Activin C expressed in nociceptive afferent neurons is required for suppressing inflammatory pain

Xing-Jun Liu,1 Fang-Xiong Zhang,1 Hui Liu,1 Kai-Cheng Li,1 Ying-Jin Lu,1 Qing-Feng Wu,1 Jia-Yin Li,1 Bin Wang,2 Qiong Wang,2 Li-Bo Lin,3 Yan-Qing Zhong,1 Hua-Sheng Xiao,3 Lan Bao2 and Xu Zhang1

1 State Key Laboratory of Neuroscience, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
2 State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
3 National Engineering Centre for Biochip at Shanghai, Shanghai 201203, China

Correspondence to: Xu Zhang, PhD, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China
E-mail: xu.zhang@ion.ac.cn

Emerging evidence suggests that the suppressive modulators released from nociceptive afferent neurons contribute to pain regulation. However, the suppressive modulators expressed in small-diameter neurons of the dorsal root ganglion remain to be further identified. The present study shows that the activin C expressed in small dorsal root ganglion neurons is required for suppressing inflammation-induced nociceptive responses. The expression of activin C in small dorsal root ganglion neurons of rats was markedly downregulated during the early days of peripheral inflammation induced by intraplantar injection of the complete Freund’s adjuvant. Intrathecally applied activin C could reduce nociceptive responses induced by formalin or complete Freund’s adjuvant. Moreover, activin C was found to inhibit the inflammation-induced phosphorylation of extracellular signal-regulated kinase in the dorsal root ganglia and the dorsal spinal cord. Thus, activin C functions as an endogenous suppressor of inflammatory nociceptive transmission and may have a therapeutic potential for treatment of inflammatory pain.

Keywords: activin C; inflammatory pain; dorsal root ganglion; extracellular signal-regulated kinase

Abbreviations: BKCa = large-conductance Ca2+ -activated K+ channel; CFA = complete Freund’s adjuvant; DRG = dorsal root ganglion; ERK = extracellular signal-regulated kinase; FSTL1 = follistatin-like 1; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; NPR-A = natriuretic peptide receptor-A; PGE2 = prostaglandin E2; PKG = cGMP-dependent protein kinase; TGF-β = transforming growth factor-β; TNF-α = tumour necrosis factor-α

Introduction

Small-diameter neurons in the dorsal root ganglion (DRG) convey the peripheral nociceptive signals to their afferent terminals in the spinal cord, causing the release of excitatory neurotransmitters (Hucho and Levine, 2007; Woolf and Ma, 2007). Nociceptive afferent transmission can be negatively regulated by the inhibitory neurotransmitters and neuropeptides,
such as opioid peptides released from the spinal dorsal horn neurons (Todd and Spike, 1993; Joseph and Levine, 2010; He et al., 2011). However, recent studies reveal some suppressive mechanisms in small DRG neurons, including the follistatin-like 1 (FSTL1)/sodium-potassium pump system and the B-type natriuretic peptide/natriuretic peptide receptor-A (NPR-A)/cGMP-dependent protein kinase (PKG)/large-conductance Ca\(^{2+}\)-activated \(K^{+}\) channel (BK\(_{Ca}\) channel) pathway (Zhang et al., 2010; Li et al., 2011a). The excitatory afferent neurotransmission could be presynaptically suppressed by FSTL1 and B-type natriuretic peptide released from the afferent terminals. Interestingly, the modification of these autoinhibitory mechanisms may contribute to the development of chronic pain (Zhang et al., 2010; Li et al., 2011b). We proposed that DRG neurons might secrete a group of molecules to negatively regulate the nociceptive afferent transmission via multiple signalling pathways. Therefore, we were interested in searching for these suppressive factors expressed in small DRG neurons.

Activins, which consist of two disulphide-linked \(\beta\) subunits, belong to a family of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily. Four mammalian activin/inhibin \(\beta\) subunits have been identified as \(\beta A\), \(\beta B\), \(\beta C\) and \(\beta E\) (Rodgarkia-Dara et al., 2006). Activin A, the homodimer of two \(\beta A\) subunits, is a pleiotropic cytokine that is widely expressed in many tissues and cells. Activin A is found to phosphorylate Smad2 and upregulate calci- tropic cytokine that is widely expressed in many tissues and cells. Activin A is found to phosphorylate Smad2 and upregulate calci-

tonin gene-related peptide expression in DRG neurons, leading to tactile allodynia (Ai et al., 1999; Hall et al., 2002; Xu et al., 2005; Xu and Hall, 2007). Activin A also contributes to acute thermal hyperalgesia by sensitizing the transient receptor potential channel V1 through the activin A receptor/protein kinase C (PKC) pathway (Zhu et al., 2007). Activin C, which consists of two \(\beta C\) subunits, has been found in liver, prostate, ovary, testis, adrenal gland, neurosecretory cells in posterior pituitary, endometrium and oviduct tissues, and could be distinct from other activins in subunit constitution, distribution pattern, signal transduction pathway and functions (Kron et al., 1998; Lau et al., 2000; Mellor et al., 2000; Vejda et al., 2002, 2003; Chabicovsky et al., 2003; Gold et al., 2004, 2005, 2009; Wada et al., 2004, 2005a, b; Takamura et al., 2005; Ushiro et al., 2006). However, the functions of activin C are poorly understood. Reports of its effects on the proliferation and regeneration of hepatocytes are controversial (Lau et al., 2000; Chabicovsky et al., 2003; Vejda et al., 2003; Wada et al., 2004, 2005a; Gold et al., 2009). The expression and function of activin C in the nervous system remain largely unknown.

The present study shows that activin C is mainly expressed in small DRG neurons, and suppresses inflammatory nociceptive responses. The activin C expression was downregulated after intraplantar injection with the complete Freund’s adjuvant, which induces persistent inflammation. Disruption of activin C function by either the small interfering RNA or antibodies could facilitate the inflammation-induced nociceptive responses and impair the recovery from hyperalgesia. Intrathecal treatment with activin C reduced the nociceptive responses induced by inflammation, suggesting a potential role of activin C in inflammatory pain therapy.

### Material and methods

#### Animal model of chronic inflammatory pain

Experiments were carried out in accordance with the guidelines of the International Association for the Study of Pain, and approved by the Committee of Use of Laboratory Animals, Institute of Neuroscience, Chinese Academy of Sciences. Adult male Sprague–Dawley rats (220–250 g; SLAC Laboratory Animal) were housed under a 12:12 h light/dark cycle at 22–26°C, with free access to water and rat chow. Rats were intraplantarly injected with 200\(\mu\)l complete Freund’s adjuvant (Sigma) at the hindpaw. These rats were sacrificed at 0.5, 1, 2, 4 and 7 days (20 rats in each group) after the injection, together with 20 naive rats.

#### Microarray and real-time reverse transcriptase-polymerase chain reaction

Detailed procedures for microarray and real-time reverse transcriptase-polymerase chain reaction are provided in the Supplementary material.

#### In situ hybridization

The method for in situ hybridization was modified from the previously published procedure (Wang et al., 2010). Briefly, frozen sections of lumbar (L) 4 and L5 DRGs were hybridized with the digoxigenin-labelled oligonucleotide probe for rat \(Inhbc\) gene (NM_022614) encoding activin/inhibin \(\beta C\). Detailed procedure, probes and quantitative analysis are provided in the Supplementary material.

#### Immunohistochemistry

Detailed procedure is provided in the Supplementary material. Briefly, after antigen retrieval with the Frozen Section Chemical Antigen Retrieval Reagent (GenMed Scientifics), DRG sections were processed for immunostaining.

#### Western blotting

Immunoblotting was performed according to the previously published procedure (Zhang et al., 2010). Briefly, protease inhibitor cocktail (Pierce) and radioimmunoprecipitation assay buffer (Pierce) were used for lysate preparation of DRGs and cells. Then, the samples were loaded on denatured sodium dodecyl sulphate-polyacrylamide gels, transferred to the nitrocellulose membrane, and probed with antibodies against activin C (1:5000, Abcam; 1:5000, AbD Serotec), extracellular signal-regulated kinase (ERK, 1:1000, Santa Cruz Biotechnology), phospho-ERK (1:1000, Santa Cruz Biotechnology), \(\alpha\)-actin (1:5000, Chemicon International). The immunoreactive band was detected with horseradish peroxidase-conjugated secondary antibodies and visualized with an ECL system (Roche Diagnostics).
The experiments were repeated at least three times. Immunoreactive bands were quantified with Image-Pro Plus 5.0 software.

Specificity of activin βC antibodies was tested by pre-absorption with synthetic peptide corresponding to amino acids 82–113 of activin βC at $10^{-6}$ M. Western blotting with mouse antibodies against the C-terminus of activin βC showed the monomer of activin βC in the HEK293 cells transfected with Inhbc. The same antibodies showed activin βC dimer (~25 kD) in the rat DRG extracts. The precursor and monomer of activin βC were detected in the DRG lysate prepared in a reduced condition (10% mercaptoethanol added in loading buffer and boiled for 20 min).

**Immunoprecipitation**

The procedure is described in the Supplementary material.

**Cell culture and treatment**

The detailed procedure is provided in the Supplementary material. Briefly, ND7-23 cells (ECACC) were cultured. After being starved for at least 4 h, cells were pretreated with 20 ng/ml recombinant human activin C (BlueGene) or vehicle (0.2% bovine serum albumin in phosphate-buffered saline) for 20 min, followed by 10 min incubation with 100 ng/ml nerve growth factor (Sigma), 5.0 μM prostaglandin E$_2$ (PG-E$_2$; Sigma), 10 μM glutamate (Sigma), 50 mM K$^+$ or 100 ng/ml tumour necrosis factor-α (TNF-α; Sigma).

DRG neurons were cultured for 24 h before use. After being starved for 48 h, cells were pretreated with 20 ng/ml activin C or vehicle for 30 min, followed by 5-min treatment with 100 ng/ml nerve growth factor.

**Intrathecal injection**

Detailed procedures are given in the Supplementary material. The single intrathecal injection was performed through a puncture at the theca of rat spinal cord into the subarachnoid space (Hylden and Wilcox, 1980; Mestre et al., 1994; Zhang et al., 2010). Briefly, when the needle tip reached the intervertebral space for 2–3 mm in depth, positioning of the needle tip was carefully adjusted and the entry into the subarachnoid space was evidenced by a brisk tail-flicking. Then, the reagent (20 μl) was injected into the subarachnoid space at the cauda equina region.

For multiple intrathecal administration of substances, a polyethylene-10 tube was catheterized into the subarachnoid space at the rostral level of the lumbosacral enlargement of rats under anaesthesia and aseptic surgical conditions (Yaksh and Rudy, 1976; Størksen et al., 1996; Li et al., 2011b). All tests were performed 5 days after catheterization. See the Supplementary material for a detailed procedure.

**Small interfering RNA and delivery**

Detailed procedure and small interfering RNA probes are described in the Supplementary material. Briefly, small interfering RNAs were mixed with the transfection reagent polyethyleneimine (ExGen 500; Fermentas) 10 min before injection (Xu et al., 2010). In the screening pilot study, 4.0 μg small interfering RNA or non-targeting control small interfering RNA was intrathecally delivered 72 h before DRGs were dissected for immunoblotting. In the formalin test, 4.0 μg small interfering RNA or scramble control small interfering RNA was intrathecally administrated 72 h before formalin injection. In the complete Freund’s adjuvant model, 4.0 μg small interfering RNA or scramble control small interfering RNA was intrathecally injected, whereas 50 μl complete Freund’s adjuvant was injected intraplantarly.

**Behavioural tests**

Tests were performed double-blindly. The animal model was prepared by injection of 100 μl complete Freund’s adjuvant into the left hindpaw. The procedure is detailed in the Supplementary material. Paw withdrawal latency to a noxious thermal stimulus (radiant heat test) was determined as the average of three measurements per paw over a 5-min test period. Tactile withdrawal thresholds were determined when there were three positive responses out of five stimuli with von Frey filaments presented perpendicular to the plantar surface. Activin C was intrathecally administrated.

For the formalin test, 50 μl of 5% (for activin C), 2% (for small interfering RNA) or 1% (for antibody) formalin was injected into the plantar surface of the left hindpaw. The number of flinches were counted within the first phase (1–10 min) and the second phase (10–60 min). The rats were intrathecally pretreated with 200 ng activin C (in 20 μl) for 15 min, or 4.0 μg small interfering RNA or 4.0 μg scramble control small interfering RNA (in 20 μl; GenePharma) for 72 h, or 2 μg activin C antibodies (in 20 μl; Santa Cruz Biotechnology), or activin C antibodies denatured in a boiling water bath, or 2 μg normal goat IgG for 30 min.

**Statistical analyses**

Differences between two groups were compared using Student’s paired or unpaired t-test. Differences for behavioural data were compared with two-way ANOVA followed by Tukey post hoc test. Significance for all tests was established at $P < 0.05$.

**Results**

**Activin C expression in small dorsal root ganglion neurons**

We used *in situ* hybridization and immunohistochemistry to study the expression and the cellular distribution of activin C in the rat DRGs. *In situ* hybridization showed that activin/inhbibin βC subunit messenger RNA-containing neurons contributed to ~30% of DRG neurons ($n = 1680$ out of 5536 counted neurons). About 96.7% of these DRG neurons were small-diameter neurons with the cross-sectional area $< 1000 \mu m^2$ (Fig. 1A). Immunostaining showed that the activin C-positive neurons contributed to ~30% of total DRG neurons ($n = 3517$ out of 11 750 counted neurons) and they were mainly small DRG neurons (Fig. 1B), consistent with the *in situ* hybridization result. Immunoblotting showed that the activin βC antibodies recognized activin βC expressed in HEK293 cells (Supplementary Fig. 1A). The level of activin C was markedly
reduced in the activin βC small interfering RNA-transfected ND 7–23 cells, which are derived from rat DRG neurons (Supplementary Fig. 1C). Moreover, activin βC antibodies could detect the precursor, dimer and monomer of activin βC in the lysate of rat DRGs (Fig. 1B and Supplementary Fig. 1B). The immunoreactive patterns could not be detected with the antibodies pre-absorbed with corresponding immunogenic peptides (Fig. 1B), suggesting that the immunoreactive patterns are specific for activin βC.

There are two major subsets of small DRG neurons, namely the IB4-positive subset and the peptidergic (mostly IB4-negative) subset. Double-immunofluorescent staining showed the co-existence of activin C with calcitonin gene-related peptide (CGRP) (Fig. 1C: green, activin C; red, calcitonin gene-related peptide) or IB4 (Fig. 1D: green, activin C; red, IB4) in small DRG neurons. Pie charts showed the proportion of activin C-immunoreactive neurons in rat DRGs (E; n = 11,750 neurons from 20 DRGs), and the ratio of activin C-immunoreactive neurons in peptidergic subset or IB4-positive subset of small DRG neurons (F; n = 3,517 neurons). Scale bars: A = 50 μm; B = 50 μm; C, D = 40 μm.

Figure 1  The expression of activin C in small DRG neurons. (A) Activin/inhibin βC messenger RNA was present in small DRG neurons (arrows) of rats. The cell-size distribution of activin βC messenger RNA-containing neurons was analysed in L4 and L5 DRGs of rats. (B) Immunoperoxidase staining showed that activin βC was expressed mainly in small DRG neurons of the rat. Such a staining pattern was not detected by pre-absorbed activin βC antibodies. Immunoblotting with activin βC antibodies, but not pre-absorbed antibodies, showed activin C in the rat DRG lysate. M indicates the protein marker. Double-immunofluorescent staining showed the co-existence of activin C with calcitonin gene-related peptide (CGRP) (C: green, activin C; red, calcitonin gene-related peptide) or IB4 (D: green, activin C; red, IB4) in small DRG neurons. (E and F) Pie charts showed the proportion of activin C-immunoreactive neurons in rat DRGs (E; n = 11,750 neurons from 20 DRGs), and the ratio of activin C-immunoreactive neurons in peptidergic subset or IB4-positive subset of small DRG neurons (F; n = 3,517 neurons). Scale bars: A = 50 μm; B = 50 μm; C, D = 40 μm.
subset. Double immunostaining showed that activin C was present in both calcitonin gene-related peptide- and IB4-positive subsets of small DRG neurons (Fig. 1C–F). About 60% of activin C-positive small DRG neurons contained neuropeptide calcitonin gene-related peptide (n = 2159 out of 3517 counted neurons), while ~30% of activin C-positive neurons were labelled by IB4 (n = 993 out of 3517 counted neurons) (Fig. 1F). About 45% of calcitonin gene-related peptide-immunoreactive small DRG neurons were stained for activin C (n = 2159 out of 4854 counted neurons), while ~27% of IB4-positive small DRG neurons contained activin C (n = 993 out of 3709 counted neurons). Thus, activin C is predominantly expressed in certain populations of both peptidergic and IB4-positive small DRG neurons.

**Decreased expression of activin C in dorsal root ganglia during peripheral inflammation**

Using multiple approaches, we then analysed the expression of activins in L4 and L5 DRGs of rats that were injected with complete Freund’s adjuvant into both hindpaws. Microarray analysis showed that the messenger RNA level of activin βC was significantly decreased (>2-fold change as compared with the control, P < 0.05, n = 3) in the DRGs, while the expressions of activin βA, βB, βE and inhibin-α subunits were not significantly changed in the DRGs (Fig. 2A).

The microarray result was re-evaluated by real-time reverse transcriptase-polymerase chain reaction and in situ hybridization. The quantitative polymerase chain reaction analysis showed that the messenger RNA level of activin βC in the DRGs was reduced to ~50% on post-complete Freund’s adjuvant Day 2, and gradually recovered from post-complete Freund’s adjuvant Day 2 to Day 7 (Fig. 2B). In situ hybridization analysis of activin/inhibin-βC in rat DRGs was conducted in the rats with the complete Freund’s adjuvant injection into the left hindpaw. The percentage of DRG neurons expressing activin βC was reduced from ~30% of neurons in control DRGs to ~19% of neurons (n = 1180 out of 6224 counted neurons) in the ipsilateral L4 and L5 DRGs on post-complete Freund’s adjuvant Day 2, without any shifted cell-size distribution (Fig. 2C–E). The proportion of activin βC messenger RNA-containing neurons in the contralateral DRGs was similar to that in the DRGs of control rats (data not shown). The reduction in the number of activin βC messenger RNA-containing DRG neurons was partially recovered on post-complete Freund’s adjuvant Day 4, and was returned to the number similar to that in control DRGs on post-complete Freund’s adjuvant Day 7 (Fig. 2E).

We further evaluated the inflammation-induced change in activin C expression with the immunoblotting method. Indeed, the reduction of activin βC messenger RNA was accompanied by decreased protein levels of activin C in the DRGs (Fig. 2F).

Using two independent antibodies against the activin βC, we identified an immunoblot with a molecular size at ~25 kD in the lysate of the rat DRGs (Fig. 2F), consistent with the notion that activin C is comprised of two activin βC subunits. Using a strongly reduced condition for sample preparation, we could detect the monomer of activin βC (Supplementary Fig. 1B). The activin βC precursor was also present (Supplementary Fig. 1B). Immunoblotting showed that the protein level of activin C began to be reduced on post-complete Freund’s adjuvant Day 1, remained ~50% of pre-inflammatory level on post-complete Freund’s adjuvant Day 2 and was nearly restored to the pre-inflammatory level on post-complete Freund’s adjuvant Day 7 (Fig. 2F). Consistently, levels of the precursor and monomer of activin βC were also reduced during the inflammation (Supplementary Fig. 1B).

Taken together, the activin C expression in small DRG neurons was markedly reduced during the first 2 days of peripheral inflammation, and then gradually returned to the basal level 1 week after inflammation. Thus, activin C in nociceptive afferent neurons is remarkably reduced during the early days of persistent peripheral inflammation.

**Suppressive effects of endogenous activin C on inflammatory nociceptive responses**

To examine the potential role of activin C in pain modulation, we tested whether activin C exerted anti-nociceptive effects at the spinal cord level using the formalin test and the complete Freund’s adjuvant-induced persistent inflammation model. We firstly investigated the function of endogenous activin C in the acute inflammatory pain model induced by intraplantar injection with formalin, which causes a stereotypic two-phase pattern of nociceptive response. Intrathecal treatment with the small interfering RNA targeting activin βC strongly reduced the protein level of activin C in L4 and L5 DRGs, while the number of flinches was markedly increased in both the first and second phase of the formalin test (Fig. 3A and B). Consistently, intrathecally applied activin C antibodies, but not the antibodies denatured in a boiling water bath, increased the number of flinches in both the first and second phase of the formalin test (Fig. 3C and D; Supplementary Fig. 2A and B).

Furthermore, intrathecally injected small interfering RNA targeting activin βC subunit significantly impaired the recovery from the complete Freund’s adjuvant-induced thermal hyperalgesia in rats, and such an inhibitory action lasted >2 weeks (Fig. 3E). However, neither the small interfering RNA targeting activin βC (Fig. 3F) nor the antibodies against activin C were found to change the basal response to heat stimuli in normal rats. Thus, endogenous activin C may mainly suppress nociceptive transmission in both the acute inflammatory response and the recovery period of persistent inflammation, suggesting that activin C expressed in small DRG neurons contributes to a mechanism for suppressing inflammatory pain.

**Inhibition of inflammatory nociceptive responses by intrathecal treatment with activin C**

Next, we examined the effects of exogenously applied activin C on the hyperalgesia induced by peripheral inflammation. Using the radiant heat test, we found that intrathecal injection with activin C (200 ng) significantly reduced the number of flinches
in both the first and the second phase of nociceptive response during the formalin test (Fig. 4A and B). Furthermore, the inflammation-induced thermal hyperalgesia of rats on post-complete Freund’s adjuvant Day 2 could be dose-dependently alleviated by a single intrathecal injection with activin C (50, 100 or 200 ng in 20 μl vehicle) (Fig. 4C and D). However, both the basal nociceptive response and the complete Freund’s adjuvant-induced nociceptive response were not apparently altered by intraplantarly injected activin C (Supplementary Fig. 2C–F).

Notably, the inhibitory effect of activin C on the complete Freund’s adjuvant-induced nociceptive response stayed at the
high level during 1–2 h after the intrathecal injection of activin C, with a peak effect at the 1.5-h interval (Fig. 4C and D). The inhibitory effect induced by a low dose (50 ng) of activin C lasted for 2 h, and was then reduced to a low level 3 h after injection (Fig. 4D). However, the anti-nociceptive effect induced by high doses (100 or 200 ng) of activin C reached a peak level 90 min after injection, and could partially remain (~68% of the peak effect induced by 100 ng activin C, and 55% of that by 200 ng activin C) at 3 h, which is the longest time interval allowed in our experiments to protect the animals. Therefore, a single

**Figure 3** Anti-nociceptive effects of endogenous activin C on inflammatory pain. (A) Immunoblotting showed that the treatment with small interfering RNA (si.), but not scramble control small interfering RNA (Scr.), reduced activin C protein in L4 and L5 DRGs dissected from the rats after the formalin test (n = 8). Intrathecal injection of small interfering RNA targeting activin βC enhanced the flinch behaviour induced by intraplantar injection of 2% formalin. (B) Time course of the effect of activin βC small interfering RNA on formalin-induced flinch behaviour. (C) Intrathecal treatment with anti-activin C antibodies, but not goat IgG, enhanced the flinch behaviour induced by intraplantar injection of 1% formalin. (D) Time course of the effect of activin C antibodies on the formalin-induced flinch behaviour. (E) Intrathecally applied small interfering RNA targeting activin βC (4.0 μg), but not scramble small interfering RNA, impaired the recovery from the complete Freund’s adjuvant (CFA)-induced thermal hyperalgesia. (F) Intrathecal injection with small interfering RNA targeting activin βC (4.0 μg) did not affect the baseline of thermal threshold in normal rats. *P < 0.05, **P < 0.01 and ***P < 0.001, compared with vehicle or IgG control. Data are shown as mean ± SEM.
injection of activin C could strongly relieve the nociceptive hypersensitivity during peripheral tissue inflammation.

**Analgesic effects of daily injected activin C without apparent tolerance**

We also investigated the effect of long-term treatment with activin C on the nociceptive responses evoked by intraplantar injection with complete Freund’s adjuvant. Intrathecal injection with activin C (100 ng/day) every day for 14 days could reduce the complete Freund’s adjuvant-induced thermal hyperalgesia without any apparent reduction in the inhibitory effect, leading to an earlier recovery from the hyperalgesia (Fig. 5A). However, the thermal hyperalgesia was re-established when the activin C injection was stopped at the time interval of 1 week (Fig. 5A).

In contrast to the gradual recovery from thermal hyperalgesia, the mechanical allodynia induced by complete Freund’s adjuvant injection was long-lasting during the 2-week time-course (Fig. 5B). We found that daily injection with activin C (100 ng/day, intrathecal treatment) for 14 days could continuously reduce the mechanical allodynia (Fig. 5B). The mechanical allodynia was re-established when the activin C injection was stopped on the 7th day (Fig. 5B). Activin C had no effect on complete Freund’s adjuvant-induced paw oedema even with long-term intrathecal administration (Fig. 5C), suggesting that it is unlikely that activin C acts through reducing peripheral tissue inflammation. Thus, the long-term treatment with activin C could reduce the thermal hyperalgesia and mechanical allodynia induced by peripheral inflammation without apparent anti-nociceptive tolerance.

**Activin C-induced inhibition of extracellular signal-regulated kinase phosphorylation**

To find the potential signalling mechanism for activin C function, we first examined whether activin C might interact with the activin A/Smad2 signalling pathway. Co-immunoprecipitation showed that activin βA interacted with follistatin but not activin βC in
the DRG lysate (Supplementary Fig. 3A). Immunoblotting with ND 7–23 cells showed that Smad2 was phosphorylated by activin A, but not by activin C (Supplementary Fig. 3B). Moreover, activin A-induced Smad2 phosphorylation was not blocked by activin C (Supplementary Fig. 3B). Therefore, activin C-signalling pathways could be different from that of activin A.

Then, we tested whether ERKs might be one of the targeting molecules for activin C, because the ERK signalling pathway plays an important role in inflammatory responses and nociceptive signalling pathways (Ji et al., 1999, 2009; Dai et al., 2002, 2004; Obata and Noguchi, 2004; Obata et al., 2004; Zhuang et al., 2004; Xu et al., 2008, 2010; Stamboulian et al., 2010). After being starved for at least 4 h, the ND 7–23 cells were exposed to activin C (20 ng/ml) before being challenged by several pro-inflammatory mediators. We found that activin C inhibited the ERK1/2 phosphorylation evoked by nerve growth factor in both ND 7–23 cells (Fig. 6A) and DRG neurons cultured from adult rats (Fig. 6B). In addition to the inhibitory effect on the nerve growth factor-induced ERK phosphorylation, activin C could inhibit the ERK phosphorylation induced by PGE2, glutamate, bradykinin, K+ or TNF-α in ND 7–23 cells (Fig. 6C–G). Furthermore, the inflammation-induced ERK phosphorylation in the rat DRGs and dorsal spinal cord could be reduced by intrathecally applied activin C (Fig. 6H and I). These results suggest that the analgesic effects of activin C are, at least partially, due to inhibiting the activation of ERK signalling pathway.

**Discussion**

The present study reveals the regulated expression and the function of activin C in the spinal sensory pathway. Activin C expressed in small DRG neurons was required for suppressing inflammation-induced nociceptive responses. However, activin C was reduced during early days of persistent tissue inflammation. Intrathecally applied activin C could inhibit inflammation-induced hyperalgesia. Moreover, the activin C action could be partially attributed to its inhibitory effect on the pro-inflammatory mediator-induced ERK phosphorylation. Therefore, activin C contributes to the intrinsic suppressive system in nociceptive afferent neurons, and may have a therapeutic potential for treatment of inflammatory pain.

**Activin C, a suppressive molecule expressed by nociceptive sensory neurons**

Recent studies show that small DRG neurons can express and secrete some suppressive molecules, such as FSTL1 and B-type natriuretic peptide, to negatively regulate the nociceptive afferent transmission (Zhang et al., 2010; Li et al., 2011a). Interestingly, these molecules are found to exert their inhibitory functions through independent signalling mechanisms. FSTL1 functions by activating the presynaptic sodium–potassium pump, while B-type natriuretic peptide functions via the NPR-A/PKG/BKCa channel pathway (Zhang et al., 2010; Li et al., 2011a). Thus, small DRG neurons may have a suppressive system composed of several secretory molecules, which could negatively regulate the nociceptive afferent transmission through distinct signalling pathways. The present finding of activin C function in the inhibition of
inflammation-induced nociceptive responses provides additional evidence for the operation of such a suppressive system in nociceptive afferent neurons. Interestingly, activin C was found to reduce not only the thermal hyperalgesia but also the long-lasting mechanical allodynia, which could not be reduced by activating the B-type natriuretic peptide signalling pathway (Zhang et al., 2010). Thus, activin C may play a unique role in the presynaptic inhibitory system of nociceptive sensory neurons.

Our immunoblotting and immunostaining assays showed the presence of activin C protein in the cell bodies of DRG neurons, but not in their afferent fibres. This might be due to the possibility that the antibodies were not good enough to detect low amounts
of activin C or activin C protein with conformational changes. The finding that activin C inhibited the pro-inflammatory mediator-induced ERK phosphorylation supports the idea that activin C may mainly suppress inflammation-induced nociceptive transmission, but not apparently affect the baseline of nociceptive threshold in normal rats. However, the mechanism of activin C action could not be fully understood at the current stage without the identification of the specific receptor for activin C. 

**Activin C versus other transforming growth factor-β superfamily members in pain modulation**

Several lines of evidence show that some members of the TGF-β superfamily, such as glial cell line-derived neurotrophic factor family members (glial cell line-derived neurotrophic factor, neuratin and artemin), activin A and TGF-β1 (Chang et al., 2002), could be involved in pain modulation. DRG neurons express glial cell line-derived neurotrophic factor receptors (Bennett et al., 2000; Xiao et al., 2002). Giallal cell line-derived neurotrophic factor was applied to reduce ectopic discharges in damaged sensory neurons by regulating sodium channels, and reverse the established neuropathy (Boucher et al., 2000; Boucher and McMahan, 2001; Sah et al., 2003). However, peripheral application of glial cell line-derived neurotrophic factor family members could induce nociceptor sensitization and thermal hyperalgesia (Lindfors et al., 2006; Malin et al., 2006; Bogen et al., 2008). Intrathecally infused TGF-β1 has multipotent effects on neurons and glial cells to regain homeostasis of the dorsal spinal cord, and therefore reduces nociceptive hypersensitivity (Echeverry et al., 2009). However, neither glial cell line-derived neurotrophic factor family members nor TGF-β1 has been found in DRG neurons and spinal dorsal horn cells (Stark et al., 2001; Echeverry et al., 2009).

Activin A is expressed in the skin and could induce pain hypersensitivity by either increasing calcitonin gene-related peptide expression in DRG neurons (Ai et al., 1999; Hall et al., 2002; Xu et al., 2005; Xu and Hall, 2007; Zhu et al., 2007). In contrast, activin C inhibited ERK phosphorylation induced by either acute treatment with pro-inflammatory mediators or persistent tissue inflammation. Therefore, our results suggest that inhibition of ERK activation could be a mechanism for activin C function in pain modulation.

It was reported that activin C overexpressed in some cultured cells could intracellularly form inactive heterodimers with activin A, thereby reducing the level of bioactive activin A (Mellor et al., 2000, 2003; Vejda et al., 2002; Wada et al., 2004). In the present study, endogenous activin A was not found to interact with activin C in the DRG lysate, suggesting that these two subunits are unlikely to form heterodimers in DRG neurons. Interestingly, peripheral tissue inflammation led to a reduced expression of activin C in small DRG neurons, while activin A expression was not apparently changed. Such an unbalanced activin-system in nociceptive afferent neurons may contribute to a mechanism for the development of hyperalgesia.

We conclude that activin C expressed in small DRG neurons is an endogenous suppressor of inflammatory nociceptive transmission. However, the activin C expression is reduced during the early days of peripheral tissue inflammation. Importantly, intrathecally applied activin C inhibits both thermal hyperalgesia and mechanical allodynia induced by inflammation, suggesting a potential role of activin C in inflammatory pain therapy.

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**Supplementary material**

Supplementary material is available at *Brain* online.
References


