Special Section on 50 Years of Opioid Research

Spinal Opioid Tolerance Depends upon Platelet-Derived Growth Factor Receptor- β Signaling, Not μ -Opioid Receptor Internalization

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ABSTRACT

Opioids are some of the most potent analgesics available. However, their effectiveness is limited by the development of analgesic tolerance. Traditionally, tolerance was thought to occur by termination of μ -opioid receptor (MOR) signaling via desensitization and internalization. Contradictory findings led to a more recent proposal that sustained MOR signaling caused analgesic tolerance. However, this view has also been called into question. We recently discovered that the platelet-derived growth factor receptor (PDGFR)- β signaling system is both necessary and sufficient to cause opioid tolerance. We therefore propose a completely new hypothesis: that opioid tolerance is mediated by selective cellular signals and is independent of MOR internalization. To test this hypothesis, we developed an automated software-based method to perform unbiased analyses of opioid-induced MOR internalization in the rat substantia gelatinosa. We induced tolerance with either morphine, which did not cause MOR internalization, or fentanyl, which did. We also blocked tolerance by administering morphine or fentanyl with the PDGFR- β inhibitor imatinib. We found that imatinib blocked tolerance without altering receptor internalization induced

by either morphine or fentanyl. We also showed that imatinib blocked tolerance to other clinically used opioids. Our findings indicate that opioid tolerance is not dependent upon MOR internalization and support the novel hypothesis that opioid tolerance is mediated by intracellular signaling that can be selectively targeted. This suggests the exciting possibility that undesirable opioid side effects can be selectively eliminated, dramatically improving the safety and efficacy of opioids.

SIGNIFICANCE STATEMENT

Classically, it was thought that analgesic tolerance to opioids was caused by desensitization and internalization of μ -opioid receptors (MORs). More recently, it was proposed that sustained, rather than reduced, MOR signaling caused tolerance. Here, we present conclusive evidence that opioid tolerance occurs independently of MOR internalization and that it is selectively mediated by platelet-derived growth factor receptor signaling. This novel hypothesis suggests that dangerous opioid side effects can be selectively targeted and blocked, improving the safety and efficacy of opioids.

Introduction

For centuries, opioids have been first-line drugs for the treatment of severe pain. However, their effectiveness is

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limited by the development of opioid analgesic tolerance. Analgesic tolerance develops more rapidly than tolerance to other severe and potentially life-threatening opioid effects, such as itching, constipation, and respiratory depression (Gutstein and Akil, 2006). Thus, the severity of side effects and risk of lethal overdose increases as opioid doses escalate, limiting their prolonged use. Better understanding of the mechanisms underlying tolerance is critical for the development of safer and more effective treatments for chronic pain.

Opioids induce cellular signaling through the μ -opioid receptor (MOR), a $G_{i/o}$ protein-coupled protein receptor (GPCR) (Pasternak, 2010). MOR is expressed in pain-modulating

ABBREVIATIONS: 3D, 3-dimensional; DAMGO, [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; 4',6-diamidino-2-phenylindole dihydrochloride; EGFR, epidermal growth factor receptor; GPCR, $G_{i/o}$ protein-coupled protein receptor; IHC, immunohistochemistry; MOR, μ -opioid receptor; NeuN, neuronal nuclear antigen; NGS, normal goat serum; PDGFR, platelet-derived growth factor receptor β ; RTK, receptor tyrosine kinase; TFL, tail-flick latency.

neuronal pathways in peripheral sensory neurons, spinal cord, and brain (Mansour et al., 1994, 1995). Initially, it was postulated that opioid tolerance was caused by termination of opioid signaling by opioid-induced receptor desensitization and downregulation (internalization) (Cox, 1991). However, it was observed that chronic morphine administration caused tolerance without inducing receptor internalization (the “morphine paradox”) (Keith et al., 1996; Gutstein and Akil, 2006). It was subsequently proposed that sustained receptor signaling, rather than termination of signaling by internalization, mediated analgesic tolerance (Kieffer and Evans, 2002; He and Whistler, 2005). This hypothesis has also been called into question by conflicting data (Bailey et al., 2003; Koch et al., 2005). Contradicting both theories, all opioids, regardless of whether they induce MOR internalization or not, induce analgesic tolerance when administered at equianalgesic doses (Duttaroy and Yoburn, 1995; Raehal et al., 2011). We recently discovered that the platelet-derived growth factor receptor (PDGFR)- β signaling system is both necessary and sufficient to cause opioid tolerance. We also demonstrated that the analgesic properties of opioids could be completely dissociated from opioid tolerance (Wang et al., 2012). Based on these findings, we propose a completely new hypothesis for the mechanisms underlying opioid tolerance; namely, that opioid tolerance is caused by selective intracellular signaling and occurs independently of MOR internalization.

To test this hypothesis, we developed an automated software-based method that allowed us to perform an unbiased analysis of the amount of MOR internalization induced by opioids in the dorsal horn of the spinal cord of rats. We induced opioid tolerance in rats with morphine, which did not cause MOR internalization, and with fentanyl, which did cause receptor internalization. We also blocked the development of tolerance by treating rats with morphine or fentanyl in combination with the PDGFR- β inhibitor imatinib. We then analyzed the degree of MOR internalization induced in each treatment group. We also treated rats with several clinically used opioids to determine whether PDGFR- β antagonism would block analgesic tolerance to a wider range of clinically used opioids.

Methods

Animals. Male Sprague-Dawley rats (150–200 g; Envigo) were housed in groups of two and were maintained on a 12-hour light/dark cycle with ad libitum access to food and water. Rats were habituated to the colony room for 1 week prior to experimental manipulations. All experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Drugs and Routes of Administration. Morphine sulfate, [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO), hydromorphone, oxycodone, and sufentanil were obtained from Sigma Chemicals (St. Louis, MO). Fentanyl was obtained from Henry Schein (Melville, NY). Imatinib was obtained from LC Laboratories (Woburn, MA). All drugs were dissolved in a solution of 10% β -cyclodextrin sulfobutyl ether (Captisol; CyDex, Lenexa, KS) in 0.9% saline. The doses of opioids used were identified as equianalgesic in preliminary experiments (data not shown). For subcutaneous injections, rats were lightly restrained without anesthesia and injected subcutaneously between the shoulder blades with a 27-gauge needle in a volume of 1 ml/kg body

weight. For lumbar intrathecal injections, rats were anesthetized with 2% isoflurane and injected using a 30-gauge needle attached to a Hamilton syringe as previously described (Xu et al., 2006).

Nociceptive Testing. Analgesia was assessed using the radiant heat tail-flick latency (TFL) test. Animals were placed in Plexiglas boxes on a modified Hargreaves device (University of California, San Diego) kept at a constant surface temperature of 30°C (Dirig et al., 1997). Rats were habituated to the device for 30 minutes per day for 3 days before testing. A hot lamp was focused on the tail, and the TFL was determined by a photocell. A cutoff of 10 seconds was used to avoid tissue damage. Measurements were performed at the time of peak analgesic effect of each opioid determined in preliminary experiments (data not shown). The experimenter performing the behavioral experiments was blinded to the treatment group.

Immunohistochemistry. Immediately after behavioral testing, rats were anesthetized, and their spinal cords were quickly removed by hydroextrusion using ice-cold PBS. The lumbar enlargement was isolated, cut into 3-mm sections, and immediately transferred to ice-cold 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) diluted in PBS and slowly agitated for 48 hours at 4°C. Fixed tissue was then transferred to 20% and 30% sucrose diluted in PBS. The tissue was then equilibrated in embedding matrix (OCT, TissueTek), snap frozen in isopentane (−55°C), and stored at −80°C until use. Tissue samples were cut into 25- μ m sections. IHC was performed on floating samples in PBS at room temperature unless otherwise indicated. Sections were blocked in 10% normal goat serum (NGS) with 0.2% Triton X-100 for 1 hour and then incubated with rabbit anti- μ -opioid receptor polyclonal antibody (1:500, MOR UMB3; Abcam, Cambridge, UK) and mouse anti-neuronal nuclear antigen (NeuN) monoclonal antibody (1:2000; Millipore, Billerica, MA) diluted in 2% NGS and 0.2% Triton X-100 at 4°C overnight. Tissues were then rinsed 3 $\times$ 5 minutes with PBS and incubated with Alexa Fluor 647 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen Molecular Probes, Eugene, OR) secondary antibodies diluted in 2% NGS for 1 hour. Tissue was then rinsed 4 $\times$ 5 minutes with PBS, incubated with DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, 4',6-Diamidino-2-phenylindole dihydrochloride) (50 ng/ml in PBS; Cell Signaling Technologies) for 5 minutes and rinsed 3 $\times$ 5 minutes. Finally, sections were mounted, air dried, cover slipped in gelvatol (Center for Biologic Imaging, Pittsburgh, PA), and stored at 4°C. Imaging was performed using a Nikon A1 confocal microscope and a multi-immersion 60 $\times$ objective lens. All exposure times and processing procedures were identical for each specimen. One spinal cord slice was imaged per rat. The medial, central, and lateral regions of both the left and right substantia gelatinosa were imaged in each slice, generating a total of six images for each specimen (Fig. 1, A and B). In total, 21 to 25 Z-plane sections (Z-stacks) of 0.3 μ m were acquired for each image. Image names were coded to ensure that the experimenter was blinded to treatment group.

Internalization Analysis and Quantification. To analyze the amount of MOR internalized in neuronal cell bodies of the substantia gelatinosa of the spinal cord, we developed an automated, unbiased software workflow using Imaris $\times$ 64 9.0 software (Bitplane, St. Paul, MN). Confocal images were imported into Imaris. Two preprocessing steps were then applied to most objectively identify neuronal cell bodies and

Fig. 1. Novel, automated, unbiased method to quantify MOR internalization. (A) Schematic of a cross section of the lumbar spinal cord of rats. Black squares represent the fields that were imaged from each spinal cord section from each rat using a Nikon A1 confocal microscope. (B) Representative field of interest in the substantia gelatinosa. Neuronal cell bodies (NeuN, red) and MORs (green) are visualized after the IHC procedure. Original magnification, $60\times$ objective lens. Scale bar, $50\ \mu\text{m}$. Dotted-line square delineates the neuron shown in panel (C). (C) Image analysis workflow. 1) Import and preprocess images; 2) identify neuronal cell bodies as surfaces; 3) identify receptor clusters as spots; 4) count spots within each surface (i.e., MOR clusters internalized within each neuron).

groups of MORs. First, local contrast settings were automatically generated by Imaris based on local maximum and minimum intensity threshold values of the red (anti-NeuN) and green (anti-MOR) channels. We then established anti-NeuN and anti-MOR signal thresholds to be applied to all the images of each experiment by averaging the automatically generated signal thresholds for anti-NeuN and anti-MOR signals in all vehicle control group images. Second, average neuron and MOR cluster diameters were set manually, with cells averaging $10\ \mu\text{m}$ in diameter and MOR cluster size $0.6\ \mu\text{m}$ in diameter. These intensity threshold and diameter criteria allowed Imaris to reconstruct three-dimensional “surfaces,” representing neuronal cell bodies, and three-dimensional “spots,” representing clusters of MORs. Ratios of the number of spots (MOR clusters) located within the volume of each surface (neuronal cell body) identified were calculated using MATLAB (Natick, MA). The algorithm averaged the results for each treatment group and expressed the data as spots per $100\ \mu\text{m}^3$ cell volume. One limitation to this calculation is that the amount of receptor internalization may be underestimated, as the spots identified do not account for larger groups of MORs; however, this automated procedure was necessary so that surface and spot identification parameters were consistently applied to all images. Figure 1

presents a schematic outline of this approach. “Neuronal surfaces” that did not contain any “MOR spots” were excluded. On average, 25 neurons per field contained at least one MOR spot (Table 1).

Statistical Analyses. Data were analyzed using GraphPad 7.0 and considered statistically significant if $P < 0.05$. Behavioral data were analyzed using two-way mixed-effect ANOVA (time, treatment), followed by Tukey’s multiple comparison post hoc analysis. Internalization data were analyzed by first square root transforming the data to normalize variance (see Quantile-Quantile plots, Fig. 2). An average value for all fields was then calculated for each rat, followed by one-way ANOVA analysis to compare treatment groups.

Results

We initially wanted to determine whether imatinib blocked the development of morphine tolerance by altering MOR internalization. Rats received daily intrathecal injections of either morphine, vehicle, imatinib, or morphine + imatinib for 5 days. Since morphine is thought to poorly internalize the MOR (McPherson et al., 2010), a group of rats also received a high dose of DAMGO, a peptide known to induce MOR

TABLE 1
Sample size summary for each treatment group

	Acute Morphine			Chronic Morphine		
	# Fields	Total # Neurons	Average Neurons/Field	# Fields	Total # Neurons	Average Neurons/Field
Veh	22	274	13	22	470	21
MS	22	313	14	24	483	19
Im	24	428	19	24	451	19
MS + Im	22	248	12	27	499	17
D	21	706	32	24	795	33

	Acute Fentanyl			Chronic Fentanyl		
	# Fields	Total # Neurons	Average Neurons/Field	# Fields	Total # Neurons	Average Neurons/Field
Veh	19	690	36	19	415	33
F	18	709	42	17	558	19
Im	17	406	21	19	420	21
F + Im	18	743	41	18	689	28

D, DAMGO; F, fentanyl; Im, imatinib; MS, morphine sulfate; Veh, vehicle.

internalization (Trafton and Basbaum, 2004), as a positive control for subsequent immunohistochemical studies. TFL was then determined 40 minutes after each daily injection. Tolerance to morphine analgesia developed over 5 days, and tolerance was blocked by imatinib (Fig. 3A). Neither vehicle nor imatinib caused analgesia, whereas DAMGO elicited strong analgesia up to the TFL cutoff on all days. It is important to note that on day 1, morphine and morphine + imatinib caused equivalent amounts of analgesia (Fig. 3A). Taken together, these results confirm that imatinib does not have an analgesic effect and does not block tolerance by augmenting morphine analgesia, as previously reported (Wang et al., 2012).

To determine whether the inhibition of morphine analgesic tolerance caused by imatinib involved changes in receptor internalization, rat lumbar spinal cords were collected

40 minutes after one intrathecal injection with vehicle, morphine, imatinib, morphine + imatinib, or DAMGO. For chronic experiments, spinal cords were collected immediately after behavioral testing 40 minutes after injection on day 5. Lumbar spinal cord slices were immunostained as described in the *Materials and Methods* to specifically label neurons (anti-NeuN) and MORs (anti-MOR). Immunofluorescent images were acquired using a confocal microscope (Nikon A1) equipped with a 60 $\times$ oil immersion objective lens. Images from the medial, central, and lateral regions of left and right dorsal horns were imaged from each rat. In total, 21 to 25 Z-plane sections (Z-stacks) of 0.3 μ m were acquired (each image = 6.3–7.5 μ m). All exposure times and processing procedures were identical. Confocal images were then imported to Imaris $\times$ 64 9.0 software (Bitplane). NeuN and MOR signals were automatically reconstructed by the

Fig. 2. QQ plots of square root transformed MOR internalization data. All treatment groups had α values >0.05 , calculated using the Shapiro-Wilk test. F, fentanyl; F + Im, fentanyl + imatinib; Im, imatinib; MS, morphine; MS + Im, morphine + imatinib; Veh, vehicle.

Fig. 3. Imatinib blocks morphine tolerance without altering MOR internalization. (A) Imatinib prevents morphine tolerance. Rats were injected intrathecally with vehicle (Veh, 10% captisol), morphine (MS, 0.6 nmol), imatinib (Im, 10 µg), MS + Im, or DAMGO (1 µmol) for five consecutive days. TFLs were measured daily 40 minutes after injection. $N = 4$ rats per group. Data are presented as means $\pm$ S.D. (B) MOR internalization after acute morphine. Spinal cords were collected 40 minutes after a single intrathecal injection with vehicle (Veh, 10% captisol), morphine (MS, 0.6 nmol), imatinib (Im, 10 µg), MS + Im, or DAMGO (1 µmol; positive control); processed for IHC; and analyzed as described in the text. $N = 4$ rats per group, with six to seven fields analyzed per rat. Data are presented as means $\pm$ S.D. One-way ANOVA on square root transformed data, $F(4, 15) = 7.30$, $P < 0.002$. Dunnett's post hoc test; ** $P < 0.001$ vs. vehicle. (C) Representative neurons from each acute morphine treatment group. (D) MOR internalization after chronic morphine. Rats were injected for 5 days with either vehicle (Veh, 10% captisol), morphine (MS, 0.6 nmol), imatinib (Im, 10 µg), MS + Im, or DAMGO (1 µmol; positive control). Spinal cords were collected 40 minutes after the last injection, processed for IHC, and analyzed as described in the text. $N = 4$ rats per group, with six to seven fields analyzed per rat. Data are presented as means $\pm$ S.D.; one-way ANOVA on square root transformed data, $F(4, 15) = 2.00$, $P < 0.145$. (E) Representative neurons from each chronic morphine treatment group. BSL, baseline.

software. To analyze the amount of MOR internalized in neuronal cell bodies, we developed an automated, unbiased software workflow. In the first stage, neurons were identified based on the anti-NeuN fluorescent signal, using the surface tool as follows: 1) Local contrast settings were automatically generated by Imaris based on local maximum and minimum intensity threshold values of the anti-NeuN signal. 2) Neuronal cell body sizes were manually estimated over a sampler of neurons from vehicle control group images. Average cell size was then set at 10 µm, and surface smoothing was set at 1 µm. These settings were applied to all images of the entire current study. 3) The anti-NeuN signal threshold was calculated by averaging the automatically generated signal thresholds from anti-NeuN signal in vehicle control group images. This average threshold was calculated for each experiment and applied to all the images of the same experiment. 4) Once generated, three-dimensional (3D) neuronal surfaces were manually verified. Neuronal clusters, identified by the presence of more than one DAPI nuclei per surface, were manually divided using the Imaris cutting tool.

In the second stage, MOR clusters were identified based on the anti-MOR signal, using the spots identification tool in the following manner: 1) Similar to the protocol used above for surfaces, local contrast settings for spots were automatically generated by Imaris based on local maximum and minimum intensity threshold values of the anti-MOR signal. 2) The

average size of MOR clusters was estimated by manually measuring the size of anti-MOR signal dots from a sample of images in the vehicle control group. Average cluster size was set at 0.6 µm and applied to all images of the entire current study. After generating 3D neuronal surfaces and 3D MOR spots, two MATLAB algorithms (paired with Imaris) were subsequently applied. First, we used the "surface identification" function, which enabled us to individually identify each surface and measure its volume (cubic micrometer). Second, we used the "split spots into surfaces" function to accurately count the number of MOR spots present in each neuronal surface previously individually identified. To finish, we proceeded to average the number of MOR spots per 100 µm³ neuronal cell volume in each image analyzed. Neuronal surfaces that did not contain any MOR spots were excluded. On average, 25 neurons per field contained at least one MOR spot (Table 1). Our method enabled us to analyze over 200 neurons in each treatment group (Table 1).

We found that the MOR was not internalized by acute (Fig. 3, B and C) or chronic (Fig. 3, D and E) morphine injections. Although imatinib was able to completely block morphine tolerance (Fig. 3A), it did not alter the amount of MOR internalization when coadministered with morphine acutely (Fig. 3, B and C) or chronically (Fig. 3, D and E). Acute and chronic injections of imatinib did not alter MOR internalization, whereas acute, but not chronic, DAMGO injection induced significant internalization.

We then wanted to determine whether imatinib could block tolerance to fentanyl, an opioid known to induce MOR internalization. If tolerance was inhibited, we then wanted to determine whether this was associated with alterations in MOR internalization. Fentanyl is a synthetic opioid that causes MOR internalization (Hashimoto et al., 2006). To explore this issue, rats underwent daily subcutaneous injections of vehicle, fentanyl, imatinib, or fentanyl + imatinib for 4 days. TFL was tested 15 minutes after the daily injections. Repeated fentanyl injections induced complete tolerance by day 4, whereas imatinib blocked this effect (Fig. 4A). As with morphine, imatinib did not alter the analgesic effect of fentanyl on day 1, again indicating that imatinib does not appear to inhibit tolerance by augmenting morphine analgesia.

To determine whether fentanyl tolerance inhibition by imatinib involved changes in the degree of receptor internalization, rat lumbar spinal cords were collected 15 minutes after 1 day of subcutaneous injections with vehicle, fentanyl, imatinib, or fentanyl + imatinib for acute experiments. For chronic studies, spinal cords were collected immediately after behavioral testing 15 minutes after injection on day 4. Spinal

cord slices were immunostained with anti-NeuN and anti-MOR antibodies and analyzed as described in the *Materials and Methods*. We found that the MOR internalization was significantly induced by acute (Fig. 4, B and C), but not chronic (Fig. 4, D and E), fentanyl injections. Imatinib did not significantly alter the degree of MOR internalization induced by fentanyl when coadministered with fentanyl acutely (Fig. 4, B and C) or chronically (Fig. 4, D and E). Again, acute and chronic injections of imatinib did not alter MOR internalization.

We then tested whether imatinib could block tolerance development to other clinically used opioids with varying pharmacologic and internalization-inducing properties. We evaluated two semisynthetic/poorly internalizing opioids, hydromorphone and oxycodone, and one full synthetic/highly internalizing opioid, sufentanil (Koch et al., 2005; Gutstein and Akil, 2006). As before, animals were injected subcutaneously with either vehicle, an opioid, imatinib, or opioid + imatinib daily for 4 days. TFL was measured daily at the time after injection that peak analgesia was observed in preliminary experiments (hydromorphone: 30 minutes, oxycodone: 15 minutes, sufentanil: 15 minutes). We found that imatinib

Fig. 4. Imatinib blocks fentanyl tolerance without altering MOR internalization. (A) Imatinib prevents fentanyl tolerance. Rats were injected subcutaneously with vehicle (Veh; 10% captisol), fentanyl (F, 25 $\mu\text{g/kg}$), imatinib (Im, 5 mg/kg), or fentanyl + imatinib (F + Im) for four consecutive days. TFLs were measured daily 15 minutes after injection. $N = 6$ rats per group. Data are presented as means $\pm$ S.D. (B) MOR internalization after acute fentanyl. Spinal cords were collected 15 minutes after a single subcutaneous injection with vehicle (Veh, 10% captisol), fentanyl (F, 25 $\mu\text{g/kg}$), imatinib (Im, 5 mg/kg), or fentanyl + imatinib (F + Im); processed for IHC; and analyzed as described in the text. $N = 3$ rats per group, with six to seven fields per rat. Data are presented as means $\pm$ S.D. One-way ANOVA on square root transformed data, $F(3, 8) = 12.59$, $P = 0.0021$. Dunnett's post hoc test; * $P < 0.05$; ** $P < 0.001$ vs. vehicle. (C) Representative neurons from each acute fentanyl treatment group. (D) MOR internalization after chronic fentanyl. Rats were injected subcutaneously with vehicle (Veh; 10% Captisol), fentanyl (F, 25 $\mu\text{g/kg}$), imatinib (Im, 5 mg/kg), or fentanyl + imatinib (F + Im) for four consecutive days. Spinal cords were collected 15 minutes after the last injection, processed for IHC, and analyzed as described in the text. $N = 4$ rats per group, with six to seven fields per rat. Data are presented as means $\pm$ S.D. One-way ANOVA on square root transformed data, $F(3, 8) = 6.11$, $P = 0.0182$. Dunnett's post hoc test; * $P < 0.05$ vs. vehicle. (E) Representative neurons from each chronic fentanyl treatment group.

Fig. 5. Imatinib prevents analgesic tolerance to clinically used opioids with differing pharmacological properties. (A) Imatinib prevents tolerance to hydromorphone. Rats received daily subcutaneous injections of either hydromorphone (0.4 mg/kg), imatinib (5 mg/kg), or hydromorphone + imatinib for 4 days. TFLs were measured daily 30 minutes after injection. $N = 6$ rats per group. Two-way mixed-effects ANOVA, time $\times$ treatment: $F(8, 60) = 33.00$, $P < 0.0001$. (B) Imatinib prevents tolerance to oxycodone. Rats received daily subcutaneous injections of either oxycodone (1 mg/kg), imatinib (5 mg/kg), or oxycodone + imatinib for 4 days. TFLs were measured daily 15 minutes after injection. $N = 7$ to 8 rats per group. Two-way mixed-effects ANOVA, time $\times$ treatment: $F(8, 60) = 21.09$, $P < 0.0001$. (C) Imatinib prevents tolerance to sufentanil. Rats received daily subcutaneous injections of either sufentanil (2.5 μ g/kg), imatinib (5 mg/kg), or sufentanil + imatinib for 4 days. TFL were measured daily 15 minutes after injection. $N = 6$ rats per group. Two-way mixed-effects ANOVA, time $\times$ treatment: $F(8, 60) = 15.56$, $P < 0.0001$. For all figures, data are presented as means $\pm$ S.D.

blocked the development of tolerance to hydromorphone (Fig. 5A), oxycodone (Fig. 5B), and sufentanil (Fig. 5C).

Discussion

In our study, we developed an automated, unbiased method to analyze opioid-induced MOR internalization (Fig. 1). We studied morphine, an opioid known to poorly induce MOR internalization (Abbadie and Pasternak, 2001), and fentanyl, an opioid that robustly induces MOR internalization (Koch et al., 2005). We demonstrated that morphine tolerance was completely inhibited by imatinib, a PDGFR inhibitor, and that imatinib did not alter the degree of MOR internalization caused by morphine after acute or chronic morphine administration (Fig. 3). We also showed that analgesic tolerance to fentanyl was completely eliminated by imatinib and that imatinib did not alter the degree of MOR internalization induced by fentanyl (Fig. 4). We also found that imatinib blocked the development of tolerance to three other clinically used opioids with varying pharmacological and internalization properties (Drewes et al., 2013) (Fig. 4). Taken together, our results indicate that 1) the development of opioid tolerance in the spinal cord occurs independently of changes in MOR internalization, 2) tolerance can be blocked without altering MOR internalization, and 3) PDGFR signaling mediates tolerance development for a wide range of clinically used opioids. However, our observations do not preclude the possibility that MOR internalization could play a role in the development of opioid tolerance in higher brain regions (Cox and Crowder, 2004; Williams et al., 2013). It is unclear why chronic administration of agonists known to induce internalization acutely did not significantly increase internalization in our study. There are two possibilities: First,

we may have missed the peak time of internalization in the chronic study, because of either the delay induced by behavioral testing or the possibility that the kinetics of the internalization response could be altered by chronic drug administration. Second, it is also possible that chronic administration of DAMGO or fentanyl could decrease the internalization response. However, there is not clear data in support of this hypothesis (Koch and Holtt, 2008; Anselmi et al., 2013).

Studies investigating MOR internalization are painstaking and require a tremendous amount of personal effort. Automated methods have been recommended to improve reproducibility in preclinical science (McNutt, 2014; Begley and Ioannidis, 2015). A previous study developed an approach called quantitative automated microscopy to evaluate RTK-mediated signaling in pain sensitization (Andres et al., 2010). Although their analytical approach was somewhat different than ours, they identified two key concepts: namely, that the “eye-based” methods of identifying cells positive or negative for a marker (in our case, immunofluorescence) may not be accurate and that it is critical to define threshold criterion to distinguish signal from background (Andres et al., 2010). These concepts suggest that any manual evaluation of marker intensity could be suspect but do not provide a direct explanation for the contradictory findings of previous studies. Another possible confound is that, given the heterogeneity of cells in living tissue, large sample sizes will likely be needed to detect statistically significant changes in a population. Our automated analyses were based on sample sizes from approximately 250 to greater than 750 neurons per treatment group (Table 1). Previous important studies of the effects of internalization upon opioid tolerance evaluated 10-fold fewer cells, with the majority studying between 10 and 20 cells per

treatment group (Trafton et al., 2000; He et al., 2002; Trafton and Basbaum, 2004; Silva-Moreno et al., 2012).

The role MOR internalization plays in opioid tolerance is complicated and controversial. Classically, opioid tolerance was thought to be due to agonist-induced desensitization and internalization of the receptor. After agonist binding, G-protein coupled receptor kinases are rapidly recruited to and phosphorylate the MOR. This increases the affinity of the receptor for β -arrestins, which uncouple the receptors from G proteins, leading to desensitization and receptor internalization (Williams et al., 2013). However, it is clear that tolerance can be induced by opioid agonists with varying abilities to induce receptor internalization. For example, morphine can cause profound tolerance without inducing receptor internalization. It has been suggested that this could be due to preferential engagement of β -arrestin 2 by morphine (Eisinger et al., 2002), whereas drugs with high intrinsic efficacy that induce strong MOR internalization interact with both β -arrestin 1 and β -arrestin 2 (Groer et al., 2011). However, morphine has been shown to induce MOR internalization in adrenergic medullary neurons (Drake et al., 2005) and the dendrites but not the cell bodies of nucleus accumbens neurons (Haberstock-Debic et al., 2003, 2005), suggesting that morphine does have the ability to cause MOR internalization depending on the expression levels and/or subcellular localization of relevant signaling molecules (e.g., G-protein coupled receptor kinases, β -arrestins) present in specific regions.

The concept of biased agonism is an evolution of these findings. It has been postulated that different opioid agonists induce unique conformational changes in the receptor, leading to preferential interactions with different subsets of cellular signaling molecules (Pradhan et al., 2012; Ehrlich et al., 2019), which could preferentially mediate different opioid effects. Importantly, Schmid et al. (2017) synthesized a series of MOR agonists that exhibited marked functional selectivity for either G protein- or β -arrestin-mediated signaling. The investigators found that β -arrestin-preferring compounds were more likely to induce respiratory depression, whereas bias toward G protein signaling decreased the amount of respiratory depression relative to analgesia, markedly improving the therapeutic index.

An assumption implicit in the classic model of opioid tolerance is that receptor internalization leads to receptor degradation in lysosomes and loss of functional surface receptors. However, studies have shown that internalized MOR receptors mainly undergo a cycle of dephosphorylation, reactivation, and recycling of active receptors into the cell membrane (Law et al., 2000; Tanowitz and von Zastrow, 2003). This concept underlies the hypothesis introduced by Whistler and colleagues (He et al. 2002) that sustained MOR signaling, rather than termination of signaling, causes opioid tolerance. They posited that the tolerance liability of a MOR agonist would be its relative activity versus its ability to induce endocytosis ("RA/VE") (Kieffer and Evans, 2002). In this formulation, an effective agonist that poorly induced internalization (such as morphine) would induce profound tolerance, whereas an agonist that induced robust internalization would have a lower tolerance liability. To support this hypothesis, He et al. (2002) presented data showing that administering a small, subanalgesic dose of the strongly internalizing MOR agonist DAMGO with morphine markedly increased MOR internalization in the spinal cord and

decreased tolerance. However, other studies were unable to replicate these findings (Bailey et al., 2003), and additional work indicated that chronic morphine treatment caused decreased, rather than sustained, MOR signaling (Bailey et al., 2004; Dang and Williams, 2005). A core assumption of the RA/VE hypothesis is that endocytosis has a direct relationship to tolerance. Our work, and that of other investigators, suggests that robust tolerance can occur regardless of the receptor internalizing properties of an opioid agonist. For example, Narita et al. (2006) showed that the highly internalizing agonist etorphine induced a number of receptor trafficking proteins, whereas morphine did not. In contrast, morphine, but not etorphine, induced elevations of glial fibrillary acidic protein. Treatment with the glial inhibitor pentophylline blocked morphine, but not etorphine, tolerance. Conversely, intrathecal injection of astrocytes and conditioned medium accelerated morphine tolerance but did not impact tolerance to etorphine. A review by Cahill et al. (2016) nicely summarizes the roles of neural circuit adaptations in the genesis of opioid tolerance.

In sum, the literature suggests that depending on the neural context, multiple mechanisms may be responsible for the reduction in opioid efficacy observed as tolerance develops to different opioid agonists. Our findings suggest that although opioid tolerance in the spinal cord is not dependent upon MOR internalization, both prior theories of tolerance development could be compatible with our findings. Given that imatinib blocked tolerance to a variety of internalizing and noninternalizing opioid agonists, it appears that PDGFR- β signaling is engaged by MOR activation regardless of the ligand's internalization properties or signaling bias. This supports the idea that PDGFR signaling is a central mediator underlying opioid analgesic tolerance. Additional experiments will be required to test this hypothesis.

It is important to note that chronic opioid administration induces sexually dimorphic responses. Observed differences can be inconsistent between studies and may depend upon the species or animal strain studied, drug dosing, route of administration, testing paradigm used, and hormonal state of the animals (Dahan et al., 2008). For example, systemic administration of morphine to rats resulted in more tolerance development in male than female rats (Lloyd et al., 2008; Zhang et al., 2012). However, spinal administration of morphine to mice induced greater tolerance to a systemic challenge of morphine in female animals (Hopkins et al., 2004), whereas intracerebroventricular injection induced the same magnitude of tolerance in male and female mice (Kest and Hopkins, 2001). Gonadal hormones may also play an important role in tolerance development, as castration has been shown to eliminate sex differences in tolerance (Mousavi et al., 2007). An important direction for future studies will be to determine whether our observations that tolerance is blocked by PDGFR inhibitors and does not appear to require receptor internalization also apply to female animals.

The PDGFR- β is a member of the RTK family (Heldin and Lennartsson, 2013). GPCRs are known to transactivate RTKs to induce downstream signaling (Shah and Catt, 2004). The MOR has previously been shown to activate the RTK's epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor in vitro (Belcheva et al., 2001, 2002), and we previously demonstrated MOR activation of the PDGFR- β (Wang et al., 2012). It is also known that RTKs

can directly feed back and affect GPCR signaling (Delcourt et al., 2007; Di Liberto et al., 2019). Previous work has shown direct effects of EGFR signaling upon the MOR (Clayton et al., 2010). These findings further support the possibility of selective RTK mediation of opioid effects.

The RTK family is quite large, and there are several RTKs that are closely related to the PDGFR (Lemmon and Schlessinger, 2010). This raises the question of whether tolerance is a selective consequence of PDGFR signaling or whether other related RTKs could also modulate tolerance. We recently reported that vascular endothelial growth factor inhibition blocked morphine tolerance (Lopez-Bellido et al., 2019). We have also observed that inhibition of EGFR signaling also blocks tolerance (Puig et al., 2020). Other investigators have shown that inhibition of signaling by the fibroblast growth factor receptor (Fujita-Hamabe et al., 2011) and the Ephrin receptor (Ephrin B) (Liu et al., 2011) also blocks morphine tolerance. Taken together, these results indicate that opioid tolerance can be caused by MOR signaling through several related RTKs. Additional studies will be needed to further define the generalizability of this phenomenon.

In conclusion, by utilizing an automated, unbiased analysis system, we have established that spinal opioid tolerance is not dependent upon MOR internalization. We have also shown that inhibition of PDGFR- β signaling blocks tolerance development in the spinal cord for a wide variety of opioid agonists with diverse properties, indicating that PDGFR- β signaling could be a central mechanism underlying opioid tolerance. These findings suggest the exciting possibility that opioid tolerance can be directly eliminated without altering opioid analgesia. This could potentially eliminate the dose escalation caused by tolerance, thereby dramatically increasing the safety and efficacy of opioid treatment.

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Authorship Contributions

Participated in research design: Puig, Barker, Gutstein.

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