ACS Chemical Neuroscience

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The δ Opioid Receptor Agonist SNC80 Selectively Activates Heteromeric μ–δ Opioid Receptors

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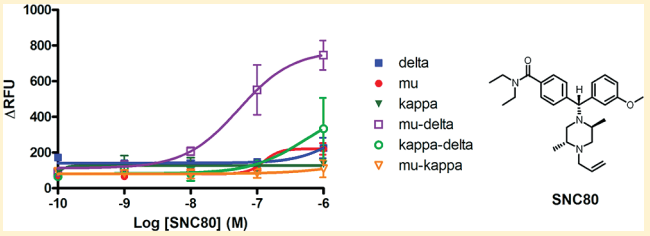
Supporting Information

ABSTRACT: Coexpressed and colocalized μ- and δ-opioid receptors have been established to exist as heteromers in cultured cells and *in vivo*. However the biological significance of opioid receptor heteromer activation is less clear. To explore this significance, the efficacy of selective activation of opioid receptors by SNC80 was assessed *in vitro* in cells singly and coexpressing opioid receptors using a chimeric G-protein-mediated calcium fluorescence assay, SNC80 produced a substantially more robust response in cells expressing μ–δ heteromers than in all other cell lines. Intrathecal SNC80 administration in μ- and δ-opioid receptor knockout mice produced diminished antinociceptive activity compared with wild type. The combined *in vivo* and *in vitro* results suggest that SNC80 selectively activates μ–δ heteromers to produce maximal antinociception. These data contrast with the current view that SNC80 selectively activates δ-opioid receptor homomers to produce antinociception. Thus, the data suggest that heteromeric μ–δ receptors should be considered as a target when SNC80 is employed as a pharmacological tool *in vivo*.



KEYWORDS: *μ opioid receptor, δ opioid receptor, μ–δ heteromer, SNC80, knockout, antinociception*

Compound SNC80 (Figure 1) has been employed as the prototypic nonpeptide, selective δ-opioid receptor agonist

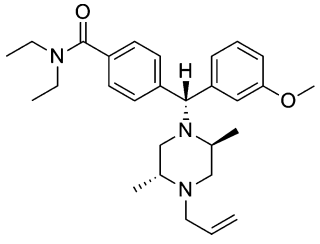


Figure 1. Structure of SNC80.

for nearly 20 years. The experimental techniques employed to define the δ-selectivity of SNC80 included radioligand binding, smooth muscle assays, and *in vivo* antagonism studies employing selective δ-antagonists.[1−4] SNC80 has found broad recognition and adoption as a selective δ-agonist standard, occupying the previously vacant nonpeptide selective δ-agonist niche in the opioid pharmacological toolbox.[3]

However, in view of the discovery of opioid receptor heteromers as targets for ligands over the past decade, it is possible that opioid receptor heteromers may be activated by SNC80. In this regard, three heteromers, μ–δ,[5] μ–κ,[6] and κ–δ,[7] have been established in heterologous cell lines, [8] and selective ligands that target these heteromers have been identified for each of the above heteromers.[9−11] Moreover, heteromeric μ–δ receptors have been detected using selective antibodies in both cultured cells and DRG neurons.[12] Thus, δ-receptors organized as heteromers could be relevant in the action of SNC80.

In this regard, SNC80-treated μ-opioid receptor knockout (μ-KO) mice have been found to be without effect in a visceral antiwrithing test.[13] Also, SNC80 produced an antihyperalgesic effect in WT mice but lacked this effect in μ-KO mice,[14] an effect similar to a separate study in δ-KO mice.[15] Additionally, *in vitro* studies on the effects of SNC80 on opioid receptors suggested the involvement of μ-opioid receptors on the activity of SNC80. These included the finding that colocalized μ- and δ-opioid receptors in large and small dorsal root ganglion (DRG) neurons [16] are cointernalized into the same subcellular compartment for lysosomal degradation upon treatment with

Received: March 30, 2012
Accepted: May 22, 2012
Published: May 22, 2012

SNC80.[17] These reports point to the involvement of both μ- and δ-opioid receptors in the activity of SNC80.

In the present study, the opioid activity of SNC80 at $\mu-\delta$ heteromers was investigated using both HEK-293 cells and knockout (μ-KO and δ-KO) studies in mice. The finding that both the *in vitro* and *in vivo* results indicate that SNC80 produces maximal efficacy only when both μ- and δ-opioid receptors are present suggests that selective activation of $\mu-\delta$ heteromers may be involved *in vivo*.

The antinociceptive activity of SNC80 was determined in mice to investigate the identity of its receptor target *in vivo*. In this regard, we hypothesized that SNC80 would have reduced activity in knockout animals lacking one of the receptors due to inability to form the $\mu-\delta$ heteromer. Wild-type, μ-KO, and δ-KO mice were employed to determine the contribution of both the μ- and δ-opioid receptors on SNC80-induced antinociception. SNC80 was administered via the intrathecal route of injection (i.t.) to mice and assessed in the warm water (52.2 °C) tail withdrawal assay (Figure 2), focusing attention on the

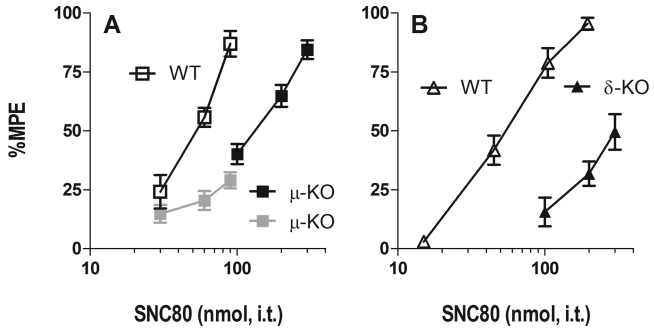


Figure 2. Antinociceptive effects of cumulative SNC80 doses in WT, μ-KO, and δ-KO mice. Dose–response curves for the cumulative spinal antinociceptive effect of SNC80 in the mouse tail flick assay. The *y*-axis represents the effect of the dose expressed in percentage of maximum possible effect (%MPE). (A) Comparison of WT (C57/129, □) and μ-KO (■) mice. (B) Comparison of WT (C57BL/6, △) and δ-KO (▲) mice. Graph A shows two cumulative dose–response curves (gray and black squares) for μ-KO mice.

spinal cord where *in vivo* data support receptor colocalization.[16] A cumulative dosing schedule was chosen partly to address the limited solubility of SNC-80, given that the three highest doses totaling 300 nmol greatly exceeded the amount of the compound that could be dissolved in a single 5 μL intrathecal injection volume. In addition, multiple comparisons with previous noncumulative dosing experiments revealed no significant differences in outcomes, validating the approach. The graph (Figure 2) shows two different dose–response curves to illustrate the lack of effect of SNC80 at low doses in μ-KO mice. In WT (C57/129) mice, SNC80 has an ED_{50} = 49 nmol, 95% CI (43–56). The dose–response curve (calculated from the second, higher dosage curve) is right-shifted in a parallel manner 2.7-fold in μ-KO mice (ED_{50} = 131 nmol, 95% CI (111–153), Figure 2A). In the WT (C57BL/6) mice, SNC80 displayed an ED_{50} = 53.6 nmol, 95% CI (47.0–61.1), which was right-shifted 6.1-fold in δ-KO mice, ED_{50} = 327 nmol, 95% CI (216–494) (~50% MPE, Figure 2B). The results indicate that the antinociceptive activity of SNC80 is reduced in either μ- or δ-opioid receptor knockout mice.

SNC80 was assayed in heterologous HEK293 cells singly or coexpressing opioid receptors, the same coexpressing cell lines where functional heteromer was established by coimmunoprecipitation.[8,18] The internal calcium release $[Ca^{2+}]_i$ assay, previously established to determine opioid receptor activation,[9,18,19] was used to determine the selective efficacy of SNC80-induced receptor activation. In this assay of opioid efficacy, the opioid receptor activation is shifted by the chimeric $\Delta 6\text{-}G_{qi4\text{-}myr}$ protein [20] to a G_q response (release of intracellular calcium), which was measured fluorescently. Importantly, opioid efficacy in the $[Ca^{2+}]_i$ assay has produced similar and consistent results when compared with opioid efficacy in the $[^{35}S]GTP\gamma S$ assay providing a convenient, nonradiological measure of whole cell efficacy.[9,19] SNC80 selectively activated $\mu-\delta$ heteromer in HEK293 cells with an EC_{50} = 52.8 ± 27.8 nM (SEM) (Figure 3), with a mean peak ΔRFU effect of 746 ±

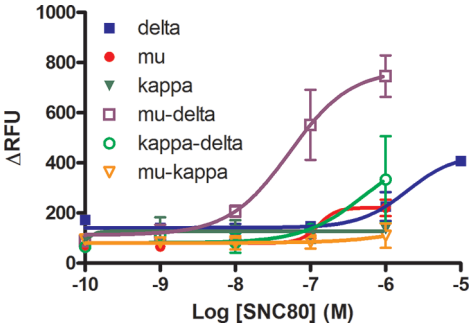


Figure 3. Activity of SNC80 on intracellular calcium ($[Ca^{2+}]_i$) release in HEK293 cells stably expressing opioid receptors with transiently expressed $\Delta 6G_{qi4\text{-}myr}$. The agonist-induced $[Ca^{2+}]_i$ response (reaction with fluorescent dye) was measured in relative fluorescence units (RFU). The *y*-axis plots change in RFU as a measure of agonist-induced $[Ca^{2+}]_i$ release (ΔRFU) ± SEM $n \geq 3$ (12) except for κ- and $\mu-\kappa$-expressing cells where $n \geq 2$ (8). The *x*-axis plots the log of the molar concentration of SNC80.

83 (SEM), n = 3 (12). Cells expressing other opioid receptors were substantially less potently activated. In this regard, SNC80 potency in cells expressing only δ-opioid receptors was at least 100-fold less than in cells that coexpressed $\mu-\delta$ heteromers.

Cells stably expressing the chimeric G_{qi4} protein and transiently expressing μ- or δ-opioid receptors or coexpressing both μ- and δ-opioid receptors were also assayed as a control for possible variability in protein-expression related artifacts. These experiments displayed similar and consistent results (Figure S1, Supporting Information) compared with cells transiently expressing the chimeric G-protein.

The *in vitro* efficacy data derived from intact HEK-293 cells in the present study support the concept that the principal targets of SNC80 are heteromeric $\mu-\delta$ receptors. As illustrated in Figure 3 and Figure S1, Supporting Information, SNC80 exhibits substantially greater activity in cells coexpressing μ- and δ-opioid receptors than in cells either singly expressing δ-opioid receptors or coexpressing δ- and κ-opioid receptors. Given the evidence for physical association of these receptors, the implication is that $\mu-\delta$ heteromers are selectively activated by SNC80, particularly since the singly expressed δ-receptor does not produce potent activation. These findings, taken together with the reported low binding affinity of SNC80 for μ-receptors,[4] suggest that it targets the δ-protomer of the $\mu-\delta$ heteromer, thereby leading to activation of the complex.

Our *in vivo* data also suggest that SNC80-induced antinociception is produced via selective activation of $\mu-\delta$ heteromer in the spinal cord.[16,17] The rightward shift in the SNC80 dose–response curve in both μ-KO and δ-KO mice

dx.doi.org/10.1021/cn3000394 | *ACS Chem. Neurosci.* 2012, 3, 505–509

demonstrates that both μ- and δ-opioid receptor protomers in the heteromeric complex contribute to the antinociceptive activity of SNC80 in wild-type mice. This result is consistent with prior studies showing that SNC80-induced antinociception possesses both δ- and μ-opioid receptor-mediated[21,22] components. The *in vivo* contribution of both receptors, as evidenced by reduced potency of SNC80 in both δ- and μ-knockout animals, is also one of three specific criteria for the establishment of the heteromeric complex established by IUPHAR guidelines.[23]

Additionally, our *in vitro* efficacy data demonstrating selective activation of μ−δ heteromers by SNC80 is further supported by trafficking and binding studies.[17,24] That these trafficking studies revealed SNC80-induced cointernalization of μ- and δ-opioid receptors is consistent with activation of a μ−δ heteromer as the signaling unit.

The present study demonstrates that potent SNC80 activity requires the presence of μ- and δ-opioid receptors both *in vitro* and *in vivo*. Considered together, and in light of prior studies, the combined results presented here suggest that SNC80, *in vivo*, interacts selectively with the δ-protomer of the μ−δ heteromer, thereby leading to the activation of the heteromeric complex. While this conclusion does not invalidate previous research demonstrating that SNC80 is a δ-selective ligand in receptor homomer models, it strongly suggests the involvement of the μ−δ heteromer as a selective target in the signaling pathway. The implications of μ−δ receptor heteromers as a target for SNC80 suggest a more complex role for this ligand *in vivo,* given that μ-selective ligands (based on binding) also target μ−δ heteromers but via the μ-protomer.[19] It therefore appears that the output of the agonist depends on which protomer in the μ−δ heteromer is targeted for activation. The fact that, unlike μ-agonists, SNC80 does not produce physical dependence [25] illustrates the complexity of the heteromeric system and suggests that different signaling pathways are dependent on the protomer that is activated in the μ−δ heteromeric complex.

METHODS

Antinociception Assays. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Drug stock solutions were dissolved in sterile water and diluted in sterile saline solution with the exception of SNC80, which was initially mixed with 1 equiv of tartaric acid before dissolution in sterile water. Antinociceptive effects of SNC-80 were assessed utilizing the warm water (52.5 °C) tail immersion assay.[26] Animals employed were 129sv/C57BL6 mice (μ-opioid receptor WT), μ-KO (−/−) mice on a 129sv/C57BL6 background,[27] C57BL/6 mice (δ-opioid receptor WT), and δ-KO (−/−) mice on a C57BL/6 background,[28] all with *ad libitum* food access and a 12 hour light/dark cycle. Intrathecal (i.t.) drug administration was accomplished by direct lumbar puncture [29] as modified, see review in ref 30. A minimum of six mice were assayed at each dose tested, each mouse was used twice, and a total of 48 mice were used in the study. No antinociceptive differences were observed between male and female mice in any of the experiments. Tail flick (TF) latencies were obtained before drug administration to establish a baseline prior to drug treatment; shortly after baseline testing, the lowest dose of drug was injected intrathecally in 5 μL of vehicle, and TF latency was determined 7 min later. Immediately after testing, the subsequent dose of a cumulative dose−response curve was administered, and TF latency was determined 5 min later; three to four doses were thereby administered in rapid succession, and a cumulative dose−response curve was determined. Antagonists were administered 7 min prior to the initial agonist injection. A 12 s cutoff was employed in cases of no detectable response to avoid tissue damage.

Data Analysis and Statistics. Results are expressed as a percentage of maximum possible effect (% MPE) calculated using the following formula using Microsoft Excel (Redmond, WA). Data plotted were concentration−effect curves and ED_{50} calculated in Prism 5.0 (San Diego, CA). Data points lacking error bars include the error within the area of the point.

$$\%\mathrm{MPE} = \frac{\text{post-drug latency} - \text{pre-drug latency} \times 100}{\text{cutoff} - \text{pre-drug latency}}$$

Intracellular Calcium Release Assay. The intracellular calcium release assay as described previously[19] was used to determine the selectivity of SNC80 in activating opioid receptors with minor modifications. Briefly, HEK293 cells stably expressing opioid receptors were grown in DMEM (10% FBS, 1% P/S) in 10% CO_2 atmosphere, transiently transfected with a chimeric G-protein Δ6-$G_{qi4\text{-}myr}$ using lipofectamine 2000 (Invitrogen, Carlsbad, CA), plated into 96 well half area plates 24 h later, and assayed for intracellular calcium release 48 h after transfection.[19] In the experiments involving HEK293 cells stably expressing the chimeric Δ6-$G_{qi4\text{-}myr}$ protein, the procedure was identical except opioid receptor DNA was transiently transfected, and the amount of lipofectamine was doubled in the cotransfected cells. Assays employed the standard explorer FLIPR calcium dye kit in a Flexstation 3 apparatus both from Molecular Devices (Sunnydale, CA). SNC80 freebase was initially dissolved in DMSO. DMSO was employed such that the final DMSO concentration did not exceed 0.1% v/v at maximum, a concentration that did not significantly alter calcium flux from basal responses. To minimize experimental variability, all experiments were conducted at least three times with four internal repetitions, $n \geq 3$ (12), except cells expressing κ- or μ−κ receptors where $n \geq 2$ (8). Assay controls (blanks and standard ligands) were performed on every plate to eliminate technical variability and ensure uniform response.

Data Analysis and Statistics. The raw data were collected in SoftMax Pro (Molecular Devices) and analyzed in GraphPad Prism 5.0, GraphPad Software (San Diego, CA). Data were represented as the change in relative fluorescence units (ΔRFU) for each concentration and plotted as sigmoidal concentration−response curves using nonlinear regression with error bars representing SEM. Points without error bars have the error contained within the plotted point.

Materials. SNC80, morphine, DAMGO, and DPDPE were obtained from the NIDA drug supply program (National Institutes on Drug Abuse).

ASSOCIATED CONTENT

Supporting Information

Supplemental figure, methods employed in the collection of the supplemental data, discussion of absence of convulsions in these assays, and associated references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Portoghese, Metcalf, Yekkirala, Powers, Wilcox, and Fairbanks participated in research design. Metcalf, Kitto, and Powers conducted experiments. Metcalf performed data analysis. Portoghese, Metcalf, and Wilcox wrote or contributed to the writing of the manuscript.

Funding
This work was supported by the National Institutes of Health, National Institutes of Drug Abuse [Grants DA01533, DA07234, and DA15438].

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Mary Lunzer and Dan Schuster for technical assistance, Dr. John Pintar for generously providing the δ-KO mice, Dr. Horace Loh for the gift of the μ-KO mice, Dr. Evi Kostenis for the gift of the Δ6-$G_{qi4\text{-}myr}$ DNA, and Dr. Jennifer Whistler for the gift of stable HEK 293 cells coexpressing opioid receptors.

■ ABBREVIATIONS

$[Ca^{2+}]_i$, intracellular calcium release; DAMGO, [D-Ala^2-*N*-Me-Phe^4-Gly^5-ol]-enkephalin; DMEM, Dubleco's modified eagle medium; DPDPE, [D-Pen^{2,5}]-enkephalin; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney cells; KO, homozygous (−/−) knockout; MPE, maximum percentage effect; P/S, penicillin/streptomycin; SNC80, 4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxypheny)methyl]-*N*,*N*-diethylbenzamide; κ−δ, kappa−delta opioid receptor heteromer; μ−δ, mu−delta opioid receptor heteromer; μ−κ, mu−kappa opioid receptor heteromer; DRG, dorsal root ganglion

■ REFERENCES

(1) Calderon, S. N., Rothman, R. B., Porreca, F., Flippen-Anderson, J. L., McNutt, R. W., Xu, H., Smith, L. E., Bilsky, E. J., Davis, P., and Rice, K. C. (1994) Probes for narcotic receptor mediated phenomena. 19. Synthesis of (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC 80): A highly selective, nonpeptide δ opioid receptor agonist. *J. Med. Chem. 37*, 2125−2128.

(2) Calderon, S. N., Rice, K. C., Rothman, R. B., Porreca, F., Flippen-Anderson, J. L., Xu, H., Becketts, K., Smith, L. E., Bilsky, E. J., Davis, P., and Horvath, R. (1997) Probes for narcotic receptor mediated phenomena. 23. Synthesis, opioid receptor binding, and bioassay of the highly selective δ agonist (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC 80) and related novel nonpeptide δ opioid receptor ligands. *J. Med. Chem. 40*, 695−704.

(3) Negus, S. S., Gatch, M. B., Mello, N. K., Zhang, X., and Rice, K. (1998) Behavioral effects of the delta-selective opioid agonist SNC80 and related compounds in rhesus monkeys. *J. Pharmacol. Exp. Ther. 286*, 362−375.

(4) Calderon, S. N., and Coop, A. (2004) SNC80 and related delta opioid agonists. *Curr. Pharm. Des. 10*, 733−742.

(5) Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, N., Nagy, V., and Devi, L. A. (2000) Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. *J. Neurosci. 20*, RC110.

(6) Wang, D., Sun, X., Bohn, L. M., and Sadee, W. (2005) Opioid receptor homo- and heterodimerization in living cells by quantitative bioluminescence resonance energy transfer. *Mol. Pharmacol. 67*, 2173−2184.

(7) Jordan, B. A., and Devi, L. A. (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature 399*, 697−700.

(8) van Rijn, R. M., Whistler, J. L., and Waldhoer, M. (2010) Opioid-receptor-heteromer-specific trafficking and pharmacology. *Curr. Opin. Pharmacol. 10*, 73−79.

(9) Yekkirala, A. S., Lunzer, M. M., McCurdy, C. R., Powers, M. D., Kalyuzhny, A. E., Roerig, S. C., and Portoghese, P. S. (2011) *N*-naphthoyl-β-naltrexamine (NNTA), a highly selective and potent activator of μ/κ-opioid heteromers. *Proc. Natl. Acad. Sci. U.S.A. 108*, 5098−5103.

(10) Daniels, D. J., Lenard, N. R., Etienne, C. L., Law, P.-Y., Roerig, S. C., and Portoghese, P. S. (2005) Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc. Natl. Acad. Sci. U.S.A. 102*, 19208−19213.

(11) Bhushan, R. G., Sharma, S. K., Xie, Z., Daniels, D. J., and Portoghese, P. S. (2004) A bivalent ligand (KDN-21) reveals spinal delta and kappa opioid receptors are organized as heterodimers that give rise to delta(1) and kappa(2) phenotypes. Selective targeting of delta-kappa heterodimers. *J. Med. Chem. 47*, 2969−2972.

(12) Gupta, A., Mulder, J., Gomes, I., Rozenfeld, R., Bushlin, I., Ong, E., Lim, M., Maillet, E., Junek, M., Cahill, C. M., Harkany, T., and Devi, L. A. (2010) Increased Abundance of Opioid Receptor Heteromers After Chronic Morphine Administration. *Sci. Signal. 3* (ra54), 51−57.

(13) Sora, I., Li, X.-F., Funada, M., Kinsey, S., and R. Uhl, G. (1999) Visceral chemical nociception in mice lacking μ-opioid receptors: effects of morphine, SNC80 and U-50,488. *Eur. J. Pharmacol. 366*, R3−R5.

(14) Gendron, L., Pintar, J. E., and Chavkin, C. (2007) Essential role of mu opioid receptor in the regulation of delta opioid receptor-mediated antihyperalgesia. *Neuroscience 150*, 807−817.

(15) Gaveriaux-Ruff, C., Karchewski, L. A., Hever, X., Matifas, A., and Kieffer, B. L. (2008) Inflammatory pain is enhanced in delta opioid receptor-knockout mice. *Eur. J. Neurosci. 27*, 2558−2567.

(16) 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., Hökfelt, T. G. M., Bao, L., and Zhang, X. (2010) Coexpression of δ- and μ-opioid receptors in nociceptive sensory neurons. *Proc. Natl. Acad. Sci. U.S.A. 107*, 13117−13122.

(17) He, S.-Q., Zhang, Z.-N., Guan, J.-S., Liu, H.-R., Zhao, B., Wang, H.-B., Li, Q., Yang, H., Luo, J., Li, Z.-Y., Wang, Q., Lu, Y.-J., Bao, L., and Zhang, X. (2011) Facilitation of μ-opioid receptor activity by preventing δ-opioid receptor-mediated codegradation. *Neuron 69*, 120−131.

(18) Waldhoer, M., Fong, J., Jones, R. M., Lunzer, M. M., Sharma, S. K., Kostenis, E., Portoghese, P. S., and Whistler, J. L. (2005) A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers. *Proc. Natl. Acad. Sci. U.S.A. 102*, 9050−9055.

(19) Yekkirala, A. S., Kalyuzhny, A. E., and Portoghese, P. S. (2009) Standard opioid agonists activate heteromeric opioid receptors: Evidence for morphine and [D-Ala^2-MePhe^4-Glyol^5]enkephalin as selective μ−δ agonists. *ACS Chem. Neurosci. 1*, 146−154.

(20) Kostenis, E. (2001) Is Gα16 the optimal tool for fishing ligands of orphan G-protein-coupled receptors? *Trends Pharmacol. Sci. 22*, 560−564.

(21) Bilsky, E. J., Calderon, S. N., Wang, T., Bernstein, R. N., Davis, P., Hruby, V. J., McNutt, R. W., Rothman, R. B., Rice, K. C., and Porreca, F. (1995) SNC80, a selective, nonpeptidic and systemically active opioid delta agonist. *J. Pharmacol. Exp. Ther. 273*, 359−366.

(22) Gaveriaux-Ruff, C., Nozaki, C., Nadal, X., Hever, X. C., Weibel, R., Matifas, A., Reiss, D., Filliol, D., Nassar, M. A., Wood, J. N., Maldonado, R., and Kieffer, B. L. (2011) Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain 152*, 1238−1248.

(23) Pin, J.-P., Neubig, R., Bouvier, M., Devi, L., Filizola, M., Javitch, J. A., Lohse, M. J., Milligan, G., Palczewski, K., Parmentier, M., and Spedding, M. (2007) International union of basic and clinical pharmacology. LXVII. Recommendations for the recognition and nomenclature of G protein-coupled receptor heteromultimers. *Pharmacol. Rev. 59*, 5−13.

(24) Kabli, N., Martin, N., Fan, T., Nguyen, T., Hasbi, A., Balboni, G., O'Dowd, B. F., and George, S. R. (2010) Agonists at the delta-opioid receptor modify the binding of mu-receptor agonists to the mu-delta receptor hetero-oligomer. *Br. J. Pharmacol. 161*, 1122−1136.

(25) Brandt, M. R., Furness, M. S., Rice, K. C., Fischer, B. D., and Negus, S. S. (2001) Studies of tolerance and dependence with the

delta-opioid agonist SNC80 in rhesus monkeys responding under a schedule of food presentation. *J. Pharmacol. Exp. Ther. 299*, 629−637.
(26) Janssen, P. A., Niemegeers, C. J., and Dony, J. G. (1963) The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawl reflex in rats. *Arzneimittelforschung. 13*, 502−507.
(27) Guo, X. H., Fairbanks, C. A., Stone, L. S., and Loh, H. H. (2003) DPDPE-UK14,304 synergy is retained in mu opioid receptor knockout mice. *Pain 104*, 209−217.
(28) Pintar, J., Ansonoff, M., Wen, T., Nitsche, J. (2011) Mouse strain-specific analgesic responses in MOR-1 and DOR-1 KO mice. Presented at the International Narcotics Research Conference, Hollywood, FL, USA, p 232.
(29) Hylden, J. L., and Wilcox, G. L. (1980) Intrathecal morphine in mice: A new technique. *Eur. J. Pharmacol. 67*, 313−316.
(30) Fairbanks, C. A. (2003) Spinal delivery of analgesics in experimental models of pain and analgesia. *Adv. Drug Delivery Rev. 55*, 1007−1041.