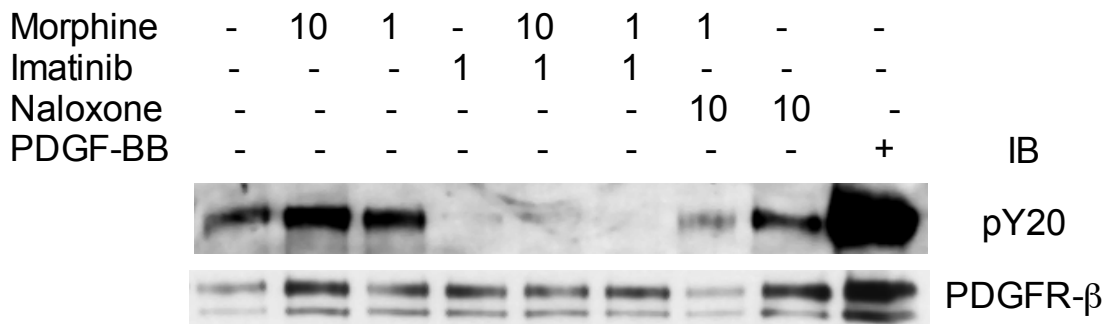


Blockade of PDGFR- β Activation Eliminates Morphine Analgesic Tolerance

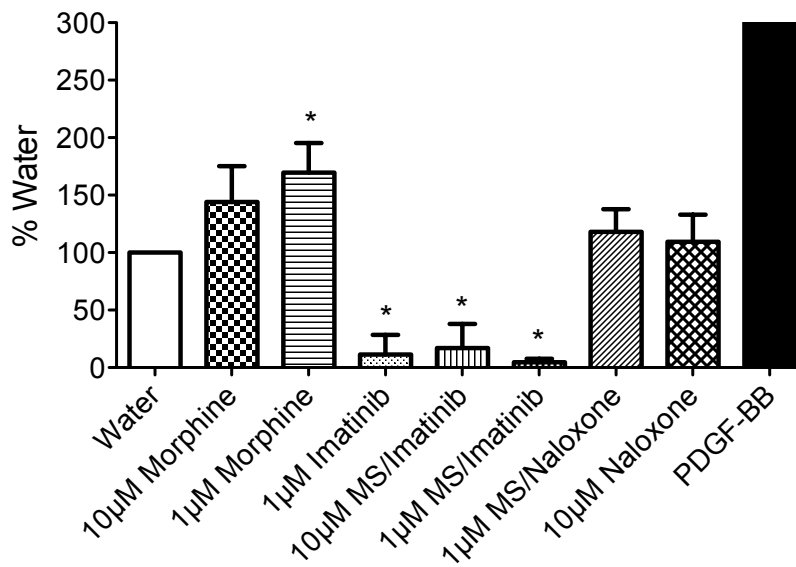
Yan Wang, Katherine Barker, Shaping Shi, Miguel Diaz, Bing Mo, and Howard B. Gutstein

Supplementary Figures and Legends Methods

a



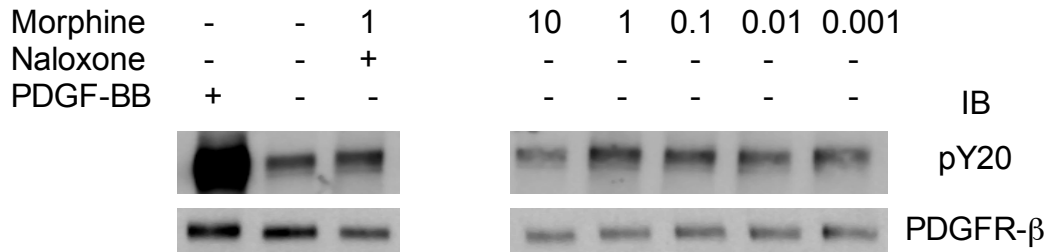
b



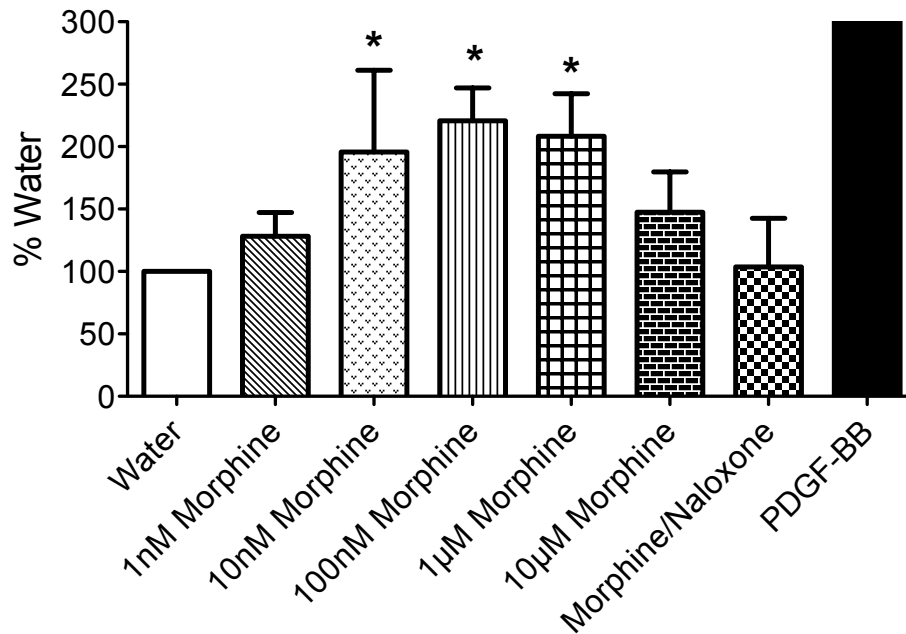
Supplementary Figure 1

Supplementary figure 1. Morphine-induced activation of PDGFR- β in C6 glioma cells was blocked by both naloxone and imatinib. Cells stably transfected with the MOR were treated with water, 10 μ M or 1 μ M morphine, 1 μ M imatinib, morphine + imatinib (MS/Imatinib), 10 μ M naloxone, or 1 μ M morphine + 10 μ M naloxone (MS/Naloxone) for 40 min. Treatment with platelet-derived growth factor-B homodimer (PDGF-BB) for 10 min. served as a positive control. Cells were then harvested, individual lysates prepared, and immunoprecipitation (IP) performed with anti-PDGFR- β . IPs were then split, with each half run on an individual SDS-PAGE gel. Gels were then immunoblotted (IB) with either anti-phosphotyrosine (pY20) or anti-PDGFR- β to control for IP efficiency. **a:** Representative IP/IB experiment. Drug doses shown in μ M above lanes. **b:** Graphic summary of the data. 1 μ M morphine caused a 70% increase in PDGFR- β phosphorylation. This activation was blocked by both naloxone and imatinib. As expected, imatinib also suppressed basal PDGFR- β phosphorylation. Data presented as mean \pm s.d. $F_{(7,16)} = 30.0$, $P < 0.0001$ (one-way ANOVA). * - $P < 0.05$ vs. water group by Dunnett's multiple comparison test. $n = 3$ independent samples per treatment group.

a



b



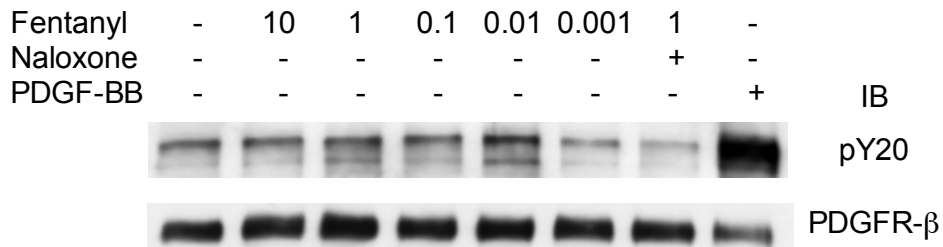
Supplementary Figure 2

Supplementary figure 2. Dose-dependence of PDGFR-β phosphorylation by

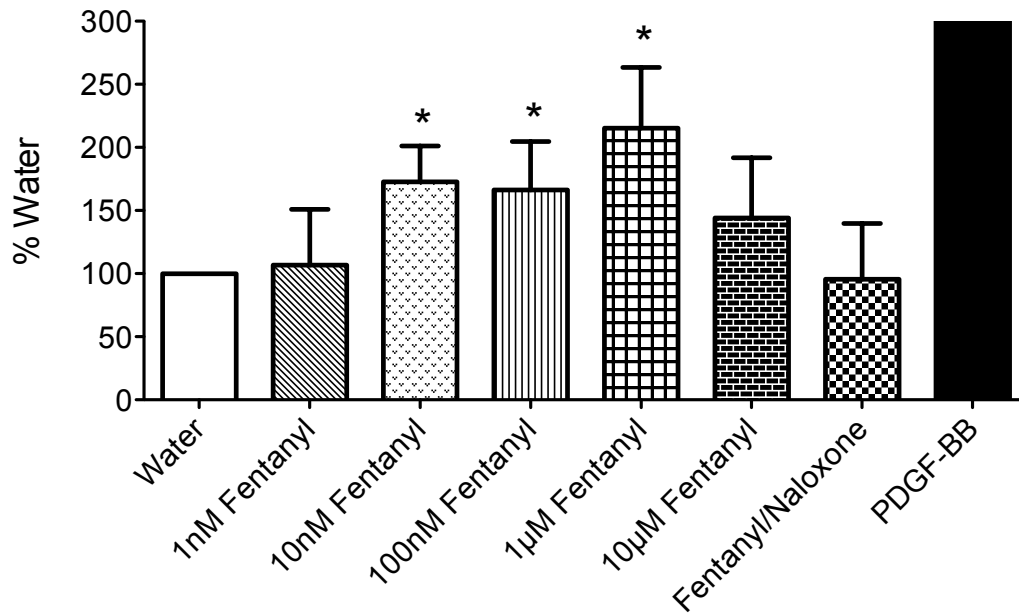
morphine: C6 cells stably expressing the MOR were treated with water or morphine

doses ranging from 0.001 μM to 10 μM for 40 min. 1 μM morphine + 10 μM naloxone was administered as a specificity control, and 4nM PDGFR-BB was applied as a positive control. Cells were then harvested, individual lysates prepared, and immunoprecipitation (IP) performed with anti-PDGFR- β . IPs were then split, with each half run on an individual SDS-PAGE gel. Gels were then immunoblotted (IB) with either anti-phosphotyrosine (pY20) or anti-PDGFR- β to control for IP efficiency. **a:** Representative IP/IB experiment with irrelevant lanes removed. Morphine concentrations in μM are indicated above the blot. **b:** Graphic summary of the data. 0.01, 0.1, and 1 μM morphine doses induced an approximately 2-fold increase in PDGFR- β phosphorylation. This stimulation was naloxone reversible. Data presented as mean \pm s.d. $F_{(6,21)} = 7.85$, $P < 0.0002$ (one-way ANOVA). * - $P < 0.05$ vs. water group by Dunnett's multiple comparison test. $n = 4$ independent samples per treatment group.

a



b

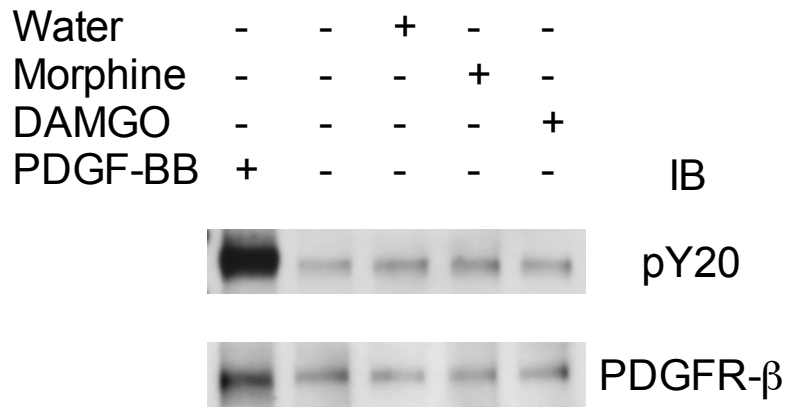


Supplementary Figure 3

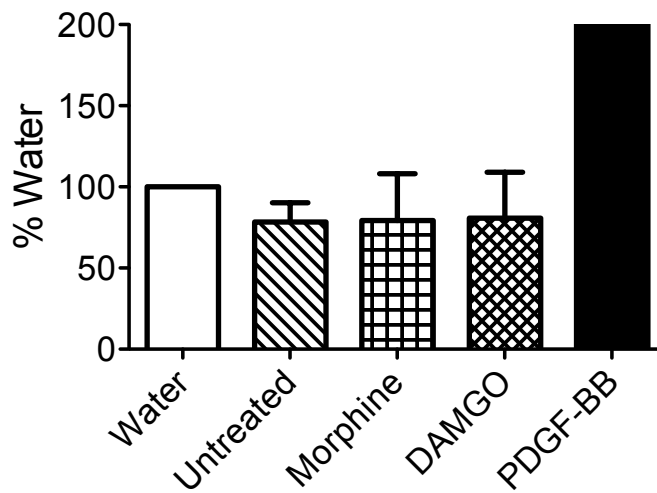
Supplementary figure 3: Dose-dependence of PDGFR- β phosphorylation by fentanyl: C6 cells stably expressing the MOR were treated with water or fentanyl doses ranging from 0.001 μ M to 10 μ M for 40 min. 1 μ M fentanyl + 10 μ M naloxone was administered as a specificity control, and 4 nM PDGFR-BB was applied as a positive

control. Cells were then harvested, individual lysates prepared, and immunoprecipitation (IP) performed with anti-PDGFR- β . IPs were then split, with each half run on an individual SDS-PAGE gel. Gels were then immunoblotted (IB) with either anti-phosphotyrosine (pY20) or anti-PDGFR- β to control for IP efficiency. **a:** Representative IP/IB experiment. Fentanyl concentrations in μM are indicated above the blot. **b:** Graphic summary of the data. 0.01, 0.1, and 1 μM fentanyl doses induced a significant increase in PDGFR- β phosphorylation. This stimulation was naloxone reversible. Data presented as mean \pm s.d. $F_{(6,33)} = 7.55$, $P < 0.0001$ (one-way ANOVA). * - $P < 0.05$ vs. water group by Dunnett's multiple comparison test. $n = 5-6$ independent samples per treatment group.

a



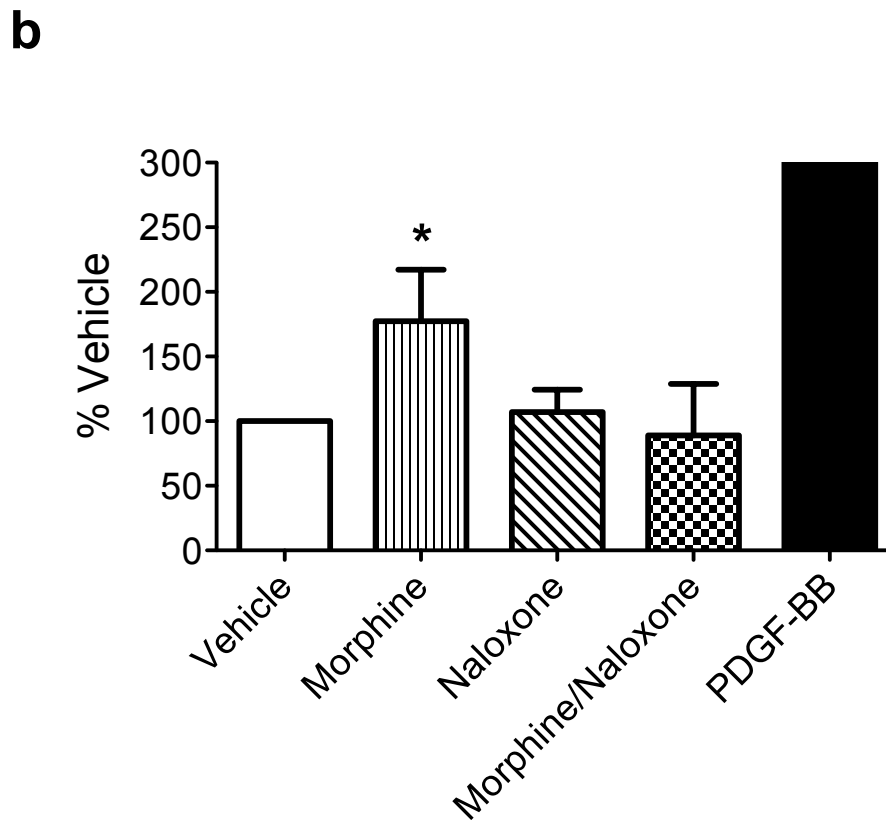
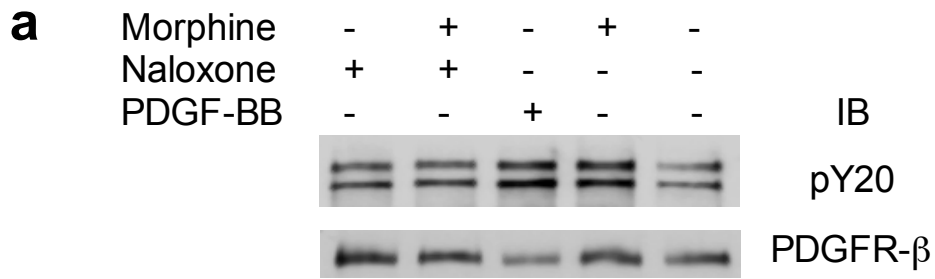
b



Supplementary Figure 4

Supplementary figure 4. MOR expression is necessary for opioid-induced phosphorylation of the PDGFR- β : C6 cells not transfected with the MOR were treated with water, 1 μ M morphine, or 1 μ M [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) for 40 min. 4nM PDGF-BB applied for 10 min. served as a positive control, and

untreated cells as an additional negative control. Cells were then harvested, individual lysates prepared, and immunoprecipitation (IP) performed with anti-PDGFR- β . Samples were then run on SDS-PAGE gels and immunoblotted (IB) with anti-phosphotyrosine (pY20). Blots were then stripped and reprobed with anti-PDGFR- β to control for IP efficiency. **a:** Representative IP/IB experiment. **b:** Graphic summary of the data. Neither morphine nor DAMGO induced PDGFR- β phosphorylation. Data presented as mean \pm s.d. $F_{(3,8)} = 0.57$ (n.s., one-way ANOVA). $n = 3$ independent samples per treatment group.

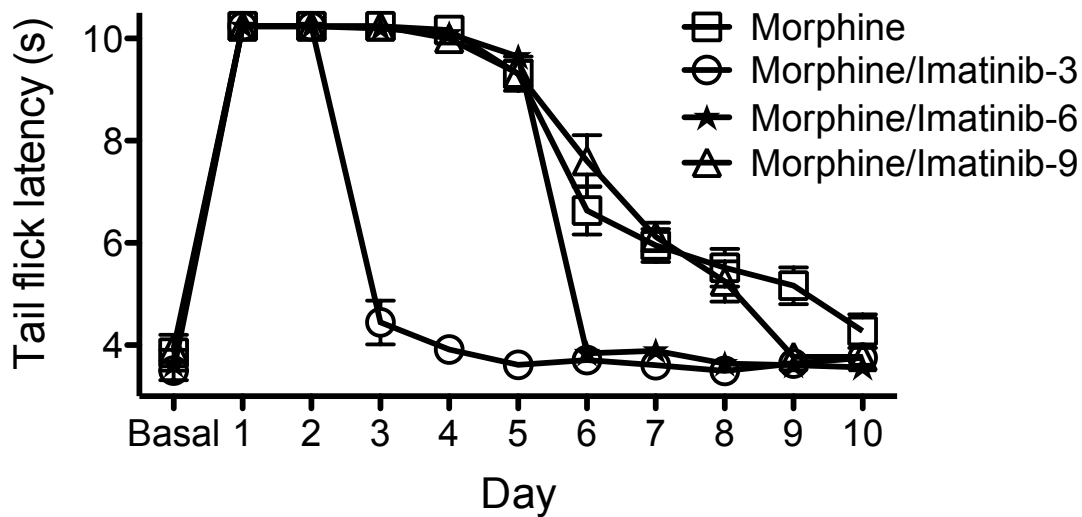


Supplementary Figure 5

Supplementary figure 5. Morphine-induced activation of PDGFR- β in rat substantia gelatinosa was blocked by naloxone. Animals received intrathecal injection of 0.6 nmol morphine, 6 nmol naloxone, morphine + naloxone, or a 1:1 mixture of aCSF/20%

sulfobutylether-7- β -cyclodextrin (vehicle) for 40 min. Intrathecal injection of 10 pmol platelet-derived growth factor-B homodimer (PDGF-BB) for 10 min. served as a positive control. Spinal cords were rapidly removed, snap frozen, and sectioned. The substantia gelatinosa was then microdissected, individual lysates prepared for each animal, and immunoprecipitation (IP) performed with anti-PDGFR- β . IPs were then split, with each half run on an individual SDS-PAGE gel. Gels were then immunoblotted (IB) with either anti-phosphotyrosine (pY20) or anti-PDGFR- β to control for IP efficiency. **a:**

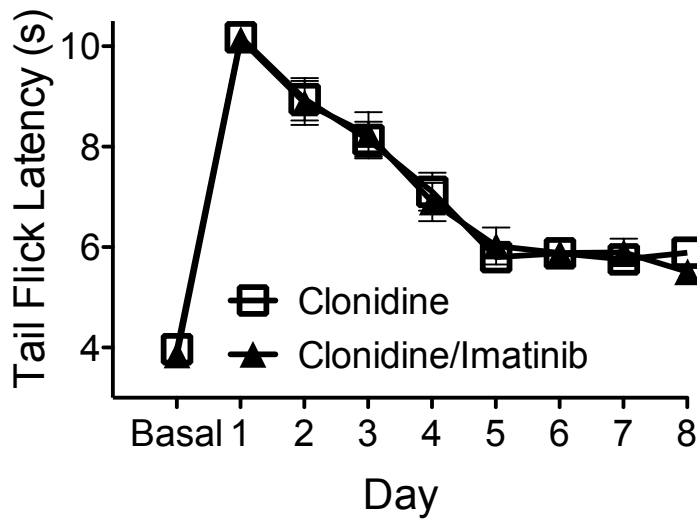
Representative IP/IB experiment. **b:** Graphic summary of the data. Morphine caused a 77% increase in PDGFR- β phosphorylation, which was naloxone reversible. Data presented as mean \pm s.d. $F_{(3,8)} = 5.51$, $P < 0.03$ (one-way ANOVA). * - $P < 0.05$ vs. vehicle group by Dunnett's multiple comparison test. $n = 3$ independent animals per treatment group.



Supplementary Figure 6

Supplementary figure 6. Imatinib did not become analgesic as tolerance

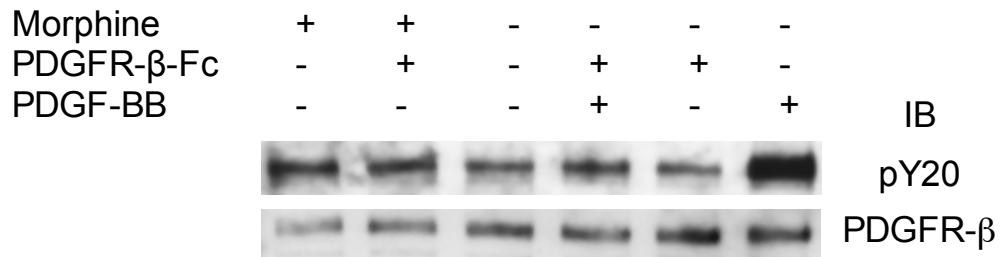
developed. Four groups of animals were treated twice daily with subcutaneous injection of either 1) 10 mg/kg morphine; 2) morphine on days 1-2 followed by 5.0 mg/kg imatinib on days 3-10 (Morphine/Imatinib-3); 3) morphine on days 1-5 followed by 5.0 mg/kg imatinib on days 6-10; or 4) morphine on days 1-8 followed by 5.0 mg/kg imatinib on days 9-10. All drugs were dissolved in 0.45% normal saline/10% sulfobutylether-7- β -cyclodextrin vehicle. All data presented as s \pm s.e.m. Treatment $F_{(3,44)} = 97.1$, Day $F_{(10,440)} = 717$, Interaction $F_{(30,440)} = 50.6$, all $P < 0.001$ (2-way ANOVA); n = 12 animals per treatment group.



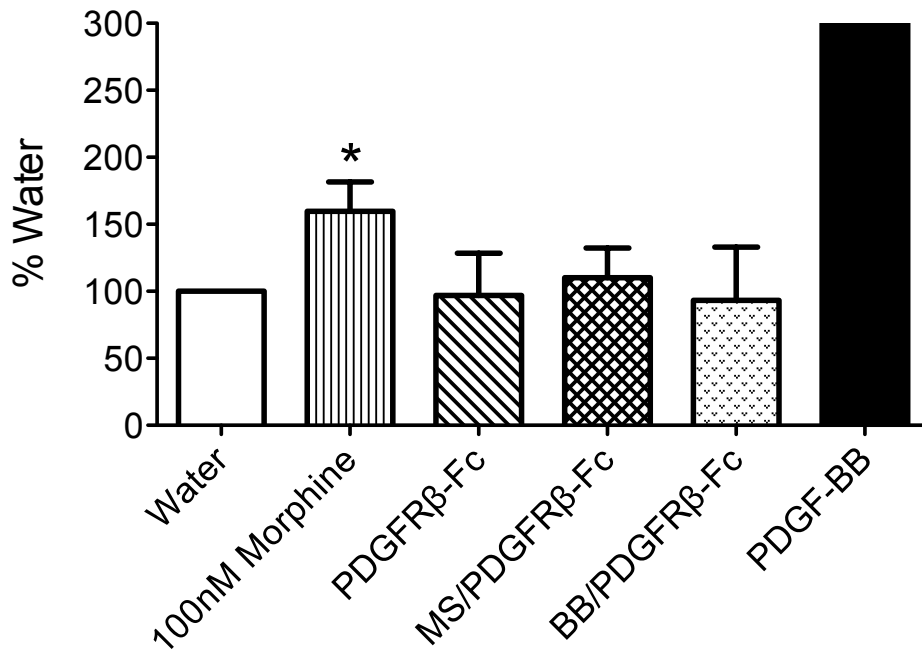
Supplementary Figure 7

Supplementary figure 7. Imatinib did not inhibit analgesic tolerance to clonidine. Animals were treated daily with i.t. injection of either 5 μ g clonidine or clonidine + 10 μ g imatinib for 10 days. All drugs were dissolved in a 1:1 mixture of aCSF/20% sulfobutylether-7- β -cyclodextrin vehicle. All data presented as s \pm s.e.m. Treatment $F_{(1,22)} = 0.02$ (n.s.), Day $F_{(8,176)} = 126$ ($P < 0.0001$), Interaction $F_{(8,176)} = 0.29$ (n.s.) (2-way ANOVA); n = 12 animals per treatment group.

a



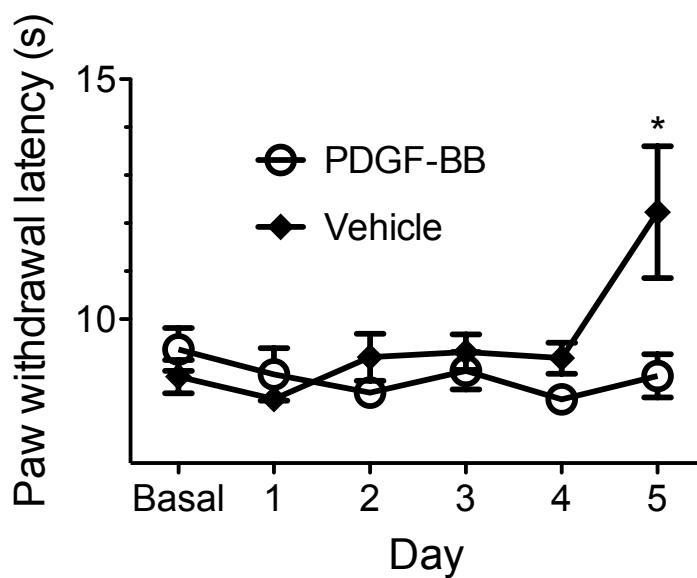
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Supplementary Figure 8

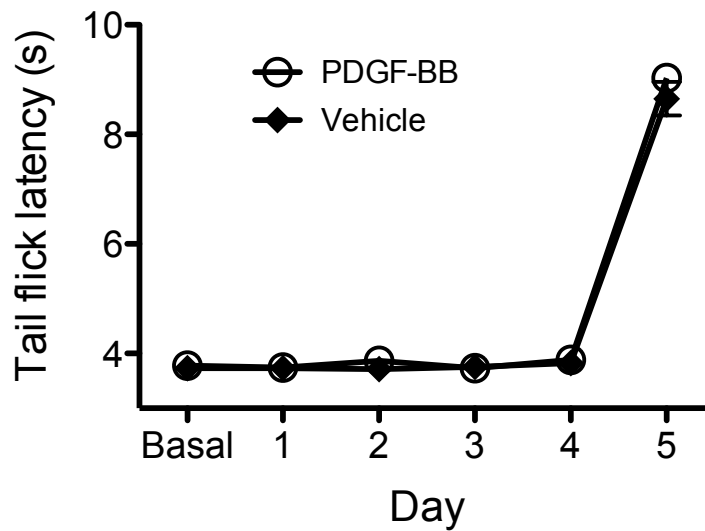
Supplementary figure 8. PDGFR- β -Fc fragment blocked morphine-induced phosphorylation of PDGFR- β in vitro: C6 cells stably expressing the MOR were treated for 40 min. with water, 100nM morphine, 5 μ g/ml PDGFR- β -Fc fragment

(PDGFR- β -Fc), morphine + PDGFR- β -Fc. Cells were also treated for 10 min. with 4nM PDGFR-BB as a positive control, and PDGF-BB + PDGFR- β -Fc as an additional control for growth factor scavenging. Cells were harvested, individual lysates prepared, and immunoprecipitation (IP) was performed with anti-PDGFR- β . IPs were split, with each half run on an individual SDS-PAGE gel. Gels were immunoblotted (IB) with either anti-phosphotyrosine (pY20) or anti-PDGFR- β to control for IP efficiency. **a:** Representative IP/IB experiment. **b:** Graphic summary of the data. Morphine caused a 60% increase in PDGFR- β activation, which was blocked by PDGFR- β -Fc fragment. PDGFR- β -Fc also completely blocked PDGF-BB-induced phosphorylation of PDGFR- β . Data presented as mean \pm s.d. $F_{(3,12)} = 6.99$, $P < 0.01$ (one-way ANOVA). * - $P < 0.05$ vs. vehicle by Dunnett's multiple comparison test. $n = 4$ independent replicates per treatment group.



Supplementary Figure 9

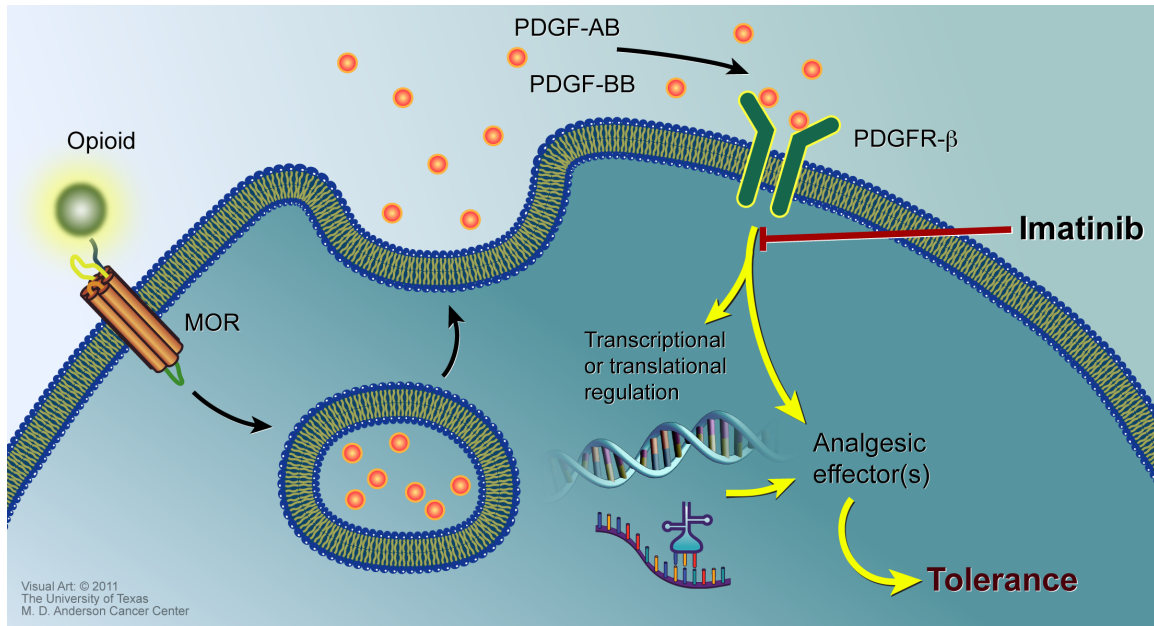
Supplementary figure 9. PDGF-BB did not cause thermal hyperalgesia. Animals were treated with intrathecal (i.t.) injection of either 5 pmol PDGF-BB or vehicle alone for four days. On day 5, all animals received i.t. injection of 0.6 nmol morphine. All drugs were dissolved in a 1:1 mixture of aCSF/20% sulfobutylether-7- β -cyclodextrin vehicle. All data presented as s \pm s.e.m. Treatment $F_{(1,54)} = 7.83$, Day $F_{(5,54)} = 3.36$, Interaction $F_{(5,54)} = 3.87$; all $P < 0.01$ (2-way ANOVA). * - $P < 0.001$ vs. vehicle group by Bonferroni post-hoc test. n = 6 animals per treatment group.



Supplementary Figure 10

Supplementary figure 10. PDGF-BB did not cause analgesic tolerance to clonidine.

Animals were treated with i.t. injection of either 5 pmol PDGF-BB or vehicle alone for four days. On day 5, all animals received 2 μ g clonidine. All drugs were dissolved in a 1:1 mixture of aCSF/20% sulfobutylether-7- β -cyclodextrin vehicle. All data presented as s \pm s.e.m. Treatment $F_{(1,95)} = 1.94$ (n.s.), Day $F_{(5,95)} = 534$ ($P < 0.0001$), Interaction $F_{(5,95)} = 0.66$ (n.s.) (2-way ANOVA); n = 9 animals per treatment group.



Supplementary Figure 11

Supplementary figure 11. Postulated relationship between PDGFR- β signaling and opioid tolerance. Opioids bind to the mu opioid receptor (MOR), which stimulates the release of the PDGFR- β receptor ligands PDGF-BB and PDGF-AB. We postulate that PDGFR- β inhibition reverses the behavioral expression of opioid tolerance via two distinct mechanisms: a rapid effect responsible for the bulk of the reversal, and a slower process that enables complete restoration of analgesia. The rapid phase could be due to post-translational modification of analgesic effector(s) after PDGFR- β antagonist administration, while changes in transcriptional or translational regulation of effector molecules could account for delayed effects. Please see the text for additional details.

Methods

Animals

Male Sprague-Dawley rats (175-200 g, Harlan) were housed in groups of three in cages on a 12-h light/dark cycle with *ad libitum* access to food and water. Rats were allowed to habituate in the colony room for one week before experimental manipulations were undertaken. All protocols were approved by our Institutional Animal Care and Use Committee.

Drug administration

Intrathecal (i.t) drug administration: Drugs were dissolved in aCSF (126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.4 mM CaCl₂, 11 mM of glucose, and 25 mM of NaHCO₃, saturated with 5% CO₂ in 95% O₂, and adjusted to a pH value of 7.3–7.4) or a 1:1 mixture of aCSF with 10% sulfobutylether-7-β-cyclodextrin (Captisol®; CyDex). Morphine was obtained from the MD Anderson Cancer Center pharmacy, clonidine from Sigma and imatinib from LC Laboratories. Recombinant rat platelet-derived growth factor B homodimer (PDGF-BB) and recombinant Human PDGFRβ-Fc chimera (both from R&D Systems) were dissolved in PBS with 0.1% BSA at 100μg/ml and stored at -80°C until use. Drugs were administered daily via intermittent lumbar puncture (LP) as previously described¹.

Subcutaneous (s.c.) drug administration: All drugs were administered in a vehicle of 0.45% normal saline/10% sulfobutylether-7-β-cyclodextrin. 75 mg morphine pellets and placebo pellets (NIDA) were implanted s.c. under isoflurane anesthesia.

Analgesic testing

Analgesia was assessed using the radiant heat tail-flick latency or paw withdrawal latency tests. Animals were placed in Plexiglas cages (9×22×25 cm) on a modified Hargreaves Device with a constant surface temperature of 30°C². Rats were habituated to the device for 3 days before testing and for 30 min before each test session. A hot lamp was focused on the tail and reflex withdrawal time was determined by a photocell. 10 sec. was used as a cutoff time to avoid damaging the tail. Three measurements were taken from 3 different areas of the tail, and the results averaged. TFLs were measured 45 min. after i.t. and 30 min. after s.c. injection. The same device and similar methods were used for PWL, except that the cutoff time was set to 20 sec.

C6 Glioma cell culture and treatment

Rat C6 glioma cells with mu opioid receptors stably introduced³ were maintained in 10% fetal calf serum (FCS) in Dulbecco's modified Eagle medium (DMEM) containing 500µg/mL of G418. Cells were grown in 15 cm dishes in 95% air, 5% carbon dioxide at 37°C until confluent. Cells were then stimulated either with morphine, fentanyl, naloxone, imatinib, or various combinations for time points ranging from 10 minutes to one hour. Water was used as a negative control, and 20nM PDGF-BB applied for 10 minutes as a positive control. After treatment, cells were washed twice with ice-cold PBS then harvested 2 ml ice cold Gentle Soft lysis buffer (10mM NaCl, 5mM EDTA pH 7.5, 20mM Pipes pH 7.5, 0.5% NP-40, protease inhibitor cocktail (Sigma), and 1 mM sodium orthovanadate). Lysates were prepared by homogenizing the cell suspension for 10 x 2s bursts on ice with a PowerGene 700 homogenizer (Fisher) and then sonicating on ice with a Sonic Dismembrator 60 (Fisher) for 20 x 2s bursts. Lysates were then cleared by centrifugation at 13,000 × g at 4°C for 10 min.

Substantia gelatinosa dissection from lumbar spinal cord

Rats were euthanized by decapitation under anesthesia and the lumbar spinal cord was quickly removed and snap-frozen in isopentane (-70 °C), then stored at -80°C until use. Frozen spinal cords were then sliced into 2mm segments and the substantia gelatinosa region microdissected using a modification of the transillumination method described by Cuello⁴. Tissues were then processed in Gentle Soft lysis buffer as described above.

Immunoprecipitation and immunoblotting.

PDGFR- α or PDGFR- β were immunoprecipitated by incubating 1ml cell or tissue lysate with 1 μ g of either anti-PDGFR- α (sc-338, Santa Cruz) or anti-PDGFR- β (sc-432, Santa Cruz) at 4°C overnight, followed by addition of 50 μ l of a 50% slurry of protein – A-Sepharose beads (Sigma) at 4°C for 2 hours. After three washes with Gentle Soft lysis buffer, pelleted beads were resuspended in 2X Gel loading buffer 20% Glycerol, 8% SDS, 0.5M DTT, 0.25 M Tris (pH=6.8), and 0.02mg/ml bromophenol blue) and denatured at 95°C for 5 minutes. Immunoprecipitated proteins were separated by 8% SDS-PAGE, and then transferred to nitrocellulose membranes by electroblotting for 2.5 h at 60V. Membranes were blocked with 3% bovine serum albumin in wash solution (0.1% Tween-20, 100 mM NaCl, 10 mM Tris-pH 7.4) for 1 h and incubated with anti-PY20 antibody (MA5-15143, Ex-Alpha) for 1 h at room temperature. Goat anti-mouse secondary antibody (115-035-003, Jackson ImmunoResearch) was then added for 1 h at room temperature, the membranes were washed 7 times in wash solution, and signal detected using ECLplus (GE Healthcare). Densitometric analysis was performed using MCID-M5⁺ imaging software (Imaging Research). Correction for IP efficiency was

performed either by stripping the blots in 62.5 mM Tris pH 6.8 and 0.7% β-mercaptoethanol at 70°C for 30 min. and then re-probing the membrane with either anti-PDGFR-α (3164, Cell Signaling) or anti-PDGFR-β (X1021, Pierce) or by splitting immunoprecipitates and immunoblotting with anti-pY20 and anti-PDGFR-β in parallel. The intensity of PY20 signal was then normalized to the amount of PDGFR-α or PDGFR-β. All data were expressed as percentage of the vehicle group in each experiment, with vehicle set to 100%.

Statistical analyses.

Data were analyzed using Graph Pad Prism v. 5.0 software (Graph Pad). P < 0.05 was required for statistical significance.

1. Xu, J.J., Walla, B.C., Diaz, M.F., Fuller, G.N. & Gutstein, H.B. Intermittent lumbar puncture in rats: a novel method for the experimental study of opioid tolerance. *Anesth. Analg.* **103**, 714-720 (2006).
2. Dirig DM, S.A., Rathbun ML, Ozaki GT, Yaksh TL. Characterization of variables defining hindpaw withdrawal latency evoked by radiant thermal stimuli. *J Neurosci Methods.* **76**, 183-191 (1997).
3. Emmerson, P.J., *et al.* Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the mu opioid receptor. *J. Pharmacol. Exp. Ther.* **278**, 1121-1127 (1996).
4. Cuello, A. & Carson, S. Microdissection of fresh rat brain tissue slices. in *Brain Microdissection Techniques* (ed. Cuello, A.) 37-126 (John Wiley and Sons, New York, 1983).