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Role of substance P signaling in enhanced nociceptive sensitization and local cytokine production after incision

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ABSTRACT

Substance P (SP) signaling facilitates nociceptive sensitization in various inflammatory and chronic pain models and we postulated that SP signaling might also contribute to the development of post-incisional hyperalgesia. These studies used mice with a deletion of the pre-protachykinin A gene (*ppt-A*$^{-/-}$) which codes for SP to determine the role of SP signaling in post-incisional pain and in the increased cytokine and nerve growth factor (NGF) expression observed in the incised skin. SP deficient *ppt-A*$^{-/-}$ mice displayed reduced mechanical allodynia and heat hyperalgesia compared to the wild-type (*wt*) mice at all post-incision time points, despite similar baseline values ($p < 0.001$). Furthermore, the NK-1 receptor antagonist LY303870 attenuated mechanical allodynia produced by incision in the *wt* mice ($p < 0.001$). Incision also up-regulated IL-6, TNF-α and KC levels but not IL-1β after 2 h in the *wt* mice skin. However, *ppt-A*$^{-/-}$ mice had more skin NGF levels 2 h post-incision. Subcutaneous hind paw SP injection produced acute and transient elevations of IL-1β, IL-6, and KC but modest elevations in TNF-α levels in the *wt* mice. Systemic LY303870 reversed the SP-induced elevations of these cytokines. Hind paw injection of IL-6 and NGF dose dependently produced less mechanical allodynia in the *ppt-A*$^{-/-}$ compared to *wt* mice. Additionally, SP produced mechanical allodynia in a dose-dependent fashion in *wt* mice. Therefore, SP supports nociceptive sensitization after hind paw incision and potentially participates directly in modulating the intensity of inflammatory response in peri-incisional tissue.

1. Introduction

The pre-protachykinin-A gene (*ppt-A*) codes for primary afferent neurotransmitters substance P (SP) and neurokinin A (NKA) [11,20], with the former being better studied. SP is a neuromodulator with a well-described role in pain signaling, possessing the unique feature of only being released upon strong nociceptive stimulation. For instance, spinal cord internalization of the NK-1 receptor, an index of SP release, occurs only to a minor extent in lamina I neurons during threshold-level heat stimulation in normal animals [11]. However, the same level of heat stimulation leads to both a greater percentage of NK-1 internalization in lamina I neurons and internalization of receptors in deeper spinal cord laminae after inflammation [1]. Interestingly, SP does not seem to be required for normal heat intensity coding or peak firing of these neurons across a range of temperatures, rather it seems to prolong heat stimulus responses [32]. It is these curious properties which may underlie the normal responses *ppt-A*$^{-/-}$ mice display to low intensity heat, mechanical and chemical stimuli with deficits only seen in paradigms involving intense or prolonged stimulation [11]. Sensitization in chronic pain models like nerve injury (neuropathic) and CFA-induced (chronic inflammatory) are normal in the *ppt-A*$^{-/-}$ mice [11]. This knockout model displays the favorable feature of having intact NK-1 receptor expression, with normal levels of expression of several primary afferent neurotransmitters such as calcitonin-gene-related peptide, dynorphin, galanin, neuropeptide Y and somatostatin [11].

Incision represents an acute, relatively intense and persistent nociceptive stimulus. The role of *ppt-A* gene products signaling in supporting nociceptive sensitization after incision has not been explored. Aside from the neurotransmitter function of SP and NKA in the spinal cord, both *ppt-A* gene products have significant peripheral actions which might impact their roles in controlling nociceptive sensitization and other characteristics of incisional wounds. For example, SP is an important participant in neurogenic extravasation.

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Limited neurogenic extravasation is seen in the *ppt-A*$^{-/-}$ mice or after the administration of NK-1 antagonists in rats [11,26,33]. Neurogenic inflammation mediated by SP has been linked to scar formation and several chronic skin conditions [2]. Additionally, SP has been shown to control the production of cytokines in skin and skin cells after injury or inflammation [20,38,39]. Moreover, SP-mediated increase in cytokine production in coordination with inflammatory cellular infiltration at the injury site appears to be necessary for adequate wound healing and the ability to resist infection [21,22,24,43] . Many of the cytokines modulated in skin by SP have also been linked to nociceptive sensitization including IL-1β, IL-6 and TNF-α [17–19]. Local and systemic administration of NGF, the cutaneous level of which is also modulated by SP, is also strong nociceptive sensitizer [4,5]. Limited information concerning NKA suggests it can interact with cytokines in enhancing airway hyperreactivity [49].

Our hypothesis in designing this work was that *ppt-A* gene product SP enhances incisional mechanical allodynia via two distinct mechanisms. The first is that SP directly participates in nociceptive signaling acting as a neurotransmitter during peripheral sensitization following incision. The second is that this neurokinin supports pro-nociceptive cytokine production in skin thus indirectly enhancing nociceptive sensitization.

2. Materials and methods

2.1. Animal use

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 Laboratories and a breeding colony was established and each mouse was genotyped according to standard procedures. These mice were derived as previously described [11]. 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*.

2.2. Hind paw incision

The hind paw incision model was used as modified for mice [47]. We have used this model previously in order to study cytokine levels and analgesic effects following incision [15,16,36]. 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 48 h after incision.

2.3. Drug administration

For some groups of mice nociceptive mediators or vehicle were injected subcutaneously into the plantar skin (*i.pl.*) of the hind paws of non-incised mice. For these injections mice were gently restrained. The injection volume was 15 μL administered through a 30 gauge needle which raised a bleb similar to the length of the incisional wounds and approximately 1 mm of surrounding tissue. For these experiments, substance P (SP), interleukin-6 (IL-6) and nerve growth factor (NGF) were obtained from Sigma Chemicals, St. Louis, MO. The selective NK-1 antagonist LY303870 was obtained from Lilly Pharmaceuticals. Cytokines and NGF were prepared in sterile 0.9% PBS which was the vehicle used for control injections. SP and LY303870 were prepared in sterile 0.9% saline.

2.4. Nociceptive testing

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 [15,35,37]. 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 min 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 for 5 s. 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 four 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 analysis [48].

Response latencies to noxious heat stimulation were measured using the method of Hargreaves [29] modified for use with mice [34]. In this assay mice were placed on a temperature controlled glass platform (23.5–24.0 °C) in a plastic enclosure as described above. After 15 min of acclimation, a beam of focused light was directed towards the same area of the hind paw as described for the von Frey assay. The time to withdraw the foot from the beam of light was measured. A 15-s cutoff was used to prevent tissue damage. Two measurements were made per animal per test session. For experiments involving extended time course measurements the animals were returned to their cages between nociceptive testing sessions.

2.5. 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 [15,16,36]. To obtain skin samples for cytokine quantification animals were first sacrificed by CO_2 asphyxiation and an ovular 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). Approximately 750 μL inhibitor containing saline was used per 25 mg tissue. 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 previously [15]. The specific cytokines were chosen based on our previously reported results and included IL-1β, IL-6, KC and TNF-α [15,16]. 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 cytokines [15]. Standard curves for each of the analyzed substances were included in each run, and sample concentrations were calculated using Bio-Plex Manager software. NGF assays were done with the ChemiKine ELISA kit (Chemicon, Billerica, MA). Sample preparation was identical to that described for the cytokines.

2.6. Statistical analysis

Data obtained from the mechanical allodynia and heat hyperalgesia experiments were analyzed by Repeated Measures one-way analysis of variance (ANOVA) followed by post hoc Dunnett's Multiple Comparison Test. Cytokine 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 the mean ± SEM unless otherwise noted.

3. Results

3.1. Mechanical allodynia and heat hypersensitivity after hind paw incision

The first step in our analyses was to determine the degree of heat hyperalgesia and mechanical allodynia generated in both wild-type *ppt-A*$^{+/+}$ and *ppt-A*$^{-/-}$ mice congenic in the C57BL/6 background after incision. The *ppt-A*$^{-/-}$ mice displayed reduced mechanical allodynia compared to the wild-type (*wt*) mice at all time points measured after incision despite having comparable baseline values ($F_{1,98} = 66.10$, $p < 0.001$; Fig. 1A). Likewise, the *wt* mice developed a more robust heat hyperalgesia after incision compared to the *ppt-A*$^{-/-}$ mice ($F_{1,66} = 20.43$, $p < 0.001$; Fig. 1B). However, significant differences between the two groups were observed for the 2 h post-incision time point only, despite the *wt* mice having significantly lower paw withdrawal latencies up to 48 h (compared to baseline values). Therefore, only assessment of mechanical sensitization was carried out for the rest of the experiments.

3.2. Effect of NK-1 receptor blockade on incision-induced mechanical allodynia

The specific question of SP participation in post-incisional sensitization was addressed by administering the NK-1 selective antagonist LY303870 (40 mg/kg *i.p.*) 30 min prior to incision. As can be seen in Fig. 2, selective blockade of this receptor produced a significant attenuation of incision-induced mechanical sensitivity for approximately the first 12 h after incision consistent with the half life of the drug in mice ($F_{1,126} = 43.41$, $p < 0.001$).

3.3. Effects of hind paw incision on peripheral cytokines and NGF levels

The demonstrated roles of cytokines in nociceptive sensitization including the sensitization of skin surrounding incisions as well as the ability of primary afferent nerve fibers to control inflammation in tissues surrounding wounds lead us to determine if the production of incision area cytokines and NGF were altered in *ppt-A*$^{-/-}$ mice. The cytokines chosen for assay were demonstrated in the previous experiments to be modulated after hind paw incision [15]. The time course of cytokine generation and the magnitude of changes in *wt* animals were similar to that seen in previous studies. Fig. 3A–E demonstrates that three of the four cytokines were generated in lesser quantities in *ppt-A*$^{-/-}$ mice, though differences from the *wt* mice were significant only at the 2 h time point.

The neurotrophin nerve growth factor (NGF) has also been linked to incisional sensitization [8], and may be under the control of SP in skin [4]. Therefore, we performed experiments in which NGF levels

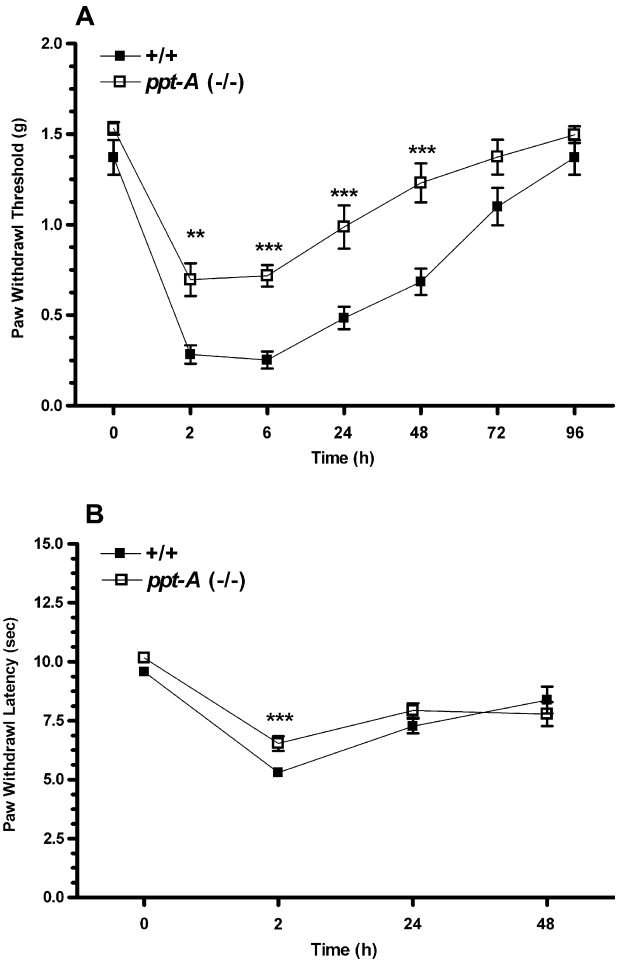

Fig. 1. Assessment of mechanical allodynia and heat hyperalgesia after hind paw incision. (A) Mechanical allodynia was measured in the wild–type ($n = 8$) and *ppt-A*$^{-/-}$ ($n = 8$) mice using calibrated von Frey filaments before and at different time points after incision. (B) Paw withdrawal latencies to heat stimuli were measured in the wild-type ($n = 12$) and *ppt-A*$^{-/-}$ ($n = 12$) mice using the Hargreaves method. Data are presented as mean ± SEM and were analyzed by two-way ANOVA with post hoc Bonferroni tests comparing strains at corresponding time points. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

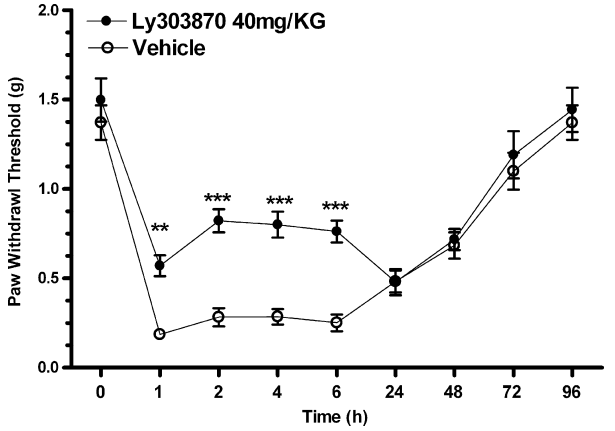

Fig. 2. Assessment of selective NK-1 receptor antagonist effects on incision-induced mechanical allodynia. Mechanical hypersensitivity was measured in two different groups of wild-type mice: vehicle pretreatment/incision, LY303870 (40 mg/kg *i.p.*) pretreatment/incision ($n = 8$ each group). Means ± SEM values of each group were analyzed by two-way ANOVA with post hoc Bonferroni tests comparing treatment groups at each time point. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

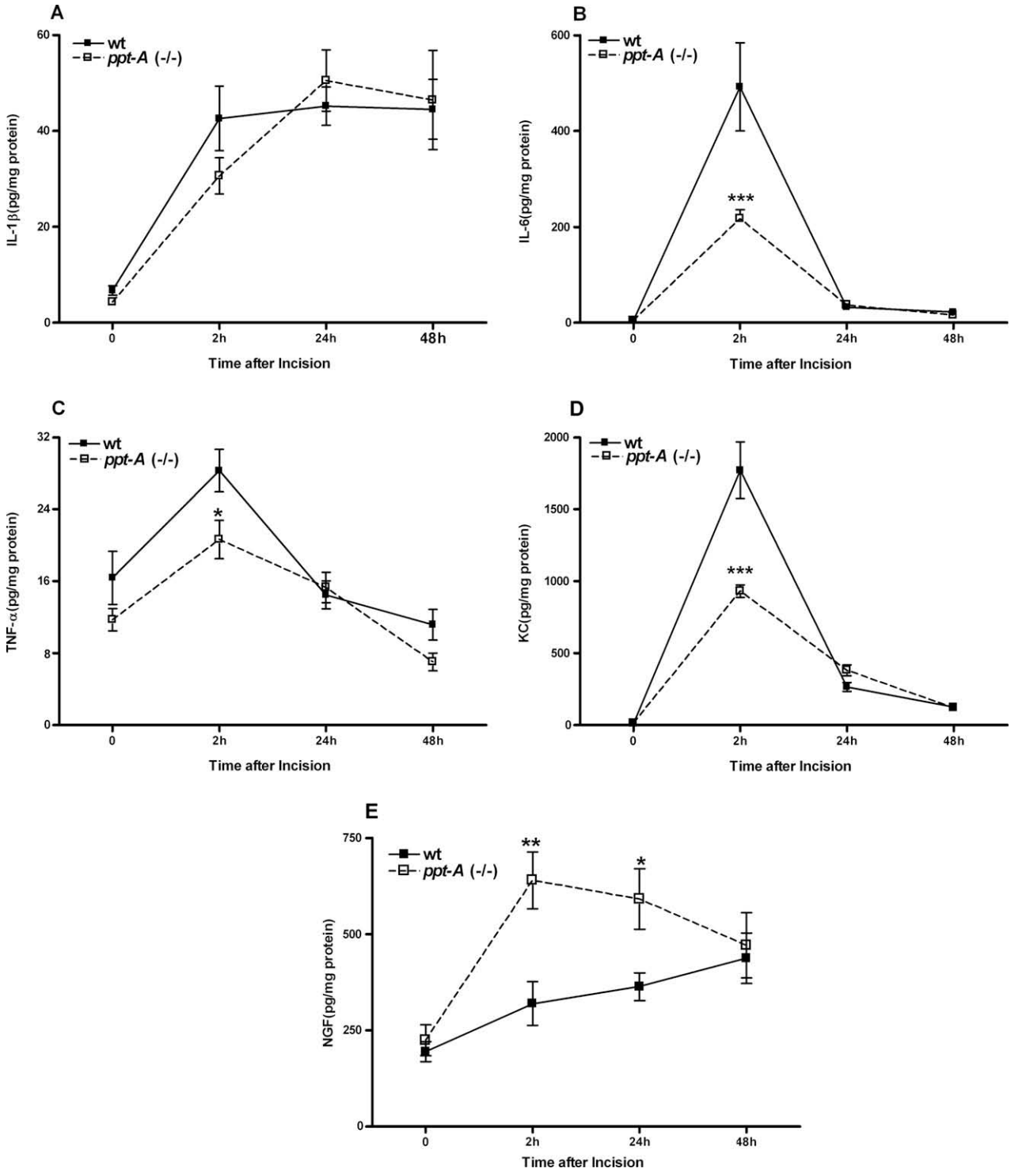

Fig. 3. Effects of hind paw incision on peripheral cytokines and NGF levels. The levels of (A) IL-1β. (B) IL-6, (C) TNF-α. (D) KC and (E) NGF were measured at baseline and at the 2–48 h time points after incision. The selected time points were based on the behavior data presented in Fig. 1. Different groups of mice of both wild-type and *ppt-A*$^{-/-}$ were used for each time point ($n = 6$ per group). Data are presented as mean ± SEM and were analyzed by two-way ANOVA with post hoc Bonferroni tests comparing strains at corresponding time points. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

in the skin surrounding wounds was measured both before and at various time points after incision in *wt* and *ppt-A*$^{-/-}$ mice. In the *wt* samples NGF levels slowly increased after incision whereas in the *ppt-A*$^{-/-}$ mice NGF levels were increased more rapidly and to a greater extent (Fig. 3F). The differences from the *wt* mice were statistically significant at the 2 and 24 h time points. Thus while *ppt*-A deficiency leads to a transient deficit in cytokine production, it leads to an over-expression of NGF in peri-incisional skin.

3.4. Effects of peripheral substance-P administration and subsequent NK-1 blockade on cytokines and NGF levels

In order to test the hypothesis that SP itself can promote cytokine production in skin, 1 μg SP was injected (*i.pl.*) in *wt* mice, followed by tissue harvest from 2 to 48 h after administration. Fig. 4 demonstrates significant elevations of IL-1β, IL-6, KC and NGF levels at the 2 h time point compared to vehicle, with

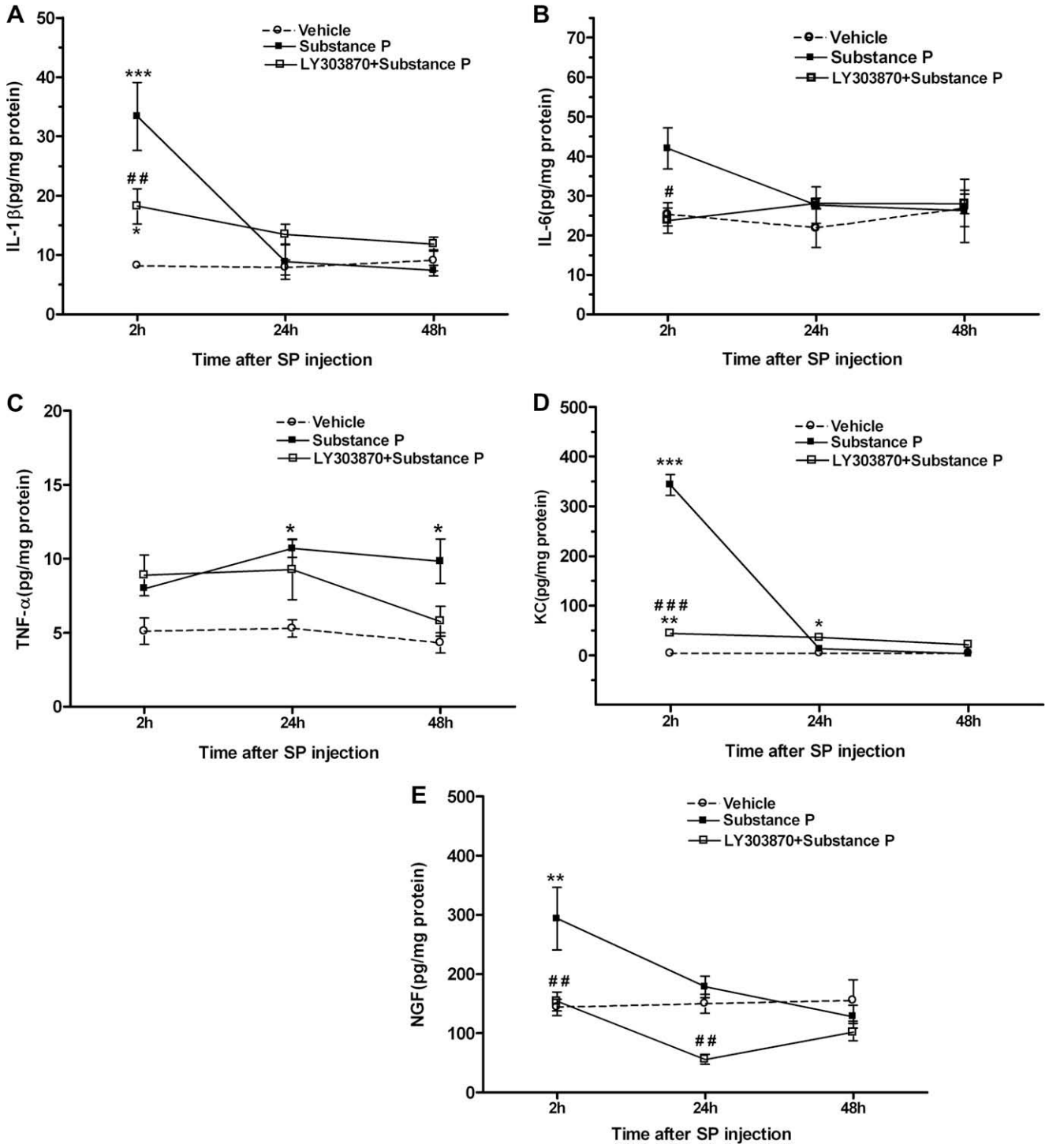

Fig. 4. Effects of intra-plantar substance P (SP: 1 μg/15 μL), LY303870 (40 mg/kg *i.p.*) pretreatment/ intra-plantar substance P (SP:1 μg/15 μL) and vehicle (15 μL saline) on peripheral cytokines and NGF levels. The levels of (A) IL-1β. (B) IL-6, (C) TNF-α. (D) KC and (E) NGF were measured at the 2–48 h time points after SP administration. Different groups of mice of both wild-type and *ppt-A*$^{-/-}$ were used for each time point (n = 4–6 per group). Data are presented as mean ± SEM and were analyzed by two-way ANOVA with post hoc Bonferroni tests comparing strains at corresponding time points. Significant difference from vehicle:$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Significant difference from SP: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

the levels returning back to baseline values by 24 h post-SP administration. However, local SP injection had a modest effect on TNF-α production. As demonstrated here, the effects of peripheral SP administration were acute and transient. The selective NK-1 receptor antagonist LY303870 (40 mg/kg *i.p.*) 30 min prior to SP (1 μg/15 μL; *i.pl.*) in *wt* mice was able to reduce the acute and transient elevations of IL-1β, IL-6, KC and NGF levels (2 h), while having no effect on the production of these cytokines at later time points (Fig. 4). In order to examine the 24 and 48 h effect of LY303870 blockade of SP cytokine production, the NK-1 receptor antagonist had to be re-administered every 12 h.

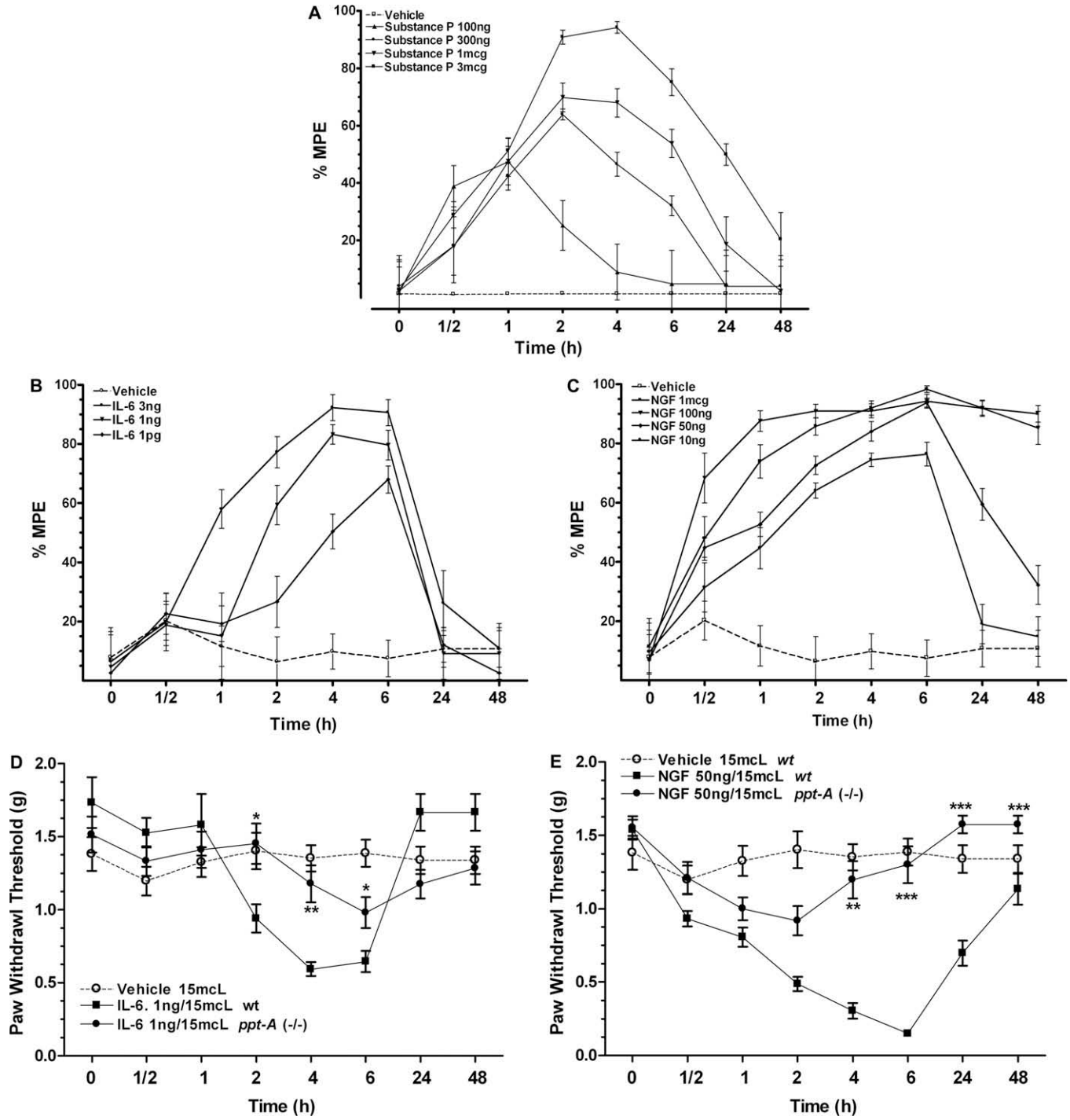

Fig. 5. Effects of peripheral administration of substance-P, IL-6 and NGF on mechanical sensitivity. (A) Intra-plantar substance P and vehicle were given after baseline mechanical paw withdrawal threshold determination. (B) and (C) Intra-plantar IL-6, NGF and vehicle given after baseline measurements of paw withdrawal threshed. Data of the effect of different doses of SP, IL-6 and NGF are presented as mean percent effect, with baseline values as being zero effect. Higher doses of NGF produced ongoing mechanical hyperalgesia for several days (data not shown). (D) and (E) Comparison of the paw withdrawal thresholds of wild-type and *ppt-A*$^{-/-}$ mice at various time points given 1 ng/15 μL of IL-6 and 50 ng/15 μL of NGF by intra-plantar injection. Data are presented as mean ± SEM and were analyzed by two-way ANOVA with post hoc Bonferroni tests comparing strains at corresponding time points. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.5. Effects of peripheral administration of substance-P, IL-6 and NGF on mechanical sensitivity

While the data obtained so far suggest possible roles for SP, several cytokines and NGF in incisional sensitization, the direct effects of these mediators on nociception needed to be determined. We therefore selected SP, IL-6 (one of the most robustly modulated cytokines) and NGF for injection into hind paw skin using the robust mechanical assay to follow nociceptive sensitization. Fig. 5A shows the dose-dependant response of peripheral SP administration on the *wt* mice, while vehicle administration had no effect in this assay.

Subsequently, we addressed the question of whether a given dose of nociceptive mediator caused as much sensitization in the

ppt-A$^{-/-}$ as in the *wt* mice. Both IL-6 and NGF dose-dependently lowered mechanical thresholds, and the thresholds remained depressed for extended periods after injection (Fig. 5B and C). Finally, Fig. 5D and E demonstrates that *ppt*-A gene deletion reduces the degree and duration of mechanical allodynia after the subcutaneous administration of IL-6 and NGF. The doses utilized in these experiments were chosen as the lowest dose that produced near maximal effect from the previous set of experiments.

4. Discussion

The *ppt*-A gene product SP has been implicated in modulating relatively high intensity nociceptive signaling occurring with the application of strong heat, mechanical and chemical stimuli. It was unclear, however, how SP participates in models of incisional pain. Earlier pharmacological testing has implicated the NK-1 SP receptor in post-incisional sensitization [25,62]. Consistent with those reports the present study demonstrated that the *ppt-A*$^{-/-}$ mice exhibit deficits in both heat and mechanical nociceptive signaling in the incisional model. Subsequently, we demonstrated that SP has at least two potential mechanisms in supporting nociception after incision thus furthering our understanding of SP function in this model. The first is the well-established role SP has as a neurotransmitter in activating the spinal cord dorsal horn neurons. Local injection of the classic pro-nociceptive cytokine (IL-6) and neurotrophin NGF sensitized the *wt* mice to a greater degree than the *ppt-A*$^{-/-}$ animals. These data are consistent with others showing that chemical stimulation of peripheral primary afferent fibers leads to less nociceptive behavior in these knockout mice [11]. Our studies, however, used endogenously produced mediators present in human wounds [13]. Interestingly, we observed that cytokine and neurotrophin production in the knock out mice incised skin were dysregulated when compared to *wt* mice; the production of several cytokines (IL-6, KC and TNF-α) was partially inhibited. Moreover, we demonstrated that increased production of skin cytokines and NGF after local SP injection could be reversed by NK-1 receptor antagonism. Thus, disruption of SP signaling lowers the responses to key nociceptive mediators and reduces the production of some of those same mediators.

Our observations of sharply reduced mechanical allodynia in *ppt-A*$^{-/-}$ mice after hind paw incision seem to fit with our current understanding of peripheral nociceptive signal transmission. Substance P is expressed predominantly by C-fibers. Detailed electrophysiological studies using the hind paw incision model have demonstrated sensitization and expansion of the receptive fields for both C- and A-delta afferent fibers [28,47]. Additional studies have provided evidence of spontaneous C-fiber activation in incisional wounds specifically [6,7]. Furthermore, capsaicin stimulation of small afferent C-fibers is attenuated in *ppt-A*$^{-/-}$ mice [11]. Finally, the unusual mammal Heterocephalus glaber (mole-rat) fails to produce detectable levels of SP in cutaneous c-fibers, thought to be responsible for it's the lack of sensitization after capsaicin injection [46]. Thus the existing literature is consistent with a role for SP expressing C-fibers supporting mechanical allodynia after incision.

Much less clear prior to undertaking these studies was whether endogenously produced substances involved in inflammatory and incisional pain, e.g. NGF and cytokines, rely on SP to support nociceptive signaling. Several cytokines and recombinant NGF have been demonstrated to cause hyperalgesia when injected intradermally in rats and mice [3,4,17–19.53,58–60]. Our own studies demonstrated that intradermal IL-6 and NGF as well as SP dose-dependently cause profound mechanical allodynia in treated paws of *wt* mice. Thus the reductions in skin cytokine abundance seen at an early post-incisional time point in the *ppt-A*$^{-/-}$ mice, might be sufficient to explain in part the longer period of reduced mechanical allodynia observed here.

Direct evidence for peripheral cytokine sensitization of C-nociceptive afferent nerve fibers is somewhat less abundant. Fu and Longhurst were able to show the sensitization of visceral C-fibers after intra-arterial administration of IL-1β [9]. Local perfusion of dorsal root ganglia (DRGs) with IL-1β, IL-6 and TNF-α also causes enhanced responsiveness of C-fibers [45,63]. Using a model of joint pain, Brenn et al. documented enhanced C-fiber activity after the application of IL-6 [10]. Other investigators failed to observe sensitization of sural nerve C-fibers after TNF-α, IL-1β and IL-6 application [42]. Thus it is not clear if all C-fiber populations are responsive to cytokine sensitization under all circumstances. Even less clear is whether the cytokines are interacting directly with cytokine receptors on peripheral fibers to cause sensitization as has been postulated by Summer et al. for IL-6, or whether complex local signaling cascades involving cytokines and prostanoids in addition to the recruitment of inflammatory neutrophils to the area is the underlying relevant pathway [18,19,53]. Much additional work needs to be completed before conclusions concerning the mechanism(s) of action of cytokines in supporting nociception in incisional wounds can be reached. One mechanism not addressed in our work involves SP-induced activation of glial cells and subsequent release of cytokines as has been implicated in the genesis of inflammatory and neuropathic pain states [27,44,54,55,61].

It is clear, however, that the *ppt-A* gene product SP can stimulate the production of cytokines in skin. In fact, SP is found in significant levels in human wound fluids [13]. Evidence supporting the hypothesis that SP can stimulate cytokine production includes the observations of Dallos et al. who demonstrated that SP applied to keratinocyte cultures increased IL-1α, IL-8 and TNF-α mRNA levels [20]. Liu et al. observed similar results when SP was applied to HaCaT cells (human epidermal keratinocyte cell line) [38]. The subcutaneous injection of SP causes nociceptive sensitization which can be blocked by NK-1 receptor antagonists, though the direct effects of SP on afferent neurons versus indirect activating effects through the stimulation of cytokine production has not been explored [12]. Our own results show that the skin administration of SP in mice causes rapid, dose-dependant but transient sensitization. It has been proposed that SP signaling via the NK-1 receptor is involved in the induction but not in the maintenance of nociception [40,56]. Yet again, the very long time course of mechanical allodynia after direct cytokine administration suggests that the deficiency in the production of multiple cytokines in *ppt-A*$^{-/-}$ mice early after incision might explain some of the diminished mechanical sensitization observed at later time points.

Our results concerning NGF levels in the skin of *wt* versus *ppt-A*$^{-/-}$ mice after incision were somewhat unexpected. While baseline levels of this neurotrophin were indistinguishable between the genotypes, incision lead to greater NGF production in the skin of knockout mice compared with *wt* controls. However, as demonstrated here, NGF is a pro-nociceptive molecule when injected into skin. To understand this apparent conflict it should be kept in mind that in the *ppt-A*$^{-/-}$ responses to many types of stimuli ranging from cytokines to formalin to capsaicin are diminished. This suggests that despite the presence of an increased level, NGF may have an impaired ability to cause maximal sensitization in *ppt-A*$^{-/-}$ mice after incision, potentially providing an explanation for our observations. Another compelling explanation for the enhanced post-incisional NGF accumulation in the skin of the *ppt-A*$^{-/-}$ mice is that denervation of the skin leads to an up-regulation of NGF expression [30,41]. This increase in NGF may be responsible both for nerve regeneration to the area and, perhaps, for enhanced nociceptive sensitivity in nerve injury models [23,50,51]. Deletion of the *ppt*-A gene might be viewed as a functional partial denervation of

the skin thus explaining the enhanced abundance of NGF after incision. Arguing against this simple view, however, is the observation that direct treatment of keratinocyte cultures with SP leads to an increase in NGF [20] and our own observation that skin SP injection increases NGF levels via a NK-1 receptor dependent mechanism. Perhaps the mechanisms linking SP release to NGF production are more complex in the setting of incision than when SP is injected in purified form to normal tissue.

The (*ppt-A*) gene codes for both SP and NKA, the contribution of the latter to cytokine and neurotrophin production, as well as, the phenotype observed in the incisional model has not been investigated. The scope of the present study has been limited to one component of the (*ppt-A*) gene product (SP) and therefore the effect of a selective NK-1 receptor antagonist has been studied. At this juncture, it is noteworthy to mention that the efficacy of SP receptor blockade in human pain conditions has not lived up to the results of animal studies [31,52]. Several mechanisms has been proposed for this lack of agreement: the role of the stress component of pain perception, species variations in NK-1 receptors and the choice of clinical paradigms influencing the analgesic efficacy of the antagonists [31,57]. Therefore, further studies to address the role of NKA signaling in the incisional model, as well as, investigating the effects of selective NK-1 and NK2 receptor antagonists on cytokine production in incision-induced hyperalgesia are required.

In summary our data are consistent with the notion that the *ppt*-A gene product SP supports nociceptive sensitization after hind paw incision. In addition to the relatively well-established roles this neuropeptide has as a neurotransmitter released into the dorsal horn of the spinal cord, it is possible that lack of this substance reduces the intensity of the inflammatory reaction surrounding incisions thereby providing a second mechanism for the reduced mechanical and heat sensitization observed in *ppt-A*$^{-/-}$ mice. Future studies might be directed at further defining the likely impact reduced cytokine production could have on pain or other parameters of wound healing. Additional experimentation is required to define direct effects versus indirect effects of cytokines on afferent neurons in incision.

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