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Activation of κ Opioid Receptors in Cutaneous Nerve Endings by Conorphin-1, a Novel Subtype-Selective Conopeptide, Does Not Mediate Peripheral Analgesia

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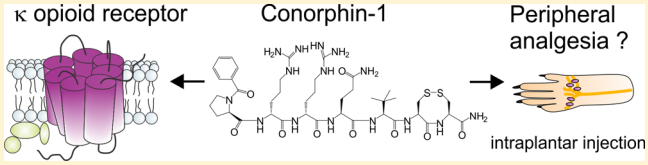
ABSTRACT: Selective activation of peripheral κ opioid receptors (KORs) may overcome the dose-limiting adverse effects of conventional opioid analgesics. We recently developed a vicinal disulfide-stabilized class of peptides with subnanomolar potency at the KOR. The aim of this study was to assess the analgesic effects of one of these peptides, named conorphin-1, in comparison with the prototypical KOR-selective small molecule agonist U-50488, in several rodent pain models. Surprisingly, neither conorphin-1 nor U-50488 were analgesic when delivered peripherally by intraplantar injection at local concentrations expected to fully activate the KOR at cutaneous nerve endings. While U-50488 was analgesic when delivered at high local concentrations, this effect could not be reversed by coadministration with the selective KOR antagonist ML190 or the nonselective opioid antagonist naloxone. Instead, U-50488 likely mediated its peripheral analgesic effect through nonselective inhibition of voltage-gated sodium channels, including peripheral sensory neuron isoforms $Na_V1.8$ and $Na_V1.7$. Our study suggests that targeting the KOR in peripheral sensory nerve endings innervating the skin is not an alternative analgesic approach.



KEYWORDS: *κ opioid receptor, U-50488, peripheral, conorphin-1, analgesia, pain*

Opioids such as morphine, oxycodone, and fentanyl remain the most effective class of analgesics in clinical use despite numerous troublesome adverse effects, including respiratory depression, sedation, constipation, and psychological dependence. These adverse effects are mostly attributed to on-target μ opioid receptor (MOR) agonist effects. Accordingly, significant efforts have been aimed at developing novel opioid analgesics that selectively target the δ opioid receptor (DOR) or κ opioid receptor (KOR). However, it soon became apparent that selective centrally penetrating KOR agonists from the arylacetamide class, including the lead compound U-50488 and its analogues spiradoline and enadoline, also produced troublesome dose-limiting adverse effects including dysphoria, sedation, and psychotomimetic effects attributed to activity at central KOR.[1,2] However, analgesia produced by systemically administered but centrally permeating opioid agonists such as morphine includes a substantial peripheral component associated with opioid receptors expressed on the central spinal terminals and peripheral terminals of the dorsal root ganglion (DRG) neurons innervating the skin, joints, muscles and internal organs.[3−6] Accordingly, selective activation of peripheral KORs has been pursued as an alternative strategy to mediate analgesia while avoiding central side effects.

Peptide-based molecules are emerging as promising therapeutics targeting peripheral pain pathways despite their typically undesirable characteristics, such as the need for administration by injection, their general inability to cross the blood-brain barrier, and their relatively unfavorable pharmacokinetics. However, peptides often display superior selectivity and potency compared with small molecules, and disulfide-bonded peptides can be surprisingly stable,[7,8] making these molecules promising alternative analgesics where exclusion from the central nervous system is desirable.

We recently discovered a novel KOR selective conopeptide and developed a vicinal disulfide-stabilized class of peptides that activate KOR with subnanomolar potency without discernible effect at MOR or DOR (unpublished experiments). Based on their physicochemical properties, we predicted these peptides might elicit KOR-mediated analgesia at receptors expressed on peripheral sensory nerve endings without crossing the blood brain barrier.[9,10] Thus, the aim of this study was to evaluate the analgesic efficacy of conorphin-1 (Figure 1) in comparison with the prototypical KOR-selective small molecule agonist U-50488 in animal models of inflammatory and neuropathic pain after intraplantar administration.

RESULTS AND DISCUSSION

Conorphin-1 and U-50488 Are Selective KOR Agonists. Intraplantar administration of compounds is a preferred route to assess the peripheral effects of small molecules and

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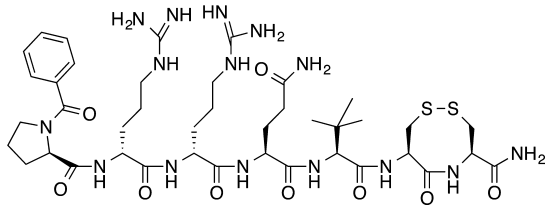

Figure 1. Chemical structure of the novel peptide KOR agonist conorphin-1.

peptides. This method relies on the administration of compounds by shallow subcutaneous injection in the footpad, which achieves a well-defined local concentration at sensory nerve endings, where the in vivo effect typically closely matches its relevant in vitro potency.[11−14] Thus, intraplantar injection achieves administration of pharmacologically relevant concentrations of test compounds to sensory nerve endings, allowing analgesic effects mediated via peripheral afferent fibers to be directly assessed. Importantly, this approach can help dissect on-target effects versus local high dose off-target effects while minimizing systemic effects, especially for compounds able to cross the blood-brain-barrier.

To establish pharmacologically relevant concentrations in vivo, we assessed the ability of conorphin-1 and U-50488 to inhibit forskolin-induced cAMP accumulation in HEK293 cells stably expressing MOR, DOR, and KOR. Conorphin-1 had low nanomolar agonist activity at KOR (pEC_{50} (M) 9.10 ± 0.07), without significantly affecting MOR or DOR at concentrations up to 1 μM (Figure 2a). U-50488 was found to be a similarly potent and selective KOR agonist, with pEC_{50} (M) values of 8.60 ± 0.07, 5.82 ± 0.22, and 4.87 ± 0.12 at KOR, MOR, and DOR, respectively (Figure 2b), in good agreement with affinities previously determined by radioligand binding.[15] Interestingly, U-50488 had partial agonist activity at MOR (58.6 ± 4.8% cAMP inhibition) versus full agonist activity at KOR and DOR.

In Vivo Analgesic Effect of Conorphin-1 and U-50488. To assess the analgesic effect of peripherally administered KOR agonists, we evaluated the antiallodynic and antihyperalgesic effects of conorphin-1 and U-50488 in rodent models of nociceptive (formalin-induced pain), inflammatory (Freund's Complete Adjuvant (FCA) and carrageenan-induced pain) and neuropathic (cisplatin-induced) pain. We administered compounds by shallow intraplantar administration using 200 nM solutions, which based on our in vitro assays would maximally activate KORs without significantly affecting MOR and DOR. Surprisingly, neither conorphin-1 nor U-50488 affected Phase I or Phase II of formalin-induced flinching when administered as 200 nM solutions by the intraplantar route, despite being delivered at a concentration at least 100-fold above their respective EC_{50}s (Figure 3a,b).

While intraplantar administration allows control over the maximum concentration of compounds delivered to sensory nerve endings, systemic redistribution and local metabolism can result in lower actual effective tissue concentrations. We thus next assessed whether a significantly higher dose of conorphin-1 and U-50488 would be able to affect formalin-induced pain behaviors. Surprisingly, even when administered at a 1000-fold higher concentration of 200 μM by local intraplantar injection, conorphin-1 failed to affect formalin-induced pain. In contrast, U-50488 at the same high dose was able to reverse Phase II of formalin-induced pain (Phase II: Control, 224 ± 16; U-50488 (200 μM), 77 ± 20; $P < 0.05$; Figure 3b).

A similar pattern of activity was seen for FCA-induced inflammation, where conorphin-1 at a high concentration (50 μM) had no significant effect on mechanical hyperalgesia, whereas U-50488 elicited analgesia, albeit only at a concentration of 11 mM (paw pressure thresholds: control, 69 ± 11 g; conorphin-1 (50 μM), 72 ± 14 g; U-50488 (11 mM), 180 ± 23 g; $P < 0.05$; Figure 3c). In addition, mechanical allodynia in a model of cisplatin-induced neuropathy[11] was also not affected by a high dose of conorphin-1 (paw withdrawal thresholds: control, 2.2 ± 0.1 g; conorphin-1 (200 μM), 1.9 ± 0.1 g; Figure 3d). In a model of carrageenan-induced inflammatory pain, intraplantar administration of conorphin-1 (200 μM) also did not affect paw withdrawal latency in the Hargreaves test (Figure 3e; Control, 2.6 ± 0.4 s; conorphin-1 (200 μM), 2.9 ± 0.6 s) or mechanical allodynia (Figure 3f; control, 1.5 ± 0.3 g; conorphin-1 (200 μM), 1.8 ± 0.3 g). Systemic administration of conorphin (2 mg/kg i.p.) also had no effect on heat allodynia (contralateral control, 12.1 ± 0.8 s; ipsilateral control, 2.6 ± 0.4 s; ipsilateral conorphin-1 (2 mg/kg i.p), 4.1 ± 1.0 s).

The Analgesic Effects of U-50488 Are Not Mediated through κ Opioid Receptor-Dependent Pain Pathways. Although conorphin-1 and U-50488 have similar in vitro functional activity at the KOR, only U-50488, administered at doses likely to achieve local concentrations far exceeding the in vitro EC_{50}, was able to produce analgesia in the animal models tested. We thus sought to explore the mechanisms by which U-

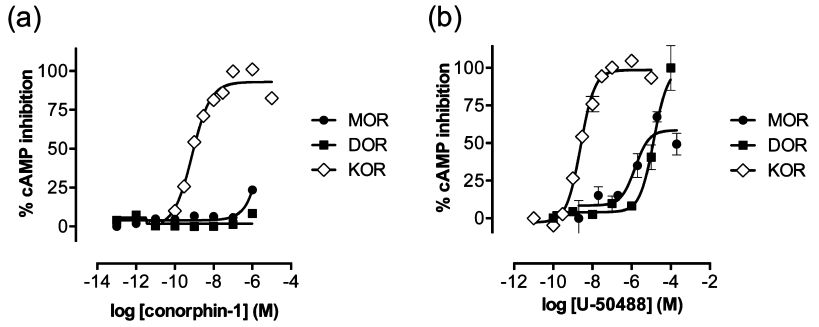

Figure 2. Inhibition of forskolin-induced cAMP accumulation by conorphin-1 and U-50488 in HEK293 cells stably expressing MOR, DOR, and KOR. (a) Conorphin-1 had low nanomolar agonist activity at KOR (pEC_{50} (M) 9.10 ± 0.07), without significantly affecting MOR or DOR at concentrations up to 1 μM. (b) U-50488 had similar potency and selectivity with pEC_{50} values (M) of 8.60 ± 0.07, 5.82 ± 0.22, and 4.87 ± 0.12 at KOR, MOR, and DOR, respectively, with only partial agonist activity at MOR. Data are presented as mean ± SEM from at least three independent experiments.

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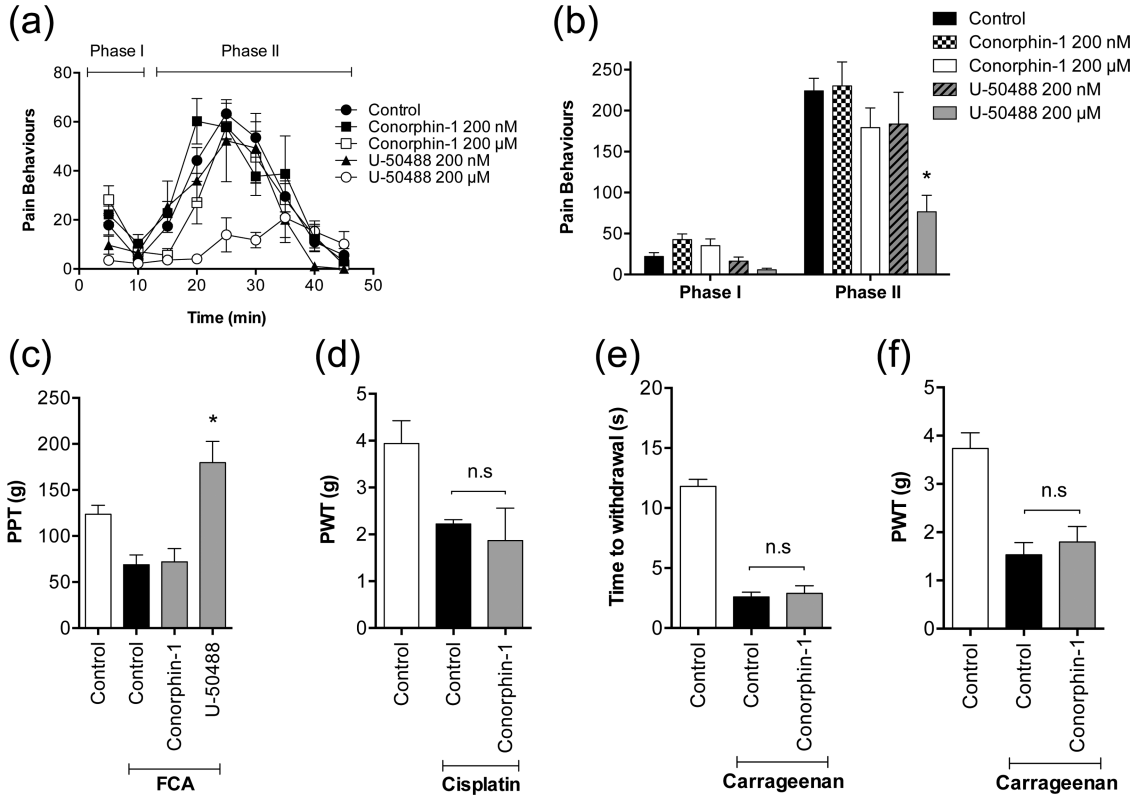

Figure 3. Effects of U-50488 and conorphin-1 in rodent models of pain. (a) Time course of formalin-induced pain behavior. Coadministration of conorphin-1 (200 nM) and U-50488 (200 nM) with formalin at a concentration expected to fully activate KOR had no effect on formalin-induced pain behaviors. At a significantly higher concentration, conorphin-1 (200 μM) still had no effect, but U-50488 (200 μM) significantly reduced formalin-induced pain behaviors. (b) Sum of pain behaviors in Phase I (0−10 min) and Phase II (10−45 min) of the formalin model. Only U-50488 (200 μM) significantly ($p < 0.05$; ANOVA with $F = 10.35$) reduced pain behaviors in Phase II of the formalin model, with no significant effect in Phase I. (c) FCA-induced mechanical hyperalgesia (white bar, contralateral control; black bar, ipsilateral control). Intraplantar administration of conorphin-1 (50 μM) had no effect on the PPT, whereas U-50488 at 11 mM, which was the minimum effective concentration, significantly increased the PPT. (d) Cisplatin-induced mechanical allodynia (white bar, contralateral control; black bar, ipsilateral control). Intraplantar administration of conorphin-1 (200 μM) had no effect on the PWT. (e) Carrageenan-induced heat allodynia (white bar, contralateral control; black bar, ipsilateral control). Intraplantar administration of conorphin-1 (200 μM; gray bar) had no effect on the paw withdrawal latency to a radiant heat stimulus. (f) Carrageenan-induced mechanical allodynia (white bar, contralateral control; black bar, ipsilateral control). Intraplantar administration of conorphin-1 (200 μM; gray bar) had no effect on the PWT. White bars represent baseline or uninjured controls; black bars represent control responses for ipsilateral paws. Data are expressed as mean ± SEM; $n = 3-8$ animals per group. *$p < 0.05$ versus control group.

50488 produces analgesia at higher doses by assessing the ability of the selective KOR antagonist ML190 to reverse the effect of U-50488. We first confirmed the selectivity of ML190 for KOR,[16] which is maintained at concentrations up to 10 μM (Figure 4a). However, the antinociceptive effect of U-50488 was not reversed by coadministration of ML190 at 10 μM or 10 μM of the nonselective opioid receptor antagonist naloxone (Phase II: U-50488 (200 μM), 77 ± 20; U-50488 (200 μM) + ML190 (10 μM), 80 ± 19; U-50488 (200 μM) + naloxone (10 μM), 81 ± 21), suggesting that the antinociceptive actions of U-50488 at higher concentrations involve opioid-independent pathways and are not generated through activation of peripheral KORs (Figure 4b,c). Although U-50488 also activates MOR at high concentrations, the lack of effect of naloxone is not surprising given U-50488 inhibits Na_V at these concentrations and this activity would lead to sustained analgesia in the presence of naloxone. We found similar results in the FCA model in rats, where U-50488 at a concentration of 11 mM, the minimum required concentration to significantly increase the paw pressure threshold, could not be reversed by the selective KOR antagonist ML190 (paw pressure thresholds: U-50488 (11 mM), 180 ± 23 g; U-50488 (11 mM) + ML190 (10 μM), 207 ± 24 g; Figure 4d).

U-50488 Inhibits Neuronal Na_V Channels at Concentrations Relevant to Those Used to Elicit Analgesia in Vivo. Structural similarity of U-50488 to local anesthetics, as well as effects on neuronal sodium currents, have been previously reported.[17] However, the effect of U-50488 on specific sodium channel isoforms has not been assessed to date. We thus systematically evaluated the Na_V subtype selectivity of U-50488 at $Na_V1.1-Na_V1.8$ using a high-throughput FLIPR^{TETRA} membrane potential assay (Figure 5a). Consistent with the structural similarity to local anesthetics, all Na_V isoforms assessed were inhibited by U-50488, although a modest selectivity for $Na_V1.4$ and $Na_V1.8$ was observed (pIC_{50}: $hNa_V1.1$, 4.38 ± 0.16; $hNa_V1.2$, 4.88 ± 0.20; $hNa_V1.3$, 4.49 ± 0.19; $hNa_V1.4$, 5.26 ± 0.19; $hNa_V1.5$, 4.37 ± 0.24; $rNa_V1.6$, 4.38 ± 0.16; $hNa_V1.7$, 4.27 ± 0.16; $hNa_V1.8$, 5.07 ± 0.18; Figure. 5b). We also assessed the effect of conorphin-1 and found no inhibition at $Na_V1.1-1.8$ at concentrations up to 1 μM (data not shown).

Analgesic effects of U-50488 after intraplantar administration in FCA- and formalin-induced pain have been reported

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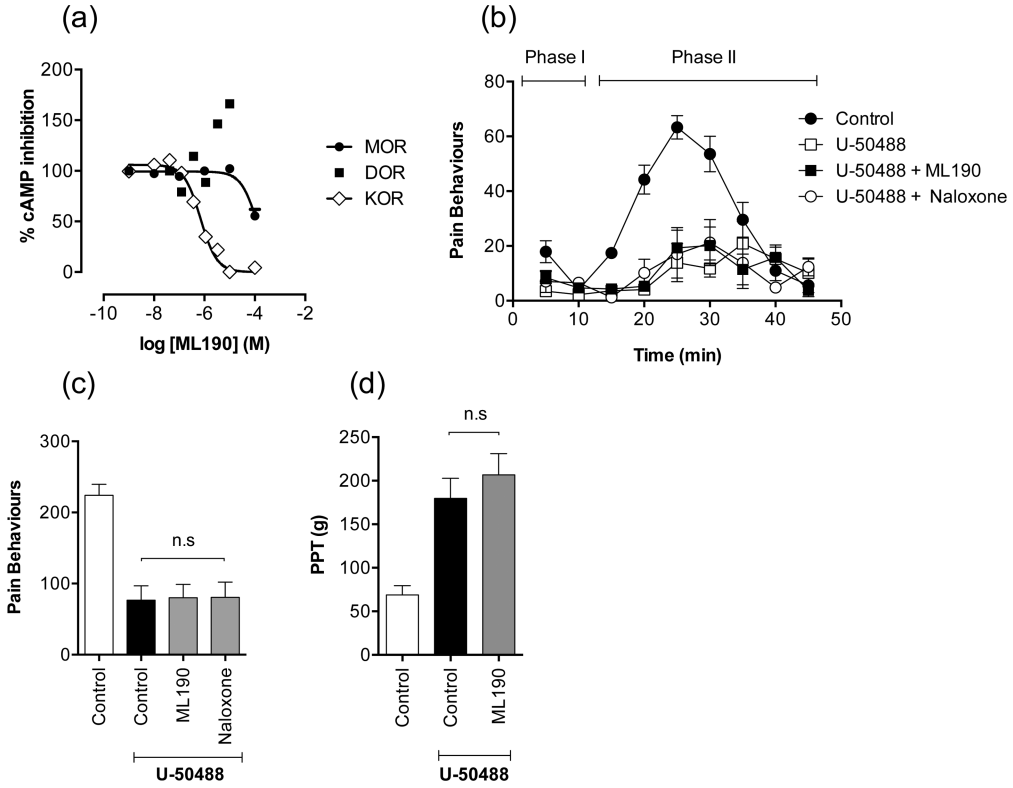

Figure 4. U-50488 mediates analgesia via KOR independent pathways. (a) ML190 is a selective KOR antagonist with minimal activity at MOR and DOR up to 10 μM. (b) Time course of formalin-induced pain behavior and (c) sum of Phase II behaviors (10−45 min) in the formalin model. Coadministration of the selective KOR antagonist ML190 (10 μM) and the nonselective opioid antagonist naloxone (10 μM) did not reverse the analgesic effect of U-50488 (200 μM) in the formalin model. (d) FCA-induced mechanical hyperalgesia. Coadministration of the selective KOR antagonist ML190 did not reverse the analgesic effect of U-50488 in the FCA-induced inflammatory model. Data are expressed as mean ± SEM; n = 3−8 animals per group.

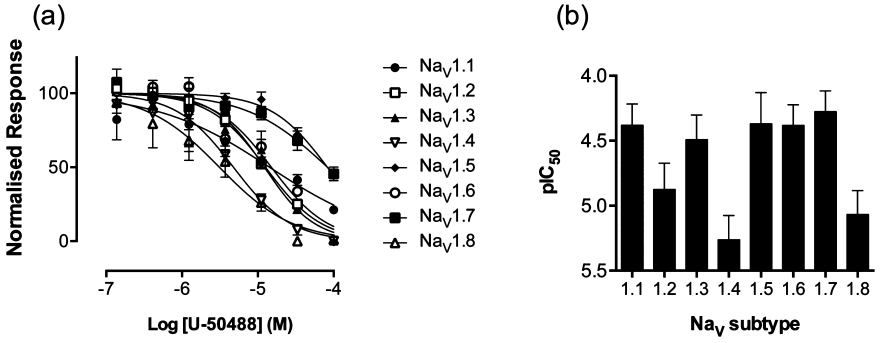

Figure 5. Na_V subtype selectivity of U-50488 assessed in HEK cells heterologously expressing $Na_V1.1$−1.8 using membrane potential assays. (a) Representative concentration−response curve of U-50488 at $Na_V1.1$−1.8. (b) U-50488 inhibited all Na_V subtypes, with modest selectivity for $hNa_V1.4$ (pIC_{50} (M) 5.26 ± 0.19) and $hNa_V1.8$ (pIC_{50} (M) 5.07 ± 0.18), followed by $hNa_V1.2$ (pIC_{50} (M) 4.88 ± 0.20) over the other subtypes (pIC_{50} (M): $hNa_V1.1$, 4.38 ± 0.16; $hNa_V1.3$, 4.49 ± 0.19; $hNa_V1.5$, 4.37 ± 0.24; $rNa_V1.6$, 4.38 ± 0.16; $hNa_V1.7$, 4.27 ± 0.16). Data are presented as mean ± SEM from at least three independent experiments.

previously,[18−21] where administration of 5−100 μg of U-50488 (190 μM to 11 mM solutions) was analgesic after intraplantar injection in the ipsilateral, but not the contralateral paw.[19] Our present results using similar doses of U-50488 confirmed these observations; however, while this activity was previously interpreted as analgesia mediated by peripheral rather than central KOR, our results suggest that inhibition of $Na_V1.8$ and $Na_V1.7$, key isoforms involved in mediating excitability of peripheral nociceptors,[22,23] likely explains the analgesic effect of U-50488 rather than activity at KOR.

Our findings therefore raise important questions about the role of peripheral KOR in pain. Although KORs are expressed in peripheral sensory neurons and presumably their peripheral projections innervating the skin and viscera, the contribution of opioid receptors to somatosensation are unclear.[24] Recently, DOR expression in myelinated mechanoreceptors that form Meissner corpuscles, Merkel cell−neurite complexes, and circumferential hair follicle endings was reported to control cutaneous mechanosensation.[25] In contrast, MORs are expressed at the peripheral terminals of nociceptors, with functional effects including decreased excitability of afferent

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fibers that is particularly apparent in inflammation.[26−28] Similarly, KOR is expressed in small diameter dorsal root ganglion neurons, but compared with MOR and DOR, the functional effect of KOR activation in peripheral sensory nerve terminals as well as the effect on neuronal excitability appear to be limited.[29] A role for peripheral KORs in pain has been suggested based on studies assessing the antinociceptive effects of KOR agonists such as U-50488 delivered locally.[19,30,31] In light of our results demonstrating significant nonopioid antinociceptive effects of U-50488 mediated through inhibition of Na_V channels, this approach needs to be reconsidered. In contrast, KOR is widely expressed throughout the central nervous system and U-50488 as well as other KOR agonists elicit potent antinociceptive effects when delivered by spinal or supraspinal routes.[32−35]

While KOR is expressed in small DRGs, it is unclear whether expression in the central or peripheral projections of these neurons mediate analgesic effects. Our data show that activation of KOR at peripheral nerve terminals innervating the skin does not mediate analgesia, corroborating an important role of KOR in spinal projections.

Interestingly, the selective tetrapeptide KOR agonist CR665 and the analogue CR845, currently being developed by Cara Therapeutics, have been shown to reduce, rather than increase, cutaneous pain thresholds in humans after local administration, providing further evidence that activation of peripheral KOR does not mediate analgesia.[36] Although CR665 is analgesic in a range of painful conditions, a lack of CNS penetration was only inferred from the absence of inhibition of the tail flick test.[37] However, without direct comparison to the effect of spinally administered KOR-selective agonists in the tail flick assay, such effects are difficult to quantify, particularly in light of evidence suggesting that tetrapeptides can indeed cross the blood brain barrier.[38−40] In addition, the centrally penetrant U-50488 also failed to affect the tail flick test[41] after systemic administration, highlighting the limitations of this approach to assess the ability of compounds to cross the blood brain barrier. Recently, it was suggested that KOR-mediated antinociception is a form of stress-induced analgesia.[45] We observed no alterations in behavior after systemic administration of conorphin-1 (2 m/kg i.p.) consistent with a relative inability of peptides to cross the blood-brain barrier. Lastly, the lack of analgesic efficacy of KOR-selective agonists in models of cutaneous pain is consistent with studies in KOR knockout mice, where behavioral deficits were only observed in the acetic acid writhing test.[42]

One limitation of pharmacological studies is that translation of in vitro findings to in vivo behaviors requires cautious interpretation. Nonetheless, this caveat is not limited to the current study as by necessity, pharmacological characterization at isolated receptor systems is frequently performed in vitro.

Given that U-50488 is structurally and functionally similar to local anesthetics and has similar analgesic activity in vivo, our data support in vivo inhibition of Na_V channels as a major mechanism of action contributing to the analgesic effect of U-50488. Thus, previous studies assessing the in vivo effects of U-50488 may need to be viewed in light of these findings.

While it is possible that a short in vivo half-life contributes to poor analgesic efficacy after systemic administration, metabolic stability likely has little effect on analgesic efficacy after intraplantar injection, as pain responses were assessed within minutes of administration. In addition, conorphin-1 analogues were effective in a model of chronic visceral hypersensitivity (unpublished experiments), demonstrating that activity in native tissue is consistent with the observed in vitro activity.

The lack of analgesic efficacy of conorphin-1 after intraplantar injection should be interpreted with particular reference to the pain modalities tested as well as the local route of administration. Activation of KOR in spinal pathways and higher brain centers is clearly associated with analgesia, as demonstrated by a number of publications assessing formalin-induced pain after systemic administration of U-50488.[32−35] In contrast, our data show that pain modalities including spontaneous pain, mechanical allodynia and hyperalgesia as well as thermal allodynia are unaffected after intraplantar administration of KOR-selective compounds.

In conclusion, our study provides evidence that targeting KOR in peripheral sensory nerve endings innervating the skin does not mediate analgesia in animal models of inflammatory and neuropathic pain, and that any peripheral analgesic effects of U-50488 are likely mediated through inhibition of Na_V channels.

METHODS

Materials. (±)-*trans*-U-50488 was obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). ML190 was obtained from Tocris distributor Bio-Scientific Pty. Ltd. (Gymea, NSW, Australia). Veratridine was obtained from Abcam (Melbourne, VIC, Australia). All other reagents, unless otherwise stated, were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

Synthesis of Conorphin-1. *Synthetic Strategy.* The peptide conorphin-1 was obtained via a medicinal chemistry program from a conopeptide analogue (unpublished experiments). The KOR agonist conorphin-1 was synthesized using Rink amide resin (0.05 mmol). Assembly of conorphin-1 was performed using HBTU in situ activation protocols[43] to couple the Fmoc-protected amino acid or unprotected acids to the resin (4 equiv excess). After chain assembly, the peptide was cleaved from the resin at room temperature (RT) in TFA/H_2O/TIPS/EDT (87.5:5:5:2.5) for 3 h. Cold diethyl ether (30 mL) was then added to the filtered cleavage mixture, and the peptide precipitated. The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether to remove scavengers. The final product was dissolved in 50% aqueous acetonitrile and lyophilized to yield a white solid. The crude, reduced peptide was examined by reversed-phase HPLC for purity and the correct molecular weight of (MH^+ = 977.2 amu) confirmed by electrospray mass spectrometry (ESMS)

Disulfide Bond Formation. Pure, reduced conorphin-1 containing only one pair of cysteine residues was oxidized by stirring at RT in 30% DMSO/5% HOAc (1 mg/mL) for 16 h. The solution was subsequently diluted to a DMSO concentration of <5%, prior to RP-HPLC purification and lyophilization.

HPLC Analysis and Purification. Analytical HPLC runs were performed using a Shimadzu HPLC system LC10A with a dual wavelength UV detector set at 214 and 254 nm. A reversed-phase C-18 column (Zorbax 300-SB C-18; 4.6 × 50 mm) with a flow rate of 2 mL/min was used. Gradient elution was performed with the following buffer systems: **A**, 0.05% TFA in water and **B**, 0.043% TFA in 90% acetonitrile in water, from 0% **B** to 80% **B** in 20 min. The crude peptides were purified by semipreparative HPLC on a Shimadzu HPLC system LC8A associated with a reversed-phase C-18 column (Vydac C-18, 25 cm × 10 mm) running at a flow rate of 5 mL/min with a 1% gradient of 0% **B** to 40% **B**. The purity of the final product was evaluated by analytical HPLC (Zorbax 300SB C-18:4.6 × 100 mm) with a flow rate of 1 mL/min and a 1.5% gradient of **B** (5−25%). The purity of synthesized conorphin-1 was 98.0%.

Electrospray Mass Spectrometry (ESMS). Electrospray mass spectra were collected inline during analytical HPLC runs on an Applied Biosystems API-150 spectrometer operating in the positive ion mode with an OR of 20, Rng of 220 and Turbospray of 350°.

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Masses between 300 and 2200 amu were detected (Step 0.2 amu, Dwell 0.3 ms).

In Vitro Activity at MOR, DOR, and KOR. HEK293 cell lines stably expressing MOR, DOR, and KOR were a kind gift from Prof. Maree T. Smith, The University of Queensland. Cells were cultured in DMEM containing 10% v/v fetal bovine serum and 500 μg/mL G-418 (Invitrogen) and passaged every 3−5 days using 0.25% trypsin/EDTA. For cAMP assays, cells were harvested using Versene (Invitrogen).

The Lance Ultra cAMP kit was used (PerkinElmer) to assess activity at KOR and DOR. All kit components were prepared according to the manufacturer's specifications. Briefly, cells (1000 cells/well) were incubated at room temperature for 15 min with either conorphin-1 or U-50488 before the addition of either buffer (control) or forskolin (50 μM; Sapphire Bioscience). For quantification of antagonist activity, ML190 and either U-50488 or SNC80 were incubated at room temperature for 15 min before the addition of either buffer (control) or forskolin (50 μM). The Eu-cAMP tracer and Ulight-anti cAMP reagents were then added for 1 h at room temperature before reading of the plate using the M1000 Pro multilabel plate reader (Tecan).

An AlphaScreen cAMP assay was used to assess activity at MOR as previously described.[44] In brief, MOR-HEK293 cells (20 000 cells/well) were resuspended in stimulation buffer containing forskolin (50 μM) and anti-cAMP acceptor beads (0.4 units/μL) and incubated with opioid agonists and antagonists in an appropriate dilution series. After 30 min incubation under low-light conditions, streptavidin donor beads (1 unit/25 μL) and biotinylated cAMP (1 unit/25 μL) were added, and the cAMP signal was determined after 16 h incubation using an EnSpire Multilabel reader (PerkinElmer, Waltham, MA).

In Vitro Activity at Na_V Channels. Na_V responses were assessed in human embryonic kidney (HEK 293) cells heterologously expressing $hNa_V1.1$, $hNa_V1.2$, $hNa_V1.3$, $hNa_V1.4$, $hNa_V1.5$, $hNa_V1.6$, $hNa_V1.7$, and $hNa_V1.8$ (SB Drug Discovery, Glasgow, UK). Cells were cultured in MEM containing 10% v/v fetal bovine serum supplemented with L-glutamine 2 mM, the selection antibiotics G-418, blasticidin (Invitrogen), and zeocin (Invitrogen) at varying concentrations as recommended by the manufacturer, and passaged every 3−4 days using 0.25% trypsin/EDTA. Cells were plated 48 h before the assay on 384-well black walled imaging plates at a density of 10 000−15 000 cells/well and were loaded with red membrane potential dye (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions for 30 min at 37 °C. After addition of varying concentrations of U-50488 or conorphin-1, cells were incubated a further 5 min before stimulating Na_V using veratridine (60 μM, $Na_V1.1$−$Na_V1.7$) or deltamethrin (150 μM, $Na_V1.8$). Changes in membrane potential were assessed using the $FLIPR^{TETRA}$ (Molecular Devices; excitation 515−545 nm, emission 565−625 nm) every second for 300 s after adding agonists

Animals. For behavioral assessment, we used adult male C57BL/6J mice or adult male Wistar rats aged 6−8 weeks. Animals were housed in groups of 3−4 per cage, under 12 h light/dark cycles and had standard rodent chow and water ad libitum.

Ethics Statement. Ethical approval for in vivo experiments in animals was obtained from the University of Queensland animal ethics committee. Experiments involving animals were conducted in accordance with the Animal Care and Protection Regulation Qld (2012), the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, eighth edition (2013) and the *International Association for the Study of Pain Guidelines for the Use of Animals in Research*.

Behavioral Assessment. Formalin (formaldehyde 16% w/v, Thermo Scientific, Australia) was diluted in saline to a 1% w/v formaldehyde solution and was administered by shallow subcutaneous (intraplantar) injection to the left hind paw of mice in a volume of 20 μL under light isoflurane anesthesia. The following agonists/antagonists were coinjected with formalin: conorphin-1 (200 nM, 200 μM), U-50488 (200 nM), U-50488 (200 μM) ± ML190 (10 μM) or naloxone (10 μM). Immediately after injection, mice were placed individually into polyvinyl boxes (10 × 10 × 10 cm^3) and spontaneous pain was quantified by counting the number of paw lifts, licks, shakes, and flinches by a blinded observer unaware of treatments received over a 45 min period in 5 min intervals from video recordings. Phase I and Phase II were defined as the cumulative pain behaviors that occurred from 0−10 min and 10−45 min postinjection, respectively.

To induce inflammation, Freund's Complete Adjuvant (FCA) was administered by shallow subcutaneous injection to the left hind paw of rats in a volume of 150 μL under light isoflurane anesthesia. After 4−6 days, rats were then administered conorphin-1 (50 μM) or U-50488 (11 mM) ± ML190 (10 μM) diluted in saline in a volume of 100 μL by intraplantar injection. Nociceptive thresholds were evaluated using an Analgesy-Meter (Ugo Basile, Italy) before and 15 min post injection. Rats were gently restrained with a towel and subjected to incremental increases in pressure (maximum 250 g) to the dorsal surface of each hind paw. The pressure at which the rat withdrew the hind paw, defined as the paw pressure threshold (PPT), was determined by the mean of three consecutive measurements, separated by at least 30 s.

To induce peripheral neuropathy, cisplatin (20 μg) was administered to the left hind paw of mice in a volume of 20 μL under light isoflurane anesthesia as previously described.[11] After 2 h, conorphin-1 (200 μM) was administered by intraplantar injection in a volume of 20 μL and mechanical allodynia was assessed using an electronic von Frey apparatus (MouseMet Electronic von Frey, TopCat Metrology). Mice were habituated in individual mouse runs for at least 10 min prior to testing. Pressure was applied to the left hind paw through a soft-tipped probe and increased slowly at a force rise rate of ∼1 g/s. The force that elicited paw withdrawal was determined using the MouseMet Software. The test was repeated three times at 5 min intervals. The force that elicited paw withdrawal from each of the three tests was averaged and designated as the paw-withdrawal threshold (PWT). The investigators remained blinded throughout all of the behavioral studies.

λ-Carrageenan (Sigma-Aldrich, Castle Hill, NSW) was dissolved in saline to a 1% w/v solution (prepared 24 h prior) and administered by intraplantar injection to the left hind paw of mice in a volume of 40 μL under light isoflurane (3%) anesthesia. At 3 h postinjection of carrageenan, conorphin-1 (200 μM) was administered by intraplantar injection to the left hind paw in a volume of 20 μL, or by intraperitoneal injection (2 mg/kg) in a volume of 10 μL/g. Mice were then placed individually into polyvinyl boxes (10 × 10 × 10 cm^3), after previously being habituated for 1 h, and heat hyperalgesia was assessed using the Hargreaves method (Plantar Analgesia Meter, IITC, Woodland Hills, CA) 15 min post administration of conorphin-1. The radiant heat light source was focused on the plantar surface of the left hind paw and the time taken for the mouse to withdraw the paw was recorded by a blinded investigator, with a cutoff of 20 s to prevent tissue damage. The mean time to withdrawal was determined from the average of three tests, separated by at least 1 min. Mechanical allodynia was assessed as described above.

Data Analysis and Statistics. Data were plotted and analyzed by GraphPad Prism, version 6.0. Statistical significance was defined as $P < 0.05$ and was determined by *t* test or one-way ANOVA analysis with Dunnett's post test. Data is expressed as the mean ± standard error of the mean.

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Author Contributions

R.J.L., P.F.A., P.J.C., and I.V. designed the study; J.R.D., E.W., A.B., M.C.I., N.H.A., and I.V. performed the experiments; J.R.D., E.W., M.C.I., and I.V. analyzed the data; J.R.D. and I.V. wrote the manuscript; all authors read and edited the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

MOR, μ opioid receptor; DOR, δ opioid receptor; KOR, κ opioid receptor; PPT, paw pressure threshold; PWT, paw withdrawal threshold; FCA, Freund's Complete Adjuvant

■ REFERENCES

(1) Walsh, S. L., Strain, E. C., Abreu, M. E., and Bigelow, G. E. (2001) Enadoline, a selective kappa opioid agonist: comparison with butorphanol and hydromorphone in humans. *Psychopharmacology (Berl.) 157*, 151−162.

(2) Rimoy, G. H., Wright, D. M., Bhaskar, N. K., and Rubin, P. C. (1994) The cardiovascular and central nervous system effects in the human of U-62066E. *Eur. J. Clin. Pharmacol. 46*, 203−207.

(3) Furst, S., Riba, P., Friedmann, T., Timar, J., Al-Khrasani, M., Obara, I., Makuch, W., Spetea, M., Schutz, J., Przewlocki, R., Przewlocka, B., and Schmidhammer, H. (2005) Peripheral versus central antinociceptive actions of 6-amino acid-substituted derivatives of 14-O-methyloxymorphone in acute and inflammatory pain in the rat. *J. Pharmacol. Exp. Ther. 312*, 609−618.

(4) Labuz, D., Mousa, S. A., Schafer, M., Stein, C., and Machelska, H. (2007) Relative contribution of peripheral versus central opioid receptors to antinociception. *Brain Res. 1160*, 30−38.

(5) Rau, K. K., Caudle, R. M., Cooper, B. Y., and Johnson, R. D. (2005) Diverse immunocytochemical expression of opioid receptors in electrophysiologically defined cells of rat dorsal root ganglia. *J. Chem. Neuroanat. 29*, 255−264.

(6) Shannon, H. E., and Lutz, E. A. (2002) Comparison of the peripheral and central effects of the opioid agonists loperamide and morphine in the formalin test in rats. *Neuropharmacology 42*, 253−261.

(7) King, G. F. (2011) Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opin. Biol. Ther. 11*, 1469−1484.

(8) Saez, N. J., Senff, S., Jensen, J. E., Er, S. Y., Herzig, V., Rash, L. D., and King, G. F. (2010) Spider-venom peptides as therapeutics. *Toxins 2*, 2851−2871.

(9) Chikhale, E. G., Ng, K. Y., Burton, P. S., and Borchardt, R. T. (1994) Hydrogen bonding potential as a determinant of the in vitro and in situ blood-brain barrier permeability of peptides. *Pharm. Res. 11*, 412−419.

(10) Egleton, R. D., and Davis, T. P. (2005) Development of neuropeptide drugs that cross the blood-brain barrier. *NeuroRx 2*, 44−53.

(11) Deuis, J. R., Lim, Y. L., Rodrigues de Sousa, S., Lewis, R. J., Alewood, P. F., Cabot, P. J., and Vetter, I. (2014) Analgesic effects of clinically used compounds in novel mouse models of polyneuropathy induced by oxaliplatin and cisplatin. *Neuro Oncol. 16*, 1324−1332.

(12) Deuis, J. R., Zimmermann, K., Romanovsky, A. A., Possani, L. D., Cabot, P. J., Lewis, R. J., and Vetter, I. (2013) An animal model of oxaliplatin-induced cold allodynia reveals a crucial role for Nav1.6 in peripheral pain pathways. *Pain 154*, 1749−1757.

(13) Mueller, A., Starobova, H., Inserra, M. C., Jin, A. H., Deuis, J. R., Dutertre, S., Lewis, R. J., Alewood, P. F., Daly, N. L., and Vetter, I. (2015) alpha-conotoxin MrIC is a biased agonist at alpha nicotinic acetylcholine receptors. *Biochem. Pharmacol. 94*, 155.

(14) Vetter, I., Touska, F., Hess, A., Hinsbey, R., Sattler, S., Lampert, A., Sergejeva, M., Sharov, A., Collins, L. S., Eberhardt, M., Engel, M., Cabot, P. J., Wood, J. N., Vlachova, V., Reeh, P. W., Lewis, R. J., and Zimmermann, K. (2012) Ciguatoxins activate specific cold pain pathways to elicit burning pain from cooling. *EMBO J. 31*, 3795−3808.

(15) Vanderah, T. W., Largent-Milnes, T., Lai, J., Porreca, F., Houghten, R. A., Menzaghi, F., Wisniewski, K., Stalewski, J., Sueiras-Diaz, J., Galyean, R., Schteingart, C., Junien, J. L., Trojnar, J., and Riviere, P. J. (2008) Novel D-amino acid tetrapeptides produce potent antinociception by selectively acting at peripheral kappa-opioid receptors. *Eur. J. Pharmacol. 583*, 62−72.

(16) Hedrick, M. P., Gosalia, P., Li, K., Frankowski, K. J., Shi, S., Prisinzano, T. E., Schoenen, F., Aube, J., Su, Y., Stonich, D., Vasile, S., Sergienko, E., Gray, W., Hariharan, S., Milan, L., Heynen-Genel, S., Vicchiarelli, M., Mangravita-Novo, A., Streicher, J. M., Smith, L. H., Chung, T. D. Y., Caron, M., Bohn, L. M., and Barak, L. S. (2010) Antagonist for the Kappa Opioid Receptor. In *Probe Reports from the NIH Molecular Libraries Program*, Bethesda, MD.

(17) Su, X., Castle, N. A., Antonio, B., Roeloffs, R., Thomas, J. B., Krafte, D. S., and Chapman, M. L. (2009) The effect of kappa-opioid receptor agonists on tetrodotoxin-resistant sodium channels in primary sensory neurons. *Anesth. Analg. 109*, 632−640.

(18) Ambriz-Tututi, M., Rocha-Gonzalez, H. I., Castaneda-Corral, G., Araiza-Saldana, C. I., Caram-Salas, N. L., Cruz, S. L., and Granados-Soto, V. (2009) Role of opioid receptors in the reduction of formalin-induced secondary allodynia and hyperalgesia in rats. *Eur. J. Pharmacol. 619*, 25−32.

(19) Auh, Q. S., and Ro, J. Y. (2012) Effects of peripheral kappa opioid receptor activation on inflammatory mechanical hyperalgesia in male and female rats. *Neurosci. Lett. 524*, 111−115.

(20) Hong, Y., and Abbott, F. V. (1995) Peripheral opioid modulation of pain and inflammation in the formalin test. *Eur. J. Pharmacol. 277*, 21−28.

(21) Joshi, S. K., and Gebhart, G. F. (2003) Nonopioid actions of U50,488 enantiomers contribute to their peripheral cutaneous antinociceptive effects. *J. Pharmacol. Exp. Ther. 305*, 919−924.

(22) Akopian, A. N., Souslova, V., England, S., Okuse, K., Ogata, N., Ure, J., Smith, A., Kerr, B. J., McMahon, S. B., Boyce, S., Hill, R., Stanfa, L. C., Dickenson, A. H., and Wood, J. N. (1999) The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. *Nat. Neurosci. 2*, 541−548.

(23) Rush, A. M., Cummins, T. R., and Waxman, S. G. (2007) Multiple sodium channels and their roles in electrogenesis within dorsal root ganglion neurons. *J. Physiol. 579*, 1−14.

(24) Schmidt, Y., and Machelska, H. (2015) Immunohistochemical analysis of opioid receptors in peripheral tissues. *Methods Mol. Biol. 1230*, 155−165.

(25) Bardoni, R., Tawfik, V. L., Wang, D., Francois, A., Solorzano, C., Shuster, S. A., Choudhury, P., Betelli, C., Cassidy, C., Smith, K., de Nooij, J. C., Mennicken, F., O'Donnell, D., Kieffer, B. L., Woodbury, C. J., Basbaum, A. I., MacDermott, A. B., and Scherrer, G. (2014) Delta opioid receptors presynaptically regulate cutaneous mechano-sensory neuron input to the spinal cord dorsal horn. *Neuron 81*, 1312−1327.

(26) Coggeshall, R. E., Zhou, S., and Carlton, S. M. (1997) Opioid receptors on peripheral sensory axons. *Brain Res. 764*, 126−132.

(27) Moshourab, R., Schmidt, Y., and Machelska, H. (2015) Skin-nerve preparation to assay the function of opioid receptors in peripheral endings of sensory neurons. *Methods Mol. Biol. 1230*, 215−228.

(28) Schmidt, Y., Labuz, D., Heppenstall, P. A., and Machelska, H. (2012) Cutaneous nociceptors lack sensitisation, but reveal mu-opioid receptor-mediated reduction in excitability to mechanical stimulation in neuropathy. *Mol. Pain 8*, 81.

(29) Wang, H. B., Zhao, B., Zhong, Y. Q., Li, K. C., Li, Z. Y., Wang, Q., Lu, Y. J., Zhang, Z. N., He, S. Q., Zheng, H. C., Wu, S. X., Hokfelt, T. G., Bao, L., and Zhang, X. (2010) Coexpression of delta- and mu-opioid receptors in nociceptive sensory neurons. *Proc. Natl. Acad. Sci. U. S. A. 107*, 13117−13122.

(30) Stein, C., Millan, M. J., Shippenberg, T. S., Peter, K., and Herz, A. (1989) Peripheral opioid receptors mediating antinociception in

DOI: 10.1021/acschemneuro.5b00113
ACS Chem. Neurosci. 2015, 6, 1751−1758

inflammation. Evidence for involvement of mu, delta and kappa receptors. *J. Pharmacol. Exp. Ther. 248*, 1269−1275.
(31) Zambelli, V. O., Fernandes, A. C., Gutierrez, V. P., Ferreira, J. C., Parada, C. A., Mochly-Rosen, D., and Cury, Y. (2014) Peripheral sensitization increases opioid receptor expression and activation by crotalphine in rats. *PLoS One 9*, e90576.
(32) Pelissier, T., Paeile, C., Soto-Moyano, R., Saavedra, H., and Hernandez, A. (1990) Analgesia produced by intrathecal administration of the kappa opioid agonist, U-50,488H, on formalin-evoked cutaneous pain in the rat. *Eur. J. Pharmacol. 190*, 287−293.
(33) Leighton, G. E., Rodriguez, R. E., Hill, R. G., and Hughes, J. (1988) kappa-Opioid agonists produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. *Br. J. Pharmacol. 93*, 553−560.
(34) Calcagnetti, D. J., Helmstetter, F. J., and Fanselow, M. S. (1988) Analgesia produced by centrally administered DAGO, DPDPE and U50488H in the formalin test. *Eur. J. Pharmacol. 153*, 117−122.
(35) Mansour, A., Fox, C. A., Burke, S., Meng, F., Thompson, R. C., Akil, H., and Watson, S. J. (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. *J. Comp. Neurol. 350*, 412−438.
(36) Arendt-Nielsen, L., Olesen, A. E., Staahl, C., Menzaghi, F., Kell, S., Wong, G. Y., and Drewes, A. M. (2009) Analgesic efficacy of peripheral kappa-opioid receptor agonist CR665 compared to oxycodone in a multi-modal, multi-tissue experimental human pain model: selective effect on visceral pain. *Anesthesiology 111*, 616−624.
(37) Hughes, F. M., Jr., Shaner, B. E., Brower, J. O., Woods, R. J., and Dix, T. A. (2013) Development of a Peptide-derived orally-active kappa-opioid receptor agonist targeting peripheral pain. *Open Med. Chem. J. 7*, 16−22.
(38) Ballet, S., Misicka, A., Kosson, P., Lemieux, C., Chung, N. N., Schiller, P. W., Lipkowski, A. W., and Tourwe, D. (2008) Blood-brain barrier penetration by two dermorphin tetrapeptide analogues: role of lipophilicity vs structural flexibility. *J. Med. Chem. 51*, 2571−2574.
(39) Guillemyn, K., Kleczkowska, P., Novoa, A., Vandormael, B., Van den Eynde, I., Kosson, P., Asim, M. F., Schiller, P. W., Spetea, M., Lipkowski, A. W., Tourwe, D., and Ballet, S. (2012) In vivo antinociception of potent mu opioid agonist tetrapeptide analogues and comparison with a compact opioid agonist-neurokinin 1 receptor antagonist chimera. *Mol. Brain 5*, 4.
(40) Zhao, K., Luo, G., Zhao, G. M., Schiller, P. W., and Szeto, H. H. (2003) Transcellular transport of a highly polar 3+ net charge opioid tetrapeptide. *J. Pharmacol. Exp. Ther. 304*, 425−432.
(41) Gallantine, E. L., and Meert, T. F. (2008) Antinociceptive and adverse effects of mu- and kappa-opioid receptor agonists: a comparison of morphine and U50488-H. *Basic Clin. Pharmacol. Toxicol. 103*, 419−427.
(42) Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., Le Meur, M., Roques, B. P., Maldonado, R., and Kieffer, B. L. (1998) Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J. 17*, 886−897.
(43) Alewood, P., Alewood, D., Miranda, L., Love, S., Meutermans, W., and Wilson, D. (1997) Rapid in situ neutralization protocols for Boc and Fmoc solid-phase chemistries. *Methods Enzymol. 289*, 14−29.
(44) Asvadi, N. H., Morgan, M., Herath, H. M., Hewavitharana, A. K., Shaw, P. N., and Cabot, P. J. (2014) Beta-endorphin 1−31 biotransformation and cAMP modulation in inflammation. *PLoS One 9*, e90380.
(45) Taylor, A. M., Roberts, K. W., Pradhan, A. A., Akbari, H. A., Walwyn, W., Lutfy, K., Carroll, F. I., Cahill, C. M., Evans, C. J. Antinociception mediated by a κ opioid receptor agonist is blocked by a δ receptor agonist. *Br. J. Pharmacol.* 2015 Jan;*172*(2):691−703.10.1111/bph.12810

DOI: 10.1021/acschemneuro.5b00113
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