[Original Research]

Pharmacological Analysis of Hydromorphone Acting as a β-Arrestin-Nonpreferred Strong μ-Opioid Receptor Ligand

Yoshinori TAKEMURA,[*1, *2, †] Yoshiyuki IWASE,[*1, †] Yusuke HAMADA,[*2, *3, †] Michiko NARITA,[*3, *4] Kensuke YAMASHITA,[*2] Moe NAKAHAMA,[*2] Yukari SUDA,[*2, *3] Kanako MIYANO,[*3] Mitsuaki YAMAZAKI,[*1] Naoko KUZUMAKI,[*2] Tomohisa MORI,[*2] and Minoru NARITA[*2, *3]

[*1] Department of Anesthesiology, Faculty of Medicine, University of Toyama
[*2] Department of Pharmacology, Hoshi University School of Pharmacy and Pharmaceutical Sciences
[*3] Division of Cancer Pathophysiology, National Cancer Center Research Institute
[*4] Department of Molecular and Cellular Medicine, Institute of Medical Science

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Abstract: It has been considered that dichotomous signal transduction pathways (G protein-coupled signaling pathway and β-arrestin recruitment pathway) can explain distinct pharmacological effects after the activation of μ-opioid receptors. Although μ-opioids have different pharmacological profiles including a prominent analgesic action, the characteristics of each opioid-oriented effect have not yet been fully described. In this study, we focused on the μ-opioid receptor agonist hydromorphone and further analyzed its pharmacological profile. By using an *in vitro* assay, we confirmed that fentanyl could predominantly activate the β-arrestin-dependent pathway, whereas hydromorphone as well as morphine and oxycodone could act as a β-arrestin-nonpreferred μ-opioid receptor ligand. In an *in vivo* analysis using mice, hydromorphone produced potent antinociceptive and locomotor-enhancing effects, and inhibited gastrointestinal transit through a pattern different from that seen with morphine and oxycodone. Under these conditions, we found for the first time that the discriminative stimulus effect of hydromorphone was distinct from that of a β-arrestin-preferred μ-opioid receptor agonist, fentanyl. These findings indicate that hydromorphone may produce a discriminative stimulus effect, which could be distinguished from that after activation of a β-arrestin-dependent signaling pathway by μ-opioids. Our present findings provide useful insight to further understand the distinct pharmacological profiles of hydromorphone in comparison to those of other standard μ-opioids.

Key words: μ-opioid, hydromorphone, G protein-coupled signaling pathway, β-arrestin

Abbreviation: cAMP, adenosine 3′, 5′-cyclic monophosphate

INTRODUCTION

μ-Opioid receptor agonists have been considered to be essential tools for combatting pain. Despite their strong analgesic effects, the side effects of μ-opioids can be a burden in clinical situations. Furthermore, loss of the analgesic potency of μ-opioids after long-term use accompanied by an increase in the required dose may lead to further side effects. The changing of prescribed opioids, which is called "opioid switching (rotation)," for the control of pain has been used to achieve an analgesic effect or reduce side effects in palliative care. To explain why opioid switching is effective for reducing side effects including tolerance induced by opioids, even though prescribed opioids target the same μ-opioid receptors, it is important to realize that opioids have distinct pharmacological profiles due to differences in their plasma concentrations,[1] permeabilities for the blood-brain barrier,[2, 3] and receptor subtypes.[4] In addition, the concept of a partial agonist to regulate a receptor may also explain the different pharmacological profiles of several opioids.[5]

Recent findings have led to a new perspective that dichotomous signal transduction pathways after the activation of G protein-coupled receptors could trigger differential pharmacological signaling (e.g., activation of a G protein-coupled signaling pathway or a β-arrestin recruitment pathway).[6] Based on the G protein-biased theory, it has been proposed that an analgesic that

Co-corresponding authors: Yoshinori Takemura, 2630 Sugitani, Toyama-shi, Toyama 930–0194, Japan, Tomohisa Mori and Minoru Narita, 2–4–41, Ebara, Shinagawa-ku, Tokyo 142–8501, Japan
[†] These authors contributed equally to this work.
E-mail address: tkmrysnr@med.u-toyama.ac.jp (Y.T.), t-mori@hoshi.ac.jp (T.M.), and narita@hoshi.ac.jp (M.N.)

selectively stimulates the G protein-dependent signaling pathway lacks many of the adverse side effects induced by μ-opioids.[7]

Morphine is an alkaloid from the opium poppy, whereas oxycodone, like hydromorphone, is a semi-synthetic opioid derivative of morphine. Fentanyl is a synthetic opioid in the phenylpiperidine family, which has a less chemically rigid structure than morphine derivatives. Based on the similarities in the chemical structures of morphine and oxycodone, they may share several pharmacological effects. However, the efficacies of oxycodone in several pain models using rodents do not overlap those of morphine and fentanyl.[8] In addition, these μ-opioids induce the inhibition of gastrointestinal transit and colonic expulsion through different mechanisms (through different binding sites or regional differences at supraspinal and spinal versus peripheral opioid receptors).[9] Here, there is another possibility that each μ-opioid may have differential efficacies at exerting antinociceptive as well as side effects through the balanced stimulation of dichotomous pathways, which in turn produces distinct pharmacological profiles.

Compared with several other prescribed μ-opioids, little information is available regarding the pharmacological effects of hydromorphone despite its long history in clinical practice. A recent study showed that hydromorphone may produce the differential activation of biased pathways after the activation of μ-opioid receptors compared with morphine or oxycodone, and fentanyl.[10] The main purpose of this study was to demonstrate the ability of hydromorphone to produce antinociceptive as well as side effects based on the differential activation of dichotomous pathways such as G protein-biased and β-arrestin-biased pathways in comparison with other prescribed μ-opioids.

MATERIALS AND METHODS

1. Animals

Male Institute of Cancer Research (ICR) mice (20–25 g) (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) and C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA), were used. Food and water were available *ad libitum* in their home cages except in the drug discrimination study. In the drug discrimination study, C57BL/6J mice were maintained at 24 g by food deprivation, whereas water was available *ad libitum* in their isolated cages. All animals were housed in a room maintained at 24 ± 1℃ with a 12-h light-dark cycle (light on 8:00 AM to 8:00 PM). The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Hoshi University, as adopted by the Committee on Animal Research of Hoshi University.

2. Examination of intracellular cAMP levels by μ-opioid receptor stimulation

We used the GloSensor™ cAMP Assay (Promega Co., WI, USA) to measure the inhibition of intracellular cAMP by μ-opioid receptor agonists according to the manufacturer's instructions with a minor modification. We used HEK293 cells stably expressing both Halo-tag μ-opioid receptor and GloSensor-22F cAMP. After the application of luciferin followed by treatment with each μ-opioid receptor agonist, intracellular cAMP levels were analyzed by measuring the intensity of luminescence.

3. Association of β-arrestin with μ-opioid receptors

To analyze β-arrestin-2 recruitment to μ-opioid receptors, a Path Hunter® express OPRM1 CHO-K1 β-arrestin GPCR Assay (Discover Co., CA, USA), where μ-opioid receptors are tagged with a small fragment of β-galactosidase in the C-terminals, was used as described previously.[11] Briefly, a quantitative analysis of β-arrestin recruitment for opioid receptors was performed by measuring the β-galactosidase activity through chemical luminescence caused by the recruitment of β-arrestin to the C-terminal side of the μ-opioid receptor after the application of each μ-opioid receptor agonist.

4. Hot plate test

After treatment with μ-opioids, mice were placed on a hot plate maintained at 55 ± 0.5℃, and the latency until pain-related behavior due to heat stimulation was measured as described previously.[11] Briefly, a cut-off time of 30 s was set to prevent tissue damage. In this study, mice were treated with morphine (2–24 mg/kg), oxycodone (1–10 mg/kg) or hydromorphone (0.1–3 mg/kg). The antinociceptive effect induced by each opioid was calculated as the % antinociception according to the following formula:

$$\%\ \text{of antinociception} = (\text{test latency} - \text{pre-drug latency}) / (\text{cut-off time} - \text{pre-drug latency}) \times 100$$

5. Inhibition of gastrointestinal transit

Gastrointestinal transit was determined according to a previous method.[9] Mice were fasted 24 h before the experiment. Briefly, morphine (0.6–24 mg/kg), oxycodone (0.3–10 mg/kg) or hydromorphone (0.1–3 mg/kg) was administered s.c. 30 min before the oral administration of blue ink (0.3 mL/mouse; Pilot Co., Ltd., Tokyo, Japan). The percentage inhibition of gastrointestinal transit was calculated as follows:

$$\%\ \text{inhibition of gastrointestinal transit} = (\text{distance traveled by the ink/distance from the pylorus to the cecum of the small intestine}) \times 100$$

6. Locomotor assay

The locomotor activity of mice was measured by a Three-points Meter (O'Hara Co., Ltd., Tokyo, Japan) as described previously.[13] To measure locomotor activity, a mouse was placed in a cage (20.5 cm × 40.5 cm × 18.5 cm) on the device. In this system, as the mouse moved horizontally (cm), it interrupted infrared beams. After a 60-min habituation period, locomotor activity was automatically measured for 180 min after the administration of hydromorphone or morphine.

7. Drug discrimination studies

As described previously,[11] experiments were conducted in operant-conditioning chambers (model ENV-307; Med Associates, Inc., VT, USA) equipped with two nose-poke holes and a food (20-mg food pellet (Bio-Serv, NJ, USA)) cup mounted midway between the holes. Briefly, in mice that had been trained to discriminate between hydromorphone (0.48 mg/kg) and saline, dose-response and substitution tests were conducted after the discrimination criterion (accuracy of at least 83% and fewer than 12 responses to obtain the first reinforcement) had been satisfied for at least 3 consecutive daily discrimination-training sessions. In the dose-response and substitution tests, mice were placed in the operant box until they had made 10 responses on either hole or 5 min had elapsed after drug administration. Performance was calculated in terms of the number of drug-appropriate nose-pokes during the first 10 responses. Drugs were considered to have substituted for the discriminative stimulus effects of hydromorphone if more than 80% of the responses were on the hydromorphone-appropriate holes. The response rate was calculated as the total number of responses before the completion of 10 responses on either hole divided by the time (minutes) taken to complete the first ratio. If the mouse did not make 10 responses within 5 min, the response was judged to have been disrupted.

8. Drugs

The drugs used in the present study were hydromorphone hydrochloride (Mallinckrodt Inc., MO, USA), morphine hydrochloride (Daiichi-Sankyo Co., Ltd., Tokyo, Japan), oxycodone hydrochloride (Shionogi Pharmaceutical Co., Inc., Osaka, Japan), and fentanyl citrate (Hisamitsu Pharmaceutical Co., Inc., Tokyo, Japan). All drugs were dissolved in saline and administered s.c. in a volume of 10 mL/kg.

9. Statistical analysis

Data are expressed as the mean ± S.E.M. The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons test. All statistical analyses were performed using Prism software (Version 8.0; GraphPad Software, CA, USA). A p value of 0.05 was considered to reflect significance.

RESULTS

1. Effects of μ-opioids on G protein-biased and β-arrestin-biased signal transduction in cells that stably expressed μ-opioid receptor

We examined the effects of several μ-opioid receptor agonists on forskolin-induced cAMP accumulation in HEK293 cells stably expressing both Halo-tag μ-opioid receptor and GloSensor-22F cAMP. cAMP accumulation induced by forskolin was dose-dependently suppressed by all of the μ-opioid receptor agonists used in this study (Fig. 1a). The relative potencies to suppress cAMP accumulation produced by μ-opioid receptor agonists were fentanyl = hydromorphone > morphine > oxycodone.

We next investigated the effects of μ-opioids on an increase in luminescence that reflected the recruitment of β-arrestin-2 in CHO-K1 cells stably expressing μ-opioid receptors. Fentanyl induced a potent recruitment of β-arrestin-2, whereas morphine (10^{-10}–10^{-5}), hydromorphone (10^{-10}–10^{-5}) and oxycodone (10^{-10}–10^{-5}) each produced a low level of β-arrestin-2 recruitment compared to that with fentanyl (Fig. 1b).

2. Pharmacological effects of hydromorphone in mice

The administration of hydromorphone produced a dose-dependent antinociceptive effect as well as the inhibition of gastrointestinal transit in mice. The dose-response curves of hydromorphone for antinociception and inhibition of gastrointestinal transit are shown in Fig. 2. Morphine (Fig. 2a) and oxycodone (Fig. 2b) inhibited gastrointestinal transit at lower doses than their respective antinociceptive doses, whereas hydromorphone inhibited gastrointestinal transit at almost the

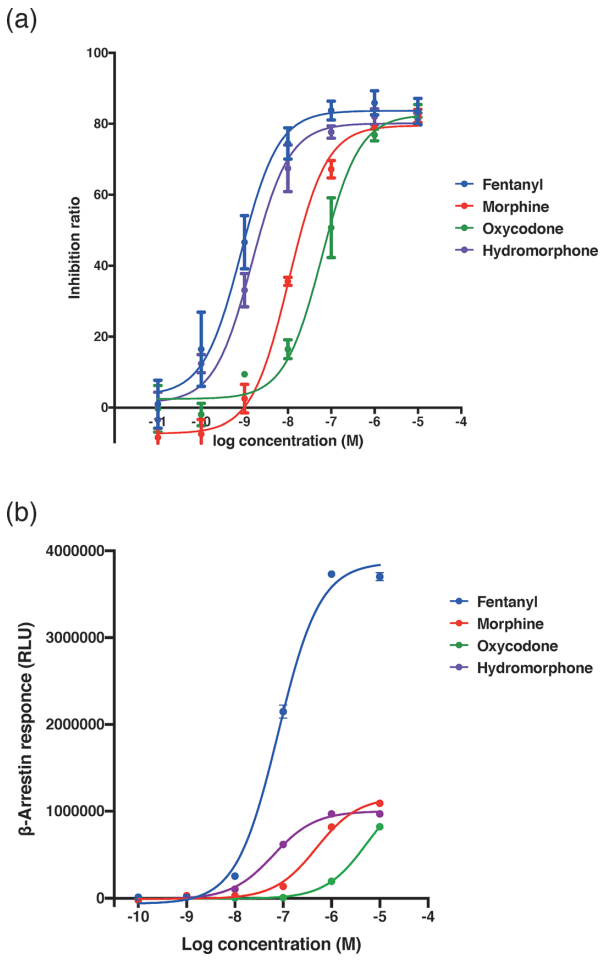

Fig. 1 Influence of μ-opioids on intracellular signaling via the activation of μ-opioid receptor. (a) Effects of morphine, oxycodone, fentanyl and hydromorphone on the inhibition ratio of intracellular cAMP levels via the activation of μ-opioid receptors. (b) Effects of morphine, oxycodone, fentanyl and hydromorphone on β-arrestin recruitment to μ-opioid receptors. RLU, relative luminescence unit. Each point represents the mean ± S.E.M.

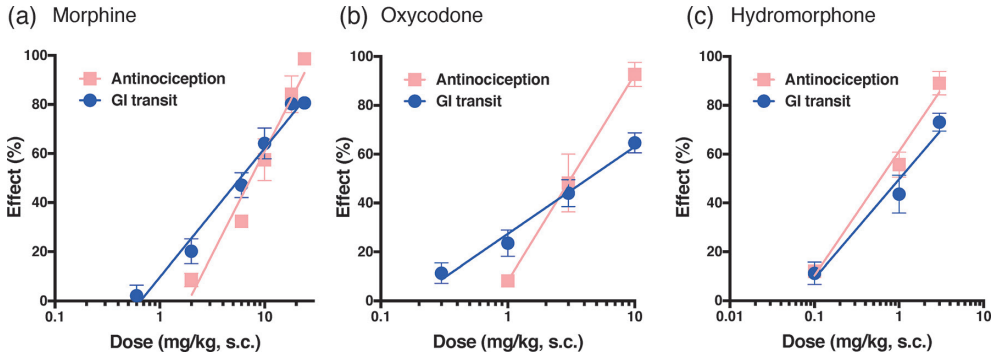

Fig. 2 Evaluation of the pharmacological effects of hydromorphone *in vivo*. (a–c) Dose-response effects of morphine (a), oxycodone (b) and hydromorphone (c) on analgesia and inhibition of gastrointestinal transit. Each data point represents the mean ± S.E.M. (n = 6–12 animals).

same dose as its lowest antinociceptive dose (Fig. 2c).

The administration of hydromorphone at 0.3–3 mg/kg produced a dose-dependent increase in locomotor activity in mice (Fig. 3), and the administration of morphine (3–30 mg/kg) induced hyperlocomotion at almost the same level as that induced by hydromorphone (0.3–3 mg/kg), which produced antinociception at almost the same level as that with morphine. With regard to hyperlocomotion, increased horizontal movement, but not vertical movement, accompanied by Straub's tail reaction, was observed with hydromorphone as well as morphine. Particularly, these behaviors induced by hydromorphone were indistinguishable from those induced by morphine (unpublished observation).

In the drug discrimination study, an initial training dose of 0.6 mg/kg was set to train the mouse to discriminate between hydromorphone and saline. However, none of the mice could nose-poke in the discrimination training phase. Therefore, mice were again trained to discriminate between hydromorphone (0.48 mg/kg) and saline. In mice that had been trained to discriminate between hydromorphone and saline, hydromorphone dose-dependently produced hydromorphone-appropriate responding, and more than 80% hydromorphone-appropriate responding was observed at 0.48 mg/kg (Fig. 4a). In the substitution test, morphine dose-dependently produced hydromorphone-appropriate responding, and mostly substituted for the discriminative stimulus effect at 5.6 mg/kg (Fig. 4b). On the other hand, fentanyl failed to produce hydromorphone-appropriate responding (less than 50%) at 10–56 μg/kg (Fig. 4c). Additionally, fentanyl at 100 μg/kg completely induced behavioral disruption (data not shown).

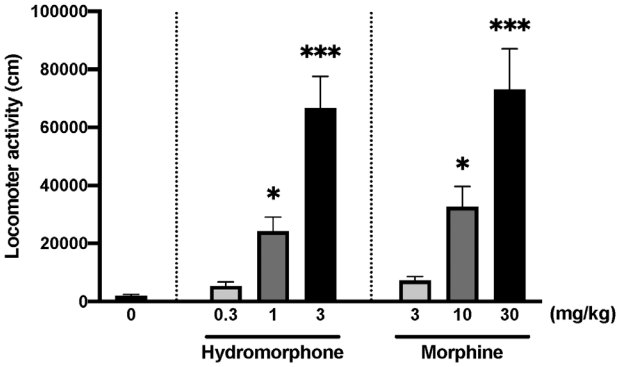

Fig. 3 Effects of hydromorphone and morphine on the locomotor activity. Changes in locomotor activity with the administration of hydromorphone and morphine. Each data point represents the mean ± S.E.M. (n = 6 animals). $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* saline group.

DISCUSSION

It is important to apply the knowledge obtained from the results of basic research to clinical research to improve health outcomes. It has been documented that ligands which selectively activate a G protein-biased pathway may have an ideal profile as an analgesic without having adverse side effects.[14,15] Consistent with a previous report that hydromorphone, unlike fentanyl, failed to produce the internalization of μ-opioid receptors,[10] we demonstrated here that hydromorphone may predominantly activate β-arrestin-independent signaling after the stimulation of μ-opioid receptors to produce potent antinociception. Interestingly, the inhibition of gastrointestinal transit was initiated by hydromorphone at almost the same dose as the lowest antinociceptive dose, whereas morphine and oxycodone inhibited gastrointestinal transit at doses lower than their antinociceptive doses. Taken together with the fact that hydromorphone has profound antinociceptive effects, while acting as a β-arrestin-nonpreferred strong μ-opioid receptor ligand, the present study provides further evidence that hydromorphone may be a useful and effective μ-opioid for opioid switching under unpleasant conditions with other μ-opioids.

An increase in the number and/or amount of prescriptions for μ-opioids including neutral μ-agonists, such as morphine and oxycodone, and the β-arrestin-preferred μ-agonist fentanyl, may have contributed to the epidemic of opioid misuse and overdose-induced deaths in the U.S. and other nations.[16–18] In the field of behavioral pharmacology, while the evaluation of a drug's effects on locomo-

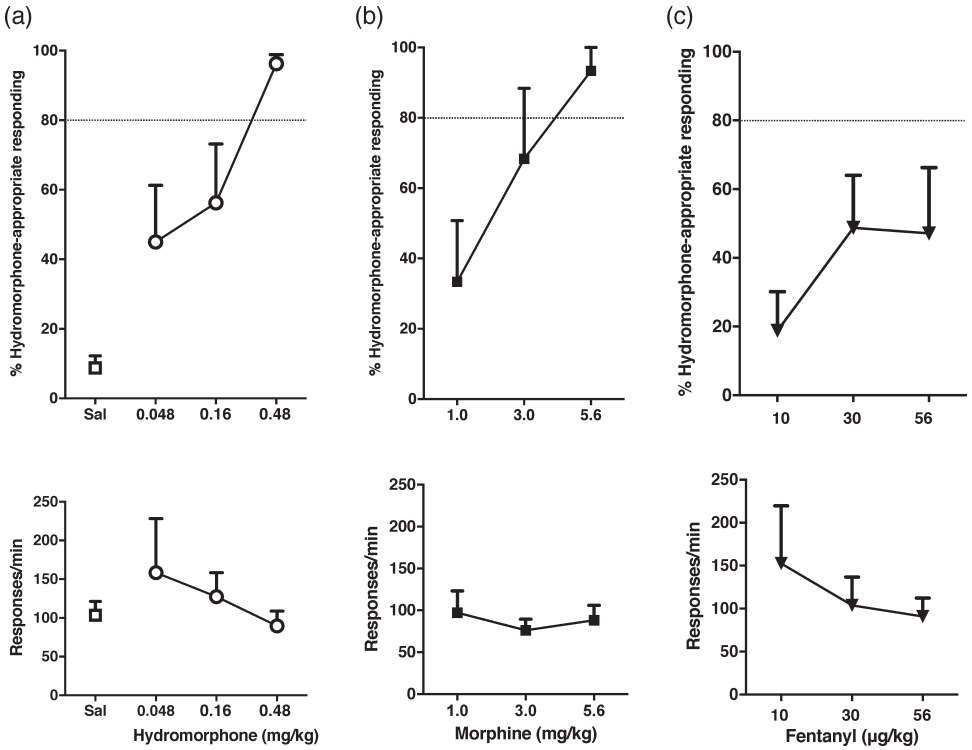

Fig. 4 Discriminative stimulus effects of hydromorphone. (a) Confirmation of the appropriate response to hydromorphone in mice trained to discriminate between hydromorphone and vehicle. (b, c) Substitution tests of morphine (b) and fentanyl (c) for the discriminative stimulus effects of hydromorphone in those mice. The data show the discriminative stimulus effects of hydromorphone (top panel) and the response rates (bottom panel). Each point represents the mean ± S. E.M. (n = 6–8 animals). Dotted line indicates the criteria for the substitution for the discriminative stimulus effects of hydromorphone.

tor activity is considered to be simplistic, it is beneficial for highlighting a drug's abuse potential.[19] It has been demonstrated that morphine, oxycodone, and fentanyl (and its related compounds) produce a robust increase in locomotor activity in mice in addition to rewarding effects,[20, 21] which could be induced by activation of the central dopaminergic pathway. On the other hand, the potent G protein-biased ligand PZM-21 induces neither hyperlocomotion nor rewarding effects in rodents.[7, 22] These results inspired the idea that activation of a β-arrestin-dependent pathway after stimulation of μ-opioid receptors could be dominantly accompanied by the abuse potential of μ-opioids. However, in the present study, we found that a β-arrestin-nonpreferred strong μ-opioid receptor agonist hydromorphone at antinociceptive doses produced hyperlocomotion, similar to morphine. Zamarripa et al. recently reported that the selective G protein-biased μ-opioid oliceridine produced self-administration under a progressive-ratio schedules of reinforcement in rats.[12] Therefore, we propose that activation of a G protein-dependent signaling pathway may not always produce antinociception without unpleasant effects related to central dopaminergic stimulation.

Subjective effects induced by abused drugs in humans may be closely related to their abuse potentials, and such subjective effects in humans are believed to reflect the discriminative stimulus effects in animals using the drug discrimination procedure. Hydromorphone shares the subjective effects of morphine and heroin in humans,[23, 24] which are closely related to its abuse. In fact, hydromorphone, like oxycodone, could be one of the most abused opioids.[25, 26] We as well as others have demonstrated that morphine and fentanyl cross-substitute for each other in rats.[11, 27, 28] However, the substitution patterns of morphine for the discriminative stimulus effects of methadone, which potently induced the recruitment of β-arrestin in cells transfected with μ-opioid receptors, were different than those of fentanyl in rats,[11] indicating that there is a subtle difference between the discriminative stimulus effects of morphine and fentanyl. In the present study, we found that morphine substituted for the discriminative stimulus effect of hydromorphone in mice trained to discriminate between 0.48 mg/kg of hydromorphone and saline, whereas fentanyl failed to substitute for the discriminative stimulus effect of hydromorphone. These findings indicate for the first time that the discriminative stimulus effect induced by hydromorphone through the activation of μ-opioid receptors under the present conditions may be distinct

from that induced by fentanyl through dominant activation of a β-arrestin-dependent pathway.

In conclusion, we have demonstrated that hydromorphone may predominantly stimulate a β-arrestin-independent pathway to produce profound antinociception. The inhibition of gastrointestinal transit was initiated by hydromorphone at almost the same dose as the lowest antinociceptive dose, which was different from the pattern seen with morphine and oxycodone, suggesting that hydromorphone may be useful and effective for opioid switching, while acting as a β-arrestin-nonpreferred strong μ-opioid receptor ligand. Furthermore, hydromorphone significantly increased locomotion, and produced a discriminative stimulus effect, which substituted for morphine, but not for fentanyl. Although further behavioral studies are still required, these results support the idea that activation of μ-opioid receptors through a β-arrestin-independent pathway may produce certain discriminative stimulus effects, which could be distinguished from those after activation of μ-opioid receptors through a β-arrestin-dependent signaling pathway. Our present findings provide critical information that could contribute to a better understanding of the pharmacological profiles of hydromorphone.

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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