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Role of bone morphogenetic protein-2/4 in astrocyte activation in neuropathic pain

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Abstract

Background: Bone morphogenetic protein-2/4 (BMP2/4) has been recognized as promoters of astrocyte activity. Substantial evidence suggests that BMP2/4 may be elevated and plays a critical role in astrocyte activation upon spinal cord injury. Although neuropathic pain is similarly associated with astrocyte activation, the participation of BMP2/4 in this regard still remains unclear.
Methods: A rat model of neuropathic pain achieved by spinal nerve ligation at L5 was used to evaluate the expression of glial fibrillary acidic protein and BMP2/4 in the spinal cord in days 1, 4, 7, 10, and 14. Next, normal rats received intrathecal exogenous BMP2/4 and the antagonist Noggin to assess the effect of BMP2/4 on astrocyte activation. In both experiments, von Frey filaments were used to evaluate the changes in paw withdrawal threshold. In addition, Western blotting and immunofluorescence were performed to assess the expression of glial fibrillary acidic protein, BMP2/4, p-Smad 1/5/8, and phospho-signal transducer and activator of transcription-3 (p-STAT3) in the spinal cord.
Results: Firstly, spinal nerve ligation caused a significant increase in the expression of BMP4, while BMP2 levels remained unchanged. Secondly, exogenous BMP4 but not BMP2 induced a significant decrease in paw withdrawal threshold, along with the upregulation of glial fibrillary acidic protein. Moreover, exogenous BMP4 stimulated both p-Smad 1/5/8 and p-STAT3, while BMP2 only upregulated p-Smad 1/5/8. Finally, exogenous Noggin alleviated the decrease in paw withdrawal threshold induced by BMP4 and reduced astrocyte activation, as well as p-STAT3 upregulation.
Conclusions: Our results indicate only BMP4—and not BMP2—intervened in allodynia in rats by eliciting glial activation probably through both p-Smad 1/5/8 and p-STAT3 signaling.

Keywords
Neuropathic pain, bone morphogenetic protein, glial activation, signal transducer and activator of transcription 3

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Introduction

Neuropathic pain (NP) is a severe debilitating disease which often arises after a traumatic injury of the central or peripheral nervous system. Allodynia, one of the most prominent features of NP, has been closely correlated with glial activation.[1] Recently, a growing body of evidence has established that once activated, glial cells—especially astrocytes[2,3]—can actively enhance inflammation and neuronal activity, which finally promotes the development of NP.[4,5] Thus, unraveling the molecular and cellular basis of astrocyte activation is essential for devising therapeutic strategies for NP.

Bone morphogenetic proteins (BMPs) are the largest class in the transforming growth factor β superfamily, containing at least 20 structurally distinct members.[6] Among them, BMP2 and BMP4 share similar amino acid sequences[7] and have displayed equivalent capability for the promotion of astrocyte differentiation both in vitro[8] and in vivo during the development of the central nervous system (CNS).[9,10] In adulthood, BMP2/4 differentially distributes in mature astrocyte in spinal cord and brain,[11,12] prompting that they may still play a role in

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the regulation of adult astrocyte activity. Previous researches from our group and others[13–15] have found BMP2/4 expressions in local astrocytes to be increased after spinal cord injury (SCI), which would in turn cause accumulation of glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycan (CSPG) and lead to astrogliosis even glial scar formation. Furthermore, application of exogenous BMP4 appears to be sufficient to induce the expression of CSPG, even in the absence of substantial tissue damage.[15] These data suggest that mature astrocytes still retain the responsiveness to BMP4.

In comparison with the rapid progress in our understanding of the role of BMP in CNS injuries, relatively little has been cleared regarding the expression patterns and effects of BMP in NP. In this study, we observed the expression patterns of BMP2/4 over time in a rat model of NP achieved through spinal nerve ligation (SNL) at L5. We also examined the effects of the administration of exogenous BMP2, BMP4, and Noggin on allodynia and GFAP expression. Finally, we assessed the downstream signaling markers such as p-Smad 1/5/8 and phospho-signal transducer and activator of transcription-3 (p-STAT3) after intrathecal administration of BMP2/4. Our findings may enhance current comprehension of the correlations between BMP2/4 and NP.

Methods and materials

Animals

Young adult male Sprague-Dawley rats weighing 200 to 250 g were purchased from the Animal Center of the Hunan Agricultural University (Changsha, Hunan, China) and housed separately with food and water ad libitum, in a temperature-controlled room with 12/12 h light/dark cycles. Animals were allowed to acclimatize to these conditions for one week before beginning the experiments. All procedures and care were approved by the Institutional Animal Care and Use Committee of the Central South University and conducted according to the Health Guidelines of the National Institutes for the Use and Care of Laboratory Animals.

Spinal nerve ligation

Fifty rats were divided into two groups using block randomization ($n = 5$ at each time point for both groups): sham or SNL group. SNL was performed according to the method described by Chung et al.[16] Briefly, the left L5 spinal nerve was isolated and then tightly ligated using a 6-0 silk thread, after careful exposure and removal of the L6 transverse process. Animals in the sham group underwent the same surgical procedure, only without ligation. Totally one rat in Sham group with evident neuromuscular dysfunction was excluded from the study.

Intrathecal catheterization

Under anesthesia with an intraperitoneal injection (i.p.) of pentobarbital (30 mg/kg), a polyethylene-10 intrathecal catheter (Smith Medical, OH, USA) was inserted through the L4–L5 intervertebral space toward the lumbar enlargement and externally fixed to the skin nearby.[17] Three days after surgery, catheter placement was confirmed by the observation of hind limb paralysis after intrathecal injection of lidocaine (2%, 10 µl). In addition, 112 rats were enrolled and 4 rats that failed to show paralysis were excluded from the experiment.

Intrathecal drug treatments

All exogenous proteins, including recombinant BMP2 (rBMP2), recombinant BMP4 (rBMP4), and recombinant Noggin (rNoggin), carrier free, were purchased from Peprotech (NJ, USA). All compounds were dissolved in a working volume of 20 µl of sterile phosphate-buffered solutions (PBS, composed of 137 mM NaCl, 2.7 mM KCl 2.7, 1.5 mM KH_2PO_4, and 8.1 mM NaH_2PO_4, pH = 7.4) for each injection. The concentrations for each drug were calculated according to the previous research[18] our preliminary observation, and the intrathecal injections were conducted once daily throughout the experiment. Rats with successful intrathecal catheterization were randomly divided into six groups ($n = 6$ at each time point for all groups): (1) *Sham*: received only the vehicle; (2) *BMP2*: received 60 µg/ml of rBMP2; (3) *BMP4*: received 2 µg/ml of rBMP4; (4) *NOG*: received 5 µg/ml of rNoggin; (5) B + NL: received 2 µg/ml of rBMP4, combined with 2 µg/ml of rNoggin; and (6) *B + NH*: received 2 µg/ml of rBMP4 with 5 µg/ml of rNoggin.

Behavioral testing: von Frey test

Von Frey filaments (Stoelting, IL, USA) was used to measure the paw withdrawal threshold (PWT) by a researcher who was unfamiliar with the grouping. All tests were conducted at a fixed time (15:00–17:00). In detail, rats were placed individually in a wire mesh cage, where they were kept for 30 min in order to acclimatize to the new environment. The plantar surface of the rats' hindpaw was pricked by researchers who were blinded to group assignment using von Frey filaments. Rapid shaking, pulling back, or licking the hind limb within 5 s were identified as a positive sign of withdrawal. The up and down method was used to determine the 50% PWT.

Immunofluorescence

Rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and then underwent transcardiac perfusion of 150 ml of PBS containing 0.1% heparin, followed by 500 ml of ice-cold 4% paraformaldehyde. Next, the lumbar enlargements were harvested, fixed in 4% paraformaldehyde for 8 h, and cryoprotected in 30% (w/v) sucrose. Serial frozen sections embedded in medium (Tissue-Tek O.C.T. Compound, Torrance, CA, USA) were cut to obtain 10 µm of thickness with CM1800 (Leica, Heidelberg, Germany). All sections were rinsed in 0.3% Triton X-100 for 15 min, and then blocked with 10% goat serum for 1 h at 37°C, before incubation with primary antibodies overnight at 4°C at the following dilutions: antimouse GFAP (1:300; Cell Signaling, MA, USA), antirabbit BMP2 (1:200; Abcam, Cambridge, UK), antirabbit BMP4 (1:200; Abcam), and antirabbit p-STAT3 rabbit IgG (1:100; Cell Signaling). This was followed by incubation for 2 h at room temperature with a mixture of fluorescein isothiocyanate-conjugated goat antimouse IgG (1:150; Proteintech, IL, USA) and rhodamine-conjugated goat antirabbit IgG (1:150; Proteintech). The tissue sections were then washed and subsequently incubated with 4′,6-diamidino-2-phenylindole for 15 min. Finally, all sections were evaluated with fluorescence microscopy (Nikon eclipse E600, Japan).

Western blotting

Rats were deeply anesthetized with pentobarbital (100 mg/kg i.p.) and then rapidly sacrificed. Next, the lumbar enlargements were harvested on ice, homogenized in liquid nitrogen, and kept in radio immunoprecipitation assay (RIPA) lysis buffer with a mixture of proteinase and phosphatase inhibitors in a freezer at −80°C. Protein concentrations were measured with the bicinchoninic acid assay. All samples containing 5 to 10 µg of protein were loaded into 10% or 15% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and then transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dried milk for 1 h, membranes were immunoblotted overnight at 4°C, with the following antibodies: antimouse GFAP (1:3000; Cell Signaling), antirabbit BMP2 (1:1000; Abcam), antirabbit BMP4 (1:1000; Abcam), antirabbit Smad1/5/9 (1:1000; Affinity, OH, USA), antirabbit p-Smad1/5/9 (1:1000; Cell Signaling), antirabbit STAT3 (1:1000; Cell Signaling), antirabbit p-STAT3 (1:1000; Cell Signaling), and antimouse glyceraldehyde 3-phosphate dehydrogenase (1:8000; Proteintech), followed by horseradish peroxidase (HRP)-conjugated goat antimouse IgG (1:5000: Proteintech), or HRP-conjugated goat antirabbit IgG (1:5000; Proteintech) for 1 h at 37°C. Each membrane was processed with Luminol Reagent (Millipore, MA, USA) and exposed to an electrochemiluminescence (ECL) film in a dark room. Finally, the films were scanned and analyzed with Image-Pro Plus 6.0 (Rockville, MD, USA).

Statistical analysis

All data were analyzed with SPSS v19.0 software (IBM, NY, USA). Continuous variables were presented as means ± standard error of the mean. Data regarding the effects of BMP2/4 on behavioral testing and Western blotting were analyzed with two-way analysis of variance, followed by Tukey's post hoc test for significance, and then charted with Prism software (GraphPad Inc., CA, USA). Results were considered to be statistically significant when $p < 0.05$.

Results

BMP4 but not BMP2 was upregulated after SNL

Rat models of SNL were established by ligating the L5 spinal nerve. As shown in Figure 1(a), SNL caused a significant decrease in PWT in the ipsilateral hind limb as early as day 1 after surgery, which persisted up to day 14 (vs. Sham group, $p < 0.01$). This finding suggested the successful establishment of NP. Moreover, GFAP expression was increased on day 4 postsurgery and persisted up to day 14 (vs. Sham group, $p < 0.01$), confirming the presence of spinal astrocytes activation in NP (Figure 1(b) and (c)).

Next, we examined the expression patterns of BMP2 and BMP4 in the spinal cord after SNL. Compared with the Sham group, BMP4 expression in the SNL group was significantly upregulated from day 1 to 14 ($p < 0.01$). Surprisingly, no significant changes were observed on BMP2 expression ($p > 0.05$, Figure 2(a) and (b)). Furthermore, double-labeling immunofluorescence further showed both BMP2 and BMP4 were abundantly localized in activated astrocytes after spinal nerve injury (Figure 2(c)), corroborating astrocytes to be an important source of BMP4 and BMP2.

Intrathecal administration of BMP4 but not BMP2 induced allodynia and glial activation

The distinct activation patterns of BMP2 and BMP4 seen in NP suggested that BMP2 and BMP4 might have differential roles in the development of NP. In order to characterize the roles of BMP2 and BMP4 on allodynia, we investigated the effect of intrathecal administration of exogenous BMP2 and BMP4 on pain hypersensitivity and astrocyte activation.

Intrathecal administration of rBMP4 was associated with a prominent decrease in bilateral PWT in normal

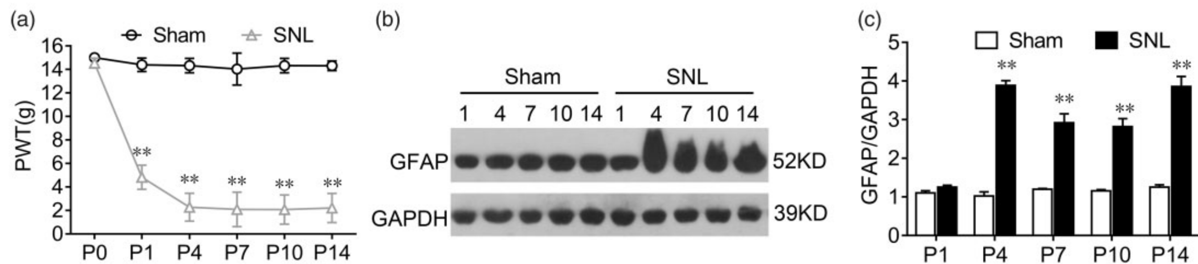

Figure 1. SNL causes allodynia and GFAP upregulation in rats. (a) Each time point indicates mean ± standard error of the mean of PWT in five rats as measured using von Frey filaments. There was a significant effect on time, $F(5, 40) = 85.62$, $p < 0.0001$, or SNL, $F(1, 8) = 954.6$, $p < 0.0001$, and interaction, $F(5, 40) = 67.07$, $p < 0.0001$, on PWT observed 14 days after SNL. Compared with the Sham group in which PWT remained at the baseline of 15 g, there was a significant decrease in PWT in day 1 (SNL: 4.82±1.03 g), day 4 (SNL: 2.27±1.19 g), day 7 (SNL: 4.82 ± 1.03 g), day 10 (SNL: 2.07 ± 1.02 g), and day 14 (SNL: 2.20 ± 1.24 g) ($p < 0.01$). **$p < 0.01$ compared with the Sham group. (b and c) Representative results from Western blotting indicated the ratio between GFAP and GAPDH remained comparable in day 1 ($p = 0.12$), but significantly increased in the SNL group in days 4, 7, 10, and 14 ($p < 0.01$), compared with the Sham group. The SNL group had significantly increased GFAP levels from day 4 to day 14. n = 3 at each time point of both groups. **$p < 0.01$ compared with the Sham group. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; PWT: paw withdrawal threshold; SNL: spinal nerve ligation.

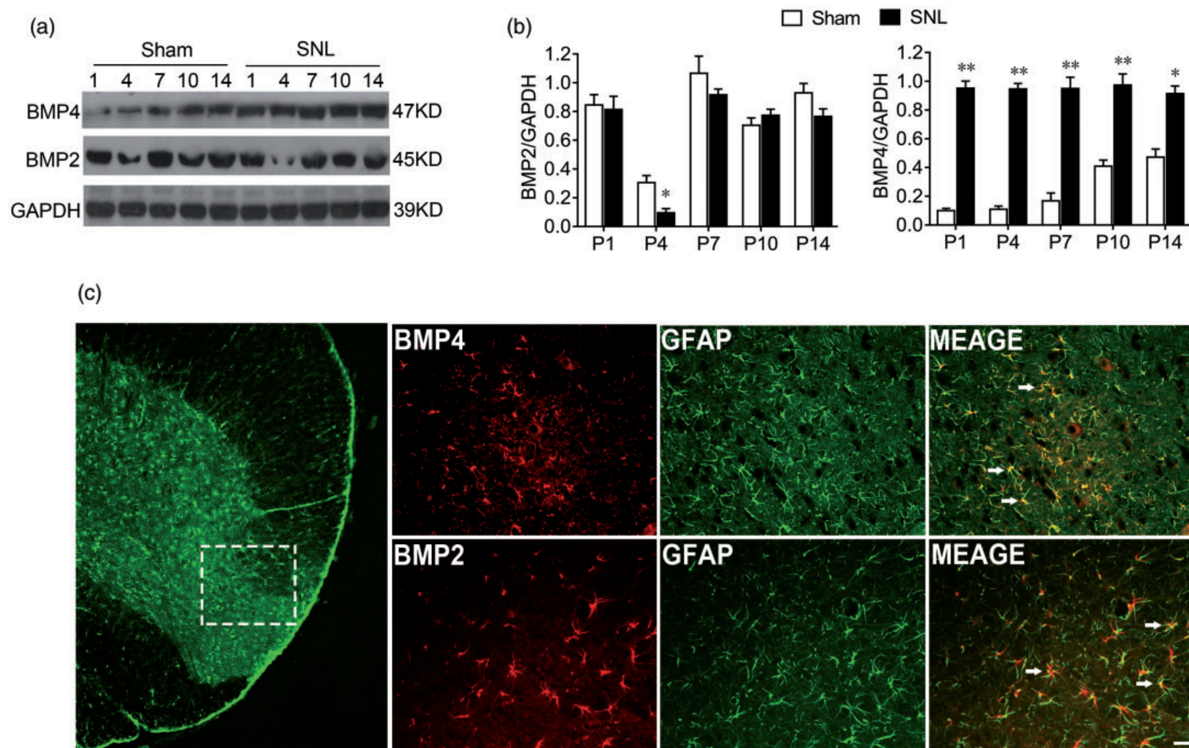

Figure 2. Expression and localization pattern of BMP2 and BMP4 after SNL. (a and b) Representative results from Western blotting, illustrating that SNL-induced BMP4 expression in the dorsal spinal cord was prominently increased in days 1, 4, 7, and 10 ($p < 0.01$), and day 14 ($p < 0.05$). In contrast, BMP2 expression in the SNL group decreased in day 4 ($p < 0.05$), yet remained comparable in day 1 ($p = 0.27$), day 7 ($p = 0.23$), day 10 ($p = 0.33$), and day 14 ($p = 0.09$). n = 3 at each time point for both groups. *$p < 0.05$ compared with the Sham group; **$p < 0.01$ compared with the Sham group. (c) Double immunofluorescence staining of GFAP (green) and BMP2 (red) or BMP4 (red) in the dorsal horn of spinal cord at day 7 after SNL; showing that both BMP2 and BMP4 were abundantly colocalized with GFAP. Scale bar = 50 μm. BMP: bone morphogenetic protein; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; SNL: spinal nerve ligation.

rats. The effect of rBMP4 on allodynia started on day 1 and lasted until day 7 ($p < 0.01$ vs. Sham group). In striking contrast, intrathecal rBMP2 did not induce significant change in bilateral PWT. Rats in the rBMP2 group basically maintained their PWT comparable with the Sham group ($p > 0.05$) (Figure 2(a)) (Figure 3(a)).

To assess whether the effect of BMP4 on allodynia was accompanied with astrocyte activation, lumbar enlargements were harvested at days 1, 4, and 7 after administration of rBMP2 or BMP4, followed by examination of GFAP expression with Western blotting. We found that rBMP4 induced a significant and lasting elevation of GFAP in the seven days following injection ($p < 0.05$ vs. Sham group). In contrast, no upregulations of GFAP was observed after treatment with rBMP2 ($p > 0.05$ vs. Sham group, Figure 2(b) and (c)) (Figure 3(b) and (c)).

In ensemble, these findings strongly indicate spinal BMP4, but not BMP2, may have a role in the regulation of hypersensitivity to mechanical pain and astrocyte activation.

BMP4 but not BMP2 activated STAT3 and Smad1/5/8

The classical downstream signaling of BMP are Smad proteins, which can be activated by both BMP2 and BMP4. The mechanisms through which BMP promotes astrogliogenesis also appear to involve interactions between Smad and Stat proteins.[19–21] In this scenario, we found the administration of both rBMP2 and rBMP4 resulted in a marked increase in p-Smad1/5/8 expression in day 1, which lasted for seven days ($p < 0.05$, vs. Sham group). However, only rBMP4, but not rBMP2, considerably enhanced p-STAT3

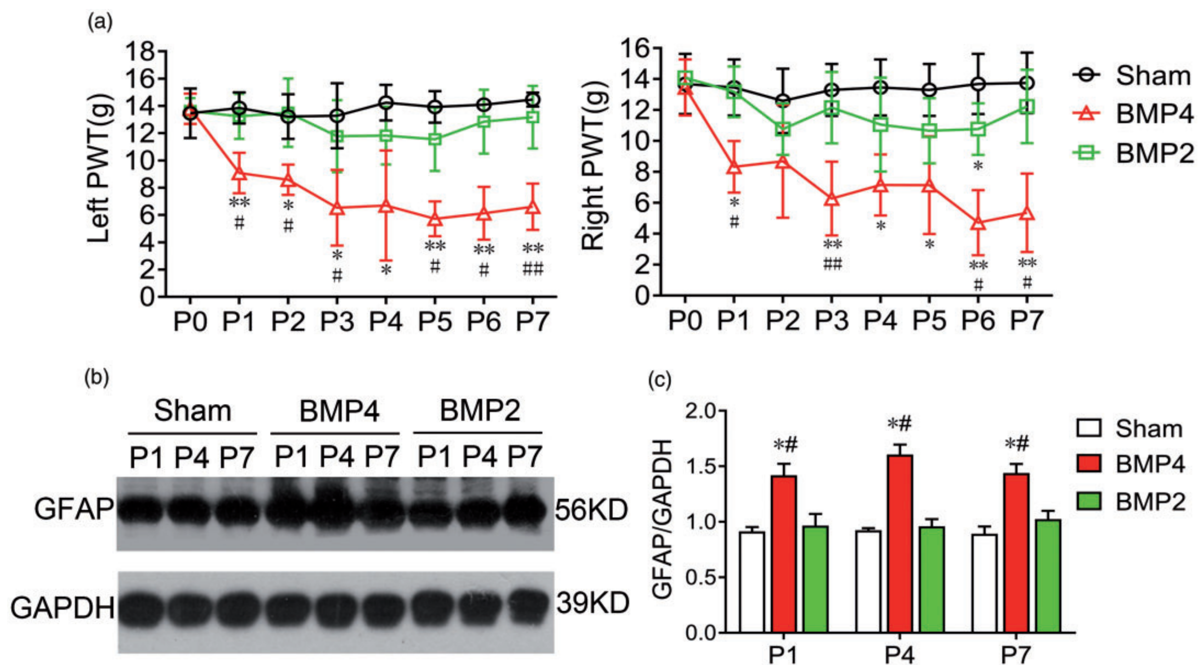

Figure 3. Intrathecal administration of BMP4 instead of BMP2-induced allodynia and glial activation. (a) Each time point indicates mean ± standard error of the mean of PWT in five rats measured with von Frey filaments, depicting the time course of the separate effects of exogenous BMP2 and BMP4. There was a significant effect on time, $F(4.030, 48.36) = 6.324$, $p = 0.0003$, left; $F(7, 32) = 4.274$, $p = 0.002$, right, or intrathecal injection, $F(2, 12) = 47.09$, $p < 0.0001$, left; $F(1.735, 55.52) = 99.72$, $p < 0.0001$, right, and interaction, $F(14, 84) = 4.498$, $p < 0.0001$, left; $F(14, 64) = 3.001$, $p = 0.0014$, right, on bilateral PMT observed seven days after intrathecal administration. Compared with the Sham group, rats in the BMP4 group showed a significant decrease in PWT from day 1 (left: 9.09 ± 1.49 g, $p < 0.01$; right: 6.61 ± 1.71 g, $p < 0.05$) to day 7 (left: 8.33 ± 1.67 g, $p < 0.01$; right: 5.36 ± 2.54 g, $p < 0.01$). In contrast, rats in the BMP2 group maintained their PWT at baseline over the following seven days (day 1 left: 13.63 ± 0.95 g, right: 13.18 ± 2.30 g; day 7: left: 14.09 ± 0.20 g, right: 12.23 ± 2.37 g, $p > 0.05$), which was comparable to the Sham group (except for the right PWT on day 6: 10.77 ± 1.67 g, $p < 0.05$). Significant statistical differences of PWT were also found between the BMP2 and BMP4 groups at each time point. $^{*}p < 0.05$ compared with the Sham group; $^{**}p < 0.01$ compared with the Sham group; $^{\#}p < 0.05$ compared with the BMP2 group; $^{\#\#}p < 0.01$ compared with the BMP2 group. (b and c) Representative Western blotting indicating GFAP expression was increased in the BMP4 group in days 1, 4, and 7 compared with the Sham group ($p < 0.05$). However, no GFAP upregulation was detected in the BMP2 group compared with the Sham group (day 1: $p = 0.51$; day 4: $p = 0.45$; day 7: $p = 0.18$). Statistical differences in GFAP expression were also found between the BMP2 and BMP4 groups at each time point ($p < 0.05$). n = 3 at each time point for both groups. $^{*}p < 0.05$ compared with the Sham group; $^{\#}p < 0.05$ compared with the BMP2 group. BMP: bone morphogenetic protein; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; PWT: paw withdrawal threshold; SNL: spinal nerve ligation.

levels from day 1 and up to day 7 ($p < 0.05$ vs. Sham and BMP2 groups, shown in Figure 3(a) and (b)) (Figure 4(a) and (b)). This suggests that STAT3 signaling may be related to the astrocyte activation effect of BMP4 only, and not BMP2.

The distribution of p-STAT3 was then visualized with immunofluorescence one day after intrathecal treatment. Figure 3(c) Figure 4(c) shows the rBMP4-induced notable p-STAT3 activation in the dorsal spinal cord, whereas rBMP2 or vehicle administration hardly increased p-STAT3 expression. Moreover, double-labeling immunofluorescence revealed that part of these p-STAT3 positive areas were accumulated in the nucleus of GFAP positive astrocytes, further demonstrating p-STAT3 may have a role on BMP4-induced glial activation.

Noggin inhibited BMP4-induced allodynia, glial activation, and p-STAT3 upregulation

Upon the finding that BMP4-STAT3 signaling in spinal astrocytes promoted mechanical allodynia, we hypothesized a BMP4 antagonist should be able to block this pronociceptive effect. In this scenario, Noggin was used as antagonist to investigate its inhibitory capacity over the pro-allodynia effect of BMP4. In the NOG group (Figure 5 (a) to (c)) (Figure 4(a) to (c)), we found that intrathecal injection of rNoggin alone at 5 µg/ml did not cause allodynia or upregulation of GFAP and p-STAT3 in comparison with the Sham group. In contrast, the combination of rNoggin with rBMP4 notably alleviated rBMP4-induced allodynia as well as GFAP upregulation and p-STAT3 activation in a dose-dependent manner ($p < 0.05$; shown in groups

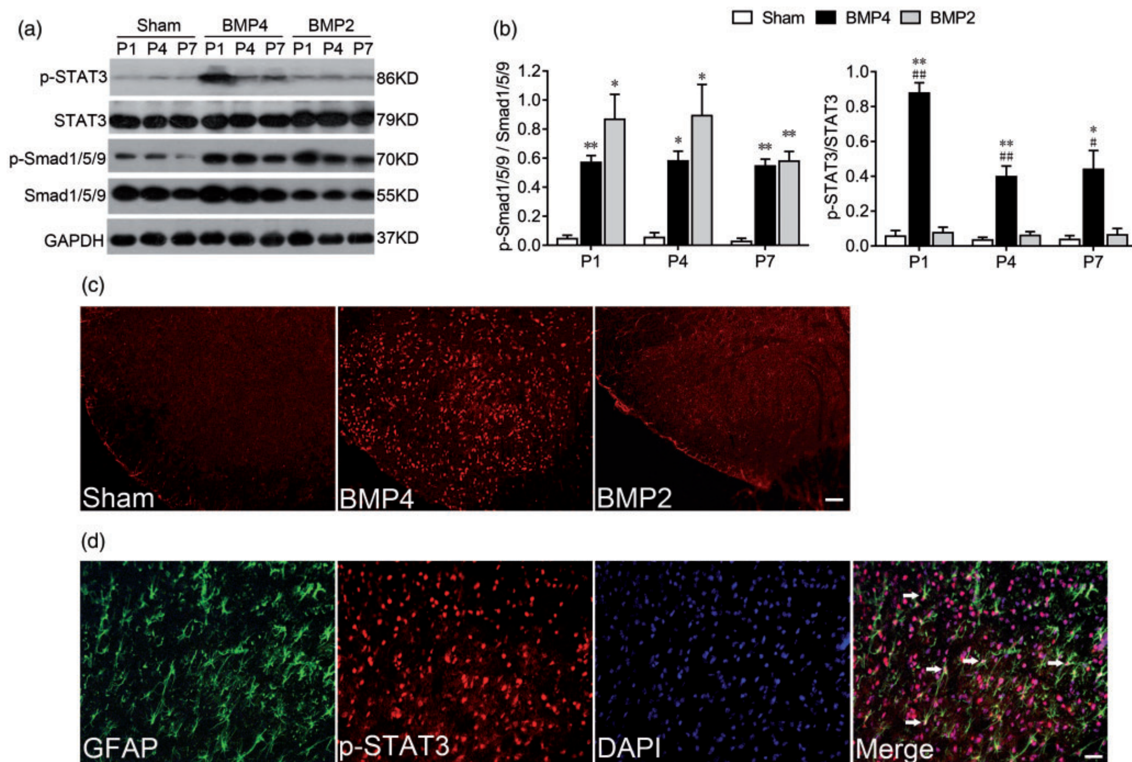

Figure 4. Exogenous BMP4 but not BMP2 activated both p-Smad1/5/9 and p-STAT3 signaling. (a and b) Western blotting showed the expression of p-Smad1/5/9 potently increased in both the BMP4 and BMP2 groups in day 1 (BMP4: $p < 0.01$; BMP2: $p < 0.05$), day 4 (BMP4: $p < 0.05$; BMP2: $p < 0.05$), and day 7 (BMP4: $p < 0.01$; BMP2: $p < 0.01$) compared with the Sham group. On the other hand, p-Smad1/5/9 expressions between the BMP4 and BMP2 groups remained similar (P1: $p = 0.1224$; P4: $p = 0.1826$; P7: $p = 0.6561$). In contrast, p-STAT3 expression in the BMP4 group considerably increased in days 1, 4, and 7 compared with the Sham group (P1: $p < 0.01$; P4: $p < 0.01$; P7: $p < 0.05$) and the BMP2 group (P1: $p < 0.01$; P4: $p < 0.01$; P7: $p < 0.05$), while p-STAT3 expression in the BMP2 group remained comparable with the Sham group (P1: $p = 0.5180$; P4: $p = 0.2867$; P7: $p = 0.0846$). n = 3 at each time point for both groups. $^{*}p < 0.05$ compared with the Sham group; $^{**}p < 0.01$ compared with the Sham group; $^{\#}p < 0.05$ compared with the BMP2 group; $^{\#\#}p < 0.01$ compared with the BMP2 group. (c) Immunofluorescence images performed one day after intrathecal administration showed the upregulated p-STAT3 in BMP4 group was prominently localized in the dorsal horn of the spinal cord, while there was hardly any expression of p-STAT3 in both the Sham and the BMP2 group. n = 2 at each time point for both groups. Scale bar = 50 µm. (d) Double-labeling immunofluorescence further showed these p-STAT3-positive cells (red) were almost completely accumulated in the nucleus (blue) of GFAP-positive astrocytes (green). n = 2 at each time point for both groups. Scale bar = 25 µm. BMP: bone morphogenetic protein; DAPI: 4′,6-diamidino-2-phenylindole; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; STAT: signal transducer and activator of transcription.

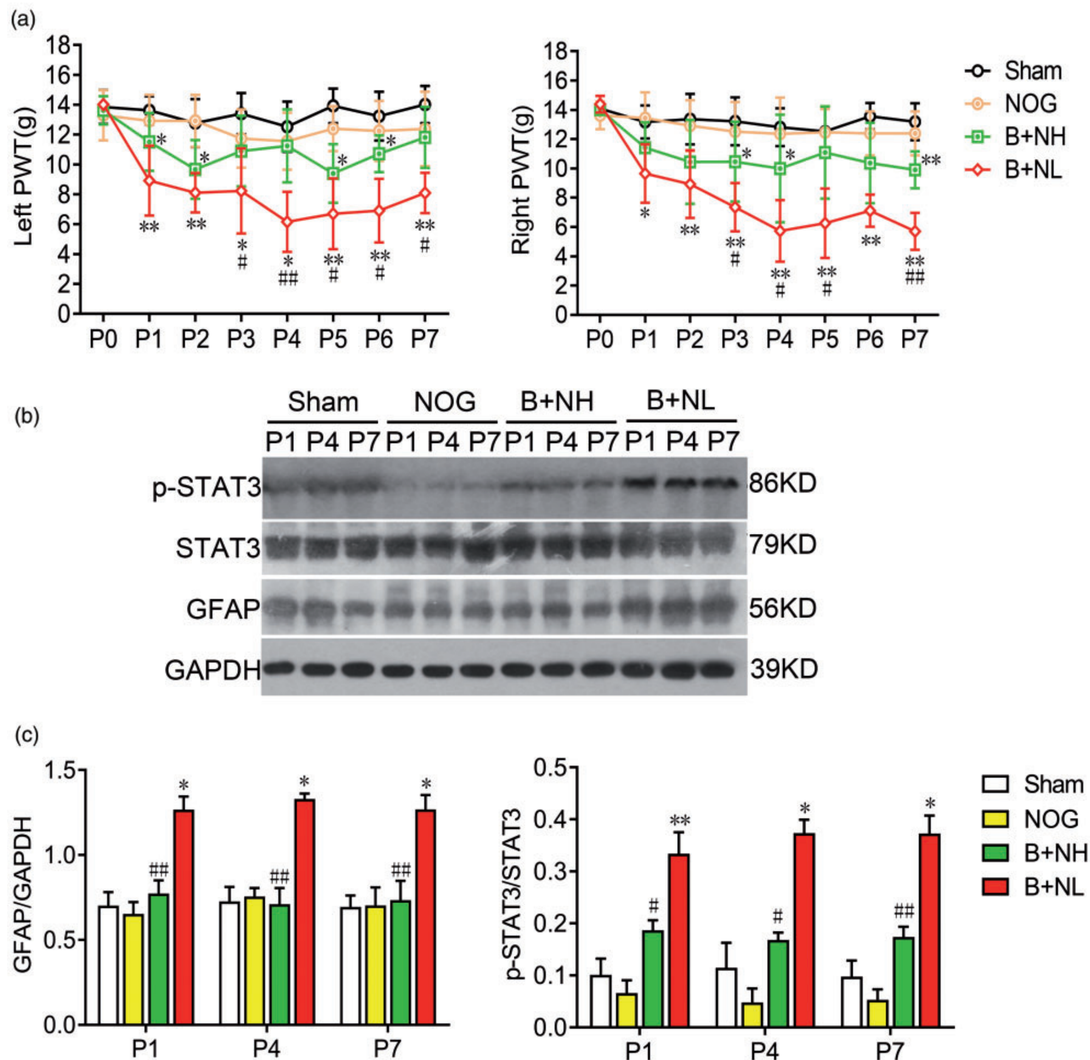

Figure 5. Noggin inhibited rBMP4-induced allodynia, glial activation, and p-STAT3 upregulation in a dose-dependent manner. (a) Each time point indicates mean $\pm$ standard error of the mean of PWT in five rats measured with von Frey filaments in order to assess the antagonistic effect of Noggin on BMP4. There was a significant effect on time, $F(7, 32) = 10.13$, $p < 0.0001$, left; $F(7, 32) = 6.492$, $p < 0.0001$, right, or intrathecal injection, $F(2.177, 69.65) = 52.25$, $p < 0.0001$, left; $F(1.869, 59.82) = 66.98$, $p < 0.0001$, right, and interaction, $F(21, 96) = 1.792$, $p = 0.0301$, left; $F(21, 96) = 2.329$, $p = 0.0029$, right, on bilateral PMT observed seven days after intrathecal administration. Firstly, the PWT of rats in the NOG group was shown to be comparable with the Sham group from day 1 to day 7 after the intrathecal treatments ($p > 0.05$). Secondly, the PWT of the B + NL group decreased significantly compared with the Sham group from day 1 (left: 6.92 ± 2.13 g, right: 9.65 ± 1.99 g) to day 7 (left: 4.02 ± 1.68 g, right: 5.71 ± 1.27 g). In addition, there was a notable increase in bilateral PWT in the B + NH group in comparison with B + NL group from day 3 (B + NH group: left: 7.60 ± 1.36 g, right: 10.46 ± 2.73 g; B + NL group: left: 4.86 ± 1.80 g, right: 7.36 ± 1.65 g) and day 7 (B + NH group: left: 7.33 ± 1.67 g right: 9.90 ± 1.27 g; B + NL group: left: 4.02 ± 1.68 g, right: 5.71 ± 1.27 g). $^{*}p < 0.05$ compared with the Sham group; $^{**}p < 0.01$ compared with the Sham group; $^{\#}p < 0.05$ compared with the B + NL group; $^{\#\#}p < 0.01$ compared with the B + NL group. (b and c) Western blotting was performed to evaluate the antagonistic effect of Noggin on BMP4-induced activation of GFAP and p-STAT3. The expression of GFAP and p-STAT3 were comparable between Sham and NOG groups (GFAP: day 1 $p = 0.608$, day 4 $p = 0.740$, day 7 $p = 0.911$; p-STAT3: day 1 $p = 0.388$, day 4 $p = 0.175$, day 7 $p = 0.139$). Furthermore, the GFAP and p-STAT3 expression in B + NL group significantly increased compared with the Sham group ($p < 0.05$). In addition, a notable decrease in GFAP and p-STAT3 expressions could be detected in B + NH group compared with B + NL group, suggesting rNoggin could dose-dependently relieve rBMP4-induced GFAP and STAT3 activation. n = 3 at each time point of all groups. $^{*}p < 0.05$ compared with the Sham group; $^{**}p < 0.01$ compared with the Sham group; $^{\#}p < 0.05$ compared with the B + NL group; $^{\#\#}p < 0.01$ compared with the B + NL group. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; NH: ■; NL: ■; NOG: ■; PWT: paw withdrawal threshold; STAT: signal transducer and activator of transcription.

B + NH and B + NL, Figure 4(a) to (c)) (Figure 5 (a) to (c)).

Discussion

BMP2/4 is known to intervene in several cellular processes, including proliferation, differentiation, and apoptosis. Their signaling network is a complex, tightly regulated system. Although substantial evidence has proven, BMP2/4 is elevated and aggravates gliosis after SCI, little is known about their role on NP. Here, we found that endogenous BMP4—and not BMP2—was upregulated in NP induced by SNL. Similarly, intrathecal administration of exogenous BMP4 but not BMP2 induced allodynia and astrocyte activation partly via both p-Smad1/5/8 and p-STAT3 signaling. In addition, exogenous Noggin alleviated the effect of BMP4 in a dose-dependent manner.

Differential effects of BMP2 and BMP4 on allodynia and astrocyte activation

Interestingly, only BMP4 but not BMP2 had an effect on allodynia and astrocyte activation. In the developmental CNS, both BMP4[22,23] and BMP2[24] are known to promote astrocyte differentiation. Transgenic overexpression of BMP4 appears to induce a remarkable increase in astrocytes in multiple brain regions.[25,26] In adulthood, BMP4 has been proved to be elevated and aggravate gliosis after SCI.[15,27] Considering BMP4 and BMP2 share 80% homology in their amino acid sequence, it is plausible that BMP2 could also induce astrocyte activation in the spinal cord. However, reports regarding the effect of BMP2 on glial activity after SCI remain conflicting. Several studies[28,29] have reported SCI could trigger BMP2 upregulation, and intrathecal administration of exogenous BMP2 to the injured spinal cord appears to aggravate gliosis and glial scarring. However, other studies have indicated BMP2 mRNA stays unchanged after SCI, while BMP4 mRNA was immediately upregulated and lasted for one month.[30] BMP2 and BMP4 have also been reported to exert opposite effects on the regulation of glial scar formation.[31] This indicates that BMP2 and BMP4 may have different roles in regulating glial activity in the adult CNS. Importantly, mature astrocytes remain responsive to BMP4, and in vivo intrathecal BMP4 injection appears to trigger significant astrocyte activation and CSPG expression in normal uninjured spinal cords.[15] Whether or not BMP2 had the similar effect and BMP4-induced astrocyte activation could induce allodynia remains unclear.

Here, using a well-characterized model of SNL, we found that nerve injury led to a significant increase in BMP4 expression. Furthermore, intrathecal administration of exogenous BMP4 induced astrocyte activation—in accordance with previous research—and allodynia in normal adult rats. In contrast, BMP2 stayed unchanged after SNL and failed to induce astrocyte activation or allodynia. Our results suggest that BMP4 but not BMP2 may be involved in astrocyte activation in allodynia.

Differential role of Smad1/5/8 and STAT3 on BMP4-induced astrocyte activation

As the canonical downstream signals of BMP2/4, Smad1/5/8 plays an essential role in the development of the CNS. Substantial evidence has proven Smad1/5/8 signaling is involved in the induction of both glial and neuron fate from neural stem cells.[32] Similarly, in adulthood, Smad1/5/8 has also been reported to either promote axon regeneration[33] or aggravate glial scar and demyelination[34,35] after SCI. How Smad signaling achieves these diverse effects remains unknown. Importantly, it has been observed that GFAP promoter does not contain known Smad consensus sited,[36] suggesting Smad signaling cannot directly promote GFAP expression. Similarly, our experiments revealed intrathecal application of both BMP2 and BMP4 elicited Smad1/5/8 phosphorylation. However, only BMP4 induced glial activation. Hence, we inferred that other signaling molecules, instead of p-Smad1/5/8, may be responsible for this BMP4-induced glial activation in normal adult rats.

Another crucial promoter of glial differentiation is STAT3.[37,38] In astrocytes, over 1200 genes have been confirmed to be controlled by STAT3, including GFAP.[39] Till now, accumulating evidences have shown that STAT3 participates in the process of NP through activating glial cells.[40,41] Notably, a synergistic effect has been described between STAT and Smad[19,20,42,43]; activation of both signals leads to the formation of a complex which translocates into the nucleus and binds to the STAT site of the GFAP promoter, triggering astrogliogenesis. Furthermore, BMP4 has been confirmed to regulate STAT3 activity by interacting with FK506-binding protein-12 (FKBP12)/rapamycin and the BMP receptor IA (BMPRIA) receptor.[21] In the present study, we firstly encountered p-STAT3 contributes to BMP4-induced glial activation and initiated both p-Smad1/5/8 and p-STAT3 signaling. By contrast, BMP2, which only activated p-Smad1/5/8 but not p-STAT3, failed to activate glial cells. In addition, BMP4 induced p-STAT3 accumulation mainly in the nucleus and was abundantly expressed in astrocytes in the spinal dorsal horn. These results may partially explain the differences between the effects of BMP2 and BMP4 on glial activation and NP initiation.

The antagonistic effect of Noggin on BMP4-induced allodynia and astrocyte activation

Noggin is an extracellular BMP antagonist which binds to BMP2, BMP4, and BMP7 with varying affinities.[44,45] In the development of the CNS, Noggin expression counteracts the glial-promoting effect of BMP4, playing a crucial role in neurogenesis.[46] In adults, CNS injury features overexpression of Noggin in the lesion site, which could promote neuron regeneration or axonal growth.[47–49] Notably, a previous study[14] had reported intrathecal application of Noggin failed to attenuate gliosis, although it markedly decreased p-Smad expression. Moreover, Noggin did not block STAT3 activation and its induction of GFAP in the injured spinal cord. Nevertheless, in another NP model—chronic constriction injury (CCI)—application of exogenous Noggin significantly alleviated mechanical allodynia and decreased GFAP expression,[50] but this effect was not further investigated regarding STAT3 activation. Similarly, in the present study, we found that intrathecal administration of exogenous Noggin relieved BMP4-induced mechanical allodynia, GFAP, and p-STAT3 upregulation in a dose-dependent fashion. The discrepancy may be attributed to the varied animal models and different mechanism of injures. For instance, in the injured spinal cord, the amount of signaling including STAT3 may be initiated by multiple molecules, rather than BMP alone. In contrast, in our study, we chose normal adult rats, thus this p-STAT3 upregulation occurred only with exogenous BMP4 application. Together, these data suggest that BMP4 signaling may be a new target for treating NP.

At present, the Food and Drug Administration has approved recombinant human BMP2 (rhBMP2) as an effective bone graft substitute.[51] Clinical research has demonstrated BMP application could obviate the need for the use of autologous bone in spine fusion.[52] However, an increasing number of complications have been reported, including its pro-inflammatory effect have been reported, probably due to the complexity of the BMP signaling network.[53–56] Notably, a recent study[57] found that implantation of rhBMP2-soaked absorbable collagen sponges (ACS) at the dorsal root ganglion triggered potent neuroinflammatory response, along with a transient change in thermal and mechanical sensitivity in rats. In contrast, another study[58] reported that rhBMP2-ACS application had a neuroprotective effect and decreased pain behavior in a rat NP model of CCI. Thus, BMP2 may have complicated role in the regulation of NP. In the present study, we found that exogenous BMP2 application failed to induce allodynia and astrocyte activation. However, we cannot exclude the possibility that glial cells could be activated by greater rhBMP2 dosage. Indeed, the potential effects of BMP2 on glial activation remain to be further elucidated.

Declaration of Conflicting Interests

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