



Deposited via The University of Leeds.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/145132/>

Version: Accepted Version

---

**Article:**

Zhang, F, Wang, Y, Liu, Y et al. (2019) Transcriptional Regulation of Voltage-Gated Sodium Channels Contributes to GM-CSF-Induced Pain. *Journal of Neuroscience*, 39 (26). pp. 5222-5233. ISSN: 1529-2401

<https://doi.org/10.1523/JNEUROSCI.2204-18.2019>

---

(c) 2019 the authors. This is an author produced version of a paper published in the *Journal of Neuroscience*. Uploaded in accordance with the publisher's self-archiving policy.

**Reuse**

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

---

1           **Transcriptional regulation of voltage-gated sodium channels**  
2                           **contributes to GM-CSF induced pain**

3  
4  
5   **Abbreviated title:** the role of Nav1.7-1.9 channel in GM-CSF induced pain

6  
7   Fan Zhang<sup>1,2†</sup>, Yiying Wang<sup>1†</sup>, Yu Liu<sup>1,2</sup>, Hao Han<sup>1</sup>, Dandan Zhang<sup>2</sup>, Xizhenzi Fan<sup>2</sup>,  
8   Xiaona Du<sup>1</sup>, Nikita Gamper<sup>1,3</sup>, Hailin Zhang<sup>1\*</sup>

9   <sup>1</sup>Department of Pharmacology, Hebei Medical University; The Key Laboratory of  
10   Neural and Vascular Biology, Ministry of Education, China; The Key Laboratory of  
11   New Drug Pharmacology and Toxicology, Hebei Province; Shijiazhuang, China.

12   <sup>2</sup>Department of Biochemistry and Molecular Biology, Hebei Medical University,  
13   Hebei Province, Shijiazhuang, China.

14   <sup>3</sup>School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds,  
15   Leeds, UK

16   \*Correspondence: Professor Hailin Zhang, Department of Pharmacology, Hebei  
17   Medical University, Shijiazhuang, China. 050017, E-mail: [zhanghl@hebmu.edu.cn](mailto:zhanghl@hebmu.edu.cn)

18   <sup>†</sup>These authors contributed equally to this work

19   **5 figures**

20   **1 table**

21   **Abstract: 185 words**

22   **Introduction: 671 words**

23   **Discussion: 924 words**

24

---

25

26 **Acknowledgments** This work is supported by the National Natural Science  
27 Foundation of China (91732108 to HZ), (31872788 and 31401199 to FZ) and  
28 (31571088 to XD); Key Basic Research Project of Applied Basic Research Program  
29 of Hebei Province (16967712D to XD); Biotechnology and Biological Sciences  
30 Research Council (Grants BB/R003068/1 and BB/R02104X/1 to NG). We greatly  
31 appreciate the collection of human osteosarcoma and chondroma tissues from Prof.  
32 Yueping Liu (The Fourth Hospital of Hebei Medical University, China).

33 **Conflict of interest** There are no conflicts of interest.

34 **Author Contributions** YW, DZ, FX-Z, YL, HH and FZ performed the experiments  
35 and data analyses. HZ, FZ, XD and NG designed the experiments. HZ, FZ and NG  
36 wrote the paper. All authors have read, edited and approved the content of the  
37 manuscript.

38

39

40

41

42

43

44

45

46

47

48

---

49 **Abstract**

50 Granulocyte-macrophage colony stimulating factor (GM-CSF) induces production of  
51 granulocyte and macrophage populations from the hematopoietic progenitor cells; it is  
52 one of the most common growth factors in the blood. GM-CSF is also involved in  
53 bone cancer pain development by regulating tumor-nerve interactions, remodeling of  
54 peripheral nerves and sensitization of damage-sensing (nociceptive) nerves. However,  
55 the precise mechanism for GM-CSF-dependent pain is unclear. In this study, we found  
56 that GM-CSF is highly expressed in human malignant osteosarcoma. Female  
57 Sprague-Dawley rats implanted with bone cancer cells develop mechanical and  
58 thermal hyperalgesia but antagonizing GM-CSF in these animals significantly  
59 reduced such hypersensitivity. The voltage gated Na<sup>+</sup> channels Nav1.7, Nav1.8 and  
60 Nav1.9 were found to be selectively up-regulated in rat DRG neurons treated with  
61 GM-CSF, which resulted in enhanced excitability. GM-CSF activated the Jak2 and  
62 Stat3 signaling pathway which promoted the transcription of Nav1.7-1.9 in DRG  
63 neurons. Accordingly, targeted knocking down of either Nav1.7-1.9 or Jak2/Stat3 in  
64 DRG neurons in vivo alleviated the hyperalgesia in male Sprague-Dawley rats. Our  
65 findings describe a novel bone cancer pain mechanism and provide a new insight into  
66 the physiological and pathological functions of GM-CSF.

67

68

69 **Significance Statement**

70 It has been reported that GM-CSF plays a key role in bone cancer pain, yet the  
71 underlying mechanisms involved in GM-CSF-mediated signaling pathway in  
72 nociceptors is not fully understood. Here, we showed that GM-CSF promotes bone  
73 cancer-associated pain by enhancing excitability of DRG neurons via the  
74 Jak2-Stat3-mediated upregulation of expression of nociceptor-specific voltage-gated  
75 sodium channels. Our study provides a detailed understanding of the roles that sodium  
76 channels and Jak2/Stat3 pathway play in the GM-CSF-mediated bone cancer pain; our  
77 data also highlight the therapeutic potential of targeting GM-CSF.

---

## 78 **Introduction**

79 Granulocyte-macrophage colony stimulating factor (GM-CSF) was originally  
80 identified as a colony stimulating factor because of its ability to induce granulocyte  
81 and macrophage populations from precursor cells. GM-CSF is also abundantly  
82 secreted by some tumor cells and plays a key role in regulating tumor-nerve  
83 interactions, remodeling of peripheral nerves and sensitization of damage-sensing  
84 nerves (nociceptors) by acting at its receptors (Schweizerhof et al., 2009). Apart of the  
85 bone metastases pain, GM–CSF was also shown to be involved in inflammatory pain,  
86 arthritic and neuropathic pains (Cook et al., 2012; Cook et al., 2013; Nicol et al.,  
87 2018). A recent study show that GM-CSF signaling contributes to pain-associated  
88 behavior that is independent of a gliosis and/or astrocyte response, suggesting that  
89 GM-CSF may directly activate sensory neurons (Nicol et al., 2018). However, the  
90 precise mechanism for GM-CSF-dependent pain is unclear.

91 Dorsal root ganglion (DRG) neurons are the peripheral somatic and visceral  
92 sensory neurons; a subset of these neurons is responsible for nociceptive signal  
93 initiation and propagation. Receptors for GM-CSF (GM-CSFR) are found to be  
94 expressed in DRG and in peripheral nerves dispersed in the periosteum of mice  
95 (Schweizerhof et al., 2009). Signaling cascades and mechanisms of action of  
96 GM-CSFR in sensory neurons are largely unknown but in hematopoietic cells,  
97 activation of GM-CSFR is known to stimulate cell signaling pathways regulating gene  
98 expression, including the Jak-Stat pathway (Janus kinase, JAK; signal transducer and  
99 activator of transcription protein, Stat) (Stosser et al., 2011). Activation of Jak leads to  
100 dimerization and translocation of Stat family transcription factors to cell nucleus to  
101 regulate gene expression (Fortin et al., 2007). The main aim of the present study was  
102 to identify molecules involved in GM-CSF-mediated signaling pathway in  
103 nociceptors and test their relevance to GM-CSF-induced pain.

104 Ion channels are the basis of sensory neuronal excitability and were previously  
105 suggested as molecular targets of GM-CSF signaling pathway (Bali et al., 2013). Our  
106 preliminary screening (see Results and Figure 3-1) revealed that GM-CSF selectively  
107 increased expression in DRG neurons of three voltage-gated sodium channels, Nav1.7,

---

108 Nav1.8 and Nav1.9. Due to the primary role of these channels in the ability of DRG  
109 neurons to generate action potentials (APs), we hypothesized that GM-CSF might  
110 promote pain and hyperalgesia by acting on voltage-gated Na<sup>+</sup> channels in  
111 nociceptors.

112 At least five different voltage-gated sodium channels are reportedly expressed in  
113 DRG, including the TTX-sensitive Nav1.1, Nav1.6 and Nav1.7 and the TTX-resistant  
114 Nav1.8 and Nav1.9 (Cummins et al., 2000). Nav1.7, Nav1.8 and Nav1.9 channels are  
115 mainly distributed in small diameter DRG neurons, most of which are involved in  
116 nociception. Nav1.7 produces a rapidly-activating and inactivating but slowly  
117 repriming current. It produces a robust ramp current in response to depolarizations,  
118 contributing to the generation and propagation of action potentials and acting as a  
119 threshold channel regulating excitability (Francois-Moutal et al., 2018; Li et al., 2018).  
120 Gain-of-function mutations within the Nav1.7 gene *SCN9A* lead to inherited pain  
121 disorders, such as erythromelalgia (IEM) and paroxysmal extreme pain disorder  
122 (PEPD) (Dib-Hajj et al., 2008; Jarecki et al., 2010; Cheng et al., 2011). Nav1.8  
123 mediates a slowly-inactivating sodium currents acting as a key component of the  
124 upstroke of the action potential and thus influences neuronal excitability and  
125 nociceptive transmission. Mutations of Nav1.8 gene, *SCN10A*, is found in patients  
126 with peripheral neuropathy (Lai et al., 2002; Choi et al., 2007; Blanchard et al., 2012).  
127 The Nav1.9 channel has a slow kinetics and is responsible for persistent Na<sup>+</sup> currents  
128 in nociceptors; together with the Nav1.7 it acts as a threshold channel for AP firing; it  
129 amplifies sub-threshold stimuli leading to AP bursts (Huang et al., 2014).  
130 Gain-of-function mutations of Nav1.9 channel gene, *SCN11A* cause familial episodic  
131 pain syndrome (Huang et al., 2014; Huang et al., 2017).

132 In this study, we found that GM-CSF significantly increased the excitability of  
133 DRG neurons in parallel with the increase the current density, mRNA and protein  
134 expression of Nav1.7, Nav1.8 and Nav1.9 channels. We further show  
135 Jak2-Stat3-mediated up-regulation of Nav1.7, Nav1.8 and Nav1.9 channel expression  
136 in nociceptors is a major factor in the GM-CSF-related component of bone cancer  
137 pain.

---

138

139 **Materials and methods**

140 *Human subjects.* The study was carried out in accordance with the ethical  
141 principles for medical research involving human subjects set out in the Helsinki  
142 Declaration, and was approved by the ethical committee at Hebei Medical University  
143 (Shijiazhuang, China). Osteosarcoma or chondroma tissues were obtained from 8  
144 patients from the Fourth Hospital of Hebei Medical University. Each specimen was  
145 fixed with 4% paraformaldehyde for immunohistochemistry study. All patients or  
146 their relatives gave informed consent prior to their participation in the study.

147

148 *Animals.* The animal protocols used in this study were approved by the Animal  
149 Care and Ethical Committee of Hebei Medical University under the International  
150 Association for the Study of Pain (IASP) guidelines for animal use. All surgeries were  
151 performed under sodium pentobarbital (Sigma) anesthesia, and all efforts were made  
152 to minimize animal suffering.

153

154 *Rat DRG neuron culture.* Dorsal root ganglion (DRG) neurons were obtained  
155 from adult Sprague-Dawley rats (provided by Experimental Animal Center of Hebei  
156 Province) based on the protocol described previously (Du X et al., 2014). Briefly, the  
157 ganglia were digested at 37°C with collagenase (2 mg/ml) with dispase (7.5 mg/ml)  
158 for 30 min. Ganglia were then mechanically triturated and washed twice with DMEM  
159 supplemented with 10% fetal calf serum. Thereafter, the DRG neurons were plated on  
160 poly-D-lysine-coated glass cover slips.

161

162 *Quantitative PCR.* Total RNA was extracted using a commercial RNA isolation  
163 kit (RNAiso, Takara). Isolated RNA was dissolved in 20 µl DEPC-treated water and  
164 reverse-transcribed using an RT reagent kit (PrimeScript with gDNA Eraser, Takara)  
165 and a thermal cycler (Mastercycler, Eppendorf). Quantitative PCR reaction was

---

166 performed using a kit (SYBR Premix Ex TaqII [Tli RNase H Plus], Takara), and the  
167 fluorescent DNA was detected and quantified with an FQD-48A(A4) system (BIOER).  
168 The PCR products were also run on a 2% agarose gel and were visualized using a gel  
169 imager (TFP-M/WL, Vilber Lourmat). For qPCR analysis, the following specific  
170 primers were used:

171 Nav1.7-Forward: GCTCCAAGGACACAAAACGAAC,

172 Nav1.7-Reverse: ATCAGACTCCCCAGGTGCAAT;

173 Nav1.8-Forward: GACCCTTTCTACAGCACACACC,

174 Nav1.8-Reverse: AAGTCCAGCCAGTTCCACG;

175 Nav1.9-Forward: GCCCCTTCACTTCCGACT,

176 Nav1.9-Reverse: GTCTTCCAGAGGCTTCGCTAC;

177 GAPDH-Forward: CCAGCCTCGTCTCATAGACA,

178 GAPDH-Reverse: CGCTCCTGGAAGATGGTGAT

179

180 ***Luciferase reporter assay.*** The Stat3 luciferase reporter vector was designed to  
181 measure the binding of transcription factors to the enhancer, and was transfected into  
182 HEK293 cells with Lipofectamine2000 reagent (Invitrogen).

183 Fragments of rat *Scn9a*, *Scn10a* and *Scn11a* gene were amplified by PCR with  
184 following primers:

185 Forward-GGCTCGAGAGCTTAAGGAAAGGAGGGTA,

186 Reverse-GTAAGCTTTTTCCCCTTTGACTCCTTAC; corresponding to the promotor  
187 region of *Scn9a* (-286/+306).

188 Forward-GGCTCGAGCCGTAGTAAGACCCTGCCTTG,

189 Reverse-GTAAGCTTGAGACCCCAGCTCTGCAAAAC; corresponding to the  
190 promotor region of *Scn10a* (749/+124) .

---

191 Forward-GGCTCGAGCTTCACATGGTTGATCCATC

192 Reverse-GTAAGCTTATTCTCGCTCTTGGCAGTA; corresponding to the promotor  
193 region of *Scn11a* (-51/+556 regions).

194 Amplified fragments were digested with appropriate restriction enzymes and cloned  
195 into pGL3 Basic (Promega). Luciferase activity was measured using a Dual  
196 Luciferase Assay Kit (Promega). Specific promoter activity was expressed as the  
197 relative activity ratio of firefly luciferase to Renilla luciferase.

198

199 **Western Blot.** The DRG neuron lysates were prepared with RIPA lysis buffer.  
200 Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide  
201 gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene fluoride  
202 membrane. Membranes were blocked with 5% non-fat dairy milk and incubated with  
203 primary antibodies against Nav1.7 (1:500, Alomone), Nav1.8 (1:500, Abcam), Nav1.9  
204 (1:200, Abcam), p-Jak1 (1:1000, Affinity), p-Jak2 (1:1000, Affinity), p-Jak3 (1:1000,  
205 Affinity), p-stat3 (1:1000, Epitomics), p-Stat5a (1:1000, Affinity) at 4°C overnight.  
206 This was followed by incubation with IRDye800-conjugated secondary antibody  
207 (1:20,000, Rockland) for 1 h at room temperature and subsequent scanning with the  
208 Odyssey Infrared Imaging System (LI-COR Biosciences). The integrated intensity for  
209 each detected band was determined using Odyssey Imager software (v3.0).

210

211 **Immunohistochemistry.** Sections from human osteosarcoma or chondroma  
212 tissues were blocked with 0.3% hydrogen peroxide, followed by preincubation with 5%  
213 normal goat serum and then incubation with primary antibodies against GM-CSF  
214 (1:200, Pepro Tech) at 4 °C overnight. Next, the sections were incubated with the  
215 biotinylated secondary antibody, followed by streptavidin-horseradish peroxidase and  
216 diaminobenzidine, and then counterstained with hematoxylin. Staining intensities  
217 were determined by measurement of the integrated optical density (IOD) by light  
218 microscopy using a computer-based Image-Pro Morphometric System.

219

---

220 **Electrophysiology.** Action potentials were recorded from dissociated rat small  
221 diameter DRG neurons (<25  $\mu\text{m}$ ) under current clamp using an Axopatch 200B  
222 amplifier and a Digidata 1322A converter (Axon Instruments). Pipettes (3–4  $\text{M}\Omega$ )  
223 were filled with solution containing (in mM): KCl 150,  $\text{MgCl}_2$  5, HEPES 10, pH 7.4  
224 adjusted with KOH. The bath solution contained (in mM): NaCl 160, KCl 2.5,  $\text{MgCl}_2$   
225 1,  $\text{CaCl}_2$  2, glucose 10, HEPES 20 and pH 7.4 adjusted with NaOH. Small DRG  
226 neurons were examined for evoked activity with a series of 1-s current injections from  
227 0 pA to 500 pA in 50 pA increments. The rheobase currents were determined by the  
228 first action potential elicited by a series of depolarizing current injections that  
229 increased in 5 pA increments. The following values were measured in this study:  
230 resting membrane potential (RMP), threshold potential (TP), AP amplitude,  
231 depolarization rate (V/s).

232 Sodium currents were recorded from these small diameter DRG neurons under  
233 the voltage clamp mode in a whole-cell configuration. Pipettes (3–4  $\text{M}\Omega$ ) were filled  
234 with solution containing (in mM): 70 CsCl, 30 NaCl, 30 TEA-Cl, 10 EGTA, 1  $\text{CaCl}_2$ ,  
235 2  $\text{MgCl}_2$ , 2  $\text{Na}_2\text{ATP}$ , 0.05 GTP, 10 HEPES, and 5 glucose, pH 7.3 with CsOH. The  
236 bath solution for DRG neurons was (in mM): 80 NaCl, 50 choline-Cl, 30 TEA-Cl, 2  
237  $\text{CaCl}_2$ , 0.2  $\text{CdCl}_2$ , 10 HEPES, and 5 glucose, pH 7.3 with NaOH. The acquisition rate  
238 was 20 kHz and signals were filtered at 5 kHz. Series resistances were compensated  
239 by 80%. Currents were elicited by a 40 ms pulses from a holding potential of -120  
240 mV to test potentials between -80 mV and +40 mV in 5 mV increments. The  
241 TTX-resistant (TTX-R) sodium currents (including both Nav1.9 and Nav1.8 currents)  
242 were recorded in the presence of 300 nm TTX in the external solution. The  
243 TTX-sensitive (TTX-S) sodium currents were obtained by digital subtraction of the  
244 TTX-R sodium currents from the total currents. Nav1.8 currents were then elicited by  
245 a prepulse of -70 mV for 500 ms before the test potentials from -80 mV to +40 mV in  
246 5 mV increments in the same neuron. The Nav1.9 currents were obtained by digital  
247 subtraction of the Nav1.8 currents from the TTX-R sodium currents (based on  
248 protocols from (Qiu *et al.*, 2016).

249

---

250        ***Rat model of tumor-evoked pain.*** The Walker 256 carcinosarcoma breast cancer  
251 cells were provided by Shanghai Cell Bank of the Chinese Academy of Sciences.  
252 Wistar rats were injected intra-peritoneally with the Walker 256 cancer cells 0.5 ml  
253 ( $2 \times 10^7$  cells/ml and 6–7 days later ascitic fluid was extracted. Sprague Dawley rats  
254 (180-200 g) were anesthetized by intraperitoneal injection of sodium pentobarbital  
255 (60-80 mg/kg). The right leg was shaved and the skin was disinfected with 70% (v/v)  
256 ethanol. A 1-cm-long rostro-caudal incision was made in the skin over the lower  
257 one-third of the tibia for exposure with minimal damage to muscles and nerves. The  
258 medullary canal was approached by inserting a 23-gauge needle proximally through a  
259 hole drilled in the tibia. The needle was then replaced with a 10- $\mu$ l microinjection  
260 syringe containing the cells to be injected. A 5- $\mu$ l volume of Walker 256 cells  
261 ( $4 \times 10^6$ /ml) or boiled cells (sham group) were injected into the bone cavity. After a  
262 2-min delay to allow cells to fill the bone cavity, the syringe was removed and the  
263 hole was sealed using bone wax. In some experiments, antibody against GM-CSF (10  
264  $\mu$ g, SCBT), antibody GM-CSFR (10  $\mu$ g, SCBT), GM-CSF antagonist, E21R (25  
265  $\mu$ g/ $\mu$ l, 3  $\mu$ l, Life Tein LLC) or vehicle was injected in the vicinity of the tibia bone.  
266 The wound was closed using 1-0 silk threads and dusted with penicillin powder. The  
267 rats were allowed unrestricted movement in their cages after recovery and their  
268 general condition was monitored during the experiment.

269

270        ***Focal application of drugs to DRG in vivo.*** All surgical procedures were  
271 performed under deep anesthesia with an i.p. injection of pentobarbital sodium (60-80  
272 mg/kg). A DRG cannula for focal application of substances to DRG was implanted as  
273 previously described (Du X et al., 2017). Briefly, a midline incision was made at the  
274 L4-L6 spinal level of adult male rats (Sprague Dawley; 180-200 g), and the L5 was  
275 identified at the midpoint of a link between both sides of the iliac crest. A 0.8-mm  
276 hole (~1 mm off the inferior edge of the transverse process) was drilled through the  
277 transverse process over the L5 DRG. Approaching of a ganglion was verified by the  
278 twitch of the paw. A hooked stainless steel blunt-tip cannula (inner diameter 0.64 mm,  
279 length 4 mm) was forced into the hole and connected to a polypropylene tube (inner

---

280 diameter 0.41 mm, length 4.5 mm). The incision was closed with sutures, and the  
281 cannula was firmly fixed in place with dental cement. Intramuscular injection of  
282 benzylpenicillin (19 mg/0.1 ml) was given immediately after surgery. Postoperatively,  
283 rats were housed individually in plastic cages with sawdust flooring and supplied with  
284 water and food *ad libitum*. Animals were left to recover for at least 24 hours before  
285 the experiments were carried out. Animals developing signs of distress were  
286 humanely sacrificed.

287

288 ***Antisense oligonucleotide knockdown.*** On the second day after DRG cannula  
289 implantation, rats were given through the cannula the antisense oligodeoxynucleotides  
290 (AS ODNs) against *Scn9a*, *Scn10a*, *Scn11a*, *Jak2*, *Stat3* or *Stat5* (each at 12.5 µg in 5  
291 µl). The AS ODNs were given consecutively twice a day for 4 days. Mismatched  
292 ODNs were also given at matched time points. On the fifth day, mechanical and  
293 thermal sensitivities were assessed at 1 h, 2 h, 5 h, 12 h, and 24 h after the focal DRG  
294 application of GM-CSF (5 µl) or saline (5 µl) , respectively. Seven groups of animals  
295 were tested, injected as follows:

296	Group	type of ODN pre-treatment	treatment type
297	1	mismatched ODN	GM-CSF or saline
298	2	AS ODN <i>Scn9a</i>	GM-CSF or saline
299	3	AS ODN <i>Scn10a</i>	GM-CSF or saline
300	4	AS ODN <i>Scn11a</i>	GM-CSF or saline
301	5	AS ODN <i>Jak2</i>	GM-CSF or saline
302	6	AS ODN <i>Stat3</i>	GM-CSF or saline
303	7	AS ODN <i>Stat5a</i>	GM-CSF or saline

304

305 The following specific ASO were used:

---

306 Mismatched ODN: TCACCCAGCACCCCCAACACATAGTT

307 ASO- *Scn9a*: CTGGATCAACATGGTCTTCA

308 ASO- *Scn10a*: CCAGAACCAAGCACAGAGGA

309 ASO- *Scn11a*: CACCATCTGCATCATCATCA

310 ASO-*Jak2*: AAGGTACAGATTCCGCAGGT

311 ASO-*Stat3*:ACATGGAGGAGTCCAACAAC

312 ASO-*Stat5a*:TCTCAGTTCAGCGTTGGCAG

313

314 ***Mechanical hyperalgesia.*** Threshold sensitivity to mechanical stimuli was  
315 assessed using the von Frey method as described previously (Chaplan et al., 1994).  
316 Briefly, calibrated nylon filaments (Von Frey hair, Stoelting Co.) with different  
317 bending forces were applied to the midplantar surface of the right hind paw of the rats.  
318 The filaments were applied starting with the softest and continuing in ascending order  
319 of stiffness. A brisk withdrawal of the right hind limb was considered a positive  
320 response.

321

322 ***Thermal hyperalgesia.*** The paw withdrawal latency in response to heat was  
323 tested using the Hargreaves method on the right hind paw of the rats using a radiant  
324 heat lamp source (Mengtai Technology Co., Ltd.). The intensity of the radiant heat  
325 stimulus was maintained at 20%. The time to withdrawal of the right hind paw (elapse  
326 time) was recorded.

327

328 ***Blinding and randomization.*** In all in vivo experiments when animal behavior  
329 test and drug administration were involved, experiments were conducted on the basis  
330 of a blind and randomized design. To achieve this, two experimenters performed  
331 every tests: one experimenter was in charge of drug injections and group  
332 randomization; second experimenter, blinded to the drug administration and grouping

---

333 schedules, conducted the mechanical and thermal sensitivity measurements.

334

335 **Statistics and analysis.** All data are given as mean  $\pm$  SEM. Differences between  
336 groups were assessed by paired or unpaired *t* test. Comparisons of the behavioral data  
337 between groups at each individual time point were conducted using a two-way  
338 ANOVA followed by Bonferroni *post hoc* tests. Comparisons of the number of action  
339 potential between groups at each injected currents were conducted using a two-way  
340 ANOVA followed by Bonferroni *post hoc* tests. Differences were considered  
341 significant at  $P \leq 0.05$ . Statistical analyses were performed using OriginPro 9.1  
342 (Originlab Corp.).

343

## 344 **Results**

345 ***GM-CSF plays a crucial role in bone metastases cancer pain.*** Osteosarcoma is  
346 the most common form of primary bone cancer, in which pain is the most common  
347 symptom and is seen in 85% of patients (Yoneda et al., 2015). Osteochondroma is the  
348 most common benign bone tumor and the majority of osteochondromas form an  
349 asymptomatic hard immobile painless palpable mass (de Mooij et al., 2012). We first  
350 collected tumor tissue from osteosarcama and osteochondroma patients after surgery,  
351 and compared the expression levels of GM-CSF in these two tumor tissue types. The  
352 H&E staining shows typical characteristics of a cancerous (Fig. 1A, low left) and a  
353 benign (Fig. 1A upper left) bone tissue. Interestingly, osteosarcoma biopsy samples  
354 demonstrated a dramatically increased levels of GM-CSF, as compared to these seen  
355 in osteochondroma samples, as revealed with immunohistochemistry (Fig. 1A right  
356 panels; summarized in Fig. 1B,  $n = 8$ ,  $*P < 0.05$ ).

357 To explore the role of GM-CSF in bone cancer pain, we first established the bone  
358 metastases cancer pain model induced by the implanted Walker 256 carcinoma cells.  
359 Consistent with previous reports (Wang et al., 2011), mice with tumors in the tibia  
360 bone displayed a significantly lower withdrawal thresholds for mechanical stimuli and  
361 a shortened withdrawal latencies for thermal stimuli at 3-21 days after tumor cell

---

362 injection (Fig. 1C, 1D). To assess the role of GM-CSF in these behavioral  
363 manifestations of pain hypersensitivity, we injected a mutant GM-CSF peptide with  
364 the glutamate-to-arginine substitution at the position 21 of a rat GM-CSF peptide  
365 sequence (E21R). E21R acts as a competitive antagonist of GM-CSF and can  
366 neutralize some of its biologic actions (Iversen et al., 1996), however its effect on  
367 GM-CSF-mediated pain has not been tested before. E21R was injected in the vicinity  
368 of the tibia bone where the cancer cells had been implanted (see Methods). Treatment  
369 of the bone cancer rats with E21R significantly alleviated both mechanical and  
370 thermal hyperalgesia, respectively (Fig. 1C). The reduction of hyperalgesia was  
371 registered starting from day 7 after the establishment of the bone cancer model and  
372 lasted for the duration of observation (21 days). To further validate the role of  
373 GM-CSF in the bone cancer pain, we also used antibodies against GM-CSF and  
374 GM-CSF receptor (GM-CSFR). Injection of anti-GM-CSF and anti-GM-CSFR  
375 antibodies also produced strong anti-hyperalgesic effect, the anti-GM-CSFR antibody  
376 was particularly efficacious, resulting in significantly stronger anti-hyperalgesia, as  
377 compared to E21R (Fig. 1C). These results indicate that GM-CSF is indeed involved  
378 in the bone cancer pain development, which is in agreement with a previous report  
379 that the bone cancer pain was attenuated following a specific knockdown of GM-CSF  
380 receptors in L4-L5 DRG of mice (Schweizerhof et al., 2009).

381 To further attest that GM-CSF is pro-algesic and the primary afferent sensory  
382 nerve are the targeted sites of GM-CSF action, we evaluated the effect of direct focal  
383 GM-CSF infusion into the L5 DRG on pain-related behavior in naïve rats (Fig. 1D).  
384 Compared with the vehicle-treated rats, GM-CSF induced significant dose-dependent  
385 thermal and mechanical hyperalgesia which persisted for at least 24 hrs after injection.  
386 Indeed, focal injection of GM-CSF (20 - 200 ng) via the DRG cannula significantly  
387 increased sensitivity of rats to thermal and mechanical stimuli as measured with the  
388 Hargreaves and Von Frey tests, respectively ( $P < 0.05$ ). The nociceptive responses in  
389 rats received 2 ng of GM-CSF also showed a tendency towards sensitization but these  
390 effects did not reach statistical significance.

391

---

392 ***GM-CSF increase the excitability of small-sized DRG neuron.*** The fact that  
393 GM-CSF enhanced pain sensitivity when injected into DRG suggests that GM-CSF  
394 might directly sensitize nociceptors by increasing their excitability. To test this  
395 hypothesis, we performed current clamp recordings from the cultured DRG neurons in  
396 control conditions and after 24 hrs treatment with GM-CSF (200 ng/ml). For this  
397 study, small diameter ( $< 25 \mu\text{M}$ ) DRG neurons were selected as these are  
398 predominantly nociceptors (Zheng *et al.*, 2013). AP firing was induced by trains of  
399 depolarizing current step from +50 to +500 pA, injected with a 50 pA increment.  
400 GM-CSF significantly increased the number of APs induced by the depolarizing  
401 current pulses from 100 to 500 pA (Fig. 2A, B). GM-CSF also significantly lowered  
402 the rheobase currents (depolarization current threshold (CT) for eliciting the 1<sup>st</sup> action  
403 potential) from  $198.6 \pm 9.8 \text{ pA}$  ( $n = 63$ ) to  $83.1 \pm 2.5 \text{ pA}$  ( $n = 39$ ,  $P < 0.05$ ); the action  
404 potential threshold voltage (TP) was also significantly decreased from  $-18.7 \pm 0.5 \text{ mV}$   
405 ( $n = 23$ ) to  $-20.4 \pm 0.6 \text{ mV}$  ( $n = 22$ ,  $P < 0.05$ ). However, the resting membrane  
406 potential was not significantly changed (Fig. 2C, 1D). We also measured the effect of  
407 GM-CSF treatment on other properties of evoked APs such as AP amplitude (mV) and  
408 rate of depolarization (V/s), which are summarized in Table 1. Together, the above  
409 data indicate that GM-CSF increases intrinsic neuronal excitability of primary sensor  
410 neurons associated with pain.

411

412 ***GM-CSF increases activity and expression level of Nav1.7 Nav1.8 and Nav1.9***  
413 ***sodium channels.*** The sensitizing effect of a single focal in vivo injection of GM-CSF  
414 was long-lasting (Fig. 1E), in addition, intracellular action of GM-CSFRs has long  
415 been linked to transcriptional effects via the activation of Jak-Stat pathway. Thus, we  
416 hypothesized that the sensitizing effect of this growth factor might be mediated by  
417 changes in the expression of some intrinsic regulator(s) of excitability. Thus, to  
418 further explore the mechanisms for GM-CSF-induced hyperactivity of DRG  
419 nociceptors, we screened the effect of GM-CSF on ion channels which have been  
420 implicated in modulation of resting excitability of DRG neurons (Liu *et al.*, 2010;  
421 Zheng *et al.*, 2013; Qiu *et al.*, 2016; Isensee *et al.*, 2017; Du *et al.*, 2018). We tested

---

422 the effect of treatment of cultured DRG neurons with 200 ng/ $\mu$ l GM-CSF (24 hrs) on  
423 the mRNA abundance of the following ion channel genes: *Scn9a* (Nav1.7), *Scn10a*  
424 (Nav1.8), *Scn9a* (Nav1.9), *Kcnd2* (Kv4.2), *Ano1* (TMEM16A), *P2rx3* (P2X3), *Kcng2*  
425 (Kv7.2), *Kcnq3* (Kv7.3). Among the transcripts tested, only the mRNAs of  
426 voltage-gated sodium channels Nav1.7, Nav1.8 and Nav1.9 were elevated (Figure 3A).  
427 Additionally, the mRNAs of Nav1.7-1.9 expression were elevated by day 7 in DRGs  
428 from bone cancer rats relative to those from sham controls (Figure 3B).

429 Next, we examined the effects of GM-CSF treatment on the current density of  
430 voltage-gated sodium currents in DRG neurons. Total, TTX- sensitive (TTX-S) and  
431 TTX-resistant Na<sup>+</sup> currents were recorded (see Method) using whole-cell patch clamp.  
432 After pretreatment of DRG neurons with GM-CSF (200 ng/ml; 24hrs), the peak  
433 current density of total Na<sup>+</sup> currents was increased from  $-101.0 \pm 8.8$  (pA/pF) (n = 44)  
434 to  $-155.8 \pm 9.9$  (pA/pF) (n = 32) at -10 mV; the peak current density of TTX-S  
435 currents (mainly Nav1.7 currents) was increased from  $-58.0 \pm 6.5$  (pA/pF) (n = 66) to  
436  $-100.6 \pm 4.7$  (pA/pF) (n = 43) at -20 mV; the peak current density of TTX-R currents  
437 was increased from  $-56.5 \pm 4.9$  (pA/pF) (n = 66) to  $-75.5 \pm 5.7$  (pA/pF) (n = 43) at -10  
438 mV (Fig 3C).

439 The TTX-R Na<sup>+</sup> currents were further separated into Nav1.8-rich currents and  
440 Nav1.9-rich current fractions (see Method). After pretreatment of DRG neurons with  
441 GM-CSF, the peak current density of Nav1.8-rich current fraction was increased from  
442  $-44.5 \pm 4.5$  (pA/pF) (n = 55) to  $-60.1 \pm 4.4$  (pA/pF) (n = 36) at 0 mV; the Nav1.9-rich  
443 current fraction was increased from  $-20.2 \pm 1.4$  (pA/pF) (n = 53) to  $-29.4 \pm 0.9$  (pA/pF)  
444 (n = 36) at -20 mV (Fig. 3C). In sum, GM-CSF significantly increased the current  
445 amplitudes of nociceptor-specific Nav1.7 Nav1.8 and Nav1.9 currents. Furthermore,  
446 the protein expression levels of Nav1.7, Nav1.8 and Nav1.9 channel in DRG neurons  
447 were also significantly increased after incubation with GM-CSF (200 ng/ml; 18 h; Fig.  
448 3D).

449

#### 450 ***Down-regulation of Nav1.7-Nav1.9 channels alleviates GM-CSF induced pain.***

451 To investigate whether the up-regulation of Nav1.7, Nav1.8 and Nav1.9 channels

---

452 contributes to the GM-CSF-mediated pain, we performed unilateral in vivo  
453 knockdown of individual sodium channel subunit in rats using the anti-sense  
454 oligodeoxynucleotides (AS ODNs). AS ODNs against *Scn9a*, *Scn10a* and *Scn11a* (or  
455 a control mismatched ODN) were injected into the L5 DRG via the DRG cannula to  
456 offset the up-regulation of these sodium channels, and then the effect of GM-CSF on  
457 pain behavior was examined. The knockdown efficiency was measured first; for this,  
458 the L5 DRGs were extracted following focal injection of saline, GM-CSF, AS ODN +  
459 Saline, AS ODN + GM-CSF, and then the mRNA expression levels of *Scn9a*, *Scn10a*  
460 and *Scn11a* were analyzed by quantitative PCR. In agreement with previous data, the  
461 mRNA expression levels of *Scn9a*, *Scn10a* and *Scn11a* in DRG neurons were  
462 significantly increased after GM-CSF injection, and importantly, these increase were  
463 totally reversed by respective AS ODNs (Fig. 4A). Consistent with our earlier  
464 conclusion that up-regulation of Nav1.7-Nav1.9 channels is a crucial factor in  
465 GM-CSF-induced hypersensitivity, AS ODNs against *Scn9a*, *Scn10a* and *Scn11a*  
466 significantly alleviated both mechanical and thermal hypersensitivity developed after  
467 the focal DRG application of GM-CSF (Fig. 4 B-C).

468

469 ***GM-CSF up-regulates Nav1.7-Nav1.9 channel expression via the Jak2-Stat3***  
470 ***signaling pathway.*** GM-CSF receptor is abundantly expressed in DRG (Schweizerhof  
471 et al., 2009). Thus we hypothesized that the GM-CSF induced up-regulation of  
472 Nav1.7-Nav1.9 in DRG neurons could be mediated by the GM-CSF receptor and the  
473 related cellular signaling pathway. To test this, we focused on the Jak-Stat3/5 pathway  
474 since this is the key pathway for GM-CSF action in hematopoietic cells (Lilly et al.,  
475 2001). Activated and phosphorylated states of Jak1, Jak2, Jak3, Stat3 and Stat5 were  
476 first measured in DRG neurons. As shown in Fig. 5A, after incubation of DRG  
477 cultures with GM-CSF for 25 min, phosphorylated Jak2 and Stat3 were significantly  
478 increased, but the level of phosphorylated Jak1 was not changed; phosphorylated Jak3  
479 and Stat5 were not detected. These results indicate that GM-CSF is able to activate  
480 Jak2-Stat3 signaling pathway in DRG neurons. Is this activated Jak2-Stat3 signaling  
481 pathway responsible for GM-CSF-induced up-regulation of Nav1.7-Nav1.9 in DRG

---

482 neurons? To test this, the acutely dissociated DRG neurons were incubated with  
483 GM-CSF with and without blockers of Jak2-Stat3 signaling pathway, AG490 (10  $\mu$ M)  
484 and stattic (20  $\mu$ M). Both compounds prevented up-regulation Nav1.7-Nav1.9 mRNA  
485 by GM-CSF (Fig 5B).

486 As Stat3 was previously demonstrated to function as a transcriptional activator  
487 (Sharma et al., 2018), thus, we designed a luciferase reporter assay to determine if  
488 Stat3 acts to regulate Nav1.7-Nav1.9 transcription. *Scn9a-Scn11a* promoter regions  
489 (relative to the transcription start site) were cloned into a luciferase reporter vector  
490 (pGL3 Basic plasmid), such that luciferase expression is driven by either of the  
491 *Scn9a-Scn11a* promoter sequences. We transfected these DNA constructs and either  
492 a control pcDNA3.1 plasmid or a pcDNA3.1-stat3 plasmid into HEK293 cells and  
493 measured the resulting luciferase activity. Luciferase activity in cells co-expressed  
494 with *Scn9a*, *Scn9a* or *Scn11a* promoter fragments and Stat3 was  $1.57 \pm 0.14$  (n = 4, P  
495 < 0.05),  $1.5 \pm 0.05$  (n = 4, P < 0.05),  $1.5 \pm 0.15$  (n = 4, P < 0.05) folds higher than that  
496 in cells co-expressed with *Scn9a*, *Scn9a* or *Scn11a* promoter fragments and the  
497 control pcDNA3.1 plasmid (Fig. 5C). These results implicate an important role for  
498 Stat3 in promoting Nav1.7, Nav1.8, and Nav1.9 gene expression.

499 Finally we assessed whether down regulation of Jak2-Stat3 signaling pathway  
500 would inhibit GM-CSF-induced up-regulation of Nav1.7-Nav1.9 and subsequently the  
501 pain behaviors. To this end, AS ODNs against Jak2 and Stat3 were injected in DRG  
502 (L5) via DRG cannula as described above. AS ODNs against Jak 2 (Fig. 5D) and  
503 Stat3 (Fig. 5E) but not against Stat5 (Fig. 5F) reduced basal mRNA levels of  
504 *Scn9a-Scn11a* and reversed the GM-CSF-induced up-regulation of *Scn9a-Scn11a*  
505 mRNA levels. Consistent with these results, AS ODNs against Jak2 (Fig. 5G) and  
506 Stat3 (Fig. 5H) but not AS ODNs against Stat5 (Fig. 5I) prevented the development of  
507 the mechanical and thermal hyperalgesia produced by focal in vivo application of  
508 GM-CSF via the DRG cannula (Fig. 5G, H, I). AS ODNs against Jak2, Stat3 and  
509 Stat5 did not significantly affect the mechanical and thermal sensitivity in rats without  
510 GM-CSF treatment (in accord with previous findings suggesting that Nav1.7,  
511 Nav1.8 or Nav1.9 knockout or knockdown does not significantly affect threshold

---

512 sensitivity in mice (Minett et al., 2013; Miao et al., 2010; Lolignier et al., 2011).  
513 Taken together, these results identified Jak2-Stat3-mediated up-regulation of  
514 Nav1.7-Nav1.9 channels as a key signaling pathway involved in the development of  
515 GM-CSF induced pain.

516

## 517 **Discussion**

518 In this study we demonstrate that GM-CSF promotes bone cancer-associated pain  
519 by enhancing excitability of DRG neurons via the Jak2-Stat3-mediated upregulation  
520 of expression of nociceptor-specific voltage-gated sodium channels. First, we show  
521 that GM-CSF is highly expressed in osteosarcoma biopsy samples from human  
522 patients. Second, we demonstrate that the competitive antagonist of GM-CSF,  
523 GM-CSF (E21R) as well as the antibodies against GM-CSF or GM-CSFR are able to  
524 reduce both thermal and mechanical hyperalgesia in a rat model of bone cancer. Third,  
525 we show that GM-CSF increases excitability of peripheral nociceptors by  
526 upregulating functional expression of nociceptor-specific Na<sup>+</sup> channels, Nav1.7-Nav1.9.  
527 Furthermore, using unilateral in vivo gene knockdown we further demonstrate that  
528 Na<sup>+</sup> channel upregulation is indeed a necessary step in the development of GM-CSF  
529 induced pain in vivo. Finally, using a set of genetic manipulations and assays, we  
530 delineated a molecular mechanism for GM-CSF induced initiation of pain in bone  
531 cancer: up-regulation of functional Nav1.7, Nav1.8 and Nav1.9 channel activity  
532 through the Jak2-Stat3 mediated activation of *Scn9a*, *Scn10a* and *Scn11a* gene  
533 transcription.

534 Several recent studies implicated contribution of GM-CSF to different types of  
535 pain, including cancer pain, neuropathic, inflammatory and osteoarthritic pain. (Cook  
536 et al., 2012; Cook et al., 2013; Nicol et al., 2018). Yet, the exact mechanism and main  
537 molecular steps of the pro-algesic action of GM-CSF remained elusive. Our study fills  
538 this gap providing a mechanistic framework for the effect.

539 Increased excitability of nociceptive neurons is a fundamental mechanism for  
540 pain. In turn, changes in excitability are ultimately linked to altered ion channel

---

541 activity. Thus, in this study we focused on ion channels controlling excitability of  
542 DRG neurons. All results pinpoint Nav.7-Nav1.9 channels as key determinants of the  
543 GM-CSF proalgesic action. 1) GM-CSF increased levels of *Scn9a*, *Scn10a* and  
544 *Scn11a* but not the other key ion channels tested. 2) Consistent with above results,  
545 protein level of Nav1.7-Nav1.9 and the appropriate Na<sup>+</sup> current fractions in  
546 nociceptive DRG neurons were also increased by GM-CSF. 3) Changes of DRG  
547 neuron excitability induced by GM-CSF were consistent with elevated Na<sup>+</sup> channel  
548 activity: lowered rheobase, lowered threshold potential, but no significant change in  
549 resting membrane potential. Notably, GM-CSF did not change amplitude of M-type  
550 K<sup>+</sup> current, which is another type of ion channels, important for setting resting  
551 excitability parameters of a neuron (Table1). 4) Down regulation of Nav1.7-Nav1.9  
552 with AS ODNs alleviated GM-CSF-induced pain behavior. While the latter evidence  
553 does not directly prove involvement of these Nav channels in GM-CSF-induced pain  
554 specifically (since down regulation of them will probably inhibit any type of pain  
555 behavior anyway), the combined evidence implicate Nav channel mechanism as the  
556 most plausible and straightforward explanation for GM-CSF induced pain nonetheless.  
557 However, contribution of other mechanisms to the GM-CSF induced pain cannot be  
558 ruled out at present; indeed involvement of other mediators, including Ccl5, Ccl3 and  
559 Il1a has been reported (Stosser et al., 2011).

560 We provide evidence that Jak2-Stat3 signaling pathway contributes to GM-CSF  
561 mediated up-regulation of Nav channels described above and, thus, to hyperalgesia  
562 associated with high GM-CSF levels, e.g. as observed in bone cancer. Activation of  
563 Jak by GM-CSF leads to activation of the Stat family transcription factors, which  
564 dimerize and translocate to the nucleus and modulate gene expression (Choi et al.,  
565 2011). In hematopoietic cells, GM-CSF exerts its biological functions mainly through  
566 activation of Jak2, which then activates Stat3 and Stat5 but not Stat2, Stat4 or Stat6  
567 (Zgheib et al., 2013). However, the signal transduction pathways mediated by  
568 GM-CSF and its receptors are cell-type specific and may differ significantly  
569 (Valdembri et al., 2002). In the present study we found that in DRG neurons Jak2 and  
570 Stat3 are selectively phosphorylated following the GM-CSF treatment;  
571 phosphorylated Jak1 was not affected and phosphorylated Jak3 and Stat5 were not

---

572 fund at all. Consistent with these results, very low levels of Jak3 and Stat5 mRNA in  
573 DRG neurons were retrieved using the iBrain big data platform (Li et al., 2016). Thus,  
574 Jak2-Stat3 is likely to be the dominant signaling pathway for GM-CSF to exert its  
575 function in DRG neurons.

576 Luciferase reporter assay provided strong evidence indicating that Stat3 is able to  
577 bind to the promoter regions of *Scn9a*, *Scn10a* and *Scn11a* genes to enhance their  
578 transcription. In accordance with this observation, down-regulation of Jak2 and Stat3  
579 with anti-sense oligodeoxynucleotides reversed the GM-CSF induced elevation of  
580 mRNA expression level of these Nav channels. Importantly, these anti-sense  
581 oligodeoxynucleotides against *Scn9a-Scn11a* also alleviated the GM-CSF elicited  
582 pain behavior. These results not only describe a clear mechanism for how  
583 Nav1.7-Nav1.9 channels are up-regulated by GM-CSF signaling pathway, but also  
584 indicate that specific Jak-Stat pathway could be targeted for pain therapeutics.

585 GM-CSF is used clinically for treatment of myelodysplastic syndromes, aplastic  
586 anemia, tumor radiotherapy and chemotherapy-induced neutropenia (Garcia et al.,  
587 2014). The most severe adverse reaction to these GM-CSF therapies is bone pain and  
588 the incidence is reported to reach up to 90% (Stosser *et al.*, 2011). These clinical  
589 observations align very well with our results showing that GM-CSF induces pain  
590 behavior in rats when injected to DRG at a concentration of 20 ng/ml GM-CSF, which  
591 is lower than the blood concentration of after a single-dose administration of  
592 GM-CAF in humans (~600 ng/ml; (Alexanderet et al., 2016)). Thus, clinically  
593 administered GM-CSF reaches sufficient blood concentrations to be able to sensitize  
594 bone periosteal nerves and nociceptive neurons through the mechanism described  
595 here.

596 In summary, in this study provides mechanistic explanation for the role of  
597 GM-CSF in pain, specifically in pain associated with the bone cancer and with the  
598 GM-CSF-based therapies. This novel mechanism should be considered as a potential  
599 target for future pain treatments.

600

---

601 **References**

602 Alexander WS (2016). In vivo at last: Demonstrating the biological credentials and  
603 clinical potential of GM-CSF. *Exp Hematol* 44 (8): 669-73.

604 Bali KK, Venkataramani V, Satagopam VP, Gupta P, Schneider R, Kuner R (2013).  
605 Transcriptional mechanisms underlying sensitization of peripheral sensory neurons by  
606 granulocyte-/granulocyte-macrophage colony stimulating factors. *Mol Pain* 9: 48.

607 Blanchard MG, Rash LD and Kellenberger S (2012). Inhibition of voltage-gated Na(+)  
608 currents in sensory neurones by the sea anemone toxin APETx2. *Br J Pharmacol* 165  
609 (7): 2167-77.

610 Cheng X, Dib-Hajj SD, Tyrrell L, Te MR, Drenth JP, Waxman SG (2011). Deletion  
611 mutation of sodium channel Na(V)1.7 in inherited erythromelalgia: enhanced slow  
612 inactivation modulates dorsal root ganglion neuron hyperexcitability. *Brain* 134 (Pt 7):  
613 1972-86.

614 Choi JK, Kim KH, Park H, Park SR, Choi BH (2011). Granulocyte macrophage-colony  
615 stimulating factor shows anti-apoptotic activity in neural progenitor cells via  
616 JAK/STAT5-Bcl-2 pathway. *Apoptosis* 16 (2): 127-34.

617 Choi JS, Dib-Hajj SD, Waxman SG (2007). Differential slow inactivation and  
618 use-dependent inhibition of Nav1.8 channels contribute to distinct firing properties in  
619 IB4+ and IB4- DRG neurons. *J Neurophysiol* 97 (2): 1258-65.

620 Cook AD, Pobjoy J, Sarros S, Steidl S, Durr M, Lacey DC, Hamilton JA. (2013).  
621 Granulocyte-macrophage colony-stimulating factor is a key mediator in inflammatory  
622 and arthritic pain. *Ann Rheum Dis* 72 (2): 265-70.

623 Cook AD, Pobjoy J, Steidl S, Durr M, Braine EL, Turner AL, Hamilton JA. (2012).  
624 Granulocyte-macrophage colony-stimulating factor is a key mediator in experimental  
625 osteoarthritis pain and disease development. *Arthritis Res Ther* 14 (5): R199.

626 Cummins TR, Black JA, Dib-Hajj SD, Waxman SG (2000). Glial-derived neurotrophic  
627 factor upregulates expression of functional SNS and NaV sodium channels and their  
628 currents in axotomized dorsal root ganglion neurons. *J Neurosci* 20 (23): 8754-61.

---

629 de Mooij T, Wuyts W, Ham J (2012). Phenotypic Differences in Multiple  
630 Osteochondromas in Monozygotic Twins: A Case Report. *JBJS Case Connect* 2 (4):  
631 e60.

632 Dib-Hajj SD, Estacion M, Jarecki BW, Tyrrell L, Fischer TZ, Lawden M, Cummins TR,  
633 Waxman SG (2008). Paroxysmal extreme pain disorder M1627K mutation in human  
634 Nav1.7 renders DRG neurons hyperexcitable. *Mol Pain* 4: 37.

635 Du X, Gao H, Jaffe D, Zhang H and Gamper N (2018). M-type K(+) channels in  
636 peripheral nociceptive pathways. *Br J Pharmacol* 175 (12): 2158-2172.

637 Du X, Hao H, Gigout S, Huang D, Yang Y, Li L, Wang C, Sundt D, Jaffe DB, Zhang HL,  
638 Gamper N (2014). Control of somatic membrane potential in nociceptive neurons and  
639 its implications for peripheral nociceptive transmission. *Pain* 155 (11): 2306-22.

640 Du X, Hao H, Yang Y, Huang S, Wang C, Gigout S, Raml R, Li X, Jaworska E,  
641 Edwards I, Deuchars J, Yanagawa Y, Qi JL, Guan BC, Jaffe DB, Zhang HL, Gamper N  
642 (2017). Local GABAergic signaling within sensory ganglia controls peripheral  
643 nociceptive transmission. *J Clin Invest* 127 (5): 1741-1756.

644 Fortin CF, Larbi A, Dupuis G, Lesur O, Fulop TJ (2007). GM-CSF activates the  
645 Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous  
646 apoptosis in young but not elderly individuals. *Biogerontology* 8 (2): 173-87.

647 Francois-Moutal L, Dustrude ET, Wang Y, Brustovetsky T, Dorame A, Ju W, Moutal A,  
648 Perez-Miller S, Brustovetsky N, Gokhale V, Khanna M, Khanna R (2018). Inhibition of  
649 the Ubc9 E2 SUMO conjugating enzyme-CRMP2 interaction decreases Nav1.7  
650 currents and reverses experimental neuropathic pain. *Pain*.

651 Garcia JA, Elson P, Tyler A, Triozzi P, Dreicer R (2014). Sargramostim (GM-CSF) and  
652 lenalidomide in castration-resistant prostate cancer (CRPC): results from a phase I-II  
653 clinical trial. *Urol Oncol* 32 (1): 33.e11-7.

654 Huang J, Han C, Estacion M, Vasylyev D, Hoeijmakers JG, Gerrits MM, Tyrrell L,  
655 Lauria G, Faber CG, Dib-Hajj SD, Merkies IS, Waxman SG (2014). Gain-of-function  
656 mutations in sodium channel Na(v)1.9 in painful neuropathy. *Brain* 137 (Pt 6):

---

657 1627-42.

658 Huang J, Vanoye CG, Cutts A, Goldberg YP, Dib-Hajj SD, Cohen CJ, Waxman SG,  
659 George AJ (2017). Sodium channel NaV1.9 mutations associated with insensitivity to  
660 pain dampen neuronal excitability. *J Clin Invest* 127 (7): 2805-2814.

661 Isensee J, Krahe L, Moeller K, Pereira V, Sexton JE, Sun X, Emery E, Wood JN, Hucho  
662 T (2017). Synergistic regulation of serotonin and opioid signaling contributes to pain  
663 insensitivity in Nav1.7 knockout mice. *Sci Signal* 10 (461).

664 Iversen PO, Rodwell RL, Pitcher L, Taylor KM, Lopez AF (1996). Inhibition of  
665 proliferation and induction of apoptosis in juvenile myelomonocytic leukemic cells by  
666 the granulocyte-macrophage colony-stimulating factor analogue E21R. *Blood* 88 (7):  
667 2634-9.

668 Jarecki BW, Piekarz AD, Jackson JN, Cummins TR (2010). Human voltage-gated  
669 sodium channel mutations that cause inherited neuronal and muscle channelopathies  
670 increase resurgent sodium currents. *J Clin Invest* 120 (1): 369-78.

671 Lai J, Gold MS, Kim CS, Bian D, Ossipov MH, Hunter JC, Porreca F (2002). Inhibition  
672 of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium  
673 channel, NaV1.8. *Pain* 95 (1-2): 143-52.

674 Li CL, Li KC, Wu D, Chen Y, Luo H, Zhao JR, Wang SS, Sun MM, Lu YJ, Zhong YQ,  
675 Hu XY, Hou R, Zhou BB, Bao L5, Xiao HS, Zhang X (2016). Somatosensory neuron  
676 types identified by high-coverage single-cell RNA-sequencing and functional  
677 heterogeneity. *Cell Research* 26(1):83-102.

678 Li Y, North RY, Rhines LD, Tatsui CE, Rao G, Edwards DD, Cassidy RM, Harrison DS,  
679 Johansson CA, Zhang H, Dougherty PM (2018). DRG Voltage-Gated Sodium Channel  
680 1.7 Is Upregulated in Paclitaxel-Induced Neuropathy in Rats and in Humans with  
681 Neuropathic Pain. *J Neurosci* 38 (5): 1124-1136.

682 Lilly MB, Zemskova M, Frankel AE, Salo J, Kraft AS (2001). Distinct domains of the  
683 human granulocyte-macrophage colony-stimulating factor receptor alpha subunit  
684 mediate activation of Jak/Stat signaling and differentiation. *Blood* 97 (6): 1662-70.

---

685 Liu B, Linley JE, Du X, Zhang X, Ooi L, Zhang H, Gamper N (2010). The acute  
686 nociceptive signals induced by bradykinin in rat sensory neurons are mediated by  
687 inhibition of M-type K<sup>+</sup> channels and activation of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels. *J Clin*  
688 *Invest* 120 (4): 1240-52.

689 Lolignier S, Amsalem M, Maingret F, Padilla F, Gabriac M, Chapuy E, Eschalier A,  
690 Delmas P, Busserolles P (2011). Nav1.9 Channel Contributes to Mechanical and Heat  
691 Pain Hypersensitivity Induced by Subacute and Chronic Inflammation. *PLoS ONE*  
692 6(8): e23083.

693 MiaoXR, GaoXF, Wu JX, Lu ZJ, Huang ZX, Li XQ, He C, Yu WF (2010). Research  
694 article Bilateral downregulation of Nav1.8 in dorsal root ganglia of rats with bone  
695 cancer pain induced by inoculation with Walker 256 breast tumor cells. *BMC Cancer*  
696 10:216

697 Minett MS, Falk S, Sonia Santana-Varela S, Bogdanov YD, Nassar MA, Heegaard AM,  
698 Wood JN (2013). Pain without Nociceptors? Nav1.7-Independent Pain Mechanisms.  
699 *Cell Reports* 6(2):301-312.

700 Nicol L, Thornton P, Hatcher JP, Glover CP, Webster CI, Burrell M, Hammett K, Jones  
701 CA, Sleeman MA, Billinton A, Chessell I (2018). Central inhibition of  
702 granulocyte-macrophage colony-stimulating factor is analgesic in experimental  
703 neuropathic pain. *Pain* 159 (3): 550-559.

704 Qiu F, Li Y, Fu Q, Fan YY, Zhu C, Liu YH, Mi WD. (2016). Stromal Cell-Derived  
705 Factor 1 Increases Tetrodotoxin-Resistant Sodium Currents Nav1.8 and Nav1.9 in Rat  
706 Dorsal Root Ganglion Neurons via Different Mechanisms. *Neurochem Res* 41 (7):  
707 1587-603.

708 Schweizerhof M, Stosser S, Kurejova M, Njoo C, Gangadharan V, Agarwal N, Schmelz  
709 M, Bali KK, Michalski CW, Brugger S, Dickenson A, Simone DA, Kuner R. (2009).  
710 Hematopoietic colony-stimulating factors mediate tumor-nerve interactions and bone  
711 cancer pain. *Nat Med* 15 (7): 802-7.

712 Sharma A, Oishi N, Boddicker RL, Hu G, Benson HK, Ketterling RP, Greipp  
713 PT, Knutson DL, Kloft-Nelson SM, He R, Eckloff BW, Jen J, Nair AA, Davila

---

714 JI, Dasari S, Lazaridis KN, Bennani NN, Wu TT, Nowakowski GS, Murray  
715 JA, Feldman AL (2018). Recurrent STAT3-JAK2 fusions in indolent T-cell  
716 lymphoproliferative disorder of the gastrointestinal tract. *Blood* 131 (20): 2262-2266.

717 Stosser S, Schweizerhof M, Kuner R (2011). Hematopoietic colony-stimulating factors:  
718 new players in tumor-nerve interactions. *J Mol Med (Berl)* 89 (4): 321-9.

719 Valdembri D, Serini G, Vacca A, Ribatti D, Bussolino F (2002). In vivo activation of  
720 JAK2/STAT-3 pathway during angiogenesis induced by GM-CSF. *FASEB J* 16 (2):  
721 225-7.

722 Wang LN, Yao M, Yang JP, Peng J, Peng Y, Li CF, Zhang YB, Ji FH, Cheng H, Xu QN,  
723 Wang XY, Zuo JL (2011). Cancer-induced bone pain sequentially activates the  
724 ERK/MAPK pathway in different cell types in the rat spinal cord. *Mol Pain* 7: 48.

725 Yoneda T, Hiasa M, Nagata Y, Okui T, White F (2015). Contribution of acidic  
726 extracellular microenvironment of cancer-colonized bone to bone pain. *Biochim*  
727 *Biophys Acta* 1848 (10 Pt B): 2677-84.

728 Zgheib A, Pelletier-Bonnie E, Levros LJ, Annabi B (2013). Selective JAK/STAT3  
729 signalling regulates transcription of colony stimulating factor-2 and -3 in  
730 Concanavalin-A-activated mesenchymal stromal cells. *Cytokine* 63 (2): 187-93.

731 Zheng Q, Fang D, Liu M, Cai J, Wan Y, Han JS, Xing GG (2013). Suppression of  
732 KCNQ/M (Kv7) potassium channels in dorsal root ganglion neurons contributes to the  
733 development of bone cancer pain in a rat model. *Pain* 154 (3): 434-48.

734

735

736

737

738

739

740

---

741 **Figure legends**

742 **Figure 1. Role of GM-CSF in bone metastases cancer pain.** (A) High expression  
743 level of GM-CSF in osteosarcoma tissue sample. H&E staining of chondroma and  
744 osteosarcoma is shown on the left; immunohistochemical staining for GM-CSF  
745 indicated by arrows in chondroma and osteosarcoma is shown on the right. (B)  
746 Summary results for immunohistochemical staining for GM-CSF (n = 8 per group,  
747 unpaired t-test:  $t=5.69$ ,  $*p=0.0013$ ). (C). Effect of antibodies against GM-CSF or  
748 GM-CSFR (10  $\mu\text{g}$ ) and GM-CSF analogue E21R (a competitive antagonist of  
749 GM-CSF, 25  $\mu\text{g}/\mu\text{l}$ , 3  $\mu\text{l}$ ) on mechanical (left panel) and thermal (right panel)  
750 nociceptive responses in bone cancer pain model of rats. The mechanical paw  
751 withdrawal threshold and thermal paw withdrawal latency were measured at 3, 7, 11,  
752 14, 17, and 21 days for the control group (black line + squares), the bone cancer group  
753 (red line + circles), the bone cancer + antibody against GM-CSF group (blue line +  
754 triangles), the bone cancer + antibody against GM-CSFR group (pink line + triangles)  
755 and the bone cancer + E21R group (green line + triangles). Two-way ANOVA  
756 followed by Bonferroni *post hoc* tests revealed a significant effect of treatment  
757 ( $F_{(4,150)}=29.49$ ,  $p=0$ ) and time ( $F_{(5,150)}=5.06$ ,  $p<0.0001$ ) but no interaction between the  
758 two ( $F_{(20,150)}=0.71$ ,  $p=0.81$ ) for the left panel; a significant effect of treatment  
759 ( $F_{(4,240)}=15.10$ ,  $p<0.0001$ ), time ( $F_{(4,240)}=2.56$ ,  $p=0.028$ ) and an interaction between the  
760 two ( $F_{(20,240)}=2.20$ ,  $p=0.003$ ) for the right panel;  $*P < 0.05$  as compared to sham group;  
761  $\#P < 0.05$  with respect to the corresponding bone cancer group. (D) Dose-dependent  
762 effects of GM-CSF on paw withdrawal threshold to mechanical stimulus (left panel)  
763 and on paw withdrawal latency to noxious heat (right panel) at 1 h, 2 h, 5 h, 12 h and  
764 24 h following focal DRG application via DRG cannula. Number of experiments is  
765 indicated as *n* in each panel. Two-way ANOVA followed by Bonferroni *post hoc* tests  
766 revealed a significant effect of dose ( $F_{(3,345)}=29.30$ ,  $p<0.0001$ ) but not of time  
767 ( $F_{(3,345)}=0.15$ ,  $p=0.96$ ), nor an interaction between the two ( $F_{(12,345)}=0.37$ ,  $p=0.97$ ) for  
768 the left panel. For the right panel, there was a significant effect of dose ( $F_{(3,350)}=70.95$ ,  
769  $p=0$ ) and an interaction between dose and time ( $F_{(12,350)}=3.71$ ,  $p<0.0001$ ) but effect of  
770 time did not reach significance ( $F_{(3,350)}=2.09$ ,  $p=0.08$ ).  $*P < 0.05$  as compared to the  
771 vehicle saline).

---

772 **Figure 2. Effect of GM-CSF on the excitability of small-sized DRG neurons. (A)**  
773 Representatives of action potentials evoked by depolarizing current pulse (left),  
774 recorded from small-sized DRG neurons. **(B)** Summary results for the effect of  
775 GM-CSF on numbers of action potential induced by increasing amplitudes of  
776 depolarizing currents. Two-way ANOVA followed by Bonferroni *post hoc* tests  
777 revealed a significant effect of treatment ( $F_{(1,1057)}=22.45$ ,  $p<0.0001$ ), injected currents  
778 ( $F_{(9,1057)}=7.82$ ,  $p<0.0001$ ) but not significant interaction between the two  
779 ( $F_{(9,1057)}=0.43$ ,  $p=0.92$ ). \* $P < 0.05$  as compared to the control. **(C)** Single action  
780 potentials from A with expanded time scales. TP, threshold potential; RMP, rest  
781 membrane potential. **(D)** Summary results for the effect of GM-CSF on the threshold  
782 potential, rheobase current and resting membrane potential (unpaired t-test, \* $p<0.05$   
783 as compared to the control).

784 **Figure 3. Effect of GM-CSF on the current amplitude and expression level of**  
785 **Nav1.7, Nav1.8 and Nav1.9 channels. (A)** Relative mRNA expression of Nav1.7,  
786 Nav1.8, Nav1.9, Kv4.2, TMEM16A, P2X3, KCNQ2 and KCNQ3 in cultured DRG  
787 cells after incubation of GM-CSF (200 ng/ml) 24h. (n=9, unpaired t-test, \* $P < 0.05$  as  
788 compared to the control) **(B)** Relative mRNA expression of Nav1.7, Nav1.8, Nav1.9  
789 in DRG neurons of bone cancer pain at the 7<sup>th</sup> day. (n=6, unpaired t-test, \* $P < 0.05$  as  
790 compared to the control) **(C)** Typical current traces and current density-voltage  
791 relationship of total TTX-S, TTX-R, Nav1.8 and Nav1.9 Na<sup>+</sup> currents in cultured  
792 DRG cells after incubation with GM-CSF (200 ng/ml) for 24h. **(D)** Western blot  
793 analysis of expression levels of Nav1.7, Nav1.8 and Nav1.9 proteins in DRG neurons  
794 treated with GM-CSF (200 ng/ml) for 18 h. (n = 3, unpaired t-test, \* $p<0.05$  as  
795 compared to the control).

796 **Figure 4. Down regulation of Nav1.7, Nav1.8 and Nav1.9 reverses nociceptive**  
797 **behavior evoked by GM-CSF. (A)** Application of antisense oligodeoxynucleotides  
798 (ASO) in DRG against Nav1.7, Nav1.8 and Nav1.9 (each ASO, 12.5  $\mu\text{g}/\text{rat}$ , 5 $\mu\text{l}$ )  
799 significantly reduced the mRNA expression level of Nav1.7, Nav1.8 and Nav1.9  
800 increased by GM-CSF treatment, and alleviated mechanical **(B)** and thermal  
801 hyperalgesia **(C)** produced by the focal GM-CSF (200 ng) application. For (A) : (n =

---

802 6, unpaired t-test,  $*P < 0.05$  as compared to control;  $^{\#}P < 0.05$  with respect to the  
803 corresponding GM-CSF). For (B): two-way ANOVA followed by Bonferroni *post*  
804 *hoc* tests revealed a significant effect of treatment ( $F_{(3,305)}=109.59$ ,  $p=0$ ) but not time  
805 ( $F_{(4,305)}=0.78$ ,  $p=0.54$ ) or interaction between the two ( $F_{(12,305)}=0.65$ ,  $p=0.80$ ) for the  
806 left panel. There was a significant effect of treatment ( $F_{(3,305)}=80.53$ ,  $p=0$ ), but not  
807 time ( $F_{(4,305)}=0.20$ ,  $p=0.94$ ) or interaction between the two ( $F_{(12,305)}=0.42$ ,  $p=0.95$ ) for  
808 the middle panel. There was a significant effect of treatment ( $F_{(3,335)}=109.87$ ,  $p=0$ ),  
809 but not time  $F_{(4,335)}=0.89$ ,  $p=0.47$  or interaction between the two ( $F_{(12,335)}=0.37$ ,  
810  $p=0.97$ ) for the right panel. For (C): two-way ANOVA followed by Bonferroni *post*  
811 *hoc* tests revealed a significant effect of treatment ( $F_{(3,295)}=168.25$ ,  $p=0$ ) and  
812 interaction between treatment and time ( $F_{(12,295)}=3.73$ ,  $p<0.0001$ ), but effect of time  
813 was not significant ( $F_{(4,295)}=1.34$ ,  $p=0.25$ ) for the left panel. There was a significant  
814 effect of treatment ( $F_{(3,295)}=336.23$ ,  $p=0$ ) and an interaction between treatment and  
815 time ( $F_{(12,295)}=2.04$ ,  $p=0.02$ ), but effect of time was not significant ( $F_{(4,295)}=1.05$ ,  
816  $p=0.38$ ) for the middle panel. There was a significant effect of treatment  
817 ( $F_{(3,250)}=274.66$ ,  $p=0$ ) and interaction between treatment and time ( $F_{(12,335)}=5.38$ ,  
818  $p<0.0001$ ), but effect of time was not significant ( $F_{(4,335)}=0.71$ ,  $p=0.74$ ) for the right  
819 panel.  $*P < 0.05$  as compared to the vehicle saline;  $n=6$ ,  $^{\#}P < 0.05$  with respect to the  
820 corresponding GM-CSF).

821 **Figure 5. GM-CSF increase the mRNA expression level of Nav1.7, Nav1.8,**  
822 **Nav1.9 channel through Jak2-Stat3 signaling pathway. (A)** Relative expression of  
823 p-Jak1, p-Jak2, p-Jak3, p-stat3 and p-stat5 in DRG neurons after incubation with  
824 GM-CSF for 25 mins. ( $n=3$ , unpaired t-test,  $*P < 0.05$  as compared to control) **(B)**  
825 Relative mRNA expression level of Nav1.7, Nav1.8, Nav1.9 in DRG neurons  
826 incubated with GM-CSF in the absence or presence of AG-490 (10  $\mu$ M) and static  
827 (20  $\mu$ M) for 4h. ( $n=4-6$ , unpaired t-test,  $*P < 0.05$  as compared to control;  $^{\#}P < 0.05$   
828 with respect to the corresponding GM-CSF). **(C)** Relative Luciferase activity in HEK  
829 293 cells transfected with reporter vector containing Nav1.7, Nav1.8, Nav1.9  
830 promoter regions (pGL3) co-expressed with either pcDNA3.1 (control) or  
831 pcDNA3.1-Stat3 cDNA. ( $n=3$ , unpaired t-test,  $*P < 0.05$  as compared to control). D-F.  
832 Relative mRNA level of Nav1.7, Nav1.8, Nav1.9 in ipsilateral DRGs (L5) of rats

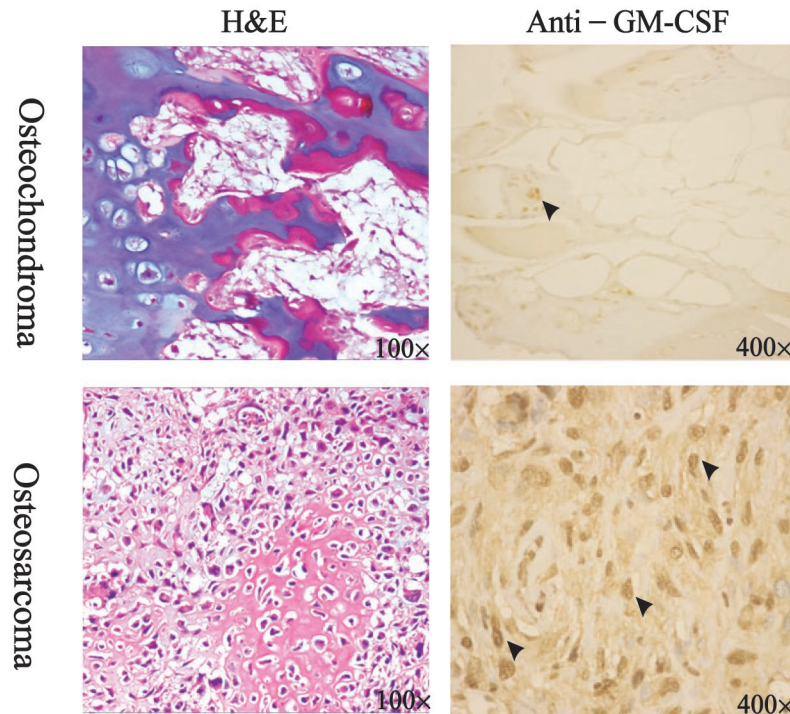
---

833 receiving anti-sense oligodeoxynucleotides (ASO) against different Jak and Stat  
834 signaling molecules (12.5 mg/rat, 5  $\mu$ l). (n = 6, unpaired t-test, \* $P$  < 0.05 as compared  
835 to control; # $P$  < 0.05 with respect to the corresponding GM-CSF). (G-I). Effect of  
836 ASOs against Jak and Stat signaling molecules (12.5 mg/rat, 5  $\mu$ l) on hyperalgesia  
837 responses to mechanical and thermal stimuli induced by GM-CSF. ASOs were given  
838 through the DRG cannula for 4 days and then GM-CSF (200ng) was given. For (G):  
839 two-way ANOVA followed by Bonferroni *post hoc* tests revealed a significant effect  
840 of treatment ( $F_{(3,415)}=125.38$ ,  $p=0$ ) but not time ( $F_{(4,415)}=0.54$ ,  $p=0.70$ ) or interaction  
841 between the two ( $F_{(12,415)}=0.73$ ,  $p=0.73$ ) for the left panel. There was a significant  
842 effect of treatment ( $F_{(3,425)}=77.18$ ,  $p=0$ ) but not time  $F_{(4,425)}=1.24$ ,  $p=0.29$  or  
843 interaction between the two ( $F_{(12,425)}=1.65$ ,  $p=0.07$ ) for the right panel. For (H):  
844 two-way ANOVA followed by Bonferroni *post hoc* tests revealed a significant effect  
845 of treatment ( $F_{(3,415)}=110.97$ ,  $p=0$ ) but not time ( $F_{(4,415)}=0.38$ ,  $p=0.82$ ) or interaction  
846 between the two ( $F_{(12,415)}=0.43$ ,  $p=0.79$ ) for the left panel. There was a significant  
847 effect of treatment ( $F_{(3,440)}=115.88$ ,  $p=0$ ) but not time ( $F_{(4,440)}=1.40$ ,  $p=0.25$ ) or  
848 interaction between the two ( $F_{(12,440)}=1.13$ ,  $p=0.33$ ) for the right panel. For (I):  
849 two-way ANOVA followed by Bonferroni *post hoc* tests revealed a significant effect  
850 of treatment ( $F_{(3,345)}=143.47$ ,  $p=0$ ) but not time ( $F_{(4,415)}=0.74$ ,  $p=0.57$ ) or interaction  
851 between the two ( $F_{(12,415)}=0.45$ ,  $p=0.94$ ) for the left panel. There was a significant  
852 effect of treatment ( $F_{(3,440)}=111.32$ ,  $p=0$ ) but not time ( $F_{(4,440)}=0.88$ ,  $p=0.47$ ) or an  
853 interaction between the two ( $F_{(12,440)}=0.94$ ,  $p=0.50$ ) for the right panel. n=6-8.\* $P$  <  
854 0.05 as compared to the vehicle saline; # $P$  < 0.05 with respect to the corresponding  
855 GM-CSF).

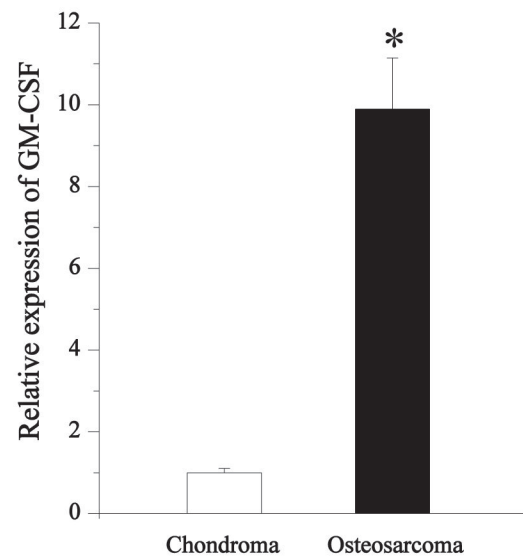
856

857

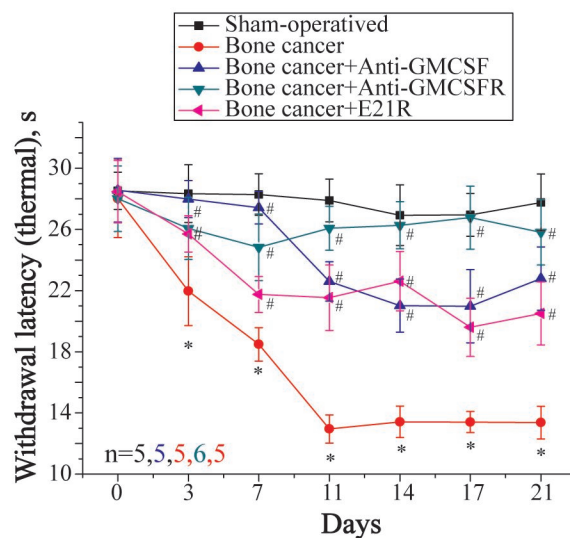
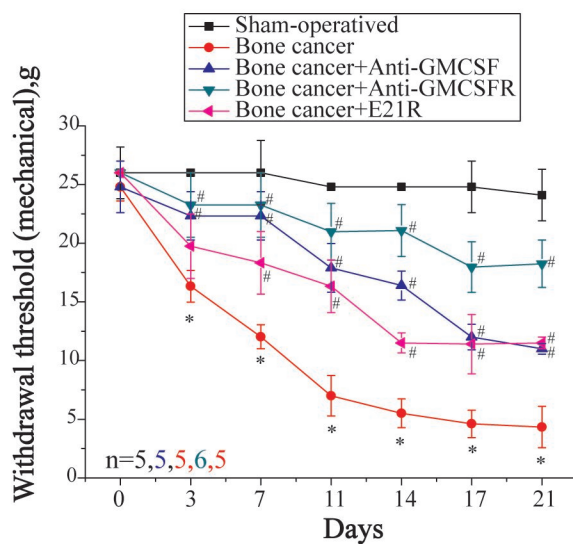
A



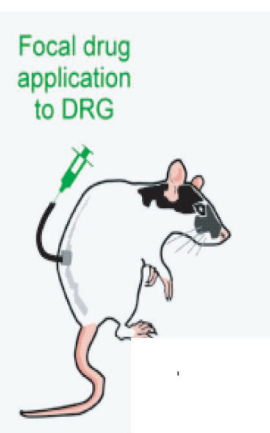
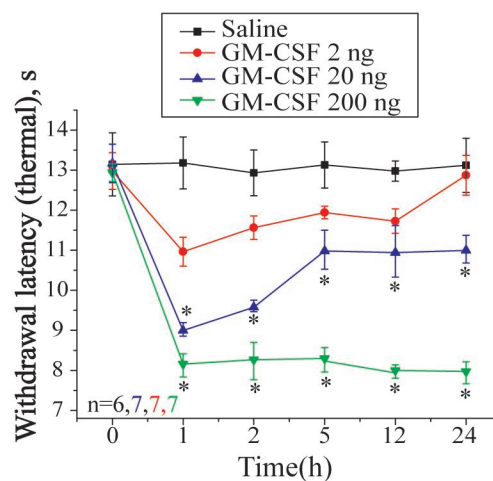
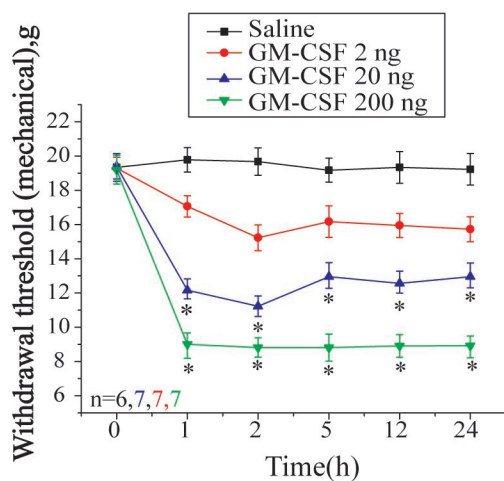
B



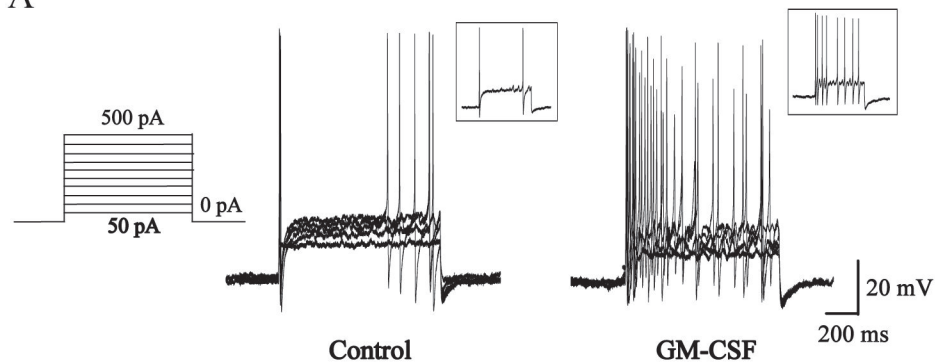
C



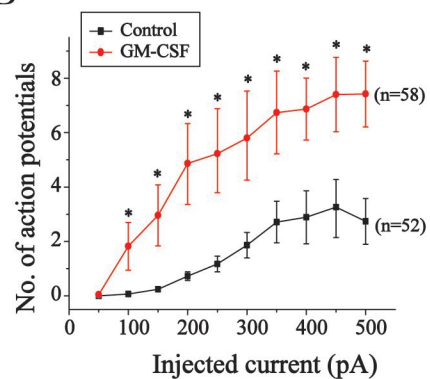
D



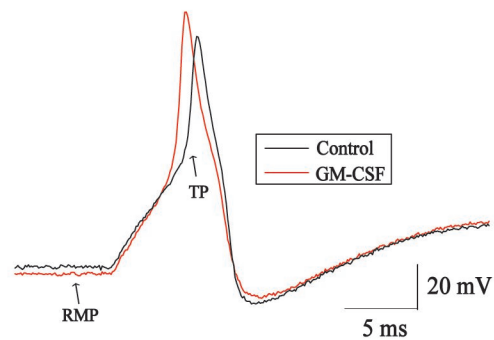
A



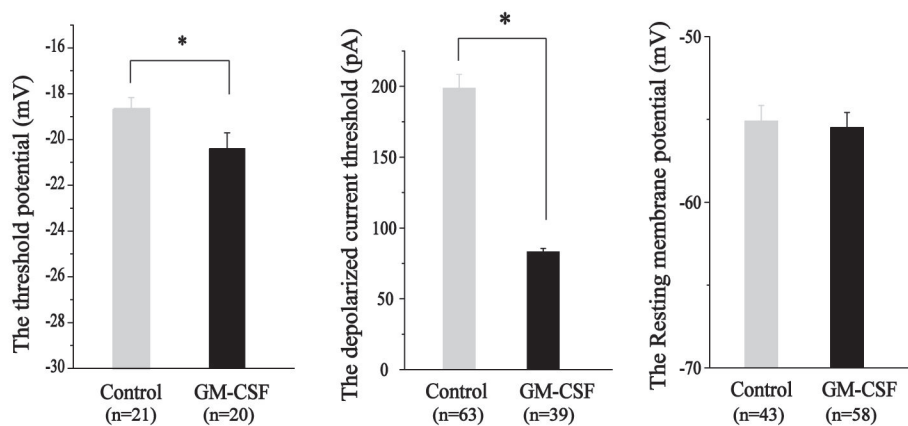
B

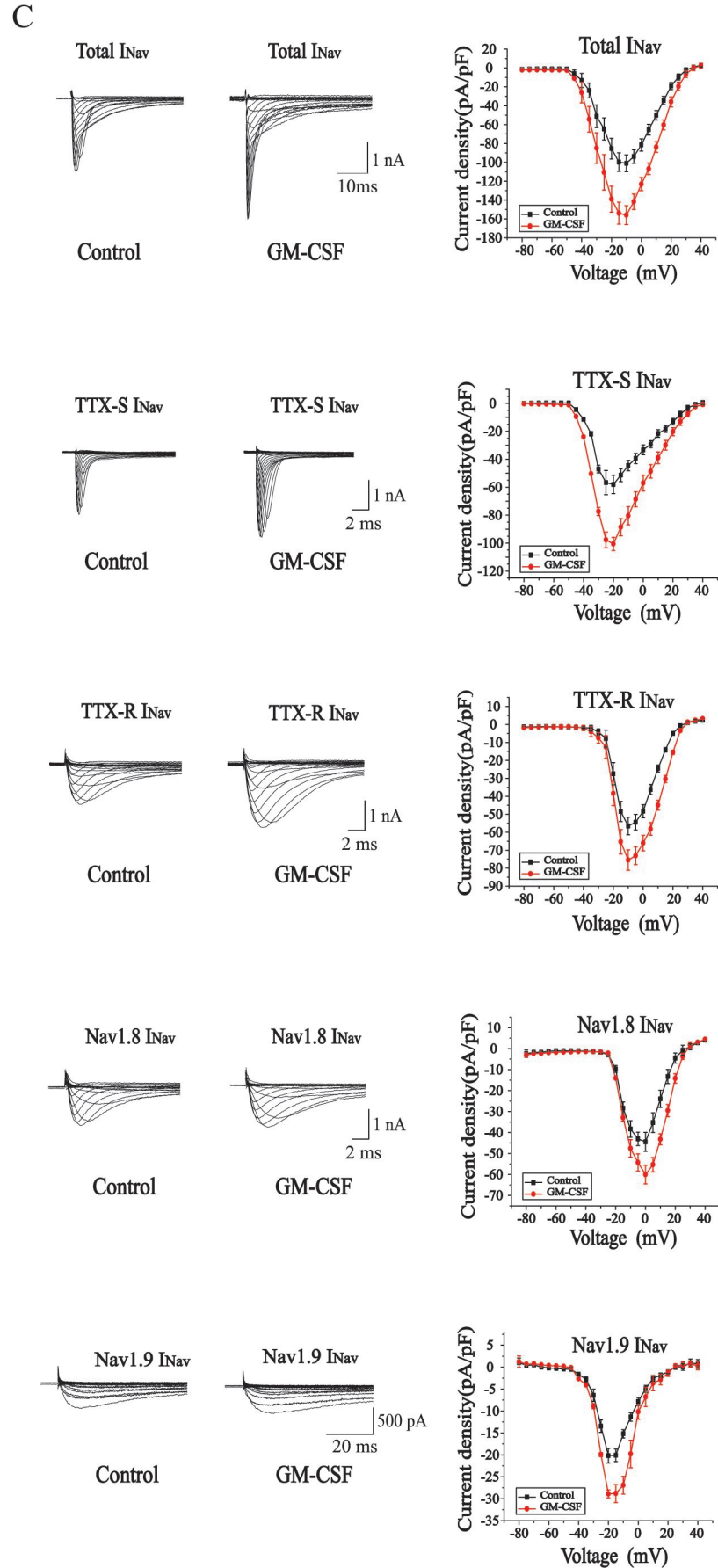
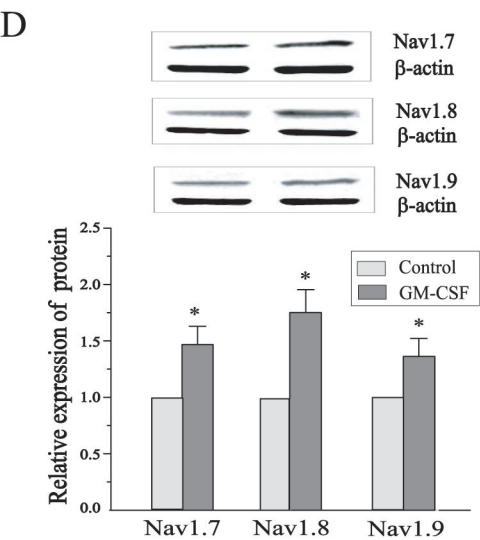
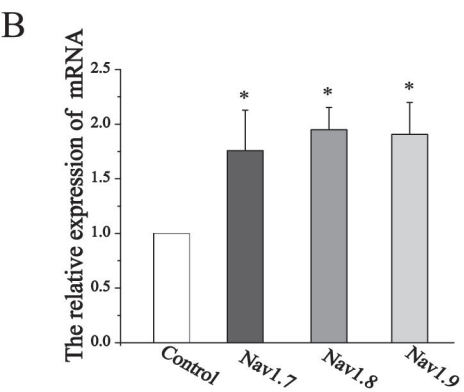
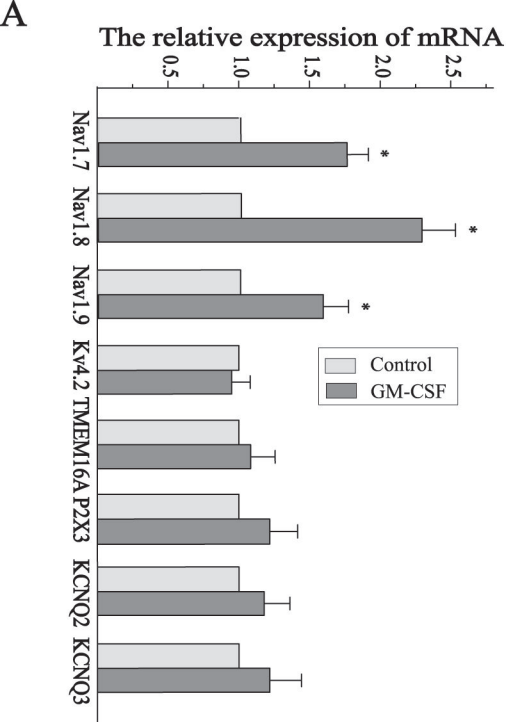


C

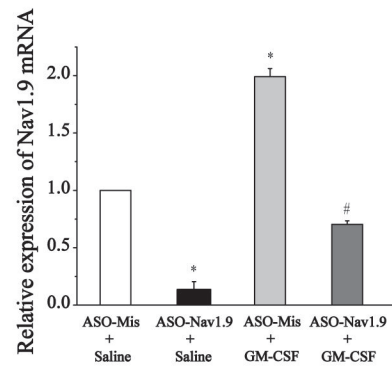
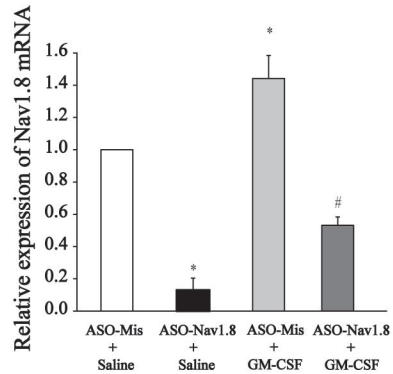
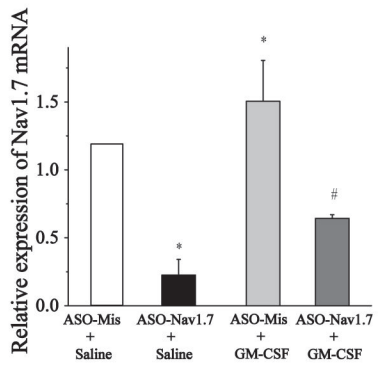


D

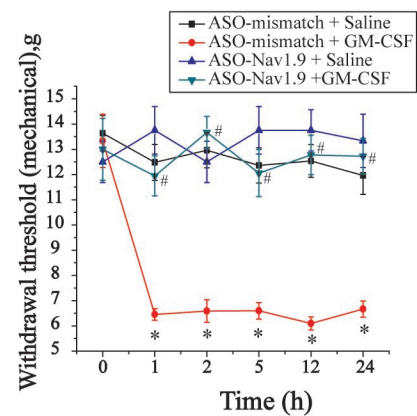
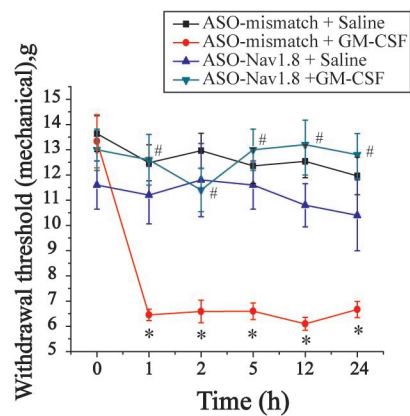
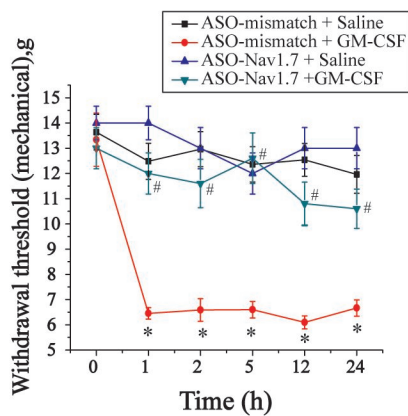




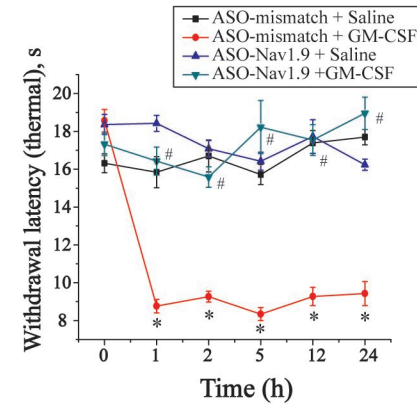
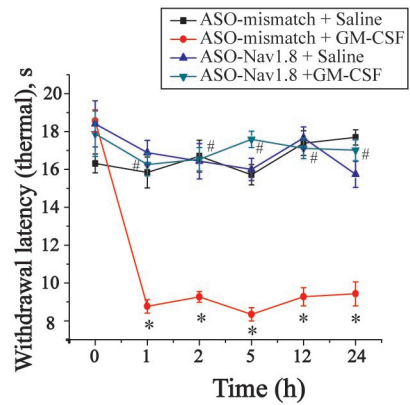
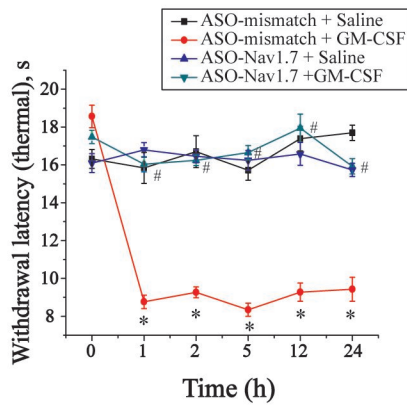
A



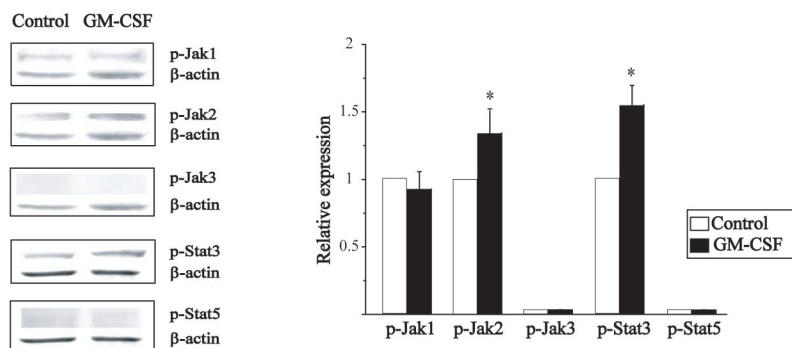
B



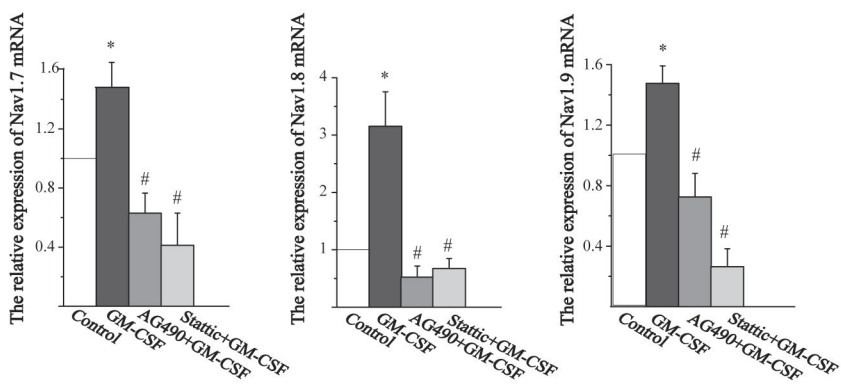
C



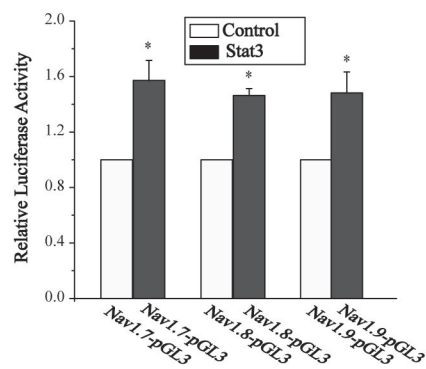
A



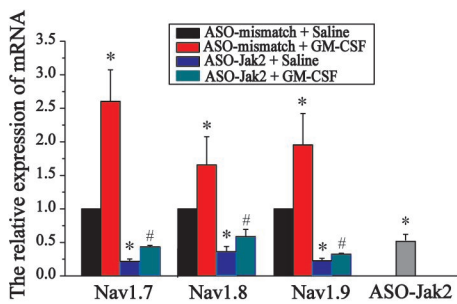
B



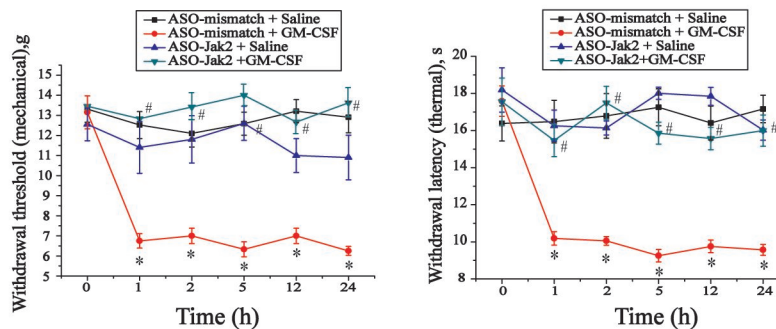
C



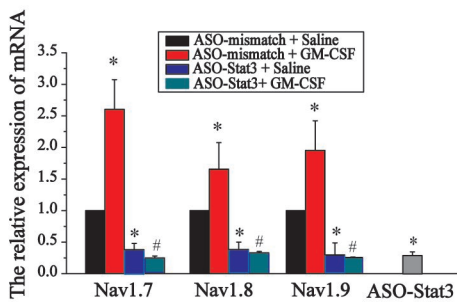
D



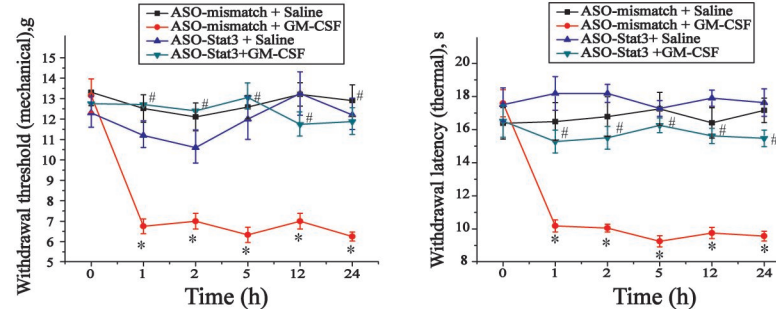
G



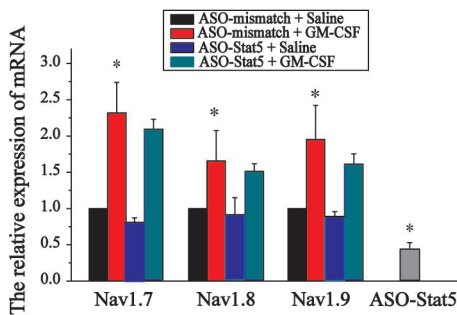
E



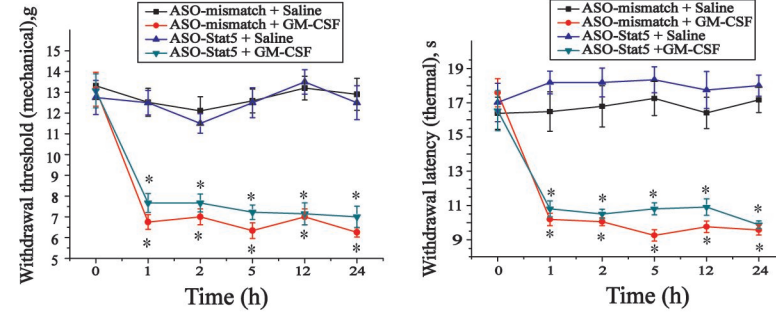
H



F



I



**Table1. Summarized effects of GM-CSF on parameters of action potential**

	Rhobase currents(pA)	TP (mV)	AP amplitude (mV)	Depolarization rate (v/s)	AHP amplitude (mV)	AHP duration (ms)	RMP (mV)	M-type K <sup>+</sup> current (pA/pF)	Rin (MΩ)
Control	198.6±9.8 (63)	-18.6±0.5 (23)	107.9±2.2 (19)	16.2±0.6 (19)	21.3±0.8 (23)	19.4±0.8 (18)	55.1±0.9 (n=43)	3.93±1.0 (9)	564.0±51.9 (32)
GM-CSF	83.1±2.5** (39)	-20.4±0.6* (22)	113.5±1.8 (22)	19.3±0.4* (21)	21.6±0.8 (26)	21.6±1.3 (18)	55.4±0.9 (58)	3.54±0.9 (8)	504.5±24.1* (25)

Rhobase currents: the depolarized current threshold for evoking the 1st action potential; TP: threshold potential; AP: action potential. RMP: resting membrane potential; Rin: input resistance. (unpaired t-test, \*p<0.05, \*\*p<0.01 compared with the control)