cowan, 2022)]. Unexpectedly, this appeared not the case, because NCP at as high as 5 mg/kg (~350xA50 of writhing test) was proven to be ineffective in inhibiting 48/80-induced scratching in MOR knockout mice as well. Thus, the mechanisms underlying the lack of anti-scratch effect of NCP remains unclear.

**NCP does get into the brain**

Despite that NCP is a full agonist at the KOR, NCP at 40 or 80 μg/kg (s.c.), which produced efficacious analgesic responses, did not cause CPA or CPP, whereas U50,488H at 2 or 5 mg/kg caused profound CPA and morphine at 10 mg/kg (sc) elicited a high level of CPP (data not shown). In addition, NCP (10-160 μg/kg, s.c.) did not depress respiratory rate although it is a partial agonist (58%) at the MOR. In contrast, morphine (10 mg/kg, sc) greatly reduce respiratory rate. The inabilities of NCP to cause CPA or CPP and to depress respiration are not due to its inability to get into the central nervous system for two reasons. In MOR-/- mice, NCP at 80 μg/kg (s.c.) induced strong CPA, indicating that it gets into the brain. Indeed, following s.c. injection of NCP (10 mg/kg) into mice, the NCP detected in the brain (0.39-0.93 μg/g) was more than morphine (<0.22 μg/g, detected in a similar way) when morphine was injected into mice at 10 mg/kg (i.m.) (Ishikawa et al., 1982), giving direct evidence that NCP gets into the brain.
Dual KOR/MOR agonists

There are precedents that compounds with KOR and MOR agonist activities are used clinically as analgesics. One example is nalbuphine. *In vitro*, nalbuphine has high-efficacy partial agonist activity at the KOR (EC$_{50}$ 27 nM, E$_{max}$ 81%, by [³⁵S]GTPγS binding) and medium efficacy partial agonist at the MOR (EC$_{50}$ 14 nM, E$_{max}$ 47%, by [³⁵S]GTPγS binding) with a 2x selectivity for the MOR over the KOR, while it showed much lower potency at the DOR (Peng et al., 2007). In humans, nalbuphine is a potent analgesic and is used to relieve pain during or after surgery and for obstetrical analgesia during labor and delivery. It was found not to have reinforcing or aversive effects in humans (Schmidt et al., 1985) and it did not produce CPP or CPA in rodents (Tao et al., 2006). Nalbuphine has much lower misuse liability in humans (Schmidt et al., 1985). Consequently, it is not scheduled under the Controlled Substances Act by Drug Enforcement Agency (DEA) in the USA. In humans, nalbuphine does not produce significant respiratory depression and produces few psychotomimetic effects, side effects typically associated with KOR agonist activities (Schmidt et al., 1985). Therefore, there are many similarities between pharmacological properties of NCP and nalbuphine, supporting the notion that compounds with dual MOR/KOR agonist activities are promising as non-addictive analgesics with fewer side effects. The optimal efficacy at either receptor and selectivity for either receptor remain to be determined.

NCP has some advantages over nalbuphine. The KOR-over-MOR selectivity of NCP vs the MOR-over-KOR selectivity of nalbuphine might render even lower misuse liability of NCP compared with nalbuphine in humans. NCP is much more potent at both the MOR and KOR, thus the dose needed is much smaller. From the standpoint of manufacture, it will be much easier for NCP to be within the quota of semisynthetic opioids synthesized from thebaine set by the DEA.

The withdrawal syndrome triggered by nalbuphine closely resembled that induced by naloxone in the methadone-dependent patients (Preston et al., 1989). Surprisingly, NCP did not precipitate morphine withdrawal in mice despite having an E$_{max}$ of 58% of DAMGO at the MOR in [³⁵S]GTPγS binding. It would be interesting to examine whether NCP induces withdrawal symptoms in opiate-dependent human subjects.

Why did we use only male mice in the current studies?

We conducted KOR pharmacological studies in male CD-1 mice in the past (Liu et al., 2019a). We found that in male C57BL/6 mice, U50,488H induced similar behavioral effects compared to those in male CD-1 mice (Huang et al., 2022). Unexpectedly, in female C57BL/6 or California mice, U50,488H induced biphasic CPA responses and higher doses (≥5 mg/kg, s.c. or i.p.) failed to cause
Conclusion

We have found that NCP, having KOR full agonist activity and MOR partial agonist activity with moderate selectivity for the KOR over MOR (6.5x), produces analgesic effects against inflammatory pain. NCP, unlike MOR agonists, was not self-administered and at analgesic doses it did not cause respiratory depression. In addition, at analgesic doses, it did not engender CPA, hypolocomotion or motor incoordination, effects typically associated with KOR activation. NCP inhibits GI transit, likely via KOR and MOR. These findings suggest that NCP warrants further investigation as a non-addictive analgesic.

Acknowledgments: We appreciate the experimental assistance from Joseph J. Meissler, Jr., Chongguang Chen, and Kathryn Bland.

The Data Availability Statement: The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions:

Participated in research design: Lee-Yuan Liu-Chen and Peng Huang
Conducted experiments: Peng Huang, Conrad K. Ho, Saadet Inan, Scott M. Rawls, E Andrew Townsend, Matthew Banks, Piyusha P. Pagare, Mengchu Li, Justin L. Poklis, and Matthew S. Halquist
Contributed new reagents or analytic tools: Boshi Huang and Yan Zhang
Performed data analysis: Peng Huang, Lee-Yuan Liu-Chen, Danni Cao, Saadet Inan, Scott M. Rawls, E Andrew Townsend, Matthew Banks, Yan Zhang
Wrote or contributed to the writing of the manuscript: Lee-Yuan Liu-Chen, Peng Huang, and Danni Cao
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Footnotes:

a) This work was supported by NIH grants R01DA056581, R01DA041359, R21DA045274, P30DA013429 and Cure Addiction Now (To LLC), R01DA024022 and UG3DA050311 (to YZ).

b) 1. Peng Huang, Danni Cao, Chongguang Chen, Saadet Inan, Boshi Huang, E. Andrew Townsend, Matthew Banks, Conrad K. Ho, Scott M. Rawls, Yan Zhang, and Lee-Yuan Liu-Chen. NCP, a dual mu and kappa opioid receptor agonist, is a potent analgesic without reinforcing or aversive properties. The International Narcotics Research Conference (INRC), July 12-14, 2021, online meeting.

2. Peng Huang, Danni Cao, Chongguang Chen, Saadet Inan, Boshi Huang, E. Andrew Townsend, Matthew Banks, Conrad K. Ho, Scott M. Rawls, Yan Zhang, and Lee-Yuan Liu-Chen. NCP, a dual mu and kappa opioid receptor agonist, is a potent analgesic without reinforcing or aversive properties and blocks stress-induced reinstatement of morphine CPP in mice. The International Narcotics Research Conference (INRC), Atlanta, GA, USA, July 9-12, 2023.

c) The person to receive reprint requests: Lee-Yuan Liu-Chen, Ph.D., Center for Substance Abuse Research, Temple University Lewis Katz School of Medicine, 3500 North Broad Street, MERB 851, Philadelphia, PA 19140, USA, Tel.: +1 215 707 4188; fax: +1 215 707 7661; lliuche@temple.edu.

d) No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

Figure 1. Chemical structure of NCP

Figure 2. NCP produced antinociceptive effects against inflammatory pain in CD-1 mice.

(A) NCP inhibited formalin-induced pain behaviors in mice. Saline, U50,488H (2.5 mg/kg), one dose of NCP was injected (s.c.) 5 min before formalin and the amount of time the animal spent licking the injected paw was counted for 20 min starting at 15 min after formalin injection. A50 doses were determined as described (Liu et al., 2019a). Data were analyzed using one-way ANOVA followed by Dunnett’s post-hoc test (for NCP) or unpaired t-test (for U50,488H). NCP: F(3,33) = 9.04, p < 0.001. *p < 0.05, ***p < 0.001, compared to saline control, by Dunnett’s post-hoc test (mean ± SEM, n = 7-11 animals/group). U50,488H: ****p < 0.0001, compared to saline control, by unpaired t-test (mean ± SEM, n = 9-11 animals/group). A50 dose of NCP is 47.6 µg/kg. A50 dose of U50,488H is 0.58 mg/kg (Liu et al., 2019a).

(B) NCP inhibited acetic acid-induced writhing in mice. Water, U50,488H (5 mg/kg) or one dose of NCP was injected (s.c.) 20 min before acetic acid was administered intraperitoneally and the number of writhes (abdominal stretches) was recorded for 15 min starting at 5 min after acetic acid injection. The writhing number for each animal was normalized by the average writhing number of the control group, which was approximately 30. Data were analyzed using one-way ANOVA followed by Dunnett’s post-hoc test (for NCP) or unpaired t-test (for U50,488H). NCP: F(4,44) = 9.74, p < 0.0001. **p < 0.01, ****p < 0.0001, compared to water control, by Dunnett’s post-hoc test (mean ± SEM, n = 7-13 animals/group). U50,488H: **p < 0.01, compared to the water control, by unpaired t-test (mean ± SEM, n = 13 animals/group for water and n = 4 for U50). A50 dose of NCP is 14.4 µg/kg.

Figure 3. Analgesic effects of NCP in the acetic acid writhing test are mediated by KOR, but not MOR.

(A) Pretreatment with β-FNA + norBNI or norBNI but not β-FNA abolished or reduced antinociceptive effects of NCP in the acetic acid-induced writhing test in CD-1 mice. norBNI (32 mg/kg, i.p.), β-FNA (32 mg/kg, s.c.) or both were pretreated 24 h or 48 h before water or NCP (40 µg/kg, s.c.) injection, respectively. Twenty min later, acetic acid (0.6%, 10 µl/g, i.p.) was injected. Five min later the number of writhes (abdominal stretches) was recorded for 15 min. Writhing number for each animal was normalized by the average writhing number (~30) of the control group. Data were analyzed using one-way ANOVA with Dunnett’s multiple comparisons test. F (4, 46) = 8.817, P<0.0001. *** p < 0.001, compared to control (column 1) (mean ± SEM, n = 9-13 animals/group).

(B) NCP reduced acetic acid-induced writhing to similar extents in MOR(-/-) and WT mice (C57BL/6 background). Saline or NCP (80 µg/kg, s.c.) was injected 20 min before acetic acid administration and the number of writhes (abdominal stretches) was recorded for 15 min starting at 5 min after acetic acid injection. Data were analyzed using two-way ANOVA. Results showed a significant main effect of drug [F(1,33) = 17.03, p < 0.001], but no significant main effects of genotype [F(1,33) = 0.0096, p > 0.05] or interaction [F(1,33) = 0.00016, p > 0.05]. *p < 0.05, compared to water control, Bonferroni post-hoc test (mean ± SEM, n = 9-10 animals/group).

Figure 4. NCP did not cause motor incoordination or conditioned place aversion (CPA) in CD-1 mice.
(A) NCP did not cause motor incoordination in the rotarod test in mice. After training the previous day, mice were injected s.c. with saline, U50,488H (2 or 5 mg/kg) or NCP (80 μg/kg) and tested on the rotarods 10, 20, 30, and 40 min after injection. The doses used produced maximal antinociception (Fig. 2). The time each stayed on the rods was recorded and normalized against the baseline. Data were analyzed with two-way ANOVA followed by Dunnett’s post-hoc test (mean ± SEM, n= 10-12/group).

NCP: Results of two-way ANOVA showed no significant main effect of treatment \[ F(1,18) = 1.23, p > 0.05 \] or time \[ F(4,72) = 1.37, p > 0.05 \] and no significant interaction \[ F(4,99) = 0.67, p > 0.05 \]. U50,488H: Results of two-way ANOVA showed a significant main effect of treatment \[ F(2,28) = 16.12, p < 0.0001 \], a significant main effect of time \[ F(4,112) = 21.09, p < 0.0001 \] and a significant interaction \[ F(8,139) = 6.47, p < 0.0001 \]. * \[ p < 0.05 \], *** \[ p < 0.001 \], **** \[ p < 0.0001 \], compared with 0 min of each group; # \[ p < 0.05 \], ## \[ p < 0.01 \], ### \[ p < 0.0001 \], compared with the saline group at the same time, by Dunnett’s post-hoc test. Data on U50,488H were from Liu et al. (2019a) and are shown for comparison.

(B) NCP did not cause CPA in mice at 80 or 800 μg/kg (s.c.). NCP at 80 μg/kg (s.c.) produced maximal antinociception (Fig. 2). On Day 0, mice were subject to pre-test. On Days 1-3, mice were injected with saline or one dose of U50,488H or NCP, and stayed in home cages for 10 min, before each 30-min conditioning session (1 saline session and 1 drug session/day) for 3 days. On Day 4 (post-test), the length of time the animal spent on the drug-paired side was measured. The graph shows the time the animal spent during the post-test subtracting the amount of time spent during the pre-test. Data were analyzed with one-way ANOVA followed by Dunnett’s post-hoc test (mean ± SEM, n = 9–10/group).

NCP, \( F (3, 35) = 1.175, P=0.3333 \); U50,488H, \( F (2,26) = 13.55, p < 0.0001 \). *** \( p < 0.001 \), compared to saline control by Dunnett’s post-hoc test. Data on U50,488H were from Liu et al. (2019a) and are shown for comparison.

Figure 5. NCP did not inhibit novelty-induced hyperlocomotion even at doses up to 56xA<sub>50</sub> in the acetic acid writhing test.

(A) and (B) NCP did not cause inhibition of novelty-induced locomotor activity or enhancement of locomotor activity in CD-1 mice. Mice were treated s.c. with saline, NCP (80 or 800 μg/kg) or U50,488H (5 mg/kg) and locomotor activities were monitored. Cumulative data between 0-30 min post-injection are shown here. Data were analyzed using one-way ANOVA followed by Dunnett’s post-hoc test (for NCP) or unpaired t-test (for U50,488H). NCP: (A) \( F (2, 21) = 0.8544, p=0.4398 \); (B) \( F (2, 21) = 0.08671, p=0.9173 \) (mean ± SEM, n = 8-12 animals/group). U50,488H: (A) *** \( p < 0.001 \), (B) *p<0.05, compared to water control, by unpaired t-test (mean ± SEM, n = 8 animals/group). Data on U50,488H were from Liu et al. (2019a) and are shown for comparison.

Figure 6. NCP was not self-administered in rats trained to self-administer heroin.

Rats were trained to self-administer heroin under an FR5 schedule of reinforcement. Heroin was then replaced with different doses of NCP (1.0, 3.2, 10, 32, 100, 320 μg/kg/infusion) or heroin (10, 32, 100 μg/kg/infusion). Filled symbols denote statistical significance \( (p < 0.05) \) compared to saline (S) training sessions before test sessions. Points above H represent heroin training sessions (32 μg/kg/infusion) before test sessions. All points represent the mean ± SEM of six rats (3 per sex).

Figure 7. NCP inhibited GI transit

Mice were injected with either saline, morphine (1 mg/kg, s.c.), or NCP (3, 10, 30, 80, 100 μg/kg, s.c.). Thirty min later, charcoal meal was administered through oral gavage and 20 min later, animals were sacrificed. Small intestine was removed from the pylorus to the ileocecal junction. The charcoal's travel distance and the total length of the intestine were measured by placing the intestine on a ruled
template. Charcoal transit was calculated as a percent of the total intestinal length. Data were analyzed with one-way ANOVA followed by Dunnett’s post-hoc test (for NCP) or unpaired t-test (for morphine). NCP: $F(5,38) = 12.47, p < 0.0001$. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, compared to saline control, by Dunnett’s post-hoc test (mean ± SEM, n = 7-8 animals/group). Morphine: ***$p < 0.001$, compared to saline control, by unpaired t-test (mean ± SEM, n = 7-8 animals/group).

**Figure 8.** NCP had no effects on (A) respiration rate or (B) oxygen saturation. Following acclimation in the individual observation boxes, mice were connected to collar sensor and injected with either saline, morphine (10 mg/kg, s.c.), or NCP (10, 20, 40, 80, 160 µg/kg, s.c.). Respiratory rate and SpO2 were recorded over 60 min. NCP up to 160 µg/kg did not have any effect on respiratory rate and SpO2, however morphine significantly reduced respiratory rate compared to vehicle (****$p<0.0001$, n=6-7, by unpaired t-test). Each column represents the group mean ± SEM. N = 6-7 for each group.

**Figure 9.** NCP induced CPA in MOR(-/-), but not in WT mice, both in C57BL/6 background. CPA was carried out following the procedure described in Fig. 4 (mean ± SEM n = 10-11). (A) For MOR(-/-) mice, **$p < 0.01$, vs saline group, by unpaired t-test. (B) For WT mice, data were analyzed by one-way ANOVA followed by Dunnett’s post-hoc test: $F(2, 28) = 0.1484, p=0.8627$.

**Figure 10.** NCP, even at doses up to 10 mg/kg (694xA50 in writhing test), did not precipitate withdrawal-associated jump in mice chronically-treated with escalating doses of morphine. Mice were subjected to twice-daily treatments for 5 consecutive days with saline or increasing doses of morphine through s.c. injections (day 1: 20 mg/kg, day 2: 40 mg/kg, day 3: 60 mg/kg, day 4: 80 mg/kg, day 5: 100 mg/kg). On the morning of day 6, mice were administered an additional injection of either 100 mg/kg morphine or saline. Two hours later, withdrawal was triggered by a s.c. injection of 10 mg/kg naloxone or NCP (0.08, 0.8 or 10 mg/kg). Subsequently, mice were placed inside transparent cylinder and observed for 30 minutes. The numbers of jumps (mean ± SEM, n = 4-7) were counted and analyzed by one-way ANOVA followed by Dunnett’s post-hoc test: F (4, 18) = 51.41, p<0.0001; ****p<0.0001, vs the saline-naloxone group.

**Figure 11.** NCP did not inhibit scratching behavior induced by compound 48/80 in CD-1 (A) and MOR(-/-) mice (B). Saline, NCP at different doses, or U50,488H (2.5 or 5 mg/kg) was injected (s.c.) 20 min before compound 48/80 injection and the bouts of scratching were counted for 30 min. Bouts of scratching were recorded and normalized to the average of saline group which was about ~300 bouts for CD-1 mice (A) or ~150 bouts for MOR(-/-) mice (B). Data were analyzed using one-way ANOVA followed by Dunnett’s post-hoc test (for NCP) or unpaired t-test (for U50,488H). NCP: (A) $F(3, 33) = 0.4798$, p=0.6986; (B) F (2, 21) = 1.061, p=0.3641. U50,488H: (A) ***$p < 0.001$, (B) **$p<0.01$, compared to saline control. Data were presented as mean ± SEM [n = 8-10 for (A), n=7-9 for (B)].
Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>5</th>
<th>10</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (μg/g)</td>
<td>0.93 ± 0.23</td>
<td>0.52 ± 0.16</td>
<td>0.39 ± 0.13</td>
</tr>
<tr>
<td>Plasma (μg/mL)</td>
<td>2.45 ± 0.47</td>
<td>0.60 ± 0.27</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>Brain-to-plasma ratio</td>
<td>0.38</td>
<td>0.87</td>
<td>0.81</td>
</tr>
</tbody>
</table>
Fig. 1.
Fig. 2.

(A) Formalin test

\[ A_{50} = 47.6 \mu g/kg \]

(B) Acetic acid writhing test

\[ A_{50} = 14.4 \mu g/kg \]
Fig. 3.

(A) Number of writhes (% average of control) for different treatments:

- NCP (μg/kg): 0, 40, 40, 40, 40
- norBNI (mg/kg): 0, 0, 32, 32, 0
- β-FNA (mg/kg): 0, 0, 32, 0, 32

(B) Number of writhes for WT and MOR(-/-) mice:

- Water
- NCP 80 μg/kg

* indicates significant difference from control.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8.
Fig. 9.

MOR(-/-) vs. WT

Preference score (sec) of paired side (posttest - pretest)

-600  -400  -200  0  200  400

saline  NCP  80 μg/kg

**

saline  80  800

NCP (μg/kg)
Fig. 10.
Fig. 11

(A) CD-1

Saline  U50 2.5 mg/kg  80  300  300

NCP (µg/kg)  

-  +  

β-FNA

Bouts of scratching (% of average of saline group)

Saline  U50 5 mg/kg  0.3  5

NCP (mg/kg)  

**

(B) MOR(-/-)

Saline  U50 2.5 mg/kg  80  300  300

NCP (µg/kg)  

-  +  

β-FNA

Bouts of scratching (% of average of saline group)