Published in final edited form as:

J Neurosci Res. 2013 June ; 91(6): 854–860. doi:10.1002/jnr.23214.

Ligand-induced μ opioid receptor internalization in enteric neurons following chronic treatment with the opiate fentanyl

Laura Anselmi^{1,*}, Ingrid Jaramillo², Michelle Palacios¹, Jennifer Huynh¹, and Catia Sternini^{1,2}

¹CURE Digestive Diseases Research Center, Digestive Diseases Division and Departments of Medicine, Los Angeles, California 90095, USA.

²Neurobiology, University of California Los Angeles, David Geffen School of Medicine, Los Angeles, California 90095, USA.

Abstract

Morphine differs from most opiates for its poor ability to internalize μ opioid receptors (μ ORs). However, chronic treatment with morphine produces adaptational changes at the dynamin level, which enhance the efficiency of acute morphine stimulation to promote μ OR internalization in enteric neurons. This study tested the effect of chronic treatment with fentanyl, a μ OR internalizing agonist, on ligand-induced endocytosis and the expression of the intracellular trafficking proteins, dynamin and β arrestin, in enteric neurons using organotypic cultures of the guinea pig ileum. In enteric neurons from guinea pigs chronically treated with fentanyl, μ OR immunoreactivity was predominantly at the cell surface following acute exposure to morphine with a low level of μ OR translocation, slightly higher than in neurons from naïve animals. This internalization was not due to morphine direct effect because it was also observed in neurons exposed to medium alone. By contrast, D-Ala2-N-Me-Phe4-Gly-ol5-enkephalin (DAMGO), a potent μ OR-internalizing agonist, induced pronounced and rapid μ OR endocytosis in enteric neurons from animals chronically treated with fentanyl or from naïve animals. Chronic fentanyl treatment did not alter dynamin or β -arrestin expression. These findings indicate that prolonged activation of μ ORs with an internalizing agonist such as fentanyl does not enhance the ability of acute morphine to trigger μ OR endocytosis nor induces changes in intracellular trafficking proteins, as observed with prolonged activation of μ ORs with a poorly internalizing agonist such as morphine. Cellular adaptations induced by opiate chronic treatment might be ligand dependent and vary with the agonist efficiency to induce receptor internalization.

Keywords

G-protein coupled receptors; endogenous opioids; tolerant and naïve animals

Opioids modulate a variety of biological processes by activating three classes of opioid receptors (μ ORs, δ ORs, κ ORs) (Kromer 1988; Law and Loh 1999; Pasternak 1988; Raynor et al. 1994). μ ORs are expressed in the nervous system (Ding et al. 1996), including the enteric nervous system, the neural network innervating the gastrointestinal tract (Bagnol et al. 1997; Ho et al. 2003). μ OR is the main target of opiate drugs used in humans for pain control (Evans 2000; Kieffer 1999; Kieffer and Gavériaux-Ruff 2002; Matthes et al. 1996). Unfortunately, the development of side effects, including tolerance and opioid bowel dysfunction (OBD) diminishes their usefulness in patients requiring chronic pain treatment

*Corresponding author: Laura Anselmi, Ph.D. CURE, Digestive Diseases Research Center, Bldg. 115, Room 224, VAGLAHS 11301 Wilshire Blvd. Los Angeles, CA, 90073 Tel: (310)-312-9477; Fax: (310)-268-4615 lauranselmi@yahoo.it.

(Brock et al. 2012; Christie 2008; Morgan and Christie 2011; Williams et al. 2001). OBD is a serious condition characterized by abdominal pain and severe constipation mediated by μ OR in enteric neurons. Whereas patients develop tolerance to the opiate analgesic effect, tolerance to GI adverse effects does not develop over time, and OBD symptoms worsen with increasing analgesic doses with a negative impact on the quality of life (Brock et al. 2012; Panchal et al. 2007).

μ OR-ligand interaction promotes phosphorylation, internalization, and recycling, which contribute to desensitization and resensitization, mechanisms regulating cellular function (Bohm et al. 1997; Gainetdinov et al. 2004; Martini and Whistler 2007; Sternini 2001). Opiates differ in their ability to induce μ OR desensitization and internalization (Martini and Whistler 2007; Sternini 2001). High efficacy opiates like etorphine and fentanyl, and endogenous opioids induce rapid μ OR endocytosis in enteric neurons (Minnis et al. 2003; Sternini et al. 1996). By contrast, morphine is a poor internalizing agonist and does not induce detectable internalization (Keith et al. 1998; Minnis et al. 2003; Sternini et al. 1996). Morphine inefficiency to internalize activated- μ ORs could be due to its low efficacy in inducing receptor phosphorylation, β -arrestin recruitment and desensitization (Alvarez et al. 2002; Kovoor et al. 1998; Whistler and von Zastrow 1999; Yu et al. 1997). However, other studies have shown that morphine promotes μ OR phosphorylation and desensitization without triggering internalization (Arttamangkul et al. 2008; Borgland et al. 2003; Yu et al. 1997; Zhang et al. 1996) and that overexpression of intracellular proteins involved in receptor trafficking increase morphine ability to induce receptor internalization (Whistler et al. 1999; Zhang et al. 1998). This agonist-selective internalization may reflect a differential ability of agonists to regulate biological effects mediated by the μ OR, including the induction of tolerance that is inversely correlated with endocytosis effectiveness (Duttaroy and Yoburn 1995; Martini and Whistler 2007; Paronis and Holtzman 1992).

Chronic opiates induce cellular adaptations, including changes in the expression of proteins implicated in receptor trafficking, which might vary depending on ligand potency, efficacy and efficiency to induce μ OR endocytosis (Duttaroy and Yoburn 1995; Martini and Whistler 2007; Paronis and Holtzman 1992). We have shown that prolonged activation of μ ORs with morphine reverses the resistance of morphine-activated μ ORs to undergo internalization in enteric neurons (Patierno et al. 2011), which depends on the increased expression of dynamin, a protein required for μ OR internalization (von Zastrow et al. 2003). In this study, we tested the hypothesis that cellular adaptations induced by prolonged activation of μ OR depend on the internalizing efficiency of μ OR agonists. Therefore, we investigated whether chronic exposure to fentanyl, an internalizing μ OR agonist, enhances morphine efficiency to induce μ OR internalization in enteric neurons and alters the expression of dynamin and β -arrestin, intracellular proteins regulating receptor trafficking.

MATERIALS AND METHODS

Male albino Porcellus guinea pigs (150-250 g) (Hartley; Charles River Laboratory, San Diego, CA, USA) were used. Care and handling of the animals were in accordance with all National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the Animal Committee at UCLA Los Angeles, CA, USA. All efforts were made to minimize the number of animals used. Guinea pigs received subcutaneous injection of saline or fentanyl (0.24 mg/kg) (35 and 26 animals per group, respectively) twice a day for 4 days (Schulz et al. 1985) for chronic treatment. The following drugs were used: fentanyl (Baxter, Deerfield, IL), which was dissolved in 0.9% sodium chloride, and tetrodotoxin (TTX) (EMD, Biosciences, CA) to block transmitter release.

Organotypic cultures of the ileum and immunohistochemistry

Guinea pigs were anesthetized with an overdose of isoflurane and euthanized by decapitation 2 hours after the last injection, and the distal ileum was rapidly removed. For the preparation of organotypic cultures, the distal ileum was dissected and washed in sterile Krebs solution (in mM: KCl, 5.9; NaCl, 118; CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 22.7; NaH₂PO₄, 1.4; glucose, 5; fungizone, 2.5 μ g/ml; penicillin, 100 IU/ml; and streptomycin, 100 μ g/ml), bubbled with 95% O₂: 5% CO₂, pH 7.4, at 37°C (Minnis et al. 2003). Tissue was incubated in either Krebs alone (unstimulated) or Krebs containing saturating doses of morphine (100 μ M) or DAMGO (10 μ M) for 1 hr at 4°C to allow ligand-receptor binding. These doses were selected on the basis of previous studies (Minnis et al. 2003). Following incubation, the ileum was opened along the mesentery, pinned flat and transferred to ligand-free medium (Dulbecco's modified Eagle's medium, containing 10% v/v fetal bovine serum, penicillin, streptomycin and fungizone) at 37°C for 30 minutes to allow receptor internalization. In some experiments, tissues were pre-incubated for 90 minutes in Krebs containing TTX 10⁻⁷ M at 37°C to block neuronal depolarization. Organotypic cultures were fixed in 0.1 M phosphate-buffered 4% paraformaldehyde, pH 7.4, for 2 hrs at room temperature and washed (Patierno et al. 2011). The longitudinal muscle with the myenteric plexus attached was separated and processed as whole mounts for immunohistochemistry as previously described using the indirect immunofluorescence technique (Ho et al. 2003). The location of the μ OR was identified using a well characterized μ OR antibody (Incstar Science, Technology and Research, Stillwater, MN) (McConalogue et al. 1999; Minnis et al. 2003). In brief, whole mount preparations were incubated in 5% normal donkey serum with 0.5% Triton X-100 for 60 min. Tissue was transferred to the same solution containing the μ OR antibody (1:2000) for 48 hrs at 4°C. Tissue was washed and incubated with Alexa Fluor 488 affinity-purified donkey anti-rabbit (1:1000; Invitrogen Molecular Probes, Eugene, OR) for 2 hrs at room temperature.

μ OR immunoreactivity distribution was analyzed with a Zeiss 510 META laser scanning confocal microscope with a 63X PlanApo 1.4 numerical aperture objective (Carl Zeiss, Inc., Thornwood, NY). Images were adjusted for brightness and contrast using Adobe Photoshop 7.0 (Adobe System, Mountain View, CA). The level of μ OR internalization was quantified using Image J software; typically a single confocal image included the nucleus and a large area of cytoplasm. For each data point, internalization was quantified for 35-60 neurons.

Dynamin and β -arrestin expression

Strips of longitudinal muscle-myenteric plexus preparations (LMMPs) were homogenized in 1 ml of ice-cold lysis buffer (5 mM Pipes/Tris buffer, pH 7.4, with 2 mM ethylenediaminetetraacetic acid and ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) and centrifuged at 14,000 rpm for 20 minutes at 4°C as described (Patierno et al. 2011). The supernatant proteins were size-separated by sodium dodecyl sulfate-polyacrylamide gel and transferred onto Immobilon membranes (Millipore, Billerica, MA). Membranes were incubated with mouse antibody to dynamin 2 (1:1,000) (Transduction Laboratories, San Diego, CA), which is ubiquitous and shares high homology with dynamin 1, more specific for the central nervous system or rabbit antiserum to β -arrestin 1 (1:75,000) (kindly provided by Robert Lefkowitz, Duke University, Durham, NC), which shares high homology with β -arrestin 2, followed by Alexa 488 anti-mouse or anti-rabbit immunoglobulin (IgG) at 1:1,000 dilution for 1 hour at room temperature.

Electrically induced Neurogenic Contractions of the ileum in vitro

To evaluate the level of tolerance induced by chronic fentanyl treatment, we measured the neurogenic contractions of the ileum in response to electrical field stimulation in LMMPs of the ileum from animals chronically treated with fentanyl or saline (12-14 animals for each

group) prepared as described (Sternini et al. 2000). Isometric contractions were recorded with a force-displacement transducer. After a 45-minute equilibration period, strips were stimulated with square wave pulses (0.5 ms) of supramaximal amplitude (60 V) at a frequency of 0.1 Hz. Concentration response-curves for the inhibitory effects of morphine (10^{-9} M- 10^{-6} M) on electrically induced contractions in preparations from animals treated with saline or chronic fentanyl were constructed in a noncumulative fashion. Strips were washed for three 5-minute periods after each agonist administration, and contractions were allowed to return to baseline before any subsequent pharmacological treatment.

Statistical analysis

For statistical analysis we used a one way ANOVA followed by the post hoc Bonferroni test or a paired Student's *t*-test (GraphPad Prism 5.0); significance was chosen at $P < 0.05$, and variability is reported as $\pm$ SEM.

RESULTS

Effect of morphine and DAMGO on enteric neurons of ileum in vitro

In organotypic cultures from animals treated with saline (naïve), μ OR immunoreactivity (IR) was mostly confined to the plasma membrane of unstimulated (Figure 1A) and morphine (100 μ M) stimulated (Figure 1B) enteric neurons, with a low level of μ OR internalization as indicated by the occasional punctate immunofluorescence in the cytosol observed in a small proportion of neurons. By contrast, DAMGO (10 μ M) stimulation induced abundant translocation of μ OR-IR into the cytoplasm (Figure 1C), confirming previous results from our group (Minnis et al. 2003). In unstimulated and morphine-stimulated neurons from animals chronically treated with fentanyl, μ OR-IR was predominantly located at the plasma membrane, with a low level of μ OR internalization, which could be detected in most neurons as sparse punctate immunofluorescence in the cytoplasm (Figures 1D, 1E). DAMGO induced strong μ OR internalization in neurons from fentanyl-treated guinea pigs (Figure 1F), which was comparable to DAMGO-induced internalization observed in neurons from naïve animals (Figure 1C).

Quantification analysis showed that the proportion of μ OR endocytosis in unstimulated enteric neurons from naïve animals was similar to the morphine-stimulated neurons ($34.3 \pm 1.6\%$ vs. $33.0 \pm 2.6\%$, $p = \text{NS}$) and was just below the threshold level previously reported in normal neurons (Patierno et al. 2004), whereas DAMGO induced pronounced μ OR internalization ($65.0 \pm 1.4\%$, $p < 0.005$ vs. unstimulated and morphine-stimulated) (Figure 2A). The levels of μ OR endocytosis in unstimulated and morphine-stimulated enteric neurons from fentanyl treated animals were also comparable ($45.0 \pm 2.3\%$ vs. $44.6 \pm 2.3\%$, $p = \text{NS}$), but slightly, though significantly, higher than in naïve animals ($p < 0.05$ for both unstimulated and morphine stimulated) (Figure 2B). This internalization was about 10% higher than the normal threshold (Patierno et al. 2004), suggesting that it was due to experimental manipulation. However, it could not be due to a direct effect of morphine on μ OR because it was comparable to the level of internalization observed in neurons only exposed to medium. Furthermore, TTX pretreatment to block neuronal depolarization did not affect the translocation of μ OR-IR in any of these conditions (data not shown), thus indicating it was not due to endogenously released opioids. Since fentanyl is an internalizing μ OR agonist, it is likely that the continued treatment induced baseline internalization in enteric neurons in the absence of acute agonist stimulation. By contrast, the levels of OR endocytosis were significantly higher in DAMGO-stimulated neurons from animals chronically treated with fentanyl ($72.6 \pm 0.9\%$, $p < 0.005$ vs. unstimulated and morphine-stimulated) (Figure 2B). Pre-treatment with TTX did not affect DAMGO-induced μ OR

internalization, indicating that it was due to a direct interaction of DAMGO with the receptor, and not to the activation of the receptor by endogenous opioids.

Effect of chronic fentanyl on dynamin and β -arrestin expression

Since dynamin and β -arrestin mediate receptor internalization of activated μ ORs (von Zastrow 2003; Whistler and von Zastrow 1999), we investigated the expression of these proteins using Western blots in LMMP preparations from both saline and chronic fentanyl treated animals. There was no change in the protein levels of either dynamin 2 or β -arrestin 1 in LMMPs from distal ileum of saline or fentanyl treated animals (Figure 3). This differs from data obtained in LMMP preparations from animals chronically treated with morphine, which induced increased expression of dynamin, but not β arrestin, suggesting different cellular responses to chronic stimulation with different μ OR agonists (Patierno et al. 2011).

Effect of chronic fentanyl on twitch contractions in electrically stimulated longitudinal muscle-myenteric plexus preparations

Morphine inhibited electrically induced twitch contractions of LMMP preparations from both saline and chronic fentanyl treated animals in a concentration-dependent manner (Figure 4). Morphine caused a rightward shift in the sigmoidal concentration-response curve from animals chronically treated with fentanyl, which is consistent with these animals being tolerant to μ OR agonists.

DISCUSSION

Chronic activation of μ ORs induces a variety of cellular adaptations at the receptor level and within receptor-associated signal transduction pathways, which are mechanisms underlying the development of tolerance and adverse side effects. We have previously shown that prolonged treatment with morphine induced upregulation of intracellular dynamin that enhances the efficiency of acute morphine stimulation to induce rapid μ OR internalization in enteric neurons (Patierno et al. 2011). By contrast, the present study shows that chronic treatment with fentanyl did not alter the expression of intracellular trafficking proteins, including dynamin, and did not improve the efficiency of acute morphine to induce μ OR endocytosis. The low level of μ OR translocation observed in morphine-stimulated neurons from animals chronically treated with fentanyl was slightly (~10%) above the normal threshold, therefore suggesting it was due to experimental manipulation. However, it was comparable to the level of internalization observed in unstimulated neurons, thus ruling out a direct effect of morphine on μ ORs. Furthermore, it was not modified by treatment to block neuronal depolarization thus arguing against an effect of endogenous opioids. It is reasonable to suggest that the baseline of μ OR translocation is induced by the continuous stimulation with fentanyl, which is an internalizing agonist (Hashimoto et al. 2006; Martini and Whistler 2007). By contrast, the magnitude of DAMGO-induced μ OR internalization in enteric neurons from animals chronically treated with fentanyl was pronounced despite the development of tolerance. Tolerance was supported by the reduction of opiate inhibitory effect on neurogenic muscle contraction, which is consistent with diminished cellular responsiveness, a typical side effect of prolonged μ OR activation (Chavkin and Goldstein 1984).

Ligand-induced receptor internalization is an important mechanism that regulates receptor-mediated functions (Dang and Christie 2012; Martini and Whistler 2007; Sternini 2001). Receptor internalization contributes to the diminution of cellular response by removing the receptors from the surface. Internalization also contributes to the recovery of cellular response by allowing the ligand-receptor complexes to traffic inside of the cell and either recycling or undergoing degradation. Since morphine, a poor internalizing opiate, has higher

propensity to induce opioid tolerance than internalizing opiates (Duttaroy and Yoburn 1995), it has been suggested that ligand-induced μ OR endocytosis serves as a protective mechanism from the development of tolerance (Eisinger et al. 2002; Koch et al. 2005; Martini and Whistler 2007). The possibility that the resistance of morphine-activated μ ORs to undergo internalization is the initial trigger for the cellular adaptations induced by prolonged activation of μ OR has gained substantial ground (Martini and Whistler 2007). However, other parameters including the different affinity of agonists to interact with G proteins, as well as the different potency, efficacy, and half-life of opiates might also play a role (Dang and Christie 2012; Martini and Whistler 2007). Furthermore, differences in the internalizing properties of morphine depend also on the cell system. Indeed, morphine fails to induce μ OR endocytosis in heterologous cells and highly differentiated neurons in the central and peripheral nervous system (Haberstock-Debic et al. 2005; Keith et al. 1996; Sternini et al. 1996). By contrast, morphine promotes rapid μ OR endocytosis in embryonic striatal neurons in cultures (Haberstock-Debic et al. 2005) as well as in adult enteric neurons in vivo following chronic treatment with morphine (Patierno et al. 2011). Furthermore, it has been proposed that differences in the intracellular concentrations of proteins regulating GPCR trafficking might influence the ability of opioids to drive μ OR endocytosis (Bohn et al. 2004). This is consonant with findings in heterologous cells showing morphine-induced μ OR endocytosis in the presence of GRKs or β arresting overexpression (Whistler and von Zastrow 1998; Zhang et al. 1998). An increased expression of dynamin is likely to be the mechanism underlying the enhanced efficiency of morphine to trigger μ OR endocytosis in enteric neurons from animals chronically treated with morphine (Patierno et al. 2011). This is supported by the inhibition of morphine-induced μ OR internalization by pretreatment with dynasore, a dynamin inhibitor (Macia et al. 2006). The lack of increase in dynamin expression in enteric neurons from animals chronically treated with fentanyl might be the basis for the failure of morphine to induce μ OR internalization observed in the present study. Intracellular trafficking proteins might influence the ability of poor internalizing μ OR agonists such as morphine to efficiently induce receptor internalization and it is likely that they differ according to the cell type and stimulation condition.

The high efficiency of DAMGO to induce μ OR internalization is not affected by chronic treatment with fentanyl as it was not affected by chronic stimulation with morphine (Patierno et al. 2011). This is in agreement with findings in the central nervous system showing that neurons that developed tolerance to morphine-induced analgesia in the spinal cord undergo the same level of μ OR endocytosis in response to DAMGO as naïve neurons (Trafton and Basbaum 2004). DAMGO-induced μ OR endocytosis is due to a direct action of DAMGO on the receptor and independent from endogenous opioids release by enteric neurons in response to chronic opioid treatment, since it was not affected by blocking neuronal depolarization. Together, these findings suggest that receptor trafficking in neurons induced by highly efficient internalizing agonists is not impaired by prolonged receptor activation independently of the type of agonist used chronically.

In summary, chronic stimulation of μ ORs with fentanyl does not induce the adaptational changes at the dynamin levels, which promote internalization of morphine-activated μ OR in enteric neurons. This supports the concept that the internalizing efficacy of the ligand affects cellular adaptations induced by prolonged receptor activation. This observation is of relevance since receptor internalization contributes to the regulation of receptor-mediated functions by participating to the attenuation and recovery of cellular response.

Acknowledgments

The authors thank Dr. Nicholas Brecha for insightful comments on the manuscript.

This work was supported by National Institutes of Health DK54155 (to C.S.) and 41301 Morphology and Cell Imaging Core (to C.S.).

REFERENCES

- Alvarez VA, Arttamangkul S, Dang V, Salem A, Whistler JL, Von Zastrow M, Grandy DK, Williams JT. μ -Opioid receptors: Ligand-dependent activation of potassium conductance, desensitization, and internalization. *J Neurosci*. 2002; 22(13):5769–5776. [PubMed: 12097530]
- Arttamangkul S, Quillinan N, Low MJ, von Zastrow M, Pintar J, Williams JT. Differential activation and trafficking of micro-opioid receptors in brain slices. *Mol Pharmacol*. 2008; 74(4):972–979. [PubMed: 18612077]
- Bagnol D, Mansour A, Akil H, Watson SJ. Cellular localization and distribution of the cloned μ and κ opioid receptors in rat gastrointestinal tract. *Neuroscience*. 1997; 81:579–591. [PubMed: 9300443]
- Bohm S, Grady EF, Bunnett NW. Mechanisms attenuating signaling by G protein-coupled receptors. *Biochem J*. 1997; 322:1–18. [PubMed: 9078236]
- Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS. Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol Pharmacol*. 2004; 66(1):106–112. [PubMed: 15213301]
- Borgland SL, Connor M, Osborne PB, Furness JB, Christie MJ. Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of μ -opioid receptors. *J Biol Chem*. 2003; 278(21):18776–18784. [PubMed: 12642578]
- Brock C, Olesen SS, Olesen AE, Frokjaer JB, Andresen T, Drewes AM. Opioid-induced bowel dysfunction: pathophysiology and management. *Drugs*. 2012; 72(14):1847–1865. [PubMed: 22950533]
- Chavkin C, Goldstein A. Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proc Natl Acad Sci USA*. 1984; 81(22):7253–7257. [PubMed: 6095280]
- Christie MJ. Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. *Br J Pharmacol*. 2008; 154(2):384–396. [PubMed: 18414400]
- Dang VC, Christie MJ. Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. *Br J Pharmacol*. 2012; 165(6):1704–1716. [PubMed: 21564086]
- Ding YQ, Kaneko T, Nomura S, Mizuno N. Immunohistochemical localization of μ opioid receptors in the central nervous system of the rat. *J Comp Neurol*. 1996; 367(3):375–402. [PubMed: 8698899]
- Duttaroy A, Yoburn BC. The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology*. 1995; 82:1226–1236. [PubMed: 7741298]
- Eisinger DA, Ammer H, Schulz R. Chronic morphine treatment inhibits opioid receptor desensitization and internalization. *J Neurosci*. 2002; 22(23):10192–10200. [PubMed: 12451120]
- Evans CJ. Agonist selective μ -opioid receptor trafficking in rat central nervous system. *Molecular psychiatry*. 2000; 5(2):121. [PubMed: 10822332]
- Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci*. 2004; 27:107–144. [PubMed: 15217328]
- Haberstock-Debic H, Kim KA, Yu YJ, von Zastrow M. Morphine promotes rapid, arrestin-dependent endocytosis of μ -opioid receptors in striatal neurons. *J Neurosci*. 2005; 25(34):7847–7857. [PubMed: 16120787]
- Hashimoto T, Saito Y, Yamada K, Hara N, Kirihara Y, Tsuchiya M. Enhancement of morphine analgesic effect with induction of μ -opioid receptor endocytosis in rats. *Anesthesiology*. 2006; 105(3):574–580. [PubMed: 16931992]
- Ho A, Lievore A, Patierno S, Kohlmeier SE, Tonini M, Sternini C. Neurochemically distinct classes of myenteric neurons express the μ -opioid receptor in the guinea pig ileum. *J Comp Neurol*. 2003; 458(4):404–411. [PubMed: 12619074]
- Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Liissin DV, Montelliet-Agius G, Stewart PL, Evans CJ, von Zastrow M. μ -opioid receptor internalization: opiate drugs have differential effects

- on a conserved endocytic mechanism in vitro and in mammalian brain. *Mol Pharmacol.* 1998; 53:377–384. [PubMed: 9495801]
- Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kag L, Evans CJ, von Zastrow M. Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem.* 1996; 271:19021–19024. [PubMed: 8702570]
- Kieffer BL. Opioids: first lessons from knockout mice. *Trends Pharmacol Sci.* 1999; 20(1):19–26. [PubMed: 10101958]
- Kieffer BL, Gaveriaux-Ruff C. Exploring the opioid system by gene knockout. *Prog Neurobiol.* 2002; 66(5):285–306. [PubMed: 12015197]
- Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G, Holtt V. Receptor endocytosis counteracts the development of opioid tolerance. *Mol Pharmacol.* 2005; 67(1):280–287. [PubMed: 15475572]
- Kovoor A, Celver JP, Wu A, Chavkin C. Agonist induced homologous desensitization of mu-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. *Mol Pharmacol.* 1998; 54(4):704–711. [PubMed: 9765514]
- Kromer W. Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. *Pharmacol Rev.* 1988; 40(2):121–162. [PubMed: 3070578]
- Law PY, Loh HH. Regulation of opioid receptor activities. *J Pharmacol Exp Ther.* 1999; 289(2):607–624. [PubMed: 10215631]
- Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. *Developmental cell.* 2006; 10(6):839–850. [PubMed: 16740485]
- Martini L, Whistler JL. The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. *Curr Opin Neurobiol.* 2007; 17(5):556–564. [PubMed: 18068348]
- Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP, Kieffer BL. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature.* 1996; 383(6603):819–823. [PubMed: 8893006]
- McConalogue K, Grady EF, Minnis J, Balestra B, Tonini M, Brecha NC, Bunnett NW, Sternini C. Activation and internalization of the μ opioid receptor by the newly described endogenous agonists, endomorphin-1 and endomorphin-2. *Neuroscience.* 1999; 90:1051–1059. [PubMed: 10218804]
- Minnis J, Patierno S, Kohlmeier SE, Brecha N, Tonini M, Sternini C. Ligand-induced μ opioid receptor endocytosis and recycling in enteric neurons. *Neuroscience.* 2003; 119:33–42. [PubMed: 12763066]
- Morgan MM, Christie MJ. Analysis of opioid efficacy, tolerance, addiction and dependence from cell culture to human. *Br J Pharmacol.* 2011; 164(4):1322–1334. [PubMed: 21434879]
- Panchal SJ, Muller-Schwefe P, Wurzelmann JI. Opioid-induced bowel dysfunction: prevalence, pathophysiology and burden. *Int J Clin Pract.* 2007
- Paronis CA, Holtzman SG. Development of tolerance to the analgesic activity of mu agonists after continuous infusion of morphine, meperidine or fentanyl in rats. *J Pharmacol Exp Ther.* 1992; 262(1):1–9. [PubMed: 1625189]
- Pasternak, GW. The opiate receptors. Humana; Totowa, NJ: 1988.
- Patierno S, Anselmi L, Jaramillo I, Scott D, Garcia R, Sternini C. Morphine induces mu opioid receptor endocytosis in guinea pig enteric neurons following prolonged receptor activation. *Gastroenterology.* 2011; 140(2):618–626. [PubMed: 21070774]
- Patierno S, Raybould HE, Sternini C. Abdominal surgery induces μ opioid receptor endocytosis in enteric neurons of the guinea pig ileum. *Neuroscience.* 2004; 123:101–109. [PubMed: 14667445]
- Raynor K, Kong H, Yasuda K, Chen Y, Yu L, Bell GI, Reisine T. Pharmacological characterization of the clones κ and μ opioid receptors. *Mol Pharmacol.* 1994; 45:330–334. [PubMed: 8114680]
- Schulz R, Seidl E, Herz A. Opioid dependence in the guinea-pig myenteric plexus is controlled by non-tolerant and tolerant opioid receptors. *Eur J Pharmacol.* 1985; 110(3):335–341. [PubMed: 2988984]
- Sternini C. Receptors and transmission in the brain-gut axis: potential for novel therapies. III. μ opioid receptors in the enteric nervous system. *Am J Physiol.* 2001; 281:G8–G15.

- Sternini C, Brecha NC, Minnis J, D'Agostino G, Balestra B, Fiori E, Tonini M. Role of agonist-dependent receptor internalization in the regulation of μ opioid receptors. *Neuroscience*. 2000; 98:233–241. [PubMed: 10854754]
- Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, von Zastrow M, Evans C, Brecha NC. Agonist-selective endocytosis of μ opioid receptor by neurons *in vivo*. *Proc Natl Acad Sci USA*. 1996; 93(17):9241–9246. [PubMed: 8799185]
- Trafton JA, Basbaum AI. [d-Ala2,N-MePhe4,Gly-ol5]enkephalin-induced internalization of the micro opioid receptor in the spinal cord of morphine tolerant rats. *Neuroscience*. 2004; 125(3):541–543. [PubMed: 15099667]
- von Zastrow M. Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. *Life Sci*. 2003; 74(2-3):217–224. [PubMed: 14607249]
- von Zastrow M, Svingos A, Habersack-Debic H, Evans C. Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr Opin Neurobiol*. 2003; 13(3):348–353. [PubMed: 12850220]
- Whistler JL, Chuang H-H, Chu P, Jan LY, von Zastrow M. Functional dissociation of μ opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron*. 1999; 23:737–746. [PubMed: 10482240]
- Whistler JL, von Zastrow M. Morphine-activated opioid receptors elude desensitization by β -arrestin. *Proc Natl Acad Sci USA*. 1998; 95:9914–9919. [PubMed: 9707575]
- Whistler JL, von Zastrow M. Dissociation of functional roles of dynamin in receptor-mediated endocytosis and mitogenic signal transduction. *J Biol Chem*. 1999; 274(35):24575–24578. [PubMed: 10455121]
- Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. *Physiol Rev*. 2001; 81(1):299–343. [PubMed: 11152760]
- Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB. Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem*. 1997; 272(46):28869–28874. [PubMed: 9360954]
- Zhang J, Ferguson SSG, Barak LS, Bodduluri SB, Laporte SA, Law P-Y, Caron MG. Role of G protein-coupled receptor kinase in agonist-specific regulation of μ -opioid receptor responsiveness. *Proc Natl Acad Sci USA*. 1998; 95:7157–7162. [PubMed: 9618555]
- Zhang J, Ferguson SSG, Barak LS, Menard L, Caron MG. Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem*. 1996; 271:18302–18305. [PubMed: 8702465]

Fig. 1.

Localization of μ OR immunoreactivity (IR) in neurons from animals chronically treated with saline (A-C) or fentanyl (D-F). μ OR-IR is confined at the cell surface (arrows) of unstimulated (A) and morphine-stimulated (B) neurons from saline treated animals, and predominantly localized at the cell surface (arrows) with low levels of μ OR-IR in the cytosol (arrowheads) of unstimulated (D) and (E) morphine-stimulated neurons from animals treated chronically with fentanyl. By contrast, receptor internalization is pronounced in DAMGO-stimulated neurons from both saline (C) and fentanyl chronically treated (F) animals as indicated by the prominent translocation of μ OR-IR in the cytoplasm. Calibration bars: A, B, D, E: 5 μ m; E and F: 4 μ m.

Fig. 2. Quantification of μ OR immunoreactivity in the cytoplasm of enteric neurons from animals chronically treated with saline (A, naïve) and fentanyl (B, chronic). The proportion of μ OR-IR translocated in the cytosol of neurons incubated with medium (Krebs; unstimulated) was comparable to the morphine-stimulated neurons in both naïve (A) and chronically treated (B). The level of μ OR-IR in the cytosol of both unstimulated and morphine-stimulated neurons from animals chronically treated with fentanyl was higher (~10%) compared to neurons from saline treated animals. The levels of μ OR-IR internalized following DAMGO stimulation is significantly higher than in unstimulated and morphine-stimulated neurons

from both naïve and chronically treated animals. Fluorescence density values are expressed as the mean $\pm$ standard error of the mean. *** $p < 0.005$.

Fig. 3. Quantification of dynamin2 and β -arrestin1 immunoreactivity in longitudinal muscle-myenteric plexus preparations of the guinea pig distal ileum from animals treated with saline (white bars) and animals chronically treated with fentanyl (black bars) measured by Western Blot. Results represent the percentage increase of dynamin2 or β -arrestin1 compared with tissue from animals treated with saline. Representative immunoblots are shown on top the histograms.

Fig. 4.

Morphine (1 nM-1 μ M) induces a dose-dependent inhibition of electrically-induced twitch contractions of longitudinal muscle-myenteric plexus preparations from animals treated with saline (white circle) or chronic fentanyl (black circle). The chronic treatment with fentanyl induces a parallel right shift of the inhibitory curve without reduction of the maximum response, indicative of tolerance. * $p < 0.05$