Short Report

Functional bias of morphine and oliceridine under conditions of minor injury

Chinwe Nwaneshiudu¹, Xiao-Yu Shi¹,², Peyman Sahbaie¹,² and J David Clark¹,²

Abstract
Recent reports suggest pain from surgical injury may influence the risks associated with exposure to opioids. In mice, hindpaw incision attenuates morphine-primed reinstatement due to kappa opioid receptor activation by dynorphin. In this focused group of studies, we examined the hypotheses that kappa-opioid receptor activation in the nucleus accumbens mediates attenuated drug-primed reinstatement after incisional surgery, and the G-protein biased mu-opioid agonist, oliceridine, leads to less priming of the dynorphin effect in comparison to morphine. To address these hypotheses, adult C57Bl/6 male mice underwent intracranial cannulation for administration of the selective kappa-opioid antagonist norBNI directly into the nucleus accumbens. After recovery, they were conditioned with morphine or oliceridine after hind-paw incisional injury, then underwent extinction followed by opioid-primed reinstatement. Intra-accumbal administration of norBNI was carried out prior to testing. The nucleus accumbens and medial prefrontal cortex were extracted and analyzed for expression of prodynorphin. We observed that animals conditioned with morphine in the setting of incisional injury demonstrated blunted responses to opioid-primed reinstatement, and that the blunted responses were reversed with intra-accumbal norBNI administration. Persistently elevated levels of prodynorphin expression in the medial prefrontal cortex and nucleus accumbens were observed in the incised morphine-treated animals. However, both behavioral and molecular changes were absent in animals with incisional injury conditioned with oliceridine. These findings suggest a role for prodynorphin expression in the nucleus accumbens with exposure to morphine after surgery that may protect individuals from relapse not shared with biased mu-opioid receptor agonists.

Keywords
Morphine, reward, reinstatement, norbinaltorphine, norBNI, TRV130, oliceridine, injury, accumbens

A significant portion of patients previously opioid naïve later develop prescription opioid use disorders after having their first exposure to opioids in treatment of acute medical and post-surgical pain.¹⁻³ These patients are now recognized to be at risk for overdose and death. Patients with prescription opioid use disorders also transition to intravenous illicit opioid use leading to greater risk of health, psychosocial and economic instability.⁴⁻⁵ Studies are needed to investigate pharmacological interventions to possibly reduce future opioid abuse liability after surgery.

Strategies to develop safer opioids include the development of mu-opioid receptor ligands with biased agonism, a means to separate desirable from some of the adverse drug responses downstream of the receptor target.⁶ Such ligands stabilize alternate G-protein-coupled receptor (GPCR) receptor conformations upon binding, with each displaying a unique pattern of activation of intracellular signaling cascades.⁷⁻⁹ Biased

¹Department of Anesthesiology, Perioperative and Pain Medicine, School of Medicine, Stanford University, Stanford, CA, USA
²Veterans Affairs Palo Alto Healthcare System, Anesthesiology Service, Palo Alto, CA, USA

Corresponding Author:
Chinwe Nwaneshiudu, 1070 Arastradero Road, Suite 200, Palo Alto, CA 94304, USA.
Email: cnwaneshi@stanford.edu
agonism at the mu-opioid receptor was based, in part, on the observation that genetic global knock-out of beta arrestin-2 in mice demonstrated enhanced analgesia to morphine while having minimal side effects such as respiratory depression, constipation and tolerance, suggesting that opioid mediated analgesia engaged the downstream G-protein coupled receptor kinase signaling pathways, while its side effects favored beta arrestin-mediated pathways. Subsequently novel biased mu-agonists have been studied including TRV 130 also known as oliceridine with its propensity to favor activating G-Protein receptor kinase-dependent pathways over beta arrestin-dependent pathways.

Reinstatement to drug seeking and reward are behavior models used to investigate human drug relapse behavior after a drug free period. Our previous studies showed in the presence of surgical hind-paw incision, there is an enhanced response to morphine’s rewarding properties but a protective or blunted response to morphine-primed conditioned reinstatement. We also showed that systemic kappa-opioid receptor blockade reversed this change in behavioral response to morphine reward, and there was a persistent elevation of prodynorphin expression in brains of incised opioid-exposed mice. What remains unclear is the site of action of the involved prodynorphin expression. Based on their significance in drug reward and expression of drug-primed reinstatement, the nucleus accumbens and medial prefrontal cortex were the selected brain regions for this study. We sought to determine this by employing pharmacological blockade of kappa opioid receptor selectively within the nucleus accumbens during expression of drug-primed reinstatement. We also used the mu-opioid receptor biased agonist oliceridine as an alternative drug of choice to examine whether these observed changes were influenced by biased activation of the mu-opioid receptor.

Male C57BL/6J mice aged 10–12 weeks were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed four per cage and maintained on a 12 hr light-dark cycle and ambient temperature of 22 degrees Celsius with ad lib access to food and water. All experimental protocols were reviewed and approved by the Veterans Affairs Palo Alto Healthcare System Institutional Animal Care and Use Committee (Palo Alto, California), and were conducted in concordance with the guidelines for the study of pain in awake animals as established by the International Association for the Study of Pain.

Intra-accumbal administration of norBNI reversed attenuation of morphine primed reinstatement as a result of injury- In this experiment, adult male C57BL/6 mice underwent placements of bilateral intra-accumbal cannulas with dummy stylets (location AP-1.9, ML+/-3, DV 4.5, 30 degrees from the midsagittal line) under isoflurane anesthesia and were allowed to recover singly in home cages for 7 days. Afterwards, they were conditioned with morphine after hind-paw incisional injury as described in prior studies. Briefly, 24 hrs after hind-paw incisional injury, and using a three chamber apparatus (MED associates Inc., St. Albans VT) in an unbiased counter-balanced design, mice were given saline and placed in their designated conditioned chamber for 30 mins and 4 hours later they were given morphine (5 mg/kg sc.) and confined to the opposite chamber for 40 mins. This was repeated for three days with total of 6 conditioning sessions. On the test day, mice were placed in the middle neutral chamber and allowed free access to all chambers for 20 mins, and time spent in the chambers were recorded (Figure 1(a)). Preference scoring was determined by subtracting the difference between time spent in the morphine paired chamber from time spent in the saline paired chamber. After establishment of conditioned place preference, mice underwent two extinction trials per day with 30 min of free access to all three chambers daily until they spent equal amount of time between the saline and drug paired chambers. Prior to reinstatement testing, the dummy stylets were carefully removed from the implanted guide cannula and inner cannula under isoflurane anesthesia and norBNI (4 μg in 0.4 μL) was slowly administered thru the guide cannulas bilaterally. After 24 hr recovery, morphine primed reinstatement was conducted. Animals were injected with morphine (5 mg/kg) and were placed in the three-chamber apparatus and allowed free access to all chambers for 20 mins, in which preference scores were determined. Cannula placements were also examined after behavioral studies and animals with misplaced cannulations were excluded from the analysis (Figure 1(e)). Animals that were conditioned with incisional injury demonstrated blunted responses to morphine primed conditioned reinstatement that were reversed with intra-accumbal norBNI (Figure 1(b), Welch’s ANOVA test, Dunnett post hoc test, F(3,11) = 3.842, alpha level = 0.05; power at 95%, p < 0.05, GraphPad Prism Software version 8, USA), in agreement with our prior studies.

Oliceridine-induced conditioned place preference with injury and drug primed reinstatement- Having established the site of action of norBNI as the nucleus accumbens in this paradigm, we then turned to examine the effects of oliceridine. In a separate cohort, 24 hrs after undergoing hind-paw incisional injury and in similar fashion to the morphine behavioral studies, mice were conditioned with either vehicle or oliceridine (1.5 mg/kg or 5 mg/kg) (Abmole Bioscience Inc, Houston, TX) using a three chamber design in an unbiased, counterbalanced fashion. The 1.5 mg/kg was selected as it is equianalgesic to the conditioning dose of morphine from our previous study.
studies, and 5 mg/kg, was included to determine whether any biased ligand effects could be overcome with a higher dose. After establishment of conditioned place preference, mice underwent daily extinction trials. Systemic norBNI (10 mg/kg, sc.) was administered prior to reinstatement testing, a dose and route of administration which attenuated morphine primed reinstatement. After incisional injury, low dose oliceridine/TRV130 becomes rewarding (t = 2.355, df = 22.53, p < 0.05; N = 12–13/group), but incisional injury causes no attenuation of oliceridine/TRV130 primed reinstatement, and is unaffected by norBNI. After conditioning and extinction, either morphine (t(8) = 2.99; p = 0.017; N = 9–12/group) or oliceridine (t(11) = 2.27; p < 0.05 N = 11–12/group) causes cross reinstatement of place preference. (g) and (h) Pro-dynorphin mRNA expression using quantitative PCR-Pro-dynorphin mRNA in the nucleus accumbens (g) and prefrontal cortex (h) increases after conditioning with morphine and incisional injury (F(5,36) = 9.61, p < 0.0001), but not in the presence of oliceridine/TRV130 with/out injury, (F(5,36) = 18.29, p < 0.0001; N = 6–7/group).

**Figure 1.** (a) Timeline of behavioral and molecular studies; (b) and (e) Morphine conditioning, extinction and drug-primed reinstatement testing with intra-accumbal administration of norBNI- After incisional injury, morphine primed reinstatement is attenuated (F(3,11) = 3.842, p < 0.05; N = 6–7 per group) and with kappa opioid receptor blockade with norBNI in the nucleus accumbens these changes are reversed; (c) and (d) Oliceridine conditioned place preference and drug-primed reinstatement- With incisional injury, low dose oliceridine/TRV130 becomes rewarding (t = 2.355, df = 22.53, p < 0.05; N = 12–13/group), but incisional injury causes no attenuation of oliceridine/TRV130 primed reinstatement, and is unaffected by norBNI; F. Morphine and oliceridine conditioning and cross-reinstatement- After conditioning and extinction, either morphine (t(8) = 2.99; p = 0.017; N = 9–12/group) or oliceridine (t(11) = 2.27; p < 0.05 N = 11–12/group) causes cross reinstatement of place preference. (g) and (h) Pro-dynorphin mRNA expression using quantitative PCR-Pro-dynorphin mRNA in the nucleus accumbens (g) and prefrontal cortex (h) increases after conditioning with morphine and incisional injury (F(5,36) = 9.61, p < 0.0001), but not in the presence of oliceridine/TRV130 with/out injury, (F(5,36) = 18.29, p < 0.0001; N = 6–7/group).
receptor agonist, oliceridine at 5 mg/kg. On the test day, they were euthanized and the nucleus accumbens and medial prefrontal cortex, a brain region with significant functional connections to the nucleus accumbens, were rapidly dissected for RNA extraction as previously described with similar reagents, primers and systems\textsuperscript{17,18} (Figure 1(a)). Briefly, the total RNA was extracted using the GeneAll Hybrid-R kit (GeneAll Biotechnology, Seoul, South Korea) and reversed transcribed into cDNA using a RT\textsuperscript{2} first strand cDNA Synthesis Kit (Qiagen, Valencia CA). Real time quantitative PCR was performed using RT\textsuperscript{2} qPCR Primer and RT\textsuperscript{2} SYBR Green ROS mastermix assays and the ABI 7900HT sequencing detection system. Relative fold of gene expression of samples was determined by the Delta delta comparative cycle threshold (CT) method with internal controls, and treatment groups were normalized to the control treatment group. From this study, conditioning with morphine in the presence of injury resulted in an increase in prodynorphin expression in the nucleus accumbens (Figure 1(g), one way ANOVA, Dunnett post hoc test $F(5,36) = 9.61; \alpha = 0.05$, power 95%, $p > 0.0001$, GraphPad Prism Software version 8, USA and medial prefrontal cortex (Figure 1(h), one way ANOVA, Dunnett post hoc test $F(5,36) = 18.29, p < 0.0001$, changes that were absent with conditioning with oliceridine and/or incisional injury (Figure 1(g) and (h)) with either brain regions.

This study revealed neural mechanisms underlying prodynorphin expression induced by minor injury and its significance with morphine reward and relapse. Conditioning with combined morphine and incisional injury demonstrated blunted morphine-primed conditioned reinstatement that was reversed with intra-accumbal norBNI refining our previous results showing the same effect after systemic norBNI administration. This corresponded to elevated prodynorphin expression in the nucleus accumbens as well as medial prefrontal cortex. These changes were largely absent during conditioning with oliceridine irrespective of incisional injury, without attenuation of drug-primed reinstatement and unaffected by systemic administration of norBNI. In situ hybridization studies well describe colocalization between preprodynorphin mRNA and prodynorphin peptide levels in the nucleus accumbens and medial prefrontal cortex.\textsuperscript{19} Further, with norBNI administration, we are able to establish functional consequence of elevations in prodynorphin expression in these regions. Our findings also suggest protection from opioid relapse induced by minor injury is lost with use of oliceridine and presents a potential disadvantage of designing novel mu-biased agonists towards reduced recruitment of beta arrestins that is worth closer inspection.

There may exist mechanisms outside of beta arrestin signaling that explain the current observations. The different efficacies of morphine and oliceridine in the behavioral assays tested may be considered, and possible effects aside from mu opioid receptor activation with oliceridine may also be considered, with loss of receptor selectivity with higher doses of oliceridine used. In addition, while morphine is a full agonist, oliceridine may act as a partial agonist\textsuperscript{12} in our behavioral assays. Differences in half-lives of the drugs with oliceridine’s shorter than morphine, and lacking an active metabolite\textsuperscript{20} could also possibly produce these behavioral and molecular observations. In a recent study by Kliewer and colleagues, knock-in mice with a series of serine and threonine-to-alanine mutations at the carboxyl terminus resulting in de-recruitment of beta-arrestins demonstrated enhanced opioid-induced analgesia and diminished analgesic tolerance. However, respiratory depression, constipation, and reward with conditioned place preference remained unaltered.\textsuperscript{21} Interestingly, respiratory depression and opioid withdrawal were accentuated in the mutant strains, which the authors suggest that opioid mediated adverse effects may not be entirely mediated by beta arrestin recruitment alone.

This study presents with some caveats. For example, diffusion of norBNI to other brain regions such as the caudate putamen cannot be ruled out. However, the volume of drug used was previously tested to have reliable confinement in the nucleus accumbens. Additional brain regions are involved in the development of conditioned place preference such as the ventral tegmental area (VTA), amygdala and caudate putamen, which may be of interest in future studies. We used morphine and oliceridine as representative of their drugs classes, but the results may not be fully generalizable to conventional and current biased opioid drugs. Also, we acknowledge that a direct comparison of the effects of morphine and oliceridine in conditioned place preference paradigms can be complex. For instance, the development and expression of conditioned place preference generally is more influenced by number of repeated drug exposure than drug dosage, and does not follow a clear dose-dependent relationship.\textsuperscript{22}

Collectively, these findings add to our understanding of opioid exposure during surgery as a pivotal period affecting future propensity for future opioid abuse. A role of kappa opioid receptor activation in the nucleus accumbens during surgery and opioid exposure may confer protection from future opioid abuse and relapse. In addition, we have further characterized novel properties of the G-protein biased agonist oliceridine in that it does not seem to confer the benefit of protection from drug-primed reinstatement as observed with morphine. These findings might support future investigations for identifying risk factors for opioid abuse and possible novel pharmacological therapeutic approaches for prevention of future opioid abuse after surgery.
Short report bottom line (s): Kappa opioid receptor activation in the nucleus accumbens by incisional injury and non-biased opioids is protective from drug-induced reinstatement of preference.

Author Contributions
- CN contributed to study design and concept, data interpretation, experiments, data analysis, drafting, editing of manuscript.
- XS contributed to conducting key experiments and neurochemical analysis.
- PS contributed to conducting key behavioral experiments, data analysis and editing of manuscript.
- JDC contributed as a supervisory role in study conception, design, data interpretation, experiments, data analysis, drafting, editing, and funding support.
- All authors wrote, reviewed, and approved the manuscript.

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ORCID iD
Chinwe Nwaneshiudu https://orcid.org/0000-0001-8792-0886

References
1. Carroll I, Barelka P, Wang CKM, Wang BM, Gillespie MJ, McCue R, Younger JW, Trafton J, Humphreys K, Goodman SB, Dirbas F, Whyte RI, Donington JS, Cannon WB, Mackey SC. A pilot cohort study of the determinants of longitudinal opioid use after surgery. Anesth Analg 2012; 115: 694–702.
2. Cicero TJ, Lynskey M, Todorov A, Iinciardi JA, Surratt HL. Co-morbid pain and psychopathology in males and females admitted to treatment for opioid analgesic abuse. Pain 2008; 139: 127–135.
3. Passik SD, Hays L, Eisner N, Kirsh KL. Psychiatric and pain characteristics of prescription drug abusers entering drug rehabilitation. J Pain Palliat Care Pharmacother 2006; 20: 5–13.
4. Hah JM, Sharifzadeh Y, Wang BM, Gillespie MJ, Goodman SB, Mackey SC, Carroll IR. Factors associated with opioid use in a cohort of patients presenting for surgery. Pain Res Treat 2015; 2015: 829696–8202016.
5. Menendez ME, Ring D, Bateman BT. Preoperative opioid misuse is associated with increased morbidity and mortality after elective orthopaedic surgery. Clin Orthop Relat Res 2015; 473: 2402–2412.
6. Rankovic Z, Brust TF, Bohn LM. Biased agonism: an emerging paradigm in GPCR drug discovery. Bioorg Med Chem Lett 2016; 26: 241–250.
7. Kenakin T. Functional selectivity and biased receptor signaling. J Pharmacol Exp Ther 2011; 336: 296–302.
8. Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, Novick S. A simple method for quantifying functional selectivity and agonist bias. ACS Chem Neurosci 2012; 3: 193–203.
9. Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB. Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther 2007; 320: 1–13.
10. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 1999; 286: 2495–2498.
11. Chen X-T, Pitis P, Liu G, Yuan C, Gotchev D, Cowan CL, Rominger DH, Koblish M, Devire SM, Crombie AL, Violin JD, Yamashita DS. Structure-activity relationships and discovery of a G protein biased mu opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl](2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro[4.5]decan-9-yl]ethyl)amine (TRV130), for the treatment of acute severe pain. J Med Chem 2013; 56: 8019–8031.
12. DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, Chen X-T, Pitis PM, Gotchev D, Yuan C, Koblish M, Lark MW, Violin JD. A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. J Pharmacol Exp Ther 2013; 344: 708–717.
13. Liang DY, Li WW, Nwaneshiudu C, Irvine KA, Clark JD. Pharmacological characteristics of oliceridine, a mu-opioid receptor G-protein-biased ligand in mice. Anesth Analg 2019; 129: 1414–1421.
14. Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue Z, Morgenweck J, Cameron MD, Bannister TD, Bohn LM. Bias factor and therapeutic window correlate to predict safer opioid analgesics. Cell 2017; 171: 1165–1175.e1113.
15. Shaham Y, Shalev U, Lu L, de Wit H, Stewart J. The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology (Berl) 2003; 168: 3–20.
16. Nwaneshiudu CA, Shi XY, Clark JD. Incisional injury modulates morphine reward and morphine-primed reinstatement: a role of kappa opioid receptor activation. Anesth Analg 2020; 130: 248–257.
17. Sahbtaie P, Liang D-Y, Shi X-Y, Sun Y, Clark JD. Epigenetic regulation of spinal cord gene expression
contributes to enhanced postoperative pain and analgesic tolerance subsequent to continuous opioid exposure. *Mol Pain* 2016; 12: 1744806916641950.

18. Poree LR, Guo TZ, Kingery WS, Maze M. The analgesic potency of dexmedetomidine is enhanced after nerve injury: a possible role for peripheral alpha2-adrenoceptors. *Anesth Analg* 1998; 87: 941–948.

19. Merchenthaler IN, Maderdrut JL, Cianchetta P, Shughrue P, Bronstein D. In situ hybridization histochemical localization of prodynorphin messenger RNA in the central nervous system of the rat. *J Comp Neurol* 1997; 384: 211–232.

20. Fossler MJ, Sadler BM, Farrell C, Burt DA, Pitsiu M, Skobieranda F, Soergel DG. Oliceridine (TRV130), a novel G protein-biased ligand at the mu-opioid receptor, demonstrates a predictable relationship between plasma concentrations and pain relief. I: development of a pharmacokinetic/pharmacodynamic model. *J Clin Pharmacol* 2018; 58: 750–761.

21. Kliewer A, Schmiedel F, Sianati S, Bailey A, Bateman JT, Levitt ES, Williams JT, Christie MJ, Schulz S. Phosphorylation-deficient G-protein-biased mu-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat Commun* 2019; 10: 367–301.

22. Tzschentke TM. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol* 1998; 56: 613–672.