Published in final edited form as:

J Pain. 2012 October ; 13(10): 997–1007. doi:10.1016/j.jpain.2012.07.009.

Preprotachykinin-A gene disruption attenuates nociceptive sensitivity after opioid administration and incision by peripheral and spinal mechanisms in mice

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Abstract

The preprotachykinin A gene (*ppt-A*) codes for Substance P (SP), supports nociceptive sensitization and modulates inflammatory responses after incision. Repeated opioid use produces paradoxical pain sensitization, termed opioid induced hyperalgesia (OIH) which can exacerbate pain after incision. Here the contribution of SP to peri-incisional nociceptive sensitization and nociceptive mediator production after opioid treatment was examined utilizing *ppt-A* knockout (–/–) mice and the neurokinin (NK1) receptor antagonist LY303870. Less mechanical allodynia was observed in *ppt-A*^{–/–} mice compared to wild types (*wt*) after morphine treatment both before and after incision. Moreover, LY303870 administered with morphine reduced incisional hyperalgesia in *wt* mice. Incision after saline or escalating morphine treatment up-regulated skin IL-1 β , IL-6, G-CSF and MIP-1 α levels in *ppt-A*^{–/–} and *wt* mice similarly. However, chronic morphine treatment greatly exacerbated increases in skin NGF levels after incision, an effect entirely dependent upon intact SP signaling. Additionally, SP dependent up-regulation of prodynorphin, NMDA1 and NK1 receptor expression in spinal cord was seen after morphine treatment and incision. A similar pattern was seen for 5-HT3 receptor expression in tissue from dorsal root ganglia. Therefore, SP may work at both central and peripheral sites to enhance nociceptive sensitization after morphine treatment and incision.

Keywords

Cytokines; NGF; Substance P; Opioid Induced Hyperalgesia; Skin Incision

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Disclosures:

This work was supported by NIH grants DA021332 and GM079126.

The authors do not have financial or other relationships that might lead to a conflict of interest.

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Introduction

Preprotachykinin A (*ppt-A*) gene codes for the primary afferent neurotransmitters Substance P (SP) and neurokinin A, with the former having better established roles in nociceptive signaling. Besides being a well-known nociceptive neurotransmitter, SP has roles in wound healing, cell proliferation and neurogenic extravasation after injury with most of these effects mediated by Neurokinin-1 (NK1) receptors^{24, 37, 49, 57, 58, 62}. One of the prominent features of nociception involving SP is its participation in most pain models characterized by intense or persistent stimulation. For example, *ppt-A* knockout (*ppt-A*^{-/-}) mice display normal thermal and mechanical nociceptive thresholds when compared with their wild-type littermates^{12, 57}. However, *ppt-A*^{-/-} mice display attenuated formalin-induced pain behaviors, and less thermal and mechanical sensitization in an incisional pain model, with no change in inflammatory pain response in the CFA model^{12, 24, 26, 35, 57}. Likewise, NK1 receptor antagonists have little effect on basal thermal and mechanical nociceptive sensitivity, but have substantial effects in pain models involving incision or persistent pain^{57, 62}. Release of SP at both central and peripheral nerve terminals participates in nociceptive sensitization in neuropathic and incisional pain models^{2, 5, 23, 37, 57}.

Paradoxical pain hypersensitivity in response to repeated administration of opioids has been described as opioid-induced hyperalgesia (OIH). This phenomenon has been studied extensively in animals, and has been noted in humans treated with opioids for the maintenance of addiction and reduction of chronic pain^{15, 19–22}. Recent reports link SP and its NK1 receptor to OIH. For example, the production of SP by sensory neurons is enhanced in DRG cultures exposed to morphine chronically⁶. The release of SP from the central terminals of afferent neurons and the spinal expression of the NK1 receptor are augmented in animals after several days of opioid treatment^{29, 33}, and the nociceptive effects of intrathecally administered SP are increased⁴⁰. Importantly, OIH was resolved after the spinal administration of NK1 antagonists or in NK1 receptor knockout mice³³. These processes together with enhanced signaling through additional changes in the central and peripheral nervous systems including NMDA, prodynorphin and serotonergic (5-HT₃) mechanisms are thought to support OIH³. Interactions between SP-NK1 signaling and other established mechanisms supporting OIH have not been well explored.

The interaction between extended exposure to opioids and responses in models of clinical pain states has been less well explored. Peri-incisional sensitization is greatly enhanced in rodents treated repeatedly with morphine prior to incision, or with very large opioid doses at the time of incision^{39, 55}. Moreover, the levels of nociception-related cytokines are enhanced in incised animals after morphine treatment⁴². Notably, SP is critical for both wound area cytokine and NGF production after incision as well as nociceptive sensitization⁵⁷. Given these observations we hypothesized that SP acting through NK1 receptors in opioid treated mice is required for, 1) enhanced nociceptive sensitization, 2) increased peripheral cytokine and NGF production, and 3) up-regulation in expression of OIH-related genes in the spinal cord and dorsal root ganglia.

Materials and Methods

Subjects

All experimental protocols were reviewed and approved by Veterans Affairs Palo Alto Healthcare System Institutional Animal Care and Use Committee prior to beginning the work. Male mice 12–14 weeks old of the C57Bl/6J strain obtained from Jackson Laboratories (Bar Harbor, MA) were kept in our facility a minimum of 1 week prior to initiating the experiments. Breeding pairs of *ppt-A*(+/-) mice congenic in the C57BL/6J background were acquired from Jackson Labs and a breeding colony was established and

each mouse was genotyped according to standard procedures. These mice were derived as previously described¹². All mice were kept under standard conditions with a 12 h light/dark cycle and an ambient temperature of 22±1°C and were allowed food and water *ad libitum*.

Hind paw Incision

The hind paw incision model was used as modified for mice⁵². We have used this model previously in order to study cytokine levels and analgesic effects following incision^{17, 18, 42}. Briefly, mice were anesthetized using isoflurane 2–3% delivered through a nose cone. After sterile preparation with alcohol, a 5 mm longitudinal incision was made with a number 11 scalpel on the plantar surface of the right hind paw. This incision was sufficiently deep to divide deep tissues including the plantaris muscle longitudinally. After controlling bleeding, a single 6-0 nylon suture was placed through the midpoint of the wound and antibiotic ointment was applied. Nociceptive testing and tissue harvest took place at time points up to 96 hours after incision.

Drug Administration

For different groups of mice, morphine or vehicle was administered prior to incision. Mice received morphine (Sigma Chemicals, St. Louis, MO) injections twice per day on an ascending schedule: Day 1, 10 mg/kg; Day 2, 20 mg/kg; Day 3, 30 mg/kg and Day 4, 40 mg/kg was injected subcutaneously. Saline vehicle injections followed the same twice daily schedule. Incision and nociceptive testing procedures began approximately 18 hours after the final dose of morphine or saline. This morphine administration protocol has been used extensively by the lab in studying opioid tolerance, dependence and hyperalgesia^{42, 43, 45}. The selective NK-1 receptor antagonist LY303870 was obtained from Lilly Pharmaceuticals and prepared in sterile 0.9% saline and administered (30 mg/kg, i.p.) concomitantly with morphine/vehicle treatments. LY303870 is highly selective for rodent NK1 receptor and is physiologically active for 24 h at systemic doses up to 30 mg/kg^{28, 32}.

Nociceptive Assay

Mechanical allodynia was assayed using nylon von Frey filaments according to the “up-down” algorithm described by Chaplan et al.¹⁴ as used previously to detect allodynia in mice after incision^{17, 41}. In these experiments, mice were placed on wire mesh platforms in clear cylindrical plastic enclosures 10 cm in diameter and 40 cm in height. After 15 minutes of acclimation, fibers of sequentially increasing stiffness were applied 1 mm lateral to the central wound edge, pressed upward to cause a slight bend in the fiber and left in place 5 sec. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained the next stiffest fiber in the series was applied to the same paw; if a response was obtained a less stiff fiber was applied. Testing proceeded in this manner until 4 fibers had been applied after the first one causing a withdrawal response. Estimation of the mechanical withdrawal threshold by data fitting algorithm permitted the use of parametric statistics for analysis⁵³.

Cytokine Analysis

Cytokines and nerve growth factor (NGF) present in the skin surrounding the wounds or injection sites were assessed in a manner similar to that described previously^{18, 42, 57}. Briefly, mice were sacrificed by CO₂ asphyxiation and an ovalar patch of full-thickness skin providing 1–1.5 mm margins surrounding the hind paw incisions was collected rapidly. These samples containing approximately 12 mg tissue per paw were placed immediately into ice cold 0.9% NaCl containing a cocktail of protease inhibitors (Complete™, Roche Applied Science, Indianapolis, IN). The samples were homogenized using a Polytron device (Brinkman Instruments Inc., Westbury, NY), then centrifuged for 10 min at 12,000 times

gravity at 4°C to remove large particles. Supernatant fractions were kept frozen at –80°C until use. An aliquot was subjected to protein assay (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA) to normalize mediator levels.

For the cytokine assays, custom Bio-Rad (Bio-Rad laboratories, Hercules, CA) Bio-Plex cytokine analysis kits were used in conjunction with the Bio-Plex system array reader according to the manufacturer's directions as described previously¹⁷. The specific cytokines were chosen based on our previously reported results^{17, 18, 57}. Samples were diluted 1:2 prior to analysis in the buffer supplied, and all samples were run in duplicate for each assay. We demonstrated previously that the dynamic range of sensitivity of this assay was sufficient to measure both baseline and incision-stimulated levels of the chosen cytokines¹⁷. Standard curves for each of the analyzed substances were included in each run, and sample concentrations were calculated using Bio-Plex Manager software. Assays of NGF were done using the ChemiKine ELISA kit (Chemicon, Billerica, MA). Sample preparation was identical to that described for the cytokines.

Expression Analysis (mRNA)

Mice were euthanized at specific time points by CO₂ asphyxiation, and spinal cord (SC) and dorsal root ganglia (DRG) tissue were harvested by dissection. Lumbar spinal cord segments L1–L5 and DRG L2–L5 were dissected on a chilled surface. Dissected tissue was then quick-frozen in liquid nitrogen and stored at –80°C until use. For PCR experiments, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity and concentration were determined spectrophotometrically as described previously for brain and spinal cord samples [44]. The cDNA (20 µL final volume) was subsequently synthesized from 1 µg RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Real time PCR reactions were conducted using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and an ABI prism 7900HT system (Applied Biosystems, Foster City, California, USA). To validate the primer sets used in this study, the dissociation curves were performed to document single product formation and the agarose gel analysis was carried out to confirm the size. The data from real time PCR experiments were analyzed by the comparative CT method (using 18S as an internal standard) as described in the manufacturer's manual.

Data Analysis

Data from the mechanical allodynia experiments were analyzed by repeated measures one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's Multiple Comparison Test. The strain comparison were done by 2-way analysis of variance (ANOVA) (strain, time) with with Bonferroni post tests. Cytokine and expression analysis data were analyzed by a two-way ANOVA followed by post-hoc Bonferroni Multiple Comparison Tests. A value of $p < 0.05$ was taken to be significant. All data are presented as mean $\pm$ S.E.M. unless otherwise noted.

Results

Mechanical Allodynia after Escalating Morphine Treatment and/or Hind paw Incision

The first step in our analyses was to determine the degree of pain hypersensitivity generated in both wild type (*wt*) C57BL/6 and *ppt-A*^{–/–} mice congenic in the C57BL/6 background after morphine treatment and/or incision. The escalating dose morphine treatment produced mechanical allodynia which lasted for at least 4 days in the *wt* mice ($F_{7, 49} = 95.42$, $p < 0.001$), in contrast to the *ppt-A*^{–/–} mice which had a lower intensity and duration of tactile hypersensitivity ($F_{7, 49} = 41.80$, $p < 0.001$). Further analysis showed a strain difference

between *ppt-A*^{-/-} and *wt* mice ($F_{1, 112}=123.80$, $p<0.001$), with the former recovering in 3 days (Fig. 1A).

Paw incision produced mechanical allodynia in *wt* and *ppt-A*^{-/-} mice ($F_{7, 49}=70.49$, $p<0.001$) lasting for 4 and 3 days respectively (Fig. 1B). The effect of paw incision on the *ppt-A*^{-/-} mice was less intense than *wt* controls (Strain effect: $F_{1, 112}=31.92$, $p<0.001$). Morphine pretreatment in *wt* mice undergoing hind paw incision resulted in a significant and long lasting tactile hypersensitivity ($F_{7, 49}=98.65$, $p<0.001$). However, the same repeated morphine pretreatment in the *ppt-A*^{-/-} mice undergoing plantar incision resulted in a lower intensity and duration of mechanical allodynia ($F_{7, 49}=23.13$, $p<0.001$). Further statistical analysis showed a significant main effect of strain between the *ppt-A*^{-/-} mice and controls in the magnitude and duration of the tactile hypersensitivity produced by morphine treatment and incision ($F_{1, 112}=123.80$, $p<0.001$), with the former recovering in 4 days (Fig. 1C).

Effect of NK-1 Receptor Blockade on Morphine and/or Incision-Induced Mechanical Allodynia

In order to address the specific question of SP participation in morphine and/or incision induced pain hypersensitivity, a selective NK-1 receptor antagonist LY303870 (30 mg/kg, *i.p.*) was utilized. As can be seen in Figure 2A, selective blockade of this receptor during morphine treatment produced a significant attenuation of morphine induced mechanical sensitivity (Group effect: $F_{1, 112}=155.94$, $p<0.001$), with complete recovery in 2 days. Similarly, simultaneous LY303870 treatment with morphine reduced the magnitude and duration of morphine plus incision induced mechanical sensitivity (Group effect: $F_{1, 112}=155.22$, $p<0.001$) with recovery in 4 days (Fig. 2B). However, LY303870 pretreatment alone had only transient effects on post-incision hyperalgesia (Fig. 2C).

Effects of Hind Paw Incision on Local Cytokine Levels 24 Hours after Escalating Morphine Treatment

In the next step, the effects of morphine pretreatment on hindpaw skin cytokine production following incision in the *ppt-A*^{-/-} mice after 24 hours were determined. The 24 hour time point was selected as incisional and opioid mediated sensitizations were maximal at this point. The cytokines chosen for assay were demonstrated in previous experiments to be up-regulated after hind paw incision¹⁷, and have been explored as mediators of pain in various models. The data in Figure 3 show significant increases in skin levels of the cytokines at the 24 hour time point after incision. Only for MIP-1 α was the enhancement reduced in *ppt-A*^{-/-} mice. At this same time point, morphine treated mice had higher IL-1 β and IL-6 levels after incision. However, for no cytokine tested was there statistical evidence of a dependence on SP for the enhancement of cytokine levels after incision in the setting of repeated morphine administration. These data therefore provided little evidence for SP working through enhanced peripheral cytokine levels to increase nociceptive sensitization after incision.

Effects of Hind Paw Incision on Peripheral NGF Levels 24 Hours after Escalating Morphine Treatment

Previously it was shown that incision causes an enhancement in peri-incisional NGF levels, and that *ppt-A* deletion slightly augments this over-expression for at least 24 hours⁵⁷. Here we went on to determine if the production of incision area NGF was altered in *ppt-A*^{-/-} mice following morphine treatment. As shown in figure 4A, incision enhances NGF levels in both genotypes after incision, while morphine treatment alone for 4 days does not alter NGF levels in unperturbed skin. However, the combination of morphine pre-treatment plus incision dramatically increased NGF levels in the WT but not *ppt-A*^{-/-} mice. To confirm that this effect was mediated by SP signaling through NK1 receptors, we again utilized

LY303870. This drug when administered along with morphine not only blocked opioid hyperalgesia, but also completely blocked the excess incision-induced NGF accumulation in peri-incisional skin (Figure 4B).

Effects Morphine, Incision and their Combination on Spinal Cord *ppt-A* Expression

Evidence from the behavioral experiments suggested that the *ppt-A* gene product SP might be involved in both post-incisional mechanical sensitization and OIH in mice. The main source of SP in the skin and spinal cord is sensory neurons. Therefore, we harvested DRG's from the WT mice 24 hours after incision, morphine treated and incisions with morphine treatment. Analysis of *ppt-A* in these samples showed modest increases in expression after morphine but significant increases following incision with/out morphine treatment (Figure 5).

Effects Morphine, Incision and their Combination on DRG and Spinal Cord Gene Expression

While *ppt-A* dependent effects on the levels of peripheral nociceptive mediators such as NGF provides one mechanism for support of prolonged sensitization after incision in opioid treated mice, changes in expression several genes in the DRG and spinal cord represent other possibilities. We selected for study the *pdyn* (prodynorphin), *grin1* (NMDA1 receptor) and *tacr1* (NK1 receptor) genes in spinal cord tissue and *htr3a* (5-HT3 receptor) gene in DRG tissue as each of the gene products has an established role in OIH^{3, 33, 44, 48, 60}. The expression of all of these genes was increased after morphine treatment and after morphine treatment plus incision in WT mice (Figure 6A–D). The expression of all genes except *pdyn* was increased by incision alone at the 24 hour time point. For *grin1*, *tacr1* and *htr3a* the incision plus morphine treatment conditions lead to an increase in expression greater than observed for incision alone. However, for no gene was any alteration in expression seen after incision, morphine treatment or the combination of the two maneuvers in *ppt-A*^{-/-} mice. Thus it appears that incision and morphine treatment may converge on a shared set of signaling genes in DRG and spinal cord tissue linked to nociceptive sensitization.

Mechanical Allodynia in Hind paw Incision after Recovery from Morphine Induced Hyperalgesia

In these experiments, hind paw incision followed recovery from OIH to temporally dissociate the effects of alterations in stress and immune responses due to withdrawal on incision induced hyperalgesia^{27, 31}. Figure 7 shows hind paw incision produced significantly increased mechanical allodynia in mice previously treated with morphine when compared to saline exposed control mice in the initial 24–72h. Thus opioid exposure causes enhanced sensitization after incision even after animals no longer display baseline evidence of sensitization.

Discussion

Once used most commonly for the management of cancer related pain and pain due to acute trauma, opioids have increased in popularity for controlling chronic non-malignant pain over the past decade⁴⁷. Though the efficacy and risks of using these drugs for more common forms of chronic pain remain somewhat controversial, it is natural that an increasing percentage of patients having surgical procedures will have histories of previous opioid use. Unfortunately, opioid-induced hyperalgesia (OIH) may both limit the efficacy of opioid use, and pose challenges for the management of these patients postoperatively^{3, 13, 38, 59}. Indeed, various studies demonstrate that chronic opioid use portends greater postoperative pain, and possibly a greater likelihood of experiencing opioid-related side effects^{25, 46, 51, 54}. To date few studies have systematically evaluated methods for preventing or controlling

postoperative pain in this population. On the other hand, several putative mechanisms supporting OIH are currently or soon will be amenable to modulation with clinically available medications. One such mechanism felt from preclinical studies to support OIH is NK1 receptor activation by the *ppt-A* gene product substance P (SP)^{33, 61}.

In these studies we sought to better establish the role SP-NK1 signaling might have in supporting OIH as it interacts with incision-related sensitization. The principal findings of our studies were, 1) that mice repeatedly treated with opioids show prolonged recovery times from mechanical sensitization after hindpaw incision, 2) that both incision and morphine treatment up-regulate *ppt-A* in DRG tissue, 3) that while incision and repeated morphine treatment elevate the levels of several pro-nociceptive cytokines, SP-NK1 signaling appears to have little influence on those increases, 4) that NGF is very strongly up-regulated in incised tissue if the animals are pretreated with morphine, and SP-NK1 signaling is critical for this effect, and 5) that the up-regulation of several other genes for which there exists substantial evidence of participation in OIH is dependent upon SP-NK1 signaling (Table 1).

The choice of the SP-NK1 system for study was supported by evidence linking this system to opioid adaptations such as tolerance and OIH in spinal cord and DRG tissues. Studies have shown an increased synthesis of SP in cultured DRG neurons, and our own data show an up-regulation of pre-protachykinin mRNA in DRG tissue from morphine treated mice^{6, 44, 45}. The spinal cord content of SP, present mostly in the terminals of afferent nerve fibers is increased in morphine treated mice⁴⁵. Additional studies have shown that the release of this neurotransmitter in response to noxious stimulation is augmented in animals undergoing sustained morphine treatment using the rate of internalization of post-synaptic NK1 receptors as an assay³³. Ablation of spinal NK1 expressing cells reduces analgesic tolerance to spinally administered morphine⁶¹. More importantly, the use of an intrathecally delivered NK1 antagonist was observed to block OIH assessed by momentary thermal stimuli³³. Prior to this time the role of SP-NK1 signaling and opioid exposure in a model of clinically relevant incisional pain had not been undertaken. Indeed, we observed *ppt-A* null mutant mice or WT mice treated with a selective NK1 antagonist to display reduced development of OIH after repeated morphine treatment, and the exacerbation of mechanical sensitization after incision in similarly pretreated animals was also reduced. It was demonstrated recently that SP-NK1 signaling controls mechanical sensitization after incision perhaps making it reasonable to hypothesize that opioid treatment might further exacerbate sensitization⁵⁷.

Though most investigations involving OIH have focused on CNS tissues, more peripheral mechanisms should not be discounted as potentially contributing. This is particularly true in models involving tissue inflammation and trauma as various peripheral tissues such as components of skin express opioid receptors as well as receptors for neurotransmitters such as SP produced in overabundance in opioid treated animals^{8, 11, 36}. Likewise, sensory neurons express opioid receptors on their peripheral terminals⁹. In fact, early studies demonstrated that very low doses of opioid receptor antagonists administered into the skin of opioid treated rats could cause hyperalgesia¹. Several studies have demonstrated effects of opioids on wound healing suggesting functionally relevant peripheral sites of action^{7, 10}. Our laboratory has examined the levels of nociception-related cytokines after incision in control and morphine treated mice and have found the levels of some of these mediators to be moderately increased in mice repeatedly treated with morphine, consistent the greater sensitization observed^{17, 57}. In the present studies we chose to analyze opioid and SP-NK1 effects on the levels of IL-1 β , IL-6, MIP-1 α and G-CSF because each of these is elevated in peri-incisional skin and each can contribute to nociceptive sensitization. For IL-1 β , IL-6, MIP-1 α and G-CSF we observed morphine to further enhance the peri-incisional levels yet

deletion of the *ppt-A* gene had no discernable effect. Thus the opioid-induced exacerbation of mechanical sensitization after incision does not appear to depend upon opioid-induced enhancement of the levels of several key cytokines.

On the other hand, we found robust effects for both morphine treatment and SP-NK1 signaling on peri-incisional NGF levels. The neurotrophin NGF is of particular interest because of its strong association with incisional pain. Several studies report the expression of NGF after incision, particularly in keratinocytes and peri-incisional fibroblasts^{4, 63}. In mice pretreated with morphine for 4 days, basal skin levels of NGF were not changed. However, morphine pretreated mice with incisions demonstrated approximately 10-fold increases in NGF and these changes were demonstrated to depend on SP-NK1 signaling, as *ppt-A* gene deletion or blocking the NK1 receptor greatly reduced augmented NGF levels. Thus the over-expression of NGF after incision in mice treated repeatedly with morphine may play a role in their prolonged sensitization after incision, though there is little evidence that changes in peripheral NGF levels participate in the sensitization to transient noxious stimuli applied to intact skin.

Alterations in gene expression in spinal cord and DRG tissues have been studied in relation to OIH and incision. We hypothesized that the expression of genes whose products have well demonstrated roles in supporting OIH would be increased by morphine treatment alone and further augmented within 24 hours of incision. We also hypothesized that these changes would be dependent on SP-NK1 signaling. Indeed morphine treatment enhanced the expression of genes coding for prodynorphin, NMDAR1 and NK1 (spinal cord)^{34, 41}, and the 5-HT₃ receptor as well as mRNA of the SP precursor itself in the DRG^{16, 44, 45}. In all cases the combination of incision plus morphine treatment leads to higher levels of expression than incision alone. The effects of incision, morphine treatment and their combination were eliminated in *ppt-A* null mutant mice. These observations suggest that SP-NK1 signaling is a mechanism on which incision and chronic morphine treatment converge to regulate the expression of genes which in turn support nociceptive sensitization.

Pain after surgery remains an unresolved yet common clinical problem. Unfortunately, the management of postoperative pain in opioid consuming patients is even more problematic, and the number of patients in this situation is likely to grow. Understanding mechanisms supporting pain in the setting of tissue trauma and opioid exposure may lead to strategies reducing excess pain. While very little work has been done to identify effective treatment approaches in this setting, one recent study demonstrated that use of the NMDA receptor blocker ketamine could reduce postoperative opioid requirements in previously opioid exposed patients undergoing spine surgery⁴⁶. The analogous laboratory model employed in the present study provides a potential role for spinal cord NMDA receptors in augmented hyperalgesia seen after incision in previously opioid exposed mice, consistent with the results of the ketamine trial. In addition, potent NK1 receptor antagonists are now available and possibly would have special value as adjuvant in opioid consuming populations undergoing surgery. Likewise, 5-HT₃ antagonists are commonly used as antiemetics though analgesic properties have not emerged in their two decades of clinical use. Perhaps in the setting of incision and chronic opioid use, these 5-HT₃ antagonists might become a more useful analgesic target. Lastly, biologic anti-NGF antibodies are being developed as analgesics, and appear to have a broad spectrum of action in pre-clinical studies^{4, 30, 50, 56, 63}. The observed link between sustained morphine exposure and enhance peripheral NGF production suggests that these anti-NGF agents may be particularly useful in treating pain in the post-surgical chronic opioid consuming patient. In any case, our observations add to the growing body of evidence that both peripheral and central mechanisms may contribute to the high pain levels experienced by opioid consuming patients after surgery.

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Perspective

These studies show that SP signaling modulates enhanced NGF production and changes in neuronal gene expression seen after incision in mice previously exposed to morphine.

Figure 1.

Assessment of mechanical allodynia after escalating morphine treatment and/or hind paw incision. Mechanical allodynia was measured in the wild type (WT) and *ppt-A*^{-/-} mice using calibrated von Frey filaments before and at different time points after morphine treatment (A), incision (B) and morphine treatment followed by hind paw incision (C).

Data are presented as Mean $\pm$ S.E.M and were analyzed by two way ANOVA with post hoc Bonferroni tests comparing strains (n=8 each) at corresponding time points. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ indicate difference from baseline for each strain and # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ represents strain differences at corresponding time points.

Figure 2.

Assessment of selective NK-1 receptor antagonist Ly303870 effects on escalating morphine and/or incision induced mechanical allodynia. Mechanical hypersensitivity was measured in four treatment groups of wild type mice: vehicle/vehicle, vehicle/morphine, LY303870/vehicle and LY303870/morphine (A). The effect of NK-1 receptor blockade was also measured in hindpaw incision alone (B) and in hindpaw incision after morphine treatment (C). LY303870 (30 mg/kg, i.p.) and morphine treatments (Escalating dose, s.c.) were carried out for 4 days (n=8 each group). Mean $\pm$ S.E.M values of each group were analyzed by a two way ANOVA with post hoc Bonferroni tests comparing treatment groups at each time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ indicate difference from baseline for each individual group and # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ represents group differences at corresponding time points.

Figure 3.

Effects of escalating morphine treatment and/or hind paw incision on local cytokines levels. The levels of cytokines were measured at 24 hours time point after incision. The 24 hour time point was selected as incisional and opioid mediated sensitizations were maximal at this point (Figure 1). Different groups of mice of both wild type and *ppt-A*^{-/-} were used (n=6 per group). Data are presented as Mean ± S.E.M and were analyzed by two way ANOVA with post hoc Bonferroni tests comparing strains at corresponding time points. **p<0.01; ***p<0.001 indicate difference from baseline for WT and ## p<0.01; ###p<0.001 represents difference from baseline for *ppt-A*^{-/-} mice. + p<0.05 represents strain differences.

Figure 4.

Effects of escalating morphine treatment and/or hind paw incision on NGF levels. NGF levels were measured at 24 hours time points after incision. Different groups of mice of both wild type and *ppt-A*(-/-) were used for assessment (A). ** $p < 0.01$; *** $p < 0.001$ indicate difference from baseline for WT and # $p < 0.05$; ### $p < 0.001$ represents difference from baseline for *ppt-A*^{-/-} mice. + $p < 0.05$ represents strain differences. Assessment of selective NK-1 receptor antagonist Ly303870 effects on NGF levels after morphine and/or incision in WT mice (B). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ indicate differences between incised and non incised in each treatment group and ### $p < 0.001$ represents differences in relation to both other treatment groups. Data are presented (n=6 per group) as Mean $\pm$ S.E.M and were

analyzed by two way ANOVA with post hoc Bonferroni tests comparing strains at corresponding time points.

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Figure 5.

Effects of hind paw incision on spinal cord *ppt-A* gene (*tac1*) expression after escalating morphine treatment in WT mice. The *ppt-A* mRNA levels in DRG were measured by real time PCR in different treatment groups (n=6 each). Data are presented as Mean ± S.E.M and were analyzed by one way ANOVA with post hoc Bonferroni tests comparing treatment groups. **p<0.01 and ***p<0.001 represents significant difference from saline/No Incision group; ## p<0.01 represents difference from saline/Incision group and +++ p<0.05 represents differences between Morphine/No Incision vs. Morphine/Incision groups.

Figure 6.

Effects of hind paw incision on DRG and Spinal Cord gene Expression after escalating morphine treatment in the wild type and *ppt-A*^{-/-} mice. The mRNA levels of *pdyn* (prodynorphin), *grin1* (NMDA1 receptor) and *tacr1* (NK1 receptor) genes (spinal cord) and *htr3a* (5-HT3 receptor) gene (DRG) were measured by real time PCR in different treatment groups (n=4–6). Data are presented as Mean ± S.E.M and were analyzed by a two way ANOVA with post hoc Bonferroni tests. * p<0.05; ** p<0.01; *** p<0.001 indicate difference from baseline for each strain and # p<0.05; ## p<0.01; ### p<0.001 represents strain differences for corresponding treatments.

Figure 7.

Assessment of mechanical allodynia after recovery from escalating morphine treatment induced hyperalgesia followed by hind paw incision. Mechanical allodynia was measured in the wild type (WT) mice using calibrated von Frey filaments before and at different time points after morphine treatment. Data are presented as Mean $\pm$ S.E.M and were analyzed by two way ANOVA with post hoc Bonferroni tests comparing saline to morphine treated groups (n=8 each) at corresponding time points. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ indicate difference treatment group differences at corresponding time points.

Table 1

Summary of expression changes in skin, DRG and spinal cord tissue after treatment with morphine, hindpaw incision or both. Cells are designated “+” if a statistically significant difference was found versus control at the p 0.05 level (ND=Not detected). Mediator levels in skin were measured at the protein level using ELISA assays. Gene expression in the DRG and spinal cord was measured using qPCR. Dependence on ppt-A refers to the requirement for an intact ppt-A gene to observe full enhancement of expression after incision in morphine treated mice.

Gene or Mediator	Δ Incision	Δ Morphine	Δ Incision/Morphine	ppt-A Dependence
<u>Skin</u>				
IL-1 β	+	–	+	–
IL-6	–	–	+	–
MIP-1 α	+	–	+	–
G-CSF	+	–	+	–
NGF	+	–	+	+
<u>DRG</u>				
5-HT3a	+	+	+	+
ppt-A	+	+	+	ND
<u>Spinal Cord</u>				
pdyn	–	+	+	+
grin1	+	+	+	+
tacr1	–	+	+	+