HHS Public Access

Author manuscript

Gastroenterology. Author manuscript; available in PMC 2019 June 01.

Published in final edited form as:

Gastroenterology. 2018 June ; 154(8): 2252–2265.e2. doi:10.1053/j.gastro.2018.02.030.

Neuronal Transforming Growth Factor beta Signaling via SMAD3 Contributes to Pain in Animal Models of Chronic Pancreatitis

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Abstract

Background & Aims—Chronic pancreatitis (CP) is characterized by pancreatic inflammation and fibrosis, associated with increased pancreatic expression of transforming growth factor beta (TGFB). It is not clear how these might contribute to pain. We investigated whether TGFB signaling via SMAD induces sensitization of pancreatic sensory neurons to increase nociception.

Methods—CP was induced in Sprague-Dawley rats by infusion of trinitrobenzene sulfonic acid; some rats were given intrathecal infusions of TGFB1. CP was induced in control mice by administration of cerulein; we also studied β1glo/Ptf1acre-ER mice, which upon induction overexpress TGFB1 in pancreatic acinar cells, and *TGFBr1*$^{f/f\text{-}CGRPcreER}$ mice, which have inducible disruption of *TGFBr1* in calcitonin gene-related peptide- positive neurons. Dominant negative forms of human TGFBR2 and SMAD3 were overexpressed from viral vectors in rat pancreas. Some rats were given the SMAD3 inhibitors SIS3 or halofuginone. After induction of CP, mice were analyzed in for pain in behavior tests or electrophysiologic studies of sensory neurons. Pancreatic nociceptor excitability was examined by patch clamp techniques and nociception was measured by Von Frey Filament tests for referred somatic hyperalgesia and behavioral responses to pancreatic electrical stimulation. Pancreata were collected from mice and rats and analyzed histologically and by ELISA and immunohistochemistry.

Results—Over-expression of TGFB in pancreatic acinar cells of mice and infusion of TGFB1 into rats resulted in sensory neuron hyperexcitability, SMAD3 activation, and increased nociception. This was accompanied by a reduction in the transient A-type current in pancreas-specific sensory neurons in rats, a characteristic of nociceptive sensitization in animal models of CP. Conversely, pancreata from *TGFBr1*$^{f/f\text{-}CGRPcreER}$ mice, rats with pancreatic expression of

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Disclosures: All authors (LL, YZ, MN, QL and PJP) have declared that no conflict of interest exists.

Author contributions: LL, YZ and QL were involved in conducting experiments, acquiring and analyzing data. MN conducted pathological examination of chronic pancreatitis. PJP conceived the project, designed the research studies, analyzed and interpreted the data and wrote the manuscript, along with QL.

dominant-negative forms of human TGFBR2 or SMAD3, and rats given small molecule inhibitors of SMAD3 had attenuated neuronal sensitization and pain behavior following induction of CP. In contrast to findings from peripheral administration of TGFB1, intrathecal infusion of TGFB1 reduced hyperalgesia in rats with CP.

Conclusions—In pancreata of mice and rats, TGFB promotes peripheral nociceptive sensitization via a direct effect on primary sensory neurons mediated by intra-neuronal SMAD3. This is distinct from the central nervous system, where TGFB reduces nociception. These results provide an explanation for the link between fibrosis and pain and pain in patients with CP. This signaling pathway might be targeted therapeutically to reduce pain in patients with CP.

Keywords

pain signal transduction; mouse model; neurobiology; pancreas

Introduction

Pain in chronic pancreatitis (CP) is one of the most disruptive symptoms, profoundly impairing quality of life in patients suffering from this disorder[1-3]. However, effective medical treatment for pain in this condition is limited and patients often undergo drastic surgery including total pancreatectomy and islet transplantation, with associated morbidity [4-6]. In recent decades, studies on the pathogenesis of pain in chronic pancreatitis have begun to provide insight into the neurobiological mechanisms underlying the sensitization of peripheral and central nociceptive sensory pathways[7-9]. Identification of the molecular drivers of this sensitization may therefore provide new opportunities for therapy. In this regard, nerve growth factor (NGF) and downstream targets have strongly been implicated in the pathogenesis of pain in both animal models and patients. However, enthusiasm for the use of anti-NGF biological agents has been significantly tempered by reports of serious adverse effects such as osteonecrosis in early trials[10].

Another promising candidate in this context is transforming growth factor beta (TGFB) which is significantly upregulated in the pancreas with chronic inflammation in patients as well as experimental models, where it has been strongly implicated in the pathogenesis of fibrosis[11-15]. TGFB activation is mediated through two receptors: the type II receptor (TGFBRII) is necessary for the initial binding of TGFB and recruitment of the type I receptor (TGFBRI). The canonical mediator of intracellular TGFB signaling is the SMAD pathway involving phosphorylation of SMAD-2 and -3 and their subsequent binding to SMAD-4 which is then transported to the nucleus where it modifies the expression of specific genes. In addition, other pathways, including MAP kinase signaling can be activated by TGFB and in turn act via both SMAD activation and independently in some cell systems at least[16, 17].

Overexpression of TGFB and SMAD3 has been demonstrated in pancreatic tissue in a rodent model of chronic pancreatitis[14]. We have previously shown that systemic neutralization of TGFB can attenuate pain in a rat model of CP and that exposure of nociceptive neurons to TGFB1 *in vitro* increased sensory neuronal excitability and decreased voltage-gated A-type K(+) currents (IA)[18]. These findings have been confirmed by others[19].

However, it is possible that the effects on pancreatic nociceptors are indirect and secondary to other mediators induced or released in their environment by TGFB. We hypothesized that TGFB acts directly on pancreatic nociceptors and that SMAD signaling within these sensory neurons is important for nociceptive sensitization in chronic pancreatitis. In this study we therefore examined the role of neuronal SMAD signaling in the pro-nociceptive effects of TGFB *in vivo* and further provide proof of concept of this pathway as a therapeutic target by the use of small molecule inhibitors.

Materials and Methods

Animals and materials

Adult male Sprague-Dawley rats (225- 250g) purchased from Harlan Laboratories (Indianapolis, IN) were housed 2-3 per cage in a temperature-, humidity-, and light-controlled room (12 h light/dark cycles). Food and water were available *ad libitum*. After pancreatic duct infusion and electrode implantation, rats were single caged. In all of the experiments, animals were randomly assigned into the experimental groups. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

Two inducible transgenic mice were generated as detailed in the Supplementary material. The $\beta1^{glo}$/Ptf1a$^{cre\text{-}ER}$ mice selectively overexpress TGFB1 in pancreatic acinar cells when induced by treatment with tamoxifen (20 mg/ml corn oil, 0.1ml/mouse PO, for 5 days). In TGFBRI$^{f/f}$-CGRP^{creER} mice, tamoxifen treatment selectively knocked out TGFBRI in calcitonin gene-related peptide (CGRP) positive neurons.

Recombinant herpes simplex viruses (HSV) containing dominant negative human TGFBRII (DN TGFBRII) and Smad3 (DN Smad3), respectively, were made at Viral Gene Transfer Core of the Brain Institute in the Massachusetts Institute of Technology (Cambridge, MA). These dominant negative sequences have been shown to knockdown rodent TGFBRII[20] and Smad3[21] respectively.

Recombinant TGFB1 was purchased from Biolegend (San Diego, CA). The SMAD3 inhibitor SIS3 (specific inhibitor of SMAD3) and halofuginone were obtained from Sigma Aldrich (St. Louis, Mo); both were dissolved in DMSO (5 mg/1.5ml and 10mg/ml, respectively). Then, further diluted with saline to 2.5mg/ml and 0.2mg/ml, respectively.

Surgical procedures

Chronic pancreatitis was induced in rats as previous described by us[22]. Briefly, 0.5 ml of 0.6% Trinitrobenzene Sulfonic Acid (TNBS) in 10% ethanol PBS was infused into the pancreatic duct via the duodenal papilla. Control rats received the same volume of 10% ethanol PBS. The animals were housed for at least three weeks to fully develop chronic pancreatitis. To provide noxious electrical stimulation, a pair of electrodes (Myo-Wires; A&E Medical, Farmingdale, NJ) were sutured into the pancreas soon after the TNBS infusion and the open ends of the wires were subcutaneously tunneled and externalized at the dorsal neck region. Pancreatic injections of DN-TGFBRII-HSV, DN-Smad3-HSV and

DiI (1,1′-dioleyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate, Life Technology, 10mg/ml methanol) were made in the head of the pancreas of rats (2μl /site, 10 sites), prior to TNBS infusion (See details in Supplementary material).

Intrathecal infusion was performed as previously described by us in detail[23]. Briefly, three weeks after TNBS infusion, an intrathecal catheter was inserted to spinal cord level of T9-T10 and connected to an osmotic pump containing 5μg TGFB1 in 200μl PBS that delivered the drug over a 2-week period.

Chronic pancreatitis in mice was generated with cerulein as previously described[24]. Cerulein (50μg/kg, 5ml/kg) or saline was injected intraperitoneally 6 times per day (at one-hour intervals) for 2 days per week for 6 weeks.

General experimental procedure

After induction of CP models and treatments as described above, animal were undergone pain behavioral tests or electrophysiological study of sensory neurons. The nociceptive tests were examined using Von Frey Filament (VFF) test and nocifensive responses to electrical stimulation of the pancreas as previously reported[22]. VFF test for mice was modified from the rat procedure. Mice were placed in individual limited Plexiglas chambers on a mesh floor. The frequency of withdrawal responses was tested using ascending order of different VFF bending strengths. The animal were then sacrificed and pancreas and DRGs (T9 and T10) were collected for ELISA and histological and immunochemical staining as detailed in the Supplementary Materials. For the electrophysiological study, DRGs (T8-12) were collected and dissociated for DRG culture and whole-cell voltage patch-clamp recordings as described previously[18] and in Supplementary Materials.

For *in vitro* the electrophysiological experiments, DRG primary culture was prepared and treated with as described previously[18] and in Supplementary materials.

Pancreatic histology and pathological analysis—To evaluate the severity of chronic pancreatitis, the pancreas was excised and fixed with PBS-neutralized10% formalin at 4°C overnight. After paraffin-embedding, the pancreas was sectioned into 5 μm slices and mounted. The tissue was stained with hematoxylin and eosin for histopathological analysis that was performed by a pathologist blinded to the study. The severity of pancreatitis was scored based upon the percentage of affected area (0=0, 1=1-5%, 2=5-25%, 3=25-50% and 4=50-100%) and a semi-quantitative score for acinar atrophy (0=None, 1=Subtotal, 2=Total), fibrosis (0=None, 1=Mild, 2=Severe) and inflammation (0=None, 1=Mild, 2=Severe).

Statistics

All of the data were presented as mean ± SEM of 6-8 animals, unless otherwise mentioned. The data were analyzed by t-test or analysis of variance analysis (ANOVA). If a significant difference ($P<0.05$) was detected, a Student-Newman-Keuls post-hoc test was used to evaluate differences between individual groups using Sigmaplot software (Systat Software Inc). The t-test and ANOVA results for all of the studies were listed in the Supplementary Materials.

Results

Infusion of TGFB in the pancreas mimics the changes in nociceptor electrophysiology and pain behavior characteristic of chronic pancreatitis

We have previously shown that intraductal infusion of exogenous TGFB results in pancreatic hyperalgesia in rats[18]. We therefore tested the hypothesis that such treatment also results in changes in pancreatic nociceptor excitability *in vivo* and further, that these changes are similar to what we have previously reported in a rat model of chronic pancreatitis[25]. We injected two doses of TGFB1 (0.1 and 1μg) into the pancreatic duct of adult male rats. Three days later, only rats that received 1 μg of TGFB1 showed an increase in pSMAD3 positive neurons in pancreas-specific DRG neurons (Figure 1A) along with pancreatic hypersensitivity to electrical stimulation (Figure 1B). This was accompanied by robust steady state changes in the excitability of labeled pancreatic sensory neurons, including a decrease in resting membrane potential and rheobase along with an increase in the number of evoked action potential spikes (Figure 1C). Further, we observed a decrease in the total voltage dependent potassium current (I_{total}), which was mainly due to a reduction in the transient 'A-type' current (I_A); ($P<0.05$, Figure 1D) with no change in the 'sustained delayed rectifier type' current (I_K). These findings are identical to our previously published study on the effects of TGFB1 on sensory neurons *in vitro* as well as what we have described in pancreatic sensory neurons isolated from the dorsal root ganglia of rats with CP, thus providing the foundation for a role of TGFB in the nociceptive sensitization seen in this condition[18, 25].

Overexpression of TGFB in the pancreas results in pancreatic hypersensitivity and SMAD3 phosphorylation in sensory neurons *in vivo*

Next we examined whether endogenous overexpression of TGFB1 in the pancreatic parenchyma could drive pancreatic hypersensitivity *in vivo*. To do this, we created a transgenic mouse ($\beta1^{glo}$/Ptf1a$^{cre\text{-}ER}$) that over-expresses TGFB1 under an inducible Cre system driven by Ptf1a, a transcription factor that is relatively specific for pancreatic acinar cells (see Supplementary material). After five days of induction with tamoxifen or vehicle, and a further 7 days of observation, animals treated with tamoxifen demonstrated increased phosphorylation of SMAD3 in TRPV1-expressing nociceptive neurons from pancreatic DRGs (T8-T12) (Figure 2-A1 and A2), indicating increased TGFB signaling in the sensory neurons, along with a significant increase in pancreatic TGFB expression (Figure 2B). This was accompanied by referred somatic hyperalgesia (a surrogate marker of pancreatic hypersensitivity in this model, Figure 2C).

TGFBR-SMAD3 neuronal signaling contributes to pancreatic hypersensitivity in chronic pancreatitis *in vivo*

Although the previous results clearly implicate a role for TGFB in pancreatic nociception, they do not indicate whether this results from a direct effect of this molecule on neurons in the setting of chronic pancreatitis. To test this hypothesis, we generated transgenic mice (TGFBRI$^{f/f}$-CGRP^{creER}) by breeding mice with a floxed TGFBRI gene with mice that expressed a fusion protein of Cre recombinase and estrogen receptor (CreER) in the CGRP, a marker for peptidergic nociceptive neurons[26]. We induced a knockout of TGFBRI in

CGRP-expressing cells by administration of tamoxifen for five day, followed by induction of chronic pancreatitis with cerulein (see Methods). In tamoxifen-treated TGFBRI$^{f/f}$-CGRP^{creER} mice, Smad3 phosphorylation was significantly reduced in TRPV1-expressing neurons in DRGs (T9-T10), as compared with animals treated with corn oil as control (Figure 3-A1 and A2). Corn oil-treated mice, but not tamxifen-treated mice with cerulein-induced CP displayed a significant increase in referred somatic hyperalgesia (measured by VFF responsiveness) when compared to animals without pancreatitis (saline controls) (Figure 3B). Furthermore, in vitro testing of DRG neurons from tamoxifen treated transgenic mice showed a lack of increase in the characteristic TGFB-induced excitability of neurons. (Figure 3C).

These results suggested that downregulation of TGFBRI in CGRP expressing neurons suppresses SMAD3 activation and pain behavior in the setting of chronic pancreatitis. We further corroborated this by directly suppressing TGFB-SMAD signaling within pancreatic sensory neurons using defective herpes simplex virus (HSV-1) constructs carrying dominant negative (DN) genes involved in TGFB signaling. In two separate studies, defective HSV-1 virus carrying either a DN-TGFBRII or DN-SMAD3 gene, was injected in rat pancreas followed by injection of DiI to label pancreas specific sensory neurons and infusion of TNBS to induce chronic pancreatitis (See detail in Supplementary material). 3 weeks later, the pain behaviors were measured in these rats. Then, the rats were sacrificed and labeled pancreatic projecting neurons in freshly dissociated DRGs were examined by patch clamping. We found that compared to control virus injections, both DN genes reduced phosphorylation of SMAD3 in pancreatic sensory neurons (Figure 4A and 5A) and attenuated pain behavior as measured by referred somatic hyperalgesia (Figure 4B and 5B). Neither viral treatment resulted in attenuation of the pathological changes of chronic pancreatitis in these rats as quantified histologically (Figures 4C and 5C). However, both treatments prevented the changes in excitability of pancreatic sensory neurons that are otherwise induced by CP, although no significant effects were observed on I_A currents. (Figure 4D and 5D).

Two small molecule SMAD3 antagonists demonstrate utility in a rat model of CP

Based on these observations, we tested the hypothesis that small molecule inhibitors of TGFB-SMAD signaling could be potentially effective in relieving pain in CP. We first tested the effects of SIS3 on DRG neurons *in vitro* and showed that it can effectively suppress the changes in excitability induced by TGFB, including decreases in IA currents (Figure 6-A-1 and A-2). We then examined the effects of this molecule on SMAD signaling in pancreatic neurons and pain behavior in rats with CP in vivo. As shown in Figure 6-A-3, pSmad3 in DiI-positive neurons were significantly decreased in rats treated with SIS3 (2.5 mg/kg, i.p. for 7 days), as compared with saline treated controls. Correspondingly, treatment with SIS3 significantly attenuated CP-induced hyperalgesia as measured by VFF and the behavioral response to pancreatic electric stimulation (Figure 6-A-4 and A-5).

We also tested the effects of halofuginone, a known inhibitor, on SMAD3 phosphorylation. Treatment with halofuginone (200μg/kg, i.p.) for 14 days significantly reduced phosphorylated SMAD3 in the DRG (Figure 6-B-1), along with attenuation of pancreatic

hyperalgesia in rats with CP (Figure 6-B-2 and B-3). Importantly, neither drug had any effect on pancreatic pathology seen with TNBS-induced CP (results not shown).

Intrathecal infusion of TGFB1 reduced the hyperalgesia in CP model

Finally, to test the effects of TGFB1 on the spinal cord, we intrathecally infused TGFB1 into T9-T10 level of spinal cord in the TNBS-induced CP rat model. After continuous infusion for 2 weeks, TGFB1 significantly reduced hyperalgesia in rats with CP as assessed by VFF and pancreatic electrode stimulation (Figure 7) but not in control rats.

Discussion

TGFB1 is a member of TGFB super-family, which are structurally related cytokines that play important roles in a wide spectrum of cellular functions. In the context of overt inflammation, TGFB1 is best known for its role in wound healing and promoting fibrosis. TGFB is also known to be upregulated in the pancreas with chronic inflammation in both patients and rodent models and is secreted in the pancreatic juice in patients with chronic pancreatitis[11-15, 27-31], but its role in the pathogenesis of pain in this condition has not been fully characterized.

Sensory neurons both produce TGFB and express its receptors[32, 33]. Our previous studies have demonstrated that incubation of DRG primary neurons with TGFB1 *in vitro* increases the excitability of these neurons, which is verified by increased spontaneous action potential frequency, reduced average rheobase (the minimum current required to evoke an action potential) and increased action potential spikes induced by 2 times of rheobase current[18]. However, it was not known whether this effect could also be seen *in vivo*. Further, although we had previously reported on a possible contribution of TGFB1 in chronic pancreatitis using systemic administration of a neutralizing antibody, this approach did not allow us to determine whether the observed suppression of hyperalgesia and associated nociceptor excitability indicated a direct action of TGFB on nerve endings or a secondary effect due to other events induced by TGFB in the pancreatic parenchyma. Finally, it is not clear whether such changes, if seen, are mediated by the canonical SMAD signaling pathway utilized by TGFB.

In this study, we provide evidence that the *in vitro* effects of TGFB on sensory neurons are mimicked in vivo by both exogenous and endogenous TGFB in the pancreas suggesting a pathogenic role for this molecule in the nociceptive sensitization observed in CP. To determine the signal pathway mediating the effects of TGFB, we then proceeded to examine this role by knocking down TGFBR1 in CGRP-expressing neurons using a transgenic mouse in which CP was induced by cerulein and observing a significant attenuation of pain behavior as well as neuronal SMAD signaling. This allowed us to localize the effects of TGFB inhibition on neurons and not glia, which do not express CGRP. To further confirm the role of TGFB is mediated by sensory neurons innervated to the pancreas, we tested another animal model, accomplished by pancreatic injection of recombinant HSV carrying dominant negative genes for TGFBRII and SMAD3. These HSV retrogradely infects sensory neurons that project to the pancreas, thereby restricting the effects to pancreatic sensory neurons. Such a local approach for targeting pancreas specific spinal neurons in

chronic pancreatitis has been validated previously by others[34]. Our results indicate that targeting either the TGFB receptor (TGFBRII) or downstream mediators (SMAD3) prevents the changes in neuronal excitability and importantly hyperalgesia, in rats with TNBS-induced CP, an animal model we have previously validated [35].

There are several possible downstream targets of TGFB-SMAD signaling that could mediate these results. Our previous *in vitro* study of the effects of TGFB on sensory neuronal excitability suggests an important though not exclusive role for voltage-dependent (Kv) potassium channels responsible for A-type (I_A) currents[18]. I_A currents are rapidly activated and inactivated and inhibit neuronal excitability (repetitive firing) by regulating the inter-spike frequency of action potentials. We have previously also demonstrated decreased I_A currents in the excitability of pancreatic nociceptive neurons in the TNBS-induced rodent model of CP[35]. In the present study, exogenous TGFB results in a similar decline in I_A current *in vivo*. However, we were unable to show that that blocking TGFB signaling by downregulating either the TGFBRII receptor or SMAD3 prevents such a decrease in I_A currents. This may reflect the role of other factors in the complex inflammatory milieu of chronic pancreatitis, such as nerve growth factor (NGF) which we have also previously shown to reduce I_A currents in this model[25, 36]. In this context, it is also noteworthy that TGFB may contribute to increase NGF expression in pancreatic stellate cells that could provide an alternative drive for peripheral sensitization despite suppression of neuronal TGG signaling[37]. On the other hand, despite this lack of change in I_A currents, animals treated with blockade of TGFB signaling demonstrate attenuation of both neuronal excitability and pain behavior, suggesting a role for channels involved in TGFB-SMAD signaling. As an example, TGFB1 has been shown to sensitize TRPV1, a key transducer of nociception, through cyclin dependent kinase 5 (Cdk5) in trigeminal neurons[38]. Thus TGFB may operate through multiple mechanisms to maintain sensitization in chronic painful inflammatory states.

Having established the role of this pathway in this condition, we then tested the potential of pharmacological inhibition for the treatment of nociceptive sensitization in animals with CP. To this end, we used the small molecule SIS3 (specific inhibitor of Smad3) which attenuates the binding of pSmad3 to DNA and consequently facilities its dephosphorylation in the nucleus[39, 40]. Furthermore, it has been reported that SIS3 does not affect phosphorylation of MEPK/p38, ERK and PI3, suggesting that the use of SIS3 is a relatively selective method to assess the contribution of SMAD signaling in TGFB induced cellular events[40]. We first demonstrated that SIS3 is capable of blocking the effects of TGFB on sensory neuronal excitability *in vitro*, including changes in excitability and I_A currents. We then showed that injection with SIS3 for 7 days significantly reduced pain behavior in rats with CP. Furthermore, when using DiI to label the DRG neurons that innervate to the pancreas, we found that SIS3 treatment prevented the increase in pSmad3-positive pancreatic neurons in rats with CP. Although we did not directly examine the non-canonical TGFB- signaling pathways in this study, the effects of SIS3 support the notion that SMAD3 signaling plays an important, if not dominant role in the development of peripheral neuronal sensitization.

As final proof of concept for TGFB signaling as a druggable target in CP, we tested the effects of halofuginone, a synthetic analog of the plant alkaloid febrifugine and a potent

inhibitor of SMAD3 signaling[41, 42]. It has been widely used to treat parasitic infections in poultry but also has potent anti-fibrotic activity (including pancreatic fibrosis) and has recently been explored for use in humans for a variety of conditions[24, 43-48]. In this study, we show for the first time that this drug is capable of attenuating pain behavior in rats with CP, suggesting another possible indication for halofuginone, if it were to be developed clinically.

Our study also has several other clinical implications. It has been postulated that pain in patients with chronic pancreatitis eventually subsides as the inflammatory component of the condition gives way to increasing fibrosis, the so-called “burn-out” theory[49]. However, other investigators have not been able to confirm this[50] and the recent North American Pancreatic Study (NAPS2) showed no correlation between either severity or frequency of pain with the duration of chronic pancreatitis[1]. The ability of TGFB to influence sensory neuronal excitability therefore provides a biological explanation for why sensitization may persist even in the presence of marked fibrosis. New knowledge about the role of TGFB in nociception also has implications for other conditions characterized by a transition from acute to chronic pain[51]. Tissue inflammation initiates a cascade of events resulting in peripheral sensitization, i.e. enhancement of the responsiveness of primary afferent neurons (nociceptors), whose bodies are housed in dorsal root ganglia (DRG) and whose central ends synapse with second order neurons in the spinal cord. While post-translational changes in key ion channels and receptors underlie the immediate/acute phase of sensitization, sustained/chronic peripheral sensitization is also accompanied by transcriptional events (“neuroplasticity”). Many factors in the inflammatory milieu, of both a physico-chemical and biological nature, contribute to these changes[52]. However, little is known about the driving factors *later in inflammation*, when tissue fibrosis is prominent. The expression of TGFB appears to be universally increased in chronic inflammation, where it plays a key role in wound healing and promoting fibrosis[53]. In this context, our study on the role of TGFB in sensory neuroplasticity assumes special importance.

Interpretation of the effect of TGFB on nociception in the past has been confounded by its role as an anti-inflammatory factor in the *central nervous system*. Thus, intrathecal infusion of TGFB1 suppresses glial activation and spinal inflammation and attenuates neuropathic pain induced by nerve injury in rats[54]. TGFB therefore has mutually contradictory central and peripheral effects on pain- this is not without precedence as it has also been reported for other peptides, such as nociceptin[56, 57]. In the present study, we confirmed that intrathecal infusion of TGFB1 reduced hyperalgesia in rats with chronic pancreatitis. This is supported by reports of attenuation of pain behavior in mice lacking BAMBI (Bone Morphogenetic Protein and Activin Membrane-Bound Inhibitor), a kinase-deficient receptor that binds TGFB without initiating downstream signaling, thus acting as a pseudoreceptor[55]. The dorsal horn expresses BAMBI at high levels and endogenous opioids are upregulated in the spinal cord by members of the TGFB as well as in BAMBI knock out mice; further, the attenuated nociception in these mice is reversed by naloxone[55]. Thus the central effects of TGFB on nociception may involve endogenous opioid signaling.

In conclusion, in the present study, we show that pancreatic TGFB1 can directly sensitize pancreatic neurons *in vivo* and result in pain behavior and these effects are mediated by

TGFB-SMAD signaling in nociceptors. Inhibiting TGFB signaling via genetic or pharmacological means attenuates pain sensitivity in two different models of chronic pancreatitis. This is distinct from its central role, where it is anti-nociceptive. Our results demonstrate a novel and more expansive role for peripheral TGFB in chronic pancreatitis and by implication, other chronic inflammatory conditions. Increased tissue levels of TGFB by acting directly on distinct cell types, may provide a common link between the fibrosis and nociceptive sensitization that often accompanies such disorders. Thus, TGFBsignaling represents a new target for their treatment that can potentially address both these features.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge Dr. Ashok Kulkarni at National Institute of Dental and Craniofacial Research and Dr. Pao-Tien Chuang at University of California San Francisco for kindly providing TGFBRI floxed mice and CGRP-Cre –ER mice, respectively. The authors also thank Dr. Rachael Neve at Massachusetts Institute of Technology for design and production of the recombinant herpes simplex viruses (HSV) containing DN TGFBRII and DN Smad3 and for related technical support.

Grant support: The studies were supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIH R01 DK073558) to Dr. Pasricha.

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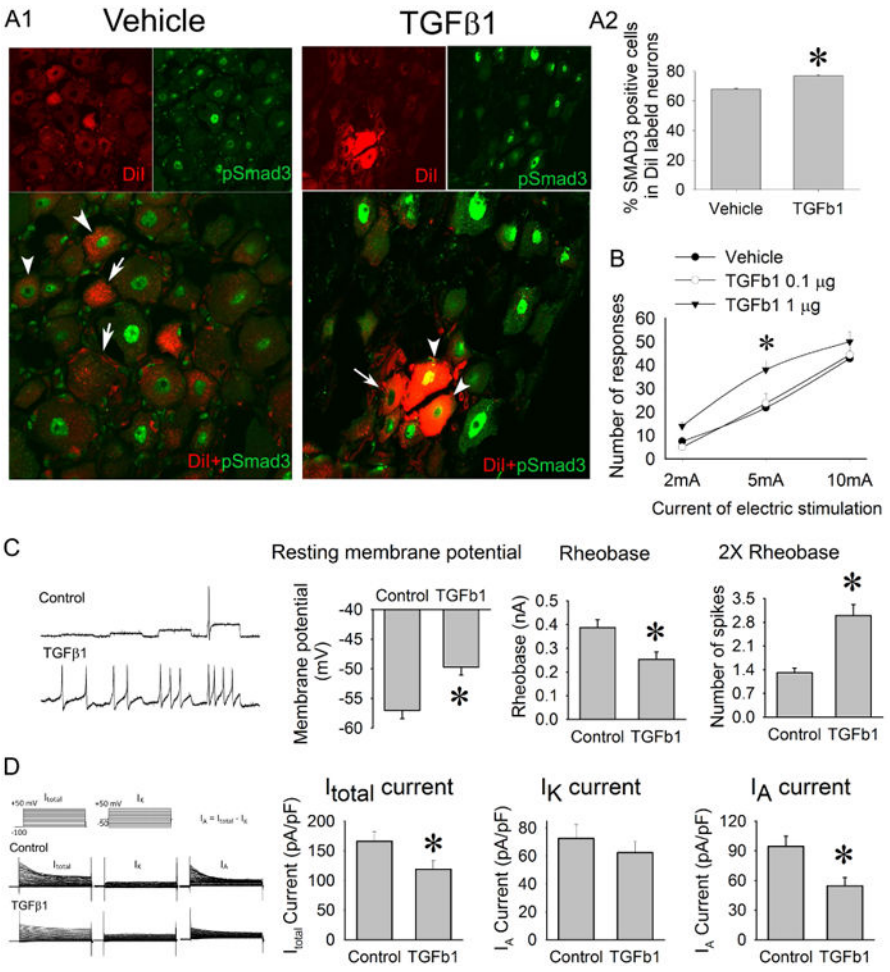

Figure 1. Effect of intra-ductal infusion of TGFB1 on SMAD signaling, pain behavior and excitability of pancreatic sensory neurons

A) *SMAD signaling in pancreatic nociceptive neuron*. **A1**: An example of pSmad3 staining (green) in DiI labeled pancreatic neurons (red). Arrow heads indicate DiI labeled pSmad3 positive neurons and arrows indicate DiI labeled pSmad3 negative neurons; **A2**: Plot showing that pSmad3 signaling was significantly increase in the pancreatic innervated DRG neurons of TGFB-infused rats (*: P<0.001 compared to Vehicle by Student t-test) (n=29-34 DRG sections from 4-5 rats).

B) Pancreatic ductal infusion of TGFB1 (1μg, but not 0.1μg) increased pancreatic pain sensitivity (*: P<0.05 compared with control group by Student-Newman-Keuls post-hoc test). Data are presented as mean ± SEM (n=6).

C & D) Whole-cell voltage patch-clamp recordings on pancreas-projecting (DiI-positive) DRG neurons. **C**. Pancreatic ductal infusion of TGFB1 (1μg) increased excitability of these neurons. *From left to right*: Example of patch-clamp recording, plots of parameters for neural excitability (*: P<0.05 compared to control group by Student T-test). **D**. Changes in voltage dependent potassium currents in pancreatic neurons. *From left to right*: Examples of the patch-clamping recording; plots of total inward potassium currents (I_{total}); I_K currents and I_A current (*: P<0.05 compared to control group by Student T-test). Data are presented as mean ± SEM (n=18-39 cells).

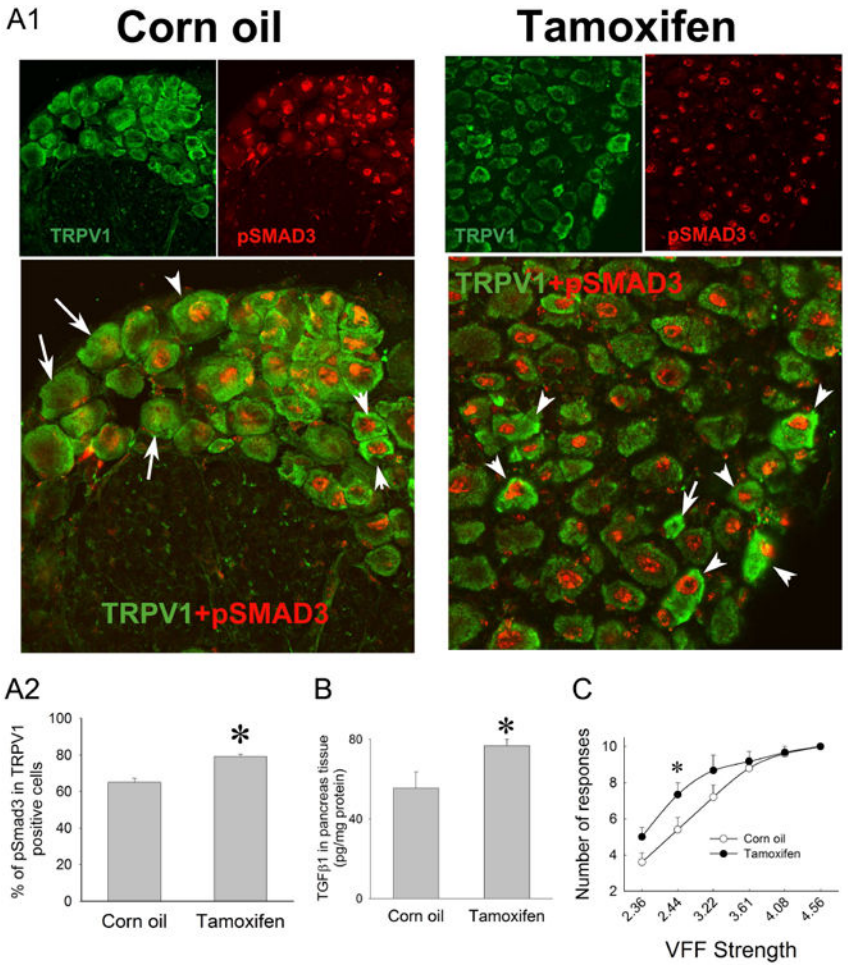

Figure 2. Effects of selective overexpression of TGFB1 in the pancreas of β1glo/Ptf1acre-ER mice (induced by tamoxifen) on SMAD signaling and pain behavior in nociceptive neurons

A) SMAD signaling in pancreatic nociceptive neurons was increased in tamoxifen-induced $\beta1^{glo}$/$Ptf1a^{cre\text{-}ER}$ mice. **A1**. An example of pSmad3 (red) and TRPV1 (green) staining in $DRG_{T8\text{-}12}$ neurons of $\beta1^{glo}$/$Ptf1a^{cre\text{-}ER}$ mice. Arrows indicate TRPV1-positive neurons expressing pSmad3; arrow-heads indicate TRPV1-positive neurons that do not express pSmad3. **A2**. Plot showing the proportion of TRPV1-positive cells in $DRG_{T8\text{-}12}$ that express pSmad3 was significantly increased in mice after tamoxifen induction (*: $P<0.05$ compared to corn oil treated group by student t-test). Data are presented as mean ± SEM (n=26-61 sections from 4-6 mice).

B) TGFB1 in the pancreas was increased after tamoxifen injection in the transgenic mice as measured by ELISA. (*: $P<0.05$ compared to corn oil group by student t-test). Data are presented as mean ± SEM (n=6-7 mice).

C) Referred somatic sensitivity, as measured by VFF testing, was increased after TGFB1 over-expression induced by tamoxifen (*: $P<0.05$ compared with corn oil treated group by Student-Newman-Keuls post hoc test). Data are presented as mean ± SEM (n=6-7 mice).

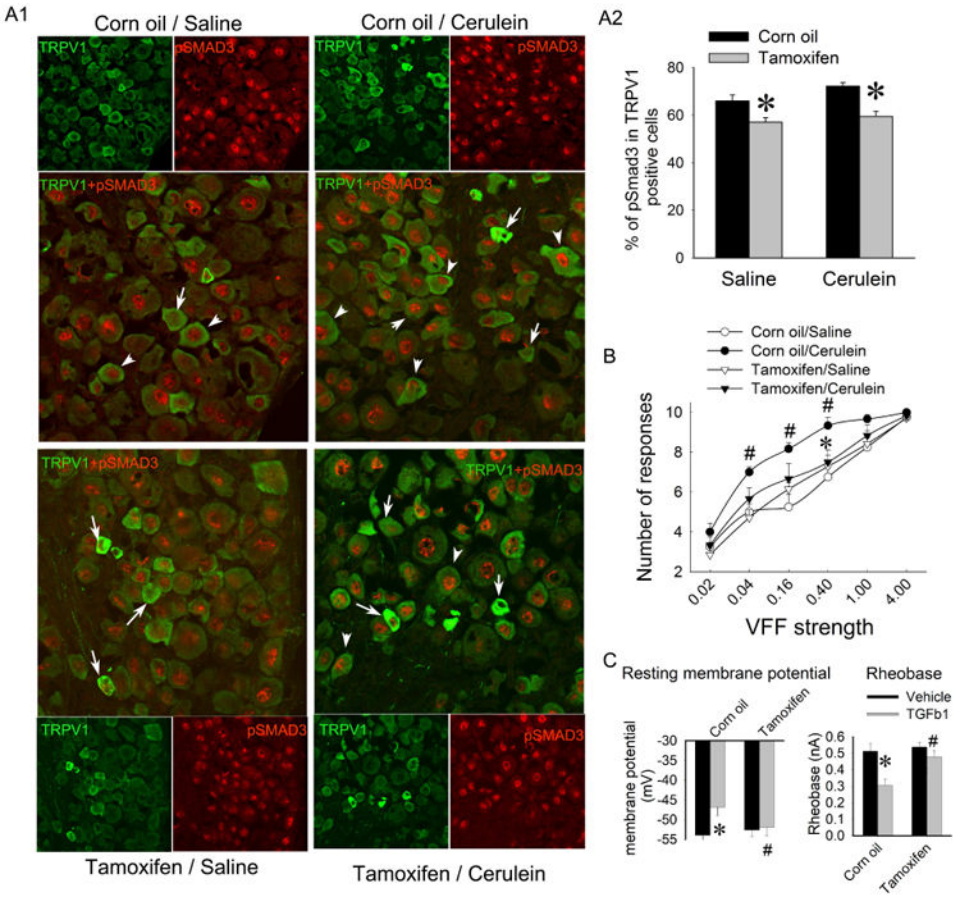

Figure 3. Effects of downregulation of TGFBRI on SMAD signaling in nociceptive neurons and pain behavior in TGFBRI$^{f/f}$-CGRP^{creER} mice with cerulein-induced CP

A1) An example of pSmad3 (red) and TRPV1 (green) staining in the $DRG_{T9\text{-}10}$ of TGFBRI$^{f/f}$-CGRP^{creER} mice. The arrows indicated same as described in Figure2. **A2**) Plot showing reduction of pSmad3 in TRPV1-positive DRG neurons after tamoxifen induction in both CP and control groups (*: P <0.05 compared to corn oil treated group in same model by Student-Newman-Keuls post hoc test). **B**) Tamoxifen-induced downregulation of TGFBRI attenuated the increased pain sensitivity in mice with CP (* P <0.05 for comparison with corn oil/cerulein group; #: P<0.05 for comparison with Corn oil/saline group at same VFF strength by Student-Newman-Keuls post hoc test). Data were presented as mean ± SEM (n=6-7 mice). **C**) In vitro TGFB1 on DRG neuronal activity in TGFBRI knockout mice. TGFBRI knockout was induced by administration of tamoxifen for 5 days. DRG neurons from the control or TGFBRI knockout mice were incubated with 10nM TGFB1 for 48 hours and electrophysiological responses were examined by whole cell voltage patch clamping. * P <0.05 for comparison with vehicle group treated with corn oil; #: P<0.05 for comparison with TGFB1 incubated cells in corn oil treated mice by Student-Newman-Keuls post hoc test). Data were presented as mean ± SEM (n=10-12 cells)

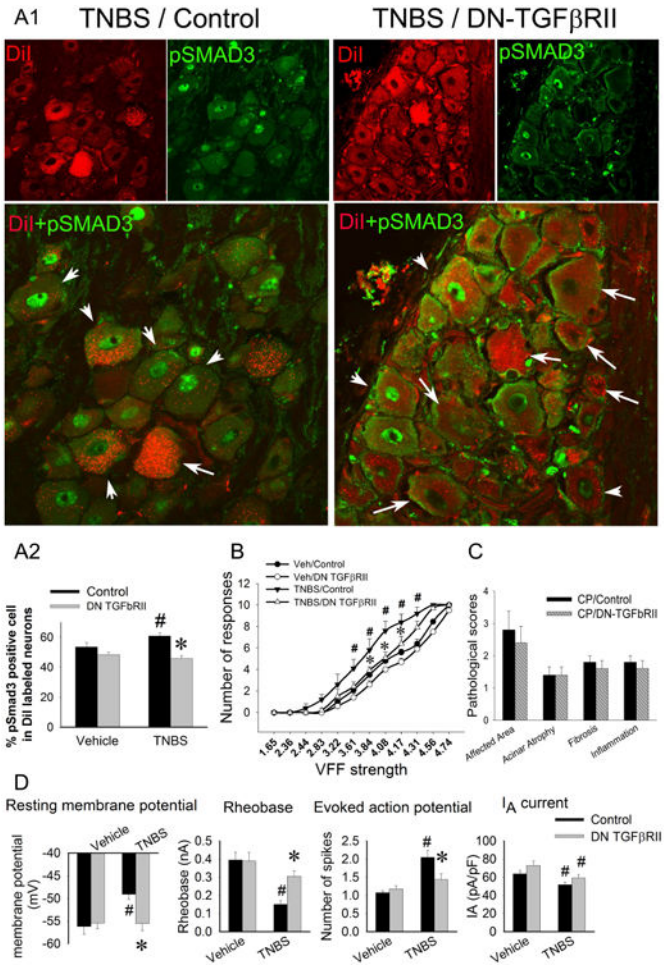

Figure 4. Effects of knock-down of TGFBRII by DN-TGFBRII-HSV on SMAD signaling, pancreatic neuronal excitability and pain behavior in CP rats

A) SMAD signaling in pancreatic nociceptive neuron. **A1**: An example of pSmad3 staining (green) in DiI labeled pancreatic neurons (red). Arrows indicated as described in Figure1; **A2**: Plot showing that CP-induced increase in pSmad3 signaling was significantly reduced in DN-TGFBRII-HSV injected rats (*: P<0.05 compared to TNBS/Control group; #: P< 0.05 compared to Saline/Control group by Student-Newman-Keuls post hoc test) (n=27-48 DRG sections from 4-5 rats).

B): CP-induced hyperalgesia was significantly reduced in rats that received DN-TGFBRII-HSV. * P<0.05 for comparison with TNBS/control group; #: P<0.05 for comparison with vehicle/control group at same VFF strength by Student-Newman-Keuls post hoc test (n=5 rats).

C): Pathological scores CP were not reversed by treatment with DN-TGFBRII-HSV. None of the parameters was significantly different between control- and DN-TGFBRII-HSV treated CP rats. Data were present as mean ± SEM (n = 4-5 rats).

D): DN-TGFBRII-HSV injection prevented the increase in the excitability of pancreatic neurons in DRG_{T8-12} of rats with TNBS-induced CP (*: P<0.05 compared to control virus treated CP rats, #: P<0.05 comparing to vehicle rats with same viral treatment by Student-Newman-Keuls post hoc test) (n=23-39 cells).

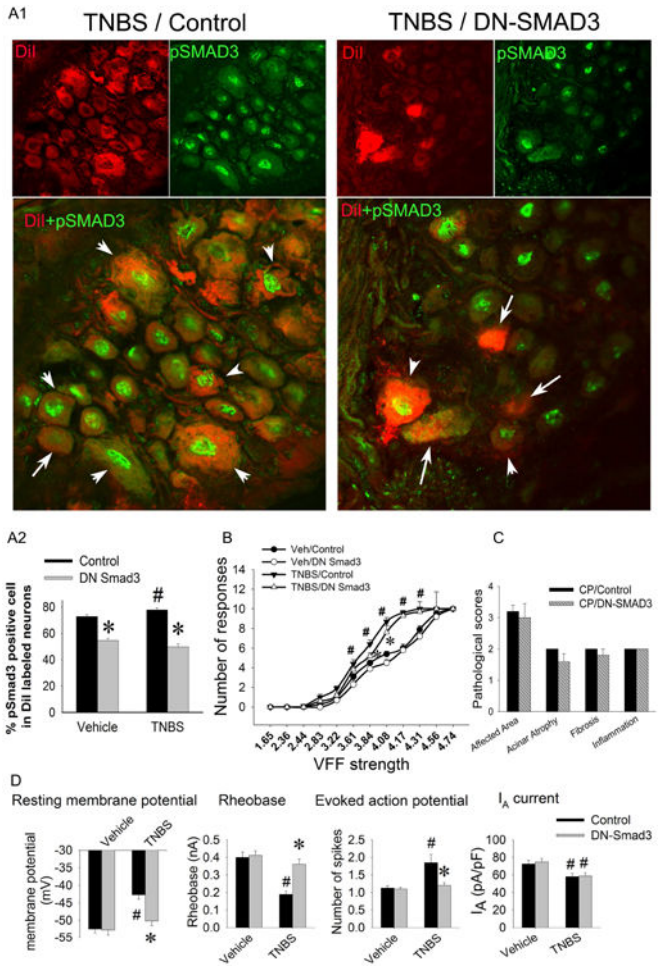

Figure 5. Effects of knock-down of SMAD3 by DN-SMAD3-HSV on SMAD signaling, pancreatic neuronal excitability, pain behavior and histology of pancreas in TNBS-CP rats

A): SMAD signaling in pancreatic neurons. **A1**: An example of pSmad3 staining (green) in Di-I labeled pancreatic neurons (red). Arrows indicated as described in Figure1; **A2**: Plot showing that CP-induced increase in pSmad3 was significantly reduced in DN-Smad3-HSV injected rats. (*: P<0.05 compared to Control virus group in the same group; #: P< 0.05 compared to Saline/Control group by Student-Newman-Keuls post hoc test) (n=48-58 DRG sections from 4-5 rats). **B)**: CP-induced hyperalgesia was significantly reduced in rats received DN-Smad3-HSV as measured by VFF responses (* P <0.05 for comparison with TNBS/control group; #: P<0.05 for comparison with vehicle/control group at same VFF strength by Student-Newman-Keuls post hoc test (n=5 rats). **C)**: Pathological scores of CP were not prevented by treatment with DN-SMAD3-HSV. None of the parameters was significantly different between control- and DN-SMAD3-HSV treated CP rats. Data were present as mean ± SEM (n = 4-5 rats) **D**): DN-Smad3-HSV injection reversed CP-induced changes in excitability of pancreatic-innervated neurons (*: P<0.05 compared to control virus treated CP rats, #: P<0.05 comparing to vehicle rats with same viral treatment by Student-Newman-Keuls post hoc test) (n=40-55 cells in 5 rats).

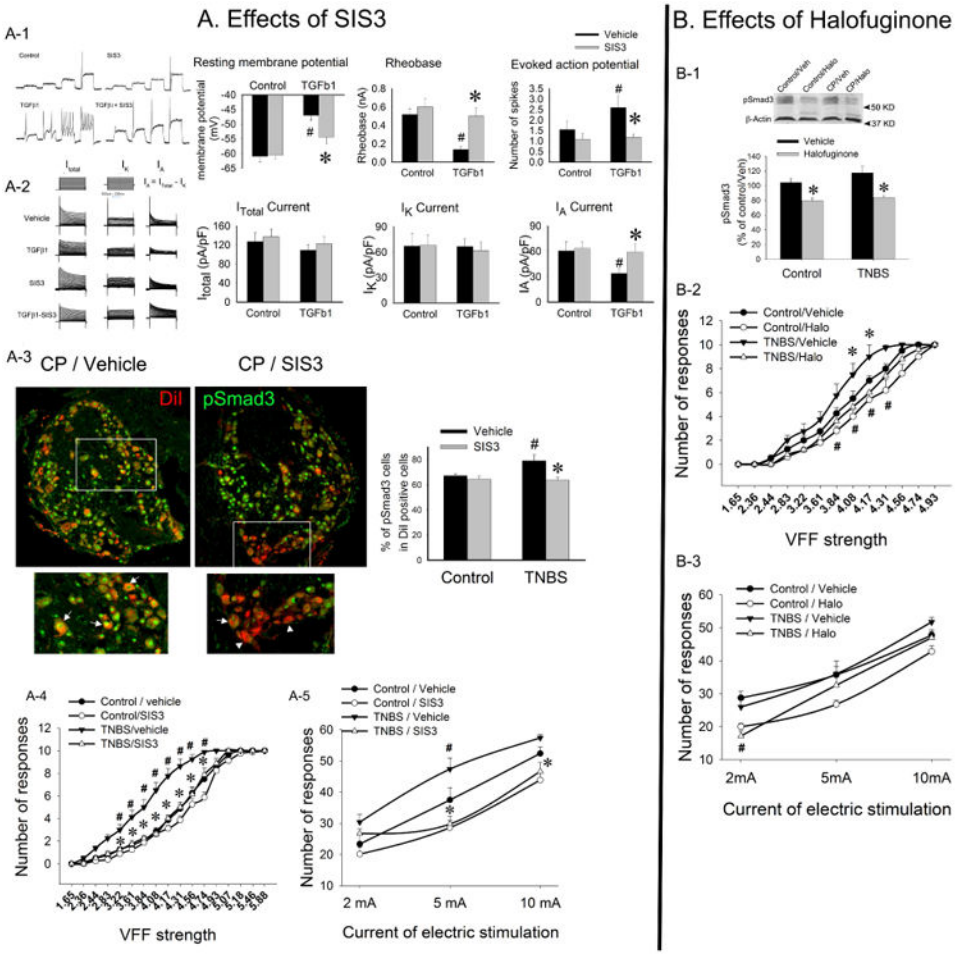

Figure 6. Effects of SIS3 (A) and halofuginone (B), two small molecule SMAD3 inhibitors, on TGFB-induced sensory neuronal excitability *in vitro*, and SMAD signaling and pain behavior in CP-rats

A-1 and A-2: Whole-cell voltage recordings of DRG neurons culture exposed to exogenous TGFB1 (10 ng/ml) with and without SIS3 (0.3μM) for 2 days. **A-1:** *Left to right:* Example of evoked action potentials after incubation with TGFB; plot of resting membrane potential, rheobase and evoked action potentials. **A-2:** *Left to right:* Examples of total inward potassium currents, I_K currents and I_A currents in sensory neuron and plots of the same. SIS3 prevented TGFB1-induced reduction of IA current (*: P<0.05 comparing to TGFB1/ vehicle group, #: P<0.05, compared to control/vehicle group by Student-Newman-Keuls post hoc test). (n=9-17 cells). **A-3:** SMAD signaling in vivo. *Left panel:* an example of pSmad3 staining (green) in DiI labeled pancreatic neurons (zoomed-in images in bottom row). Arrows indicated same as described in Figure1. *Right panel* shows the plot of analysis for pSmad3 staining (*: P<0.05 comparing to vehicle treated TNBS group, #: P<0.05 comparing to control/vehicle group) (n=45-58 DRG sections). **A-4 and A-5:** Treatment with SIS3 (2.5 mg/kg, i.p.) for 7 days attenuated responses to VFF (A-4) and electric stimulation (ES, A-5) in CP-rats (*: P<0.05 compared to CP/vehicle rats, #: P<0.05 compared to control/vehicle rats) (n=7-8 rats).

B) **Effects of halofuginone *B-1*:** Western blot showed that pSMAD3 expression was significantly reduced in the DRGs from both control and CP rats (* P<0.05, comparing to the vehicle treated rats with same pre-treatment). ***B-2 and B-3*:** Treatment with halofuginone for 14 days attenuated responses to VFF (*B-2*) and electric stimulation (B-3) in CP-rats (*: P<0.05 compared to CP/vehicle rats, #: P<0.05 compared to control/vehicle rats, by Student-Newman-Keuls post hoc test). Data were presented as mean ± SEM (n=7-8).

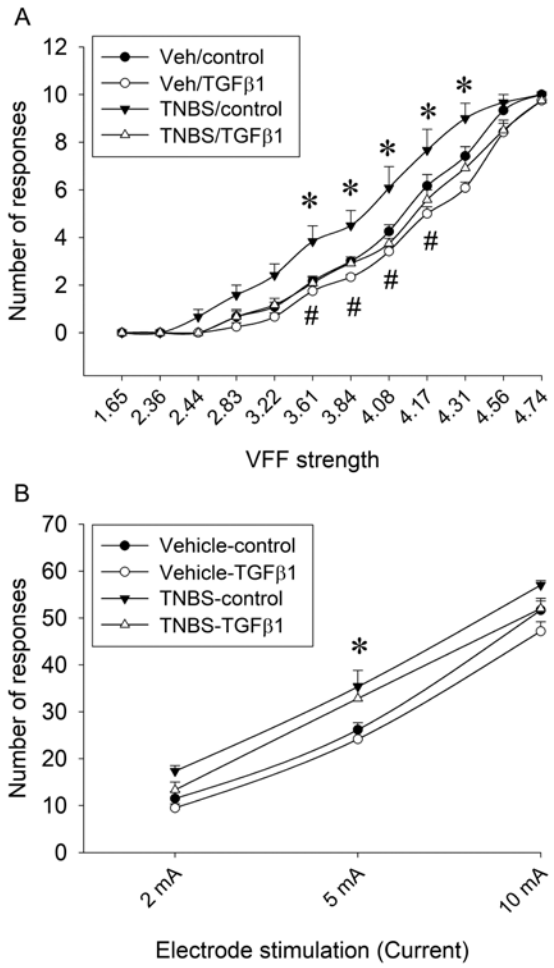

Figure 7. Intrathecal infusion of TGFB1 suppressed hyperalgesia in CP rats as examined by VFF (A) and electrode stimulation (B)

Intrathecal infusion of TGFB1 (5μg/rat) into T9-10 level of CP rats for 2 weeks significantly reduced CP-induced hyperalgesia in both VFF and ES tests (*: P<0.05 compared to CP/vehicle rats, #: P<0.05 compared to control/vehicle rats, by Student-Newman-Keuls post hoc test). Data were presented as Mean ± SEM (n=5-6).