Peripheral opioid tolerance involves skin keratinocytes and platelet-derived growth factor type B signaling.

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Abstract
Opioid analgesic tolerance is a deleterious side-effect that requires escalation of dose to overcome reduced analgesia overtime. Dose-escalation dramatically reduces opioid safety due to centrally-mediated life-threatening side-effects, including respiratory depression or addiction. Peripheral opioid delivery is a safer alternative as it provides effective local analgesia with limited central penetration. However, tolerance also occurs peripherally, via mechanisms that remain unknown. Centrally, activation of the mu-opioid receptor (MOPr) by opioids induces release of platelet-derived growth factor-B (PDGF-B); and inhibition of PDGF receptor beta (PDGFRβ) prevents opioid tolerance. In the periphery, MOPr and PDGF-B are expressed in skin keratinocytes, and PDGFRβ is expressed in peripheral sensory neurons (PSNs), known to convey tolerance. Previous studies also showed that optogenetic stimulation of keratinocytes modulates PSNs via release of keratinocytes-derived factors. Thus, we hypothesized that mechanisms of peripheral opioid tolerance could involve keratinocytes and PDGFRβ signaling. Using behavioral pharmacology, optogenetics and in situ hybridization in mice, we found that selective inhibition of PDGFRβ at the periphery, prevents peripheral morphine tolerance caused by repeated intraplantar (i.pl.) morphine injections. In addition, we show that PDGF-B is necessary and sufficient to cause peripheral morphine tolerance and repeated peripheral morphine injections lead to an increase in PDGF-B mRNA in MOPr-expressing keratinocytes. In parallel, we discovered that repeated optogenetic activation of keratinocytes is sufficient to induce peripheral morphine tolerance in a PDGF-B/PDGFRβ-dependent manner. Together, these data show that keratinocytes and PDGF-B/PDGFRβ signaling are essential components in the mechanisms of peripheral opioid tolerance.

Introduction

Opioids are powerful analgesic compounds which have long served as a cornerstone treatment for chronic severe pain [1]. However, repeated opioid use leads to tolerance, which requires escalation of doses to overcome the reduction of analgesia over time. Dose-escalation dramatically reduces opioid safety by increasing the development of deleterious centrally-mediated side effects such as physical dependence, respiratory depression, or addiction, which can lead to overdoses and death. Thus, novel strategies to increase opioid safety without affecting analgesia are needed. Clinical evidence has shown that peripheral and topical application of low doses of opioids produces effective analgesia, while limiting the central penetration [2-5] and reducing the development of adverse side effects [3, 6-9]. In fact, peripheral and topical opioid formulations are under investigation in phase III clinical trials[10, 11]. Yet, the specific mechanisms of action for peripheral opioids remain poorly understood[12]. In addition, even in the realm of peripherally administered opioids, tolerance to analgesia develops [13-18], overshadowing the clinical utility of topical/peripherally administered opioids [2, 5, 19, 20]. Targets to prevent peripheral tolerance and preserve peripheral analgesia over time, are needed. Unfortunately, peripheral tolerance is poorly understood [13, 16, 17, 21-24] and no clear mechanism of peripheral tolerance has been provided [18, 25]. This could be in part due to the fact that the majority of previous studies investigating peripheral tolerance used systemic injections of opioids and only very limited studies examined mechanisms with opioid administrations restricted to the periphery [26]. Therefore, it is still unclear if peripheral tolerance involves different mechanisms from tolerance in the CNS [22, 27, 28].

At the periphery, the mu-opioid receptor (MOPr) is expressed in primary sensory neurons (PSNs) in cell bodies located in dorsal root ganglia (DRG) [29, 30] and in the nerve endings in the dorsal horn of the spinal cord as well as in the skin [31]. In the skin, PSNs peripheral nerve endings project among keratinocytes located in the epidermis. Interestingly, keratinocytes are also known to express MOPr[31-35], which has been shown to play a role in skin homeostasis, wound healing, inflammation and itch[33, 34, 36]. The role of peripheral MOPr in tolerance remains unclear. Recent studies suggested that they were essential to develop tolerance[37, 38], while others had previously argued that they were not involved[39], illustrating the complexity of this issue. Growing evidence suggests that non-neuronal keratinocytes, communicate with peripheral cutaneous sensory neurons that convey somatosensory information[40, 41] by directly modulating their activity[42]. Communication between keratinocytes and cutaneous sensory neurons has been hypothesized to be mediated via keratinocyte-derived factors[41-44]. However, the precise mechanisms of epithelial-neuronal communication remain under investigation.

Previous work showed that, centrally, platelet-derived growth factor receptor beta (PDGFRβ) signaling is essential in the mechanisms of opioid tolerance. Indeed, spinal or systemic delivery of opioids in combination with PDGFRβ signaling inhibitors could completely block opioid tolerance. This discovery led to the notion that the development of spinal tolerance involved spinal release of platelet-derived growth factor type B (PDGF-B) and activation of the PDGFRβ in response to morphine activation of spinal MOPrs[45]. At the periphery, we showed that PDGFRβ and the PDGF-B ligand are expressed in the soma of PSNs in DRGs [46, 47]. We also reported the expression of PDGFRβ in skin terminals of PSNs [48] while PDGF-B ligand was previously found in epithelial keratinocytes in the skin [49-53]. This convergence of MOPr and PDGF-B/PDGFR-β localization in peripheral tissue prompted us to hypothesize that PDGF-B/PDGFR-β signaling could also play a role in peripheral opioid tolerance. In addition, MOPr and PDGF-B expression in keratinocytes and the recent involvement of these epithelial cells in somatosensation [41, 42], raised the possibility that they could be a component of the circuitry mediating peripheral tolerance. In this study, we used a behavioral and pharmacological approaches, to examine the role of PDGF-B/PDGFR-β signaling in tolerance from peripheral administration of morphine in mice. We also employed optogenetics to examine the impact of specific keratinocyte activation on the development of peripheral morphine analgesia and tolerance development. Finally, using fluorescence *in situ* hybridization, we examined the impact of peripheral morphine administration and

keratinocyte stimulation on the expression of MOPR and PDGF-B in keratinocytes. We discovered that morphine peripheral tolerance can be blocked by the selective inhibition of PDGFRβ or PDGF-B at the periphery. Our data also revealed that PDGFRβ and keratinocytes signaling are sufficient to induce peripheral morphine tolerance, where the activation of MOPr on keratinocytes may mediate the release of PDGF-B to activate PDGFRβ signaling and mediate peripheral morphine tolerance.

Results

Inhibition of PDGFRβ prevents peripheral morphine tolerance. To study peripheral morphine tolerance, we used a model of peripheral administration (**Fig. 1A**) where we first established a dose of intraplantar (i.pl.) morphine which would induce analgesia in the ipsilateral, but not the contralateral paw (N=6 males and females per group, 2-way ANOVA, Time x Paw, F (2, 20) = 8.954, P=0.0017, **Fig.1B**). To test the impact of the PDGFRβ inhibitor, imatinib, on peripheral morphine tolerance, female (**Fig.1C**) and male (**Fig.1D**) mice received vehicle (5µl), morphine alone (5µg/5µl), imatinib alone (10µg/5µl), or the combination of morphine + imatinib i.pl. for 5 consecutive days, and paw withdrawal latencies (PWL) were measured 20 minutes later to test analgesia and development of peripheral tolerance. Groups receiving morphine only developed peripheral morphine tolerance as shown by PWLs returned to baseline levels after 3 days of injections in both sexes (2-way ANOVAs, **Fig. 1C**: females, N=6, time x treatment, F(15, 100)=9.411, P<0.0001; **Fig.1D:** males, N=6, time x treatment, F (15, 100) = 4.917, P<0.0001). However, co-administration of morphine + imatinib i.pl. completely prevented tolerance, thus, showing that imatinib inhibition is sufficient to block peripheral morphine tolerance. A 3-way ANOVA analysis comparing sex x treatment x time showed no interaction of sex x treatment (F (3, 246) = 1.644, P = 0.180), revealing no differential sex effect of imatinib on peripheral tolerance (**Fig. 1E)**

Administration of anti-PDGFRβ mAb blocks peripheral morphine tolerance. Imatinib is a PDGFRβ inhibitor with several off-target effects[54-60]. Therefore, we sought to confirm its effect on peripheral tolerance via its actions on the PDGFRβ, using a neutralizing monoclonal antibody (mAb) anti-PDGFRβ to selectively target PDGFRβ. Strikingly, when morphine was co-administered with the PDGFRβ mAb for 5 days, peripheral morphine tolerance was completely prevented in both female and male mice (N=4-5 per sex per group, 2-way ANOVAs, **Fig. 2A:** females, time x treatment, F (15, 70) = 9.797, P<0.0001; **Fig. 2B**: males, time x treatment, F (15, 70) = 16.33, P<0.0001). No sex difference was found, as confirmed by 3-way ANOVA analysis (**Fig 2C**, N=4-5/sex/treatment, F(3, 168)= 1.532, P = 0.208). Importantly, no change in thermal pain threshold was observed over time in the groups treated with the mAb alone, showing that: 1) mAb PDGFRβ selective inhibition does not induce analgesia, and 2) that the effect observed in combination with morphine is specifically blocks peripheral morphine tolerance. To further confirm that the i.pl. presence of an IgG2a isotype was not mediating these effects, we co-administered morphine i.pl. with IgG2a kappa isotype control antibody and found that it did not affect peripheral morphine acute analgesia and development of peripheral tolerance (**Fig. 2D**, N=4-9, 2-way ANOVA, time x treatment, F (10, 90) = 19.22, P<0.0001). Collectively, these results point out the specific role of PDGFR-β in mediating peripheral morphine tolerance.

PDGF-B is necessary and sufficient to cause peripheral tolerance. We next examined whether the endogenous PDGFR-β ligand, PDGF-B, could be necessary for the establishment of peripheral morphine tolerance. For this, we used PDGFR-β-Fc chimera protein (R&D Systems), which consists of a fusion of the N-terminal ligand binding domain of PDGFR-β with the Fc fragment of an IgG_1 at the C-terminal part, to confer solubility. Mice co-administered i.pl. with morphine and PDGFRβ-Fc completely abolished peripheral morphine tolerance in females (**Fig. 3A,** 2-way ANOVA, time x treatment, F(15, 100)=14.89, P<0.0001), and males (**Fig. 3B**, 2-way ANOVA, time x treatment, F(15, 100)=13.44, P<0.0001), thus, showing that extracellular PDGF-B at the periphery is necessary to cause peripheral morphine tolerance. 3-way ANOVA analysis confirmed no sex difference between females and males (**Fig. 3C,** N=6/sex/treatment: F(3, 240) =2.012, P=0.113). Next, we determined whether PDGF-B could be sufficient to induce peripheral morphine tolerance. Vehicle or recombinant PDGF-B were administered i.pl. for 4 days. As previously showed, PDGF-B repeated injections did not change PWL thresholds in females (**Fig. 3D**) and males (**Fig. 3E**). On day 5, all mice were challenged with a dose of i.pl. morphine. As expected, vehicle mice challenged with morphine showed acute antinociception. However, morphine antinociception was completely abolished in mice pre-treated with PDGF-B for 4 days in females (**Fig. 3D**, 2-way ANOVA, time x treatment, F(5, 50)=26.72, P<0.0001) and males (**Fig. 3E**, 2-way ANOVA, time x treatment, F(5, 45)=11.43). No interaction of sex x treatment was observed (**Fig. 3F**, N=5-6/sex/treatment, 3-

way ANOVA, F(1, 114) = 0.038, P=0.845). In functional terms, these findings suggest that PDGF-B plays a pivotal role in peripheral morphine tolerance, as it is necessary and sufficient to induce morphine peripheral tolerance.

Peripheral morphine administration increases PDGF-B expression in keratinocytes. We previously showed that PDGFR-β is peripherally expressed in cutaneous sensory neurons that are IB4 or CGRP positive [46]. PDGF-B ligand is also present in the periphery, with evidence of expression in epidermal keratinocytes[49-53]. MOPr is similarly known to be expressed in the periphery in the DRG neurons [29, 30] and localized to terminals in the skin[31]. Interestingly, MOPR mRNA (*Oprm1* gene) is also expressed in keratinocytes [31-35]. Therefore, we sought to examine if PDGFR-β, PDGF-B and MOPR could be co-expressed at the periphery, with a particular focus on expression in epithelial keratinocytes. Using fluorescence in situ hybridization (RNAscope, ACDbio), we found that, in naïve mice, PDGF-B mRNA (*Pdgfb*) and *Oprm1* are co-expressed in epithelial keratinocytes, while PDGFR-β mRNA *Pdgfrb* was not detected (**Fig. 4A**). At baseline, 6.09+/-1.89% of total keratinocytes co-expressed *Pdgfb* and *Oprm1*(*Pdgfb*+*Oprm1*+), while 5.68+/-1.73% only expressed *Pdgfb* (*Pdgfb*+), 6.079+/-2.11% only expressed *Oprm1*(*Oprm1*+), and 82+/-3.35% cells did not express either of these mRNAs (other, **Fig. 4B**). Interestingly, when compared to animals that were administered with morphine for 5 consecutive days and were peripherally tolerant to morphine, the number of *Pdgfb*+ cells increased to 17.53+/-2.88%, and *Pdgfb*+*Oprm1*+ cells increased to 27.4+/-3.09%, while *other* cells decreased to 47.14+/-3.59% (**Fig. 4B,** N=3-4/sex/group, 2-way ANOVA, Cell type x Treatment, F(3, 39)=32.87, P<0.0001). Thus, showing that the total pool of cells expressing *Pdgfb* was increased by i.pl. morphine injections, causing peripheral tolerance. Furthermore, the amount of *Pdgfb* in *Pdgfb*+*Oprm1*+ keratinocytes was significantly increased in the morphine group compared to the saline group (**Fig. 4C,** Mann-Whitney test, unpaired comparisons, P=0.0022). These data indicate that repeated i.pl. morphine injections promote an increase in PDGF-B mRNA in keratinocytes which co-express both MOPR and PDGF-B.

Optogenetic stimulation of keratinocytes mediates peripheral morphine tolerance in a PDGFR-β- and PDGF-B-dependent manner. Evidence of PDGF-B and MOPR co-expression in keratinocytes and the necessity of PDGF-B signaling to mediate peripheral morphine tolerance, prompted us to hypothesize that keratinocytes could play a role in the mechanisms of peripheral opioid tolerance. Furthermore, earlier work showed that keratinocyte stimulation directly causes action potential firing in several peripheral sensory neuron subtypes in these mice [42], suggesting communication between keratinocytes and peripheral sensory neurons, which are known to be involved in opioid tolerance [37]. We first tested whether keratinocyte signaling is sufficient to drive opioid tolerance in the periphery via an optogenetic approach. In transgenic mice, blue-light sensitive channelrhodopsin-2 (ChR2) ion channel was genetically expressed in keratinocytes under the control of the Krt14 promoter (Krt14-ChR2 mice) [42]. Krt14-ChR2 mice and wild type (WT) littermates were exposed to blue light for 5 consecutive days for 15min (BL-ON groups) (**Fig. 5A**). As control another cohort of Krt14-ChR2 and WT mice underwent the same procedure, but blue light was kept off (BL-OFF groups). On day 5, mice were injected with i.pl. morphine alone or in combination with imatinib (**Fig. 5B and C)**, or with PDGFRβ-Fc (**Fig. 5C and D**). Strikingly, this was completely abolished in Krt14-ChR2 mice exposed to BL ON (**Fig. 5B left:** 3-way ANOVA, day x genotype x treatment, F (1, 24) = 6.014, P=0.0218, Sídák's post hoc test BSL vs. Day 5: WT – morphine: P < 0.0001, K14Chr2 – morphine: P = 0.8438, **and 5D left:** 3-way ANOVA, day x genotype x treatment, F (1, 60) = 0.3537, P=0.5543, Sídák's post hoc test BSL vs. Day 5: WT – morphine: P < 0.0001, K14Chr2 – morphine: P = 0.6315). Importantly, challenge of morphine + imatinib to inhibit PDGFRβ (**Fig. 5B, right**) or morphine + PDGFRβ-Fc (to scavenge the PDGF-B ligand (**Fig 5D, right)** produced a significant antinociceptive effect both in WT littermates and Krt14-ChR2 mice exposed to blue light (BL ON) for 5 days (**Fig 5B right**: Sídák's post hoc test BSL vs. Day 5: WT – morphine + Imatinib: P < 0.0001, K14Chr2 – morphine + imatinib: P = 0.0003, **Fig 5D right**: Sídák's post hoc test BSL vs. Day 5: WT – morphine + PDGFRβ-Fc: P < 0.0001, K14Chr2 – morphine + PDGFRβ-Fc: P < 0.0001,). These data show that: 1) keratinocyte optogenetic activation ablates morphine analgesia; and 2) PDGFRβ signaling plays a crucial role in regulating these

peripheral keratinocyte effects since morphine analgesia could be restored either by inhibition of PDGFRβ signaling via imatinib, or by scavenging PDGF-B with PDGFRβ-Fc. Importantly, control groups which underwent the same procedure but were not exposed to blue light (WT or Krt14-ChR2 BL-OFF) displayed full morphine analgesic effects of morphine when injected alone or in combination with imatinib (**Fig. 5C,** 3-way ANOVA, day x genotype x treatment, $F(1, 16) = 0.1330$, $P=0.7201$, Sídák's post hoc test BSL vs. Day 5: WT – morphine: $P=0.0002$, K14Chr2 – morphine: $P < 0.0001$, WT – morphine + Imatinib: $P < 0.0001$, K14Chr2 – morphine + imatinib: $P <0.0001$), or PDGFRβ-Fc (**Fig. 5E,** 3-way ANOVA, day x genotype x treatment, $F(1, 40) = 3.892$, $P=0.0555$, Sídák's post hoc test BSL vs. Day 5: WT – morphine: $P < 0.0001$, K14Chr2 – morphine: $P < 0.0001$, WT – morphine + Imatinib: $P < 0.0001$, K14Chr2 – morphine + imatinib: $P = 0.0011$). Therefore, highlighting that the precluding effect on morphine analgesia is directly due to repeated optogenetic stimulation of keratinocytes. Altogether, these data highlight our discovery that repeated keratinocytes optogenetic activation is sufficient to induce peripheral morphine tolerance in a PDGFRβ / PDGF-B-dependent manner.

Discussion

Topical and peripheral opioids produce effective analgesia with a much better safety profile for adverse effects than systemic opioids, as they relieve local pain with minimal systemic effects[12, 61]. However, despite growing clinical and pre-clinical evidence that peripherally acting opioids have many advantages [3, 8, 62, 63], repeated peripheral application of opioids leads to a rapid loss of their analgesic efficacy [13] [64]. This presents a significant disadvantage and barrier to their clinical opioid use in the periphery [2, 5, 19, 20].

We previously discovered that central mechanisms of opioid tolerance involve PDGFRβ/PDGF-B signaling through mechanisms involving the release of PDGF-B ligand upon opioid activation of MOPR by opioids [47, 65, 66]. In the current study, we sought to investigate the involvement of PDGFRβ/PDGF-B signaling in peripheral morphine analgesic tolerance. Through pharmacology experiments, we show that selective PDGFRβ inhibition can completely block tolerance to morphine injections at doses that provide local analgesia. Moreover, we show that PDGF-B ligand is both necessary and sufficient to induce peripheral morphine tolerance. Finally, we demonstrate that skin keratinocytes express PDGF-B and MOPR, but not PDGFRβ. Importantly, we find that repeated peripheral morphine administration increases the number of keratinocytes expressing PDGF-B and the average mRNA copies of PDGF-B in MOPR-expressing keratinocytes is increased. Finally, using an optogenetic approach, we have discovered that selective optogenetic stimulation of keratinocytes precludes peripheral morphine analgesia. This shows that keratinocyte stimulation is sufficient to cause peripheral morphine tolerance in a PDGFRβ/PDGF-B-dependent manner.

Prior work suggested that signaling mechanisms of peripheral tolerance could be distinct from those involved in central tolerance[22, 27, 28], which is supported by studies using topically applied opioids in animals that were tolerant to systemically administered opioids[26, 28]. However, this idea was not confirmed by others[18, 25], leaving the issue of peripherally administered opioids and tolerance unresolved. Our current work addresses the mechanisms of peripheral tolerance using a model that strictly administers opioids via a peripheral route in mice hindpaws, at doses that we confirmed have no analgesic effect on the contralateral non-injected paw, and therefore should not involve central signaling mechanisms. This model allows us to address the role of peripherally-expressed MOPr in the mechanisms of opioid antinociception and tolerance, which has been debated in previous studies [37, 67]. While some studies, suggest that opioids are required for tolerance[37, 38], others argue they were not involved[39]. Similarly, divergent findings exist regarding their role in antinociception[37, 68], highlighting the complexity of understanding their function in these processes. At the periphery, MOPr is expressed by DRG neurons[29, 30, 69] and localized to terminals in the skin[31]. Additionally, MOPr is expressed in skin cells [31-35] and we confirmed that MOPr mRNA is abundantly present in skin keratinocytes. Previous studies examining the role of MOPr in tolerance and antinociception rarely acknowledged or studied the role of MOPrs in keratinocytes [37, 38, 68, 70], therefore, possibly explaining previous discrepancies in the role of peripheral MOPr in tolerance and nociception. Here, we highlight that keratinocytes activation does not alter morphine peripheral antinociception but is sufficient to mediate peripheral opioid tolerance. We also show that repeated peripheral morphine administration induces an increase in PDGF-B ligand expression in keratinocytes. Together, these findings suggest that opioid simulation of MOPR in keratinocytes elicits cellular responses that are involved in the development of peripheral opioid tolerance and that involve PDGF-B expression in keratinocytes. Therefore, we now posit that MOPR signaling in keratinocytes play an important role in the mechanisms of opioid tolerance but not antinociception.

PDGFRβ is the receptor through which PDGF-B has been shown to signal *in vivo* [71, 72]. We found that peripheral tolerance was abolished when morphine was peripherally co-administered with the PDGFRβ blocker imatinib for five days. However, because imatinib has other potential targets[13], the specific involvement of PDGFRβ signaling in peripheral tolerance was selectively investigated. Our findings showed that co-administration of morphine together with a selective antibody anti-PDGFRβ antibody or with a scavenger to neutralize the PDGF-B ligand completely prevented peripheral tolerance. Moreover, repeated peripheral administrations of PDGF-B ligand induced peripheral tolerance in a group of mice that was never exposed to

morphine. These findings indicate that PDGF-B/PDGFRβ signaling is necessary and sufficient for peripheral tolerance. In our prior work, we showed that PDGFRβ is expressed in the soma and in skin nerve endings of peripheral DRG neurons, specifically in small diameter nociceptors, but not larger myelinated neurons[48]. Interestingly, in the current study, PDGFRβ mRNA was not found to be expressed in skin keratinocytes, showing that its involvement in peripheral tolerance is likely mediated by signaling in peripheral nociceptors. Here, we also confirmed that PDGF-B is expressed in mouse skin keratinocytes, consistent with single-cell transcriptomics analysis [52] and other studies reporting human and rodent epidermis expressing both PDGF-B mRNA and protein [51, 53, 73]. In addition, 5 days of peripheral morphine administration increased PDGF-B expression in skin keratinocytes that co-express both MOPR and PDGF-B. Together, these data indicate that PDGF-B and PDGFRβ are present in nociceptive conveying structures. We now propose a model where opioid-activated keratinocytes may engage PDGF-B/PDGFRβ signaling to cause peripheral tolerance by 1) increasing PDGF-B expression, 2) release this growth factor, and 3) activate PDGFRβ on peripheral nociceptors to mediate peripheral tolerance via signaling mechanisms that remain to be elucidated. Noteworthy, although the specific signaling mechanism by which PDGFRβ mediates peripheral tolerance remains unknown, we hypothesize that it involves trans-modulation of MOPR signaling in nociceptors (for a review see [74]).

The concept that keratinocytes may be releasing PDGF-B upon opioid activation to initiate peripheral tolerance aligns with prior studies showing that keratinocytes may release factors modulate peripheral nociceptors signaling activity. In fact, prior work has shown that keratinocyte stimulation directly elicits action potential firing [75] via keratinocyte-derived factors [44, 75], which modulate sensory and nociceptive processing [43, 76-79]. The recent advance in uncovering the involvement of keratinocytes in somatosensory processing has been allowed using optogenetics selective stimulation of keratinocytes, as the cutaneous-neural interface is complex and prior technology did not enable the separation of keratinocyte and primary afferent responses to a stimulus applied on the skin, (i.e., both cell types would be activated by any stimulus). Our study also took advantage of selective stimulation of keratinocytes using optogenetics, to study their role in peripheral opioid tolerance. We used mice which express the blue light-sensitive channelrhodopsin-2 (ChR2) ion channel selectively expressed in Krt14+ keratinocytes (Krt14-ChR2 mice). Prior work showed that activation of ChR2 in Krt14-ChR2 mice elicits depolarization and calcium transients in keratinocytes[77, 79] and causes action potential firing in nociceptor-type peripheral sensory neurons in mice[75]. Our model investigated for the first time the impact of a repeated optogenetic stimulation of keratinocytes for 5 days on the effect of peripheral morphine effects. Strikingly, we found that Krt14-ChR2 mice developed peripheral morphine tolerance even in the absence of repeated peripheral opioid injections. Importantly, keratinocyte-mediated peripheral tolerance was precluded by acute co-administration of imatinib or PDGFRβ-Fc, indicating a major role of keratinocytes/PDGFRβ signaling in peripheral tolerance. This startling finding therefore suggests that repeated keratinocyte stimulation is sufficient to mimic the effects of repeated peripheral administration of morphine, and that both keratinocyte and morphine-mediated peripheral tolerance develop in a PDGF-B/PDGFRβ-dependent manner.

In summary, we demonstrate that PDGF-B/PDGFRβ signaling is essential in the mechanisms of peripheral morphine tolerance. We also show for the first time that keratinocyte stimulation is sufficient to induce peripheral opioid tolerance, highlighting that keratinocytes are an integrative component of the peripheral somatosensory circuit that mediates opioid signaling and tolerance. Our work opens new avenues of research exploring the potential of targeting keratinocytes and PDGFRβ to preserve peripheral morphine efficacy overtime and enable the use of peripheral opioids for the treatment of local peripheral chronic pain. If successful, this approach could be leveraged to shift opioid administration from central/systemic to localized/topical, thus circumventing the dangers of opioid addiction and abuse.

Materials and Methods

Animals

Mice used for this study were originally purchased from the Jackson laboratory. C57BL/6J (stock # 000664), Krt14-Cre (stock# 004782), Ai32 (stock #012569) were purchased and bread to be used at 8–14-weeks-old. All animals were grouped and housed in a 12/12 light cycle (7 am lights on, 7 pm lights off). Water and rodent chow were provided *ad libitum* throughout the experiment. Animals were habituated to the laboratory environment for 1 week prior to experimental testing or breeding.

For optogenetic experiments, we generated Krt14-ChR2 mice by crossing mice that express Cre-recombinase under the control of the Krt14 promoter (Krt14-Cre) with mice that express the blue-light-sensitive channelrhodopsin-2 (ChR2) under the control of the CAG promoter inserted into the Rosa26 locus (Ai32). As a result, Krt14-ChR2 mice express the ChR2 ion channel in Krt14+ keratinocytes[75]. F2 generation mice were used to obtain Cre allele heterozygous and either ChR2 heterozygous or homozygous mice. Littermate animals not expressing the ChR2 allele were used as wild-type (WT) littermate controls.

All procedures were approved and performed in compliance with the Institutional Animal Care and Use Committee at Boston University and the University of Pittsburgh.

Drugs

Morphine sulfate was obtained from Sigma (cat. number: 6211-15-0), Imatinib from LC Laboratories (Cat. # I-5508), recombinant rat PDGF-BB peptide (Cat #520-BB), recombinant mouse PDGFRβ-Fc scavenger (Cat. # 1042-PR) from R&D Systems, PDGFRβ monoclonal antibody (PDGFRβ mAb) from Invitrogen (Cat #16-1402-82), IgG2a kappa Isotype Control antibody from Invitrogen (Cat #16-4321-82). Drugs were either dissolved in a solution of filtered (0.22 μm) 10% β-cyclodextrin sulfobutyl ether (Captisol, CyDex) in 0.9% saline (for Imatinib, PDGFRβ-Fc, and PDGFRβ mAb), or in filtered 4mM HCl in 0.9% saline (for PDGF-BB peptide).

Intraplantar (i.pl.) drug administration

All drugs were injected subcutaneously in the plantar area of one hind paw of awake animals that were lightly restrained. The volume of injection was 5μl using a 30-gauge needle and 20μl Hamilton syringe. The paw side was randomized within and across experiments.

Doses per injection were as follows: Morphine Sulfate: 5μg, Imatinib: 10μg, PDGF-B peptide: 0.25μg, PDGFRβ-Fc: 250ng, PDGFRβ: mAb 0.1μg, IgG2: 0.1μg. Doses used were chosen after performing dose-responses (not shown).

Nociceptive testing

Hargreaves Thermal Pain Assay

Mice were habituated to a Hargreaves apparatus (IITC Life Sciences Inc., Woodland Hills, CA) on a tempered glass maintained at 30°C for 90 minutes per day for 2 days and prior to behavioral testing. On test days, thermal nociceptive thresholds were recorded following habituation, by measuring paw withdrawal latencies (PWL) to a light source focused on the plantar surface of each hind paw. Recorded behaviors for latency included withdrawal, licking, biting, or shaking of the targeted hind paw. Measurements were repeated three times per hind paw, with at least 1-minute intervals and averaged for each hind paw. Intensity of the targeted light source was adjusted to generate baseline responses at ~8 seconds on average. A cut-off of 20 seconds was set to avoid tissue damage.

Experimental workflow

Development of peripheral tolerance with morphine

Mice were randomly assigned to drug groups. Development of tolerance and testing of pharmacological agents were performed in a 2x2 design by injecting either vehicle, morphine alone, the pharmacological agent alone, or morphine combined with the pharmacological agent. Baselines were acquired on day 0. On days 1-5, animals

were injected intraplantarly (i.pl.) with their assigned drug and placed on the Hargreaves apparatus, and PWL measured 20 minutes later (20-minute time point of testing was determined through a time course experiment to ascertain the peak analgesic effects of morphine administered i.pl., not shown). On day 5, animals were euthanized, and tissue was collected according to tissue collection procedures.

Development of peripheral tolerance with blue light

On day 0, Krt14-ChR2 mice and WT littermates were habituated to the Hargreaves apparatus. Following habituation, baseline PWL was measured using our Hargreaves apparatus. On days 1-4, animals were exposed to a blue light using LED covered tray (170 Lumens, 475 nm wavelength) placed underneath the Hargreaves apparatus tempered glass surface. Blue light exposure lasted 15 minutes per day. On day 5, animals were injected in one hind paw (ipsilateral, ipsi) with either morphine or morphine combined with the tested agent, before being exposed to 15 min blue light exposure and paw withdrawal latency testing 5 minutes later. Control groups underwent the same procedure with the blue light LED tray off.

Tissue Collection

Mice were euthanized via intracardiac perfusion. Mice were anesthetized with 4% isoflurane vapor until respiration ceased. The abdomen was then incised to expose the heart. A butterfly needle was then inserted into the left ventricle and the right atrium was cut to create an open circuit. Filtered 1X phosphate-buffered saline (PBS) was pumped through the circulatory system to remove all blood from the body. The skin from the hind paw was removed and placed in 2% paraformaldehyde (PFA) at 4°C for 48 hours. Then, tissue was transferred to 30% sucrose for 24hrs, and then to 20% sucrose until sunk and stored at 4°C. Tissue was then snap frozen in OCT, and 14µm sections were obtained using a cryostat at -18°C and were pre-mounted on glass slides (FisherBrand™ Superfrost™ Plus Microscope Slides: 1255015). Sectioned tissue was stored at -80°C for at least 24 hours before use.

Fluorescence *in situ* hybridization

Slides stored at -80°C were directly transferred to a baking oven and baked at 60°C for 60 min, before post-fixation in 4% paraformaldehyde (PFA) for 60 mins at 4°C. After 2 washes in distilled water, slides were diehydrated in 50%, 70%, and 100% ethanol each for 5 mins. A hydrophobic pen was used to draw a barrier around the tissue slices prior to incubation in target retrieval solution (ACDBio) at 95°C for 5 mins. Slides were then processed using the RNAscope kit V2 from ACDBio (Cat. 323100, Biotechne). They were first incubated in hydrogen peroxyde 10 min at RT, and protease III at 40°C for 30 mins. Probes targeting mRNAs sequences of the platelet-derived growth factor type b (Pdgfb, Mm-PDGF-B-C1 (ACD Bio Cat. 42451)), platelet-derived growth factor receptor beta (Pdgfrβ, Mm-PDGFr-B-C2 (ACD Bio Cat. 411381-C2)) and opioid receptor mu-1 (Oprm1, Mm-Oprm1-C3 (ACD Bio Cat. 315841-C3)), were added to the slides and incubated at 40°C for 2hrs. Probe signals were then amplified using manufacturer's AMP solutions 1, 2, and 3 via incubation at 40°C for 30 mins, 30 mins, and 15 mins, respectively. Probes were assigned a TSA Plus fluorophore (TSA Vivid 520 - Pdgfb, TSA vivd 570 - Pdgfrβ, TSA vivid 650 - Oprm1) and incubated for 30 min at 40°C. Slides were incubated with HRP blockers at 40°C for 15 mins following each TSA Plus fluorophore incubation. All tissue was stained with DAPI (ACD Bio Cat. 323108), and coverslipped. Slides were stored at 4°C until imaging.

RNAscope imaging and analysis

Skin tissue labelled with fluorescent mRNA probes was imaged using a BZ-8000 widefield fluorescence microscope (Keyence) using a 40X objective lens. Z-stacks were acquired (3µm total, 1µm steps). Two ROIs were imaged per tissue sample per mouse and Maximum Intensity projections were used for analysis.

All images were uploaded to QuPath for mRNA spots analysis. Cell segmentation was performed based on DAPI nuclear staining. The basal layer of keratinocytes was segmented using the following settings: 10µm background radius, Sigma 0.75, Minimum area: 2µm^2, Maximum Area 400µm^2, Cell expansion: 2µm. The upper layers of keratinocytes were segmented using the following settings: 8µm background radius, Sigma 0.75, Minimum area:

2μm^2, Maximum Area 400μm^2, Cell expansion: 4μm. Thresholds for detection of spots (subcellular detections, mRNA copies) were calculated as 35% of maximum intensity of each channel in the ROI. Minimum and maximum spot size set at 0.3 and 2μm, respectively. Background noise was calculated by averaging number of estimated spots per cell across all samples in an experiment. Cells were then considered positive for an mRNA if they contained at least the average detected spots. Cell populations were categorized as: 1) negative for both Pdgfb and Oprm1 mRNA (*Pdgfb-/Oprm1-),* negative for Pdgfb and positive for Oprm1 (Pdgfb-/Oprm1+), positive for Pdgfb and negative for Oprm1 (Pdgfb+/Oprm1-), and positive for Pdgfb and positive for Oprm1 (Pdgfb+/Oprm1+). Then, we calculated average number of Pdgfb mRNA copies in all Oprm1+ cells per treatment group.

Data Analyses and Statistics.

All results are presented as mean±SEM. Data were tested for normality and sphericity and a Greenhouse Geisser correction was applied where appropriate. Assumption of Normality was tested either with the Shapiro–Wilk test or with a Q-Q plot. Repeated Measure two-way ANOVAs were used with treatment and time as variables. Tuckey's or Sidack's were used for *post hoc* comparisons of groups. Mann-Whitney test was used to compare the morphine effect. GraphPad Prism 9.5 and RStudio were used for data treatment and statistical analysis. All statistics are summarized in the SI Appendix, Table S1. The experiments were repeated at least two times. Behavioral and cell counting assessments were performed by investigators blinded to experimental group assignments. $P<0.05$ was considered as significant.

Acknowledgments

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Figures and Legends:

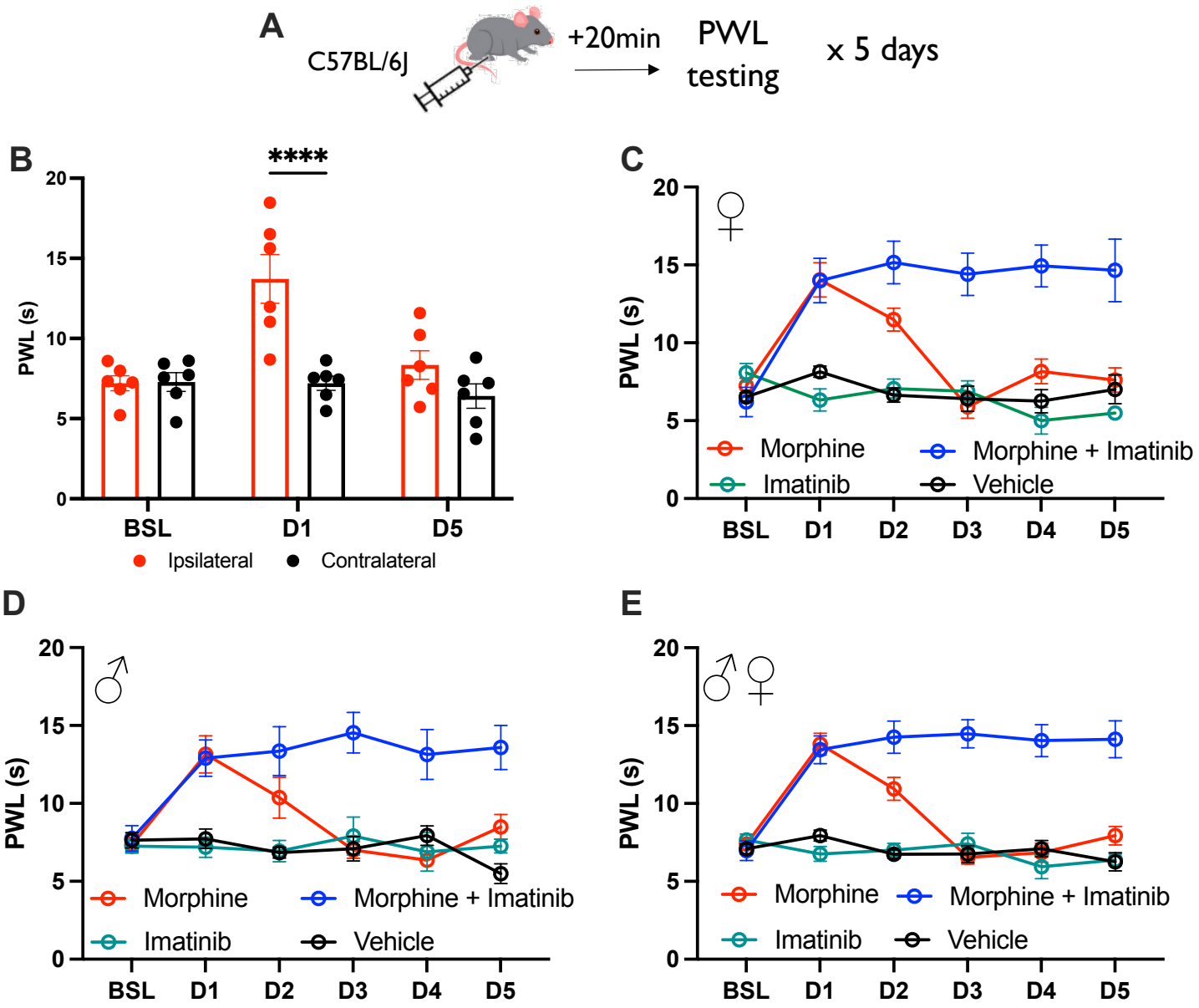

Figure 1. Imatinib blocks peripheral morphine tolerance. A. Schematic of the experimental design. **B**. Intraplantar (i.pl.) morphine administration induces ipsilateral but not contralateral acute antinociception as seen by increase in paw withdrawal latency (PWL) on day1. Tolerance develops by day5. **C-D.** Morphine peripheral tolerance develops upon repeated morphine i.pl. Co-injection of morphine + imatinib blocks peripheral tolerance in females (**C**) and males (**D**) mice. **E**. Females and males' data merged. BSL = baseline, D = day. N = 6/7 per group/sex. * p<0.05, **p<0.01, ***p<0.001 vs vehicle, # p<0.05 ## p<0.01, ### p<0.001 vs morphine. Two-way or three-way Repeated Measures ANOVA followed by Šídák's (**A**) or Tukey's (**B-D**) multiple comparisons test.

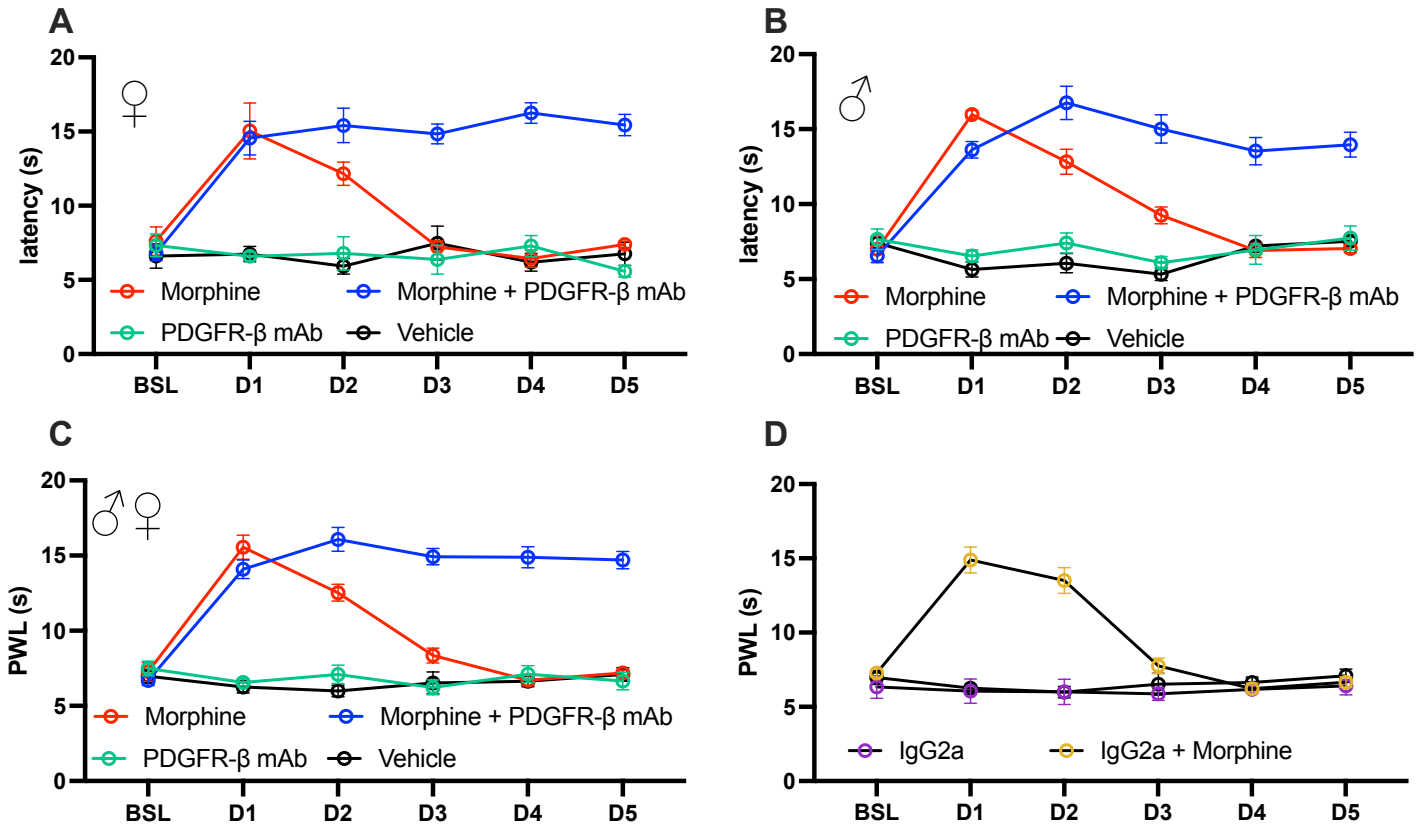

Figure 2. Selective PDGFRβ inhibition blocks peripheral morphine tolerance. Co-injection of morphine + anti-PDGFRβ monoclonal antibody (mAb) blocks peripheral tolerance in females (**A**) and males (**B**) mice. **C**. Females and males' data merged. **D.** Control experiment. Neutral IgG2a mAb does not affect morphine acute analgesia on day 1 and does not prevent the development of tolerance overtime. MS = Morphine Sulfate, BSL = baseline, D = day. N = 6/7 per group/sex. Two-way or three-way Repeated Measures ANOVA followed by Tukey's multiple comparisons test.

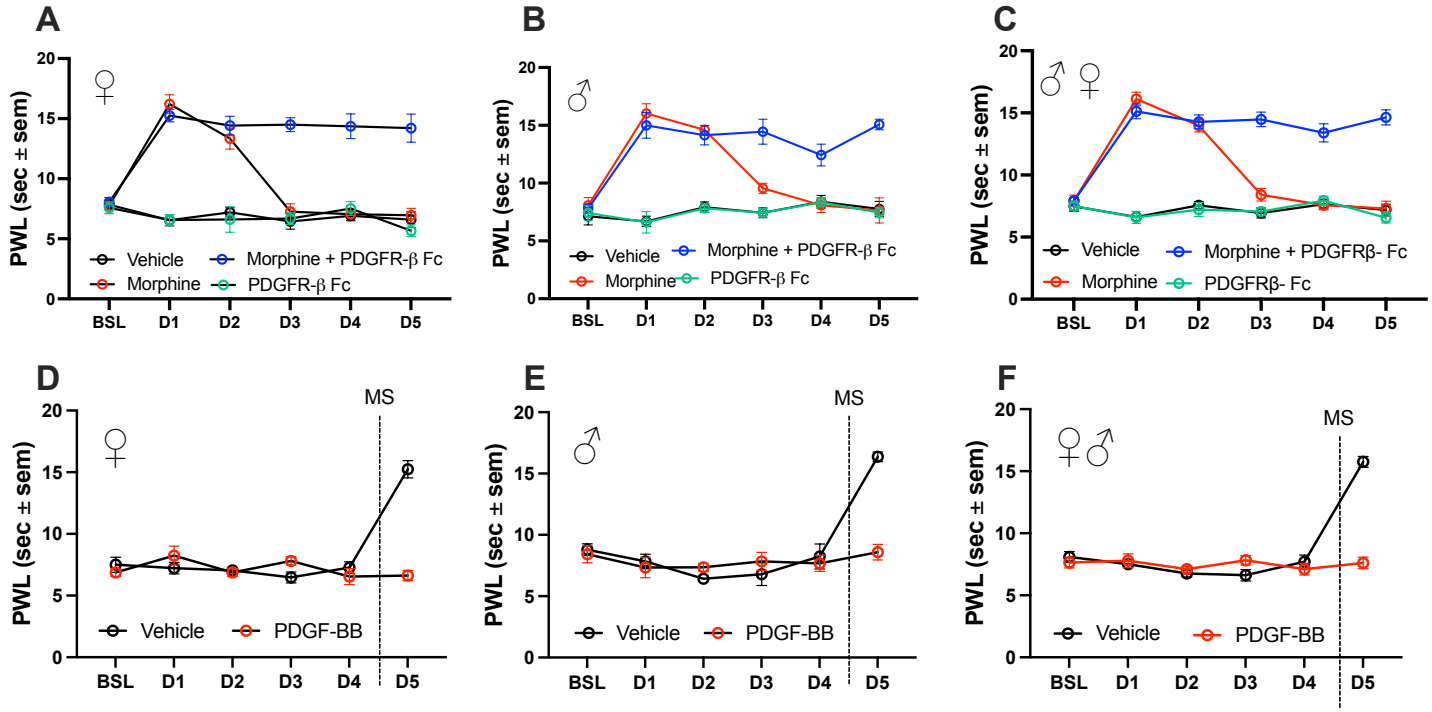

Figure 3. Selective PDGFRβ inhibition blocks peripheral morphine tolerance. A-C. Co-injection of morphine + the PDGF-B scavenger, PDGFRβ Fc, blocks peripheral tolerance in females (**A**) and males (**B**) mice. Morphine + PDGFR-β Fc females and males' data merged (**C**). **D-F.** Repeated intraplanar administration of recombinant PDGF-B blocks analgesia to a morphine challenge on day 5, in females (**D**) and males (**E**) mice. Morphine + PDGFR-β Fc females and males' data merged (**F**). MS = Morphine Sulfate, BSL = baseline, D = day. N = 6/7 per group/sex. Two-way or three-way Repeated Measures ANOVA followed by Tukey's multiple comparisons test.

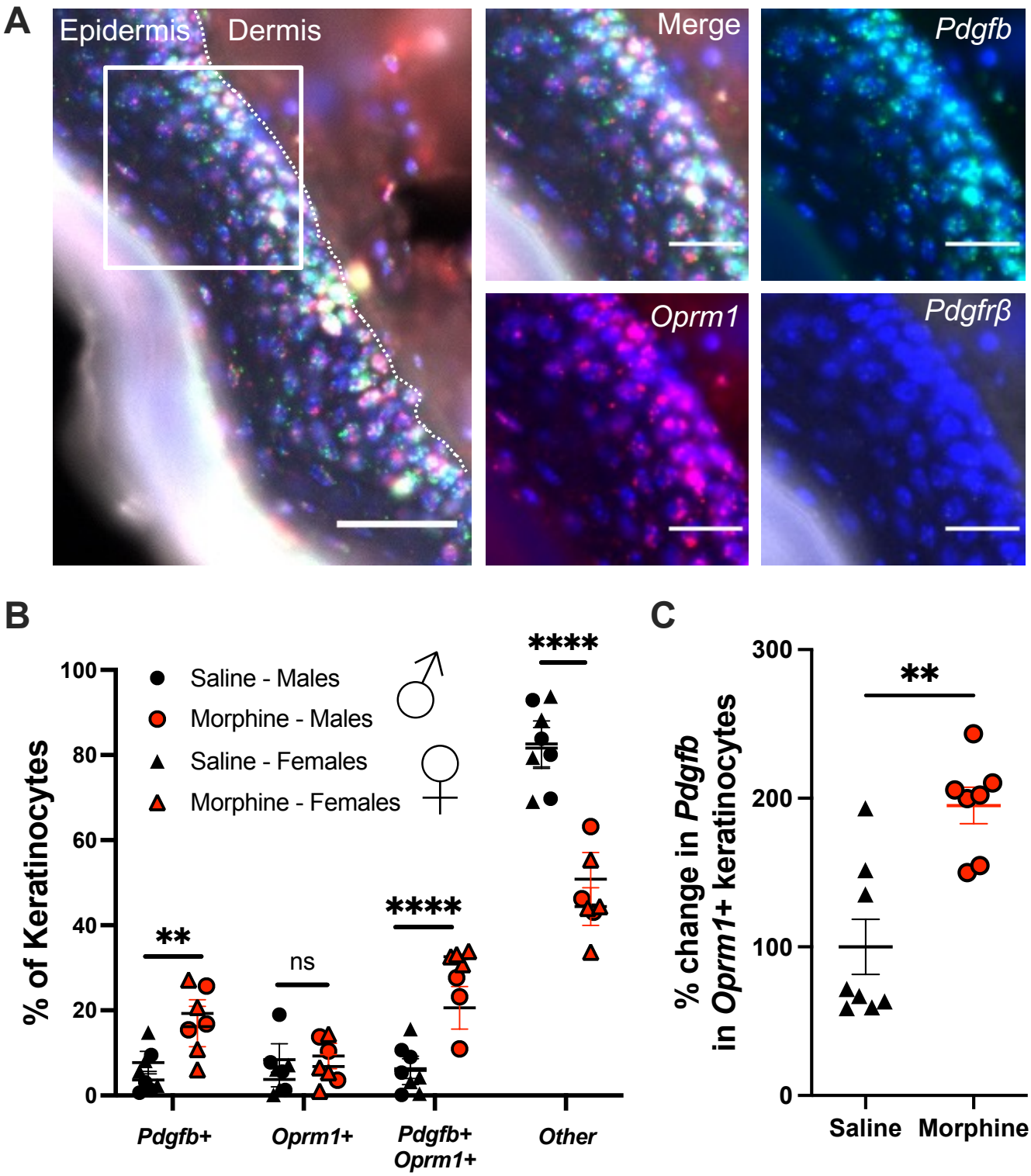

Figure 4. Peripheral morphine administration increases PDGF-B expression in keratinocytes. A. *In situ* hybridization shows co-expression of *Pdgfb* (green) and *Oprm1* (red) mRNA but not *Pdgfrb* (white) mRNA in epidermal keratinocytes. **B.** Quantification of expression highlights that morphine i.pl. for 5 days changes proportions of keratinocytes expressing *Pdgfb, Oprm1,* or both. Two-way ANOVA followed by Sidack's multiple comparison test. **C.** Amount of *Pdgfb* mRNA detected in *Oprm1* cells increases in mice injected with morphine i.pl. for 5 days. Student t-test. *Pdgfb* = PDGF-B mRNA, *Oprm1* = MOPR mRNA, *Pdgfrb* = PDGFRβ mRNA. **p<0.01, ****p<0.0001 morphine vs. vehicle

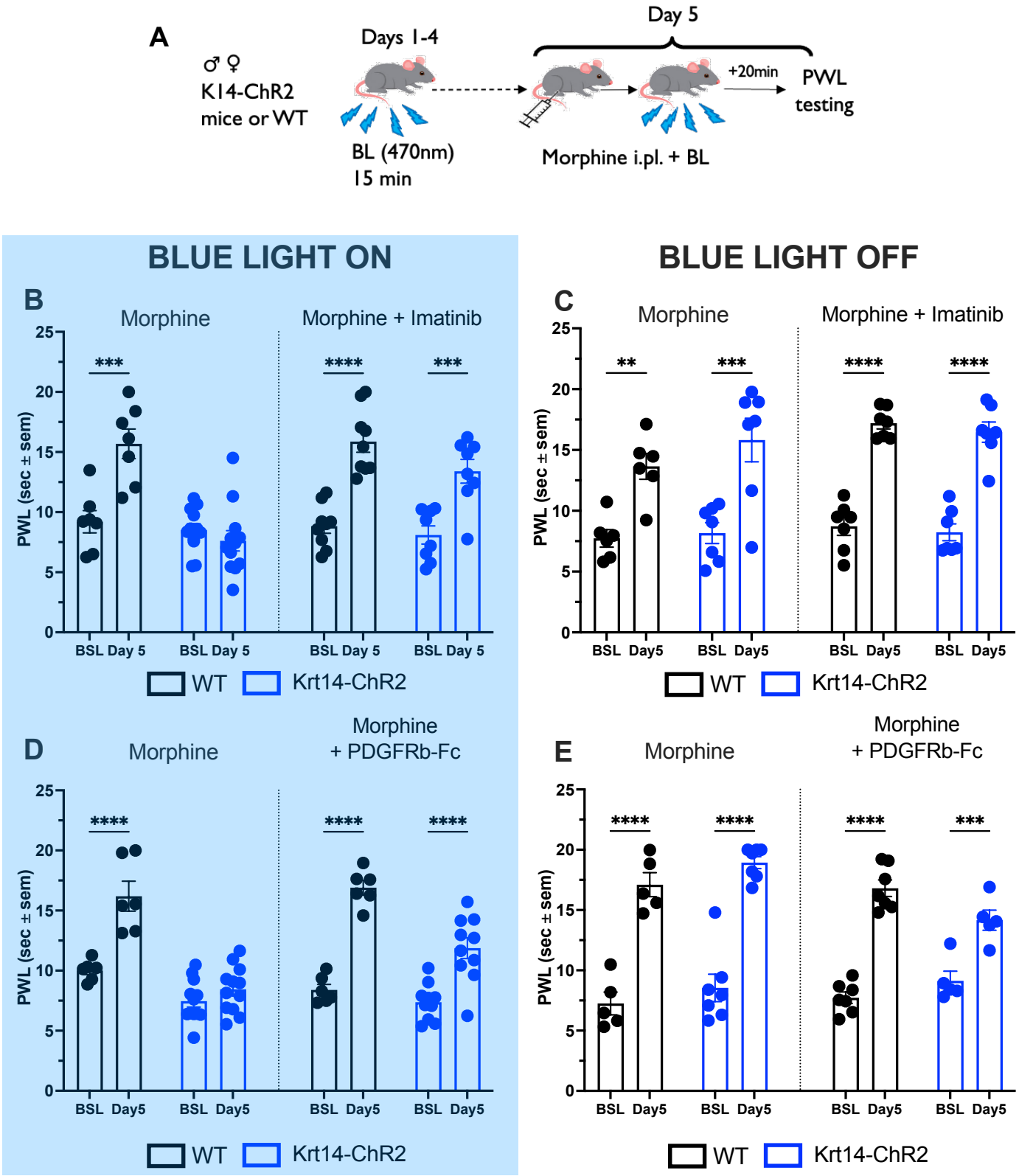

Figure 5. Optogenetic stimulation of keratinocytes mediates peripheral morphine tolerance in a PDGFR-β- and PDGF-B-dependent manner. A. Schematic of the experimental design. **B.** Blue-Light stimulation of keratinocytes for 5 days precludes antinociceptive effect of a challenge of morphine on day 5, which is restored by a co-administration of morphine + imatinib. **D.** Blue-Light stimulation of keratinocytes for 5 days precludes antinociceptive effect of a challenge of morphine on day 5, which is restored by a co-administration of morphine + the PDGF-B scavenger, PDGFRβ- Fc. **C, E.** Blue-light off controls. Three-way ANOVAs followed by Sidak's multiple comparisons test. **p<0.01, ***p<0.0001, ****p<0.0001. BSL vs. Day5. WT = Wild-Type. BSL = baseline.