Blocking EphB1 Receptor Forward Signaling in Spinal Cord Relieves Bone Cancer Pain and Rescues Analgesic Effect of Morphine Treatment in Rodents

Supplementary Data, Liu et al.

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Supplementary Figure Legends

**Figure S1.** Distribution and colocalization of expression of EphB1 and ephrinB2 with cells and primary afferents within the DH and DRG after TCI. **A,** EphB1 (green) with astrocytes (GFAP, red, left) and, in a small amount, with microglial cell (IBA, red, right). **B,** EphrinB2 (green) with astrocytes (red, left), but not microglial cell (red, right). **C, D,** EphrinB2 (green) with primary afferents CGRP (red), but not IB4 (red) in the DH (C) and with CGRP (red)-, but not IB4 (red)-nociceptive cells in the DRG (D). Magnification: 200 ×.

**Figure S2.** Blocking EphB receptor activation prevents and attenuates pain-related behaviors after TCI. **A, B,** Spontaneous guarding and flinching. **C, D,** Movement-evoked guarding and limb use. Data shows the foot ipsilateral to TCI tested on the postoperative 14 day. Eight rats were included in each group. *P<0.05, **P<0.01 indicate significant differences compared to the control group (Sham+Fc). #P<0.05, ##P<0.01 indicate significant differences compared to the corresponding TCI group.

**Figure S3.** Blocking EphB receptor activation suppresses induction of c-Fos and activation of
astrocytes and microglial cells in the DH ipsilateral to TCI. ** A, Induction of c-Fos. ** B, Activation of astrocytes (GFAP). ** C, Activation of microglial (IBA). EphB2-Fc (5 µg, i.t., once a day) were given on postoperative 7, 8 and 9 days, respectively. Tissues were collected on the 14th day after TCI. Magnifications: 200×. **P<0.01 indicates significant differences compared to the corresponding control of Sham+Fc. ##P<0.01 indicates significant differences compared to the corresponding control of TCI+Fc.

**Figure S4.** Presence of osteolytic lesions in the tibia of the rats after TCI. ** A, X-ray images show bone destruction and tumor growth on postoperative 14 and 21 days. ** B, HE staining shows bone destruction and tumor growth on the 14th day after TCI. Scale bar in A: 100 µm. EphB2-Fc (5 µg, i.t., once a day) was given on postoperative 7, 8 and 9 days, respectively.

**Figure S5.** Spinal administration of ephrinB2-Fc downregulates ephrinB2 in the spinal cord and induces thermal hypersensitivity in naïve rats. ** A, Western blot analysis shows expression of ephrinB2 and its phosphorylation, PY99. EphrinB2-Fc: 2 µg, i.t. Tissues were taken at 1h, 2h and 4h after injection. Four samples were included in each group. ** B, Single dose of ephrinB2-Fc (2 µg) caused thermal hypersensitivity. Eight rats were included in each group. **P<0.01 indicate significant differences compared to the corresponding control groups.

**Figure S6.** Repetitive spinal administration of ephrinB2-Fc increases level of EphB1, pNR1 and pNR2B and decreases ephrinB2 in the spinal cord while causing thermal hypersensitivity in naïve rats. ** A-C, Western blot shows effects of ephrinB2-Fc on expression of EphB1 and ephrinB2 and their phosphorylation and phosphorylation of NR1 and NR2B. Tissues were taken 4h after the third dose of ephrinB2-Fc. **P<0.01 indicates significant differences compared to the corresponding control groups of PBS and Fc.
**Figure S7.** Activation of EphB1 receptor is required for ephrinB2-Fc inducing thermal hypersensitivity. Pretreatment with EphB2-Fc (each 5 µg, i.t., once a day for 3 consecutive days) blocked ephrinB2-Fc (2 µg)-induced thermal hypersensitivity (A), but not the accompanied downregulation of ephrinB2 (B). Eight rats were included in each group and injection(s) was indicated by the arrow(s) in A. Four samples were included in each group and tissues were taken 4 h after injection of ephrinB2-Fc in B. **P<0.01 indicates significant differences compared to any of the control groups.

**Figure S8.** Spinal administration of EphB2-Fc or ephrinB2-Fc upregulates activity of MMP-2 and MMP-9. Gelatin zymography shows activity of MMP-2 and MMP-9 in the spinal cord in naïve rats. Intensity of both MMP-9 and active MMP-9 as well as MMP-2 and active MMP-2 are measured and included in A and B. Tissues were taken 4h after spinal injection of EphB2-Fc (5 µg) or ephrinB2-Fc (2 µg). Four samples were included in each group. *P<0.05, **P<0.01 indicates significant differences compared to the corresponding groups of PBS and Fc.

**Figure S9.** Spinal administration of EphB2-Fc inhibits morphine (Mor)-induced upregulation of EphB1 in rats. A, Mor upregulates expression of EphB1 and its phosphorylation. Mor100: a single treatment of Mor (100 mg/kg, s.c.). Mor(100-10): Mor at 10mg/kg was injected 24h after the first dose at 100 mg/kg (acute tolerance protocol). Tissues were taken 0.5h after the second injection. B, EphB2-Fc inhibits Mor-induced upregulation of EphB1. EphB2-Fc (2µg and 5µg, i.t.) was administrated 30 min before the first dose of Mor at 100 mg/kg. C, EphB2-Fc (5µg, i.t.) was administrated 30 min before each of Mor at 10 mg/kg. Mor10: chronic tolerance protocol.
Five samples were included in each group in A-C. *$p<0.05$, **$p<0.01$ indicate significant differences compared to group of Sham. #$p<0.05$, ##$p<0.01$ indicate significant differences compared to the corresponding group of Fc (B,C).
Supplementary Methods

Spontaneous and movement-evoked pain-like behaviors

Spontaneous and movement-evoked pain-like behaviors were analyzed as described previously (1). Spontaneous nocifensive behaviors were evaluated by measuring spontaneous guarding and flinching over a 2-min period of observations. Movement-evoked pain was assessed by measuring the time spent guarding over a 2-min period of observations after non-noxious palpation, and the limb use during spontaneous ambulation, which was scored on a scale of 0-4: 0 = normal use, 1 = slightly limping, 2 = clearly limping, 3 = no use of the limbs (partial), and 4 = no use of the limbs.

Immunohistochemistry

In Fig.S1, S2, S3, sections of the spinal cord or DRG tissues were incubated with polyclonal antibodies of anti-EphB1(1:20), anti-ephrinB2(1:20), rabbit anti-c-Fos(1:100, sc-52, Santa Cruz), rabbit anti-CGRP(1:1000, Millipore), mouse monoclonal anti-neuronal nuclear protein (NeuN) (1:100)(Alexa Fluor-488 conjugated, MAB377X, Chemicon), rabbit polyclonal anti-GFAP (1:500, ab7260, Abcam), anti-IBA (1:100, 019-19741, Wako Pure Chemical Industries), and anti-IB4 (1:100, FITC conjugated) (Sigma), respectively. Rabbit IgG (1:200, Vector Laboratories) was used as an isotype control. Morphologic details of the double immunofluorescent staining were examined with a confocal microscope (Leica TCS SP2, Germany). The number of Fos-like immunoreactive neurons in laminae I-VII was determined by averaging the counts made in 20 spinal cord sections for each group. To obtain quantitative measurements of GFAP and IBA immunofluorescence, 15–20 fields covering the entire DH in each group were evaluated and photographed at the same exposure time to generate the raw data. The average green
fluorescence intensity of each pixel was normalized to the background intensity in the same image. These protocols were similar to those described previously (2, 3).

**Gelatin zymography**

The protocol was similar to that previously described (2, 3). The animals were anesthetized deeply and transcardially perfused with PBS and then a length of spinal cord containing segments L₁ to L₆ was rapidly dissected and homogenized in a lysis buffer containing proteinase inhibitors. Aliquots (10 µl) of the homogenates were saved for total protein measurement (BCA kit). The homogenates were centrifuged at 12,000 RPM for 5 min. The supernatants were recovered and incubated for 60 min with gelatin-Sepharose 4B (Pharmacia Biotech) with constant shaking. After incubation, the samples were centrifuged at 500 RPM for 2 min. The pellets were resuspended in 80 µl of elution buffer for 30 min. The entire sample was loaded onto a 8% SDS gel containing 1 mg/ml gelatin. The gel was washed for 3–4 h to remove SDS and allow renaturation of MMPs. The gel was then left for 48 h in an incubation buffer to allow MMPs to degrade the gelatin in their immediate vicinity. Finally, zones of gelatin degradation representing proteolytic activity were identified by staining the gel with Coomassie blue and destaining with methanol and acetic acid in water (3:1:6).

**X-ray and histological observations**

To verify bone destruction and tumor cell infiltration, X-ray and histological observations of the affected bone were made after TCI. The X-ray images were taken on postoperative 14 and 21 days (exposure setting: 12 ms, 31KVp) using Philips Digital Radiographer System (Digital diagnost VM, Philips Medical Systems DMC GmbH, Hamburg, Germany). Histological
observations of the affected bone were made on the 14th day after TCI. Rats were anesthetized and transcardially perfused with 0.9% saline (150 ml) followed with 4% paraformaldehyde (300 ml). The tibia bones were removed and demineralized in EDTA (10%) for 2 weeks. Then the tibiae were embedded in paraffin, and 5µm sections were cut with a microtome and stained with hematoxylin and eosin (HE) for analysis under a microscope.

References