SUPPLEMENTAL MATERIAL

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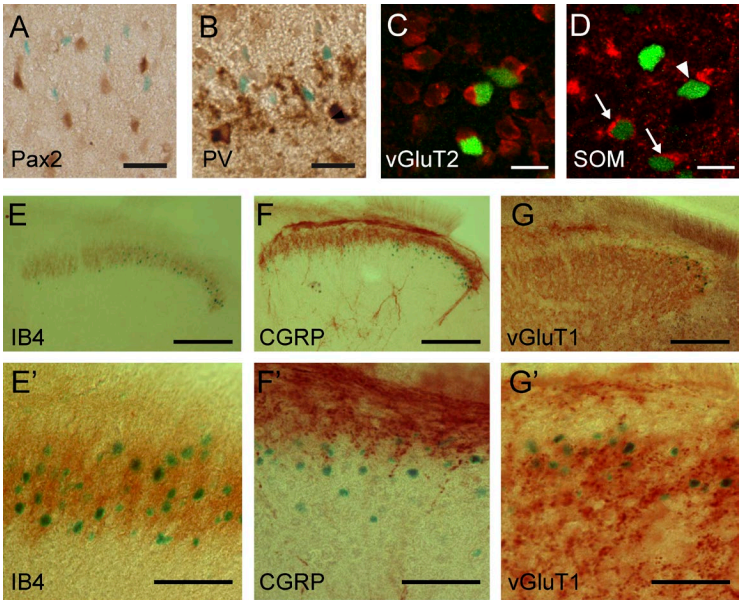

Figure S1. **Neuronal subtype and localization of Netrin-4–expressing cells in the dorsal horn.** (A and B) LacZ$^+$ nuclei (blue) in the dorsal horn of the lumbar cord were not merged with Pax2 (brown), an inhibitory neuron-specific transcription factor (A) or with parvalbumin (PV), which labels a subpopulation of inhibitory interneurons (B). (C) In contrast, LacZ$^+$ nuclei (green) were merged with excitatory neuron marker vGluT2 (red). (D) LacZ signal was observed in SOM-expressing cells (arrows), whereas there were LacZ$^+$ nuclei in SOM-negative cells (arrowhead). (E–G) Costaining of LacZ$^+$ cells (blue) and markers of primary afferents (brown). (E′–G′) Higher magnification images of E–G. LacZ$^+$ cells overlapped with IB4 staining, a marker of nonpeptidergic C fibers projecting to lamina IIi (E and E′). Calcitonin gene-related peptide (CGRP) staining, a marker of peptidergic C fibers that mainly project to lamina I and lamina IIo, did not overlap with the LacZ$^+$ nuclei (F and F′). Moreover, vGluT1 staining, a marker of myelinated A fibers, showed partial overlap with the LacZ$^+$ nuclei (G and G′). These results confirm that Netrin-4 is expressed in lamina IIi and imply that Netrin-4–expressing cells receive input from nonpeptidergic C fibers and A fibers. Bars: (A and B) 20 µm; (C and D) 10 µm; (E–G) 0.2 mm; (E′–G′) 50 µm. Results are representative from two independent experiments.

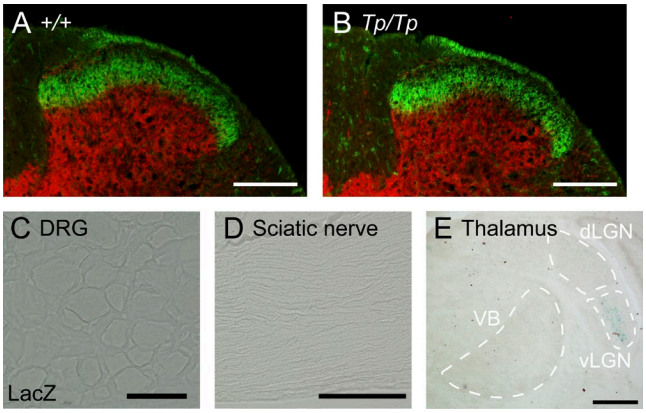

Figure S2. **Netrin-4 in nociceptive pathway.** (A and B) Projections of peripheral nerves onto the spinal cords of wild-type and Netrin-4–mutant rats. Double immunofluorescence for IB4 (green) and vGluT1 (red) in the lumbar cord is shown. No obvious differences were observed in the axonal projections of DRG neurons between wild-type (*+/+*; A) and Netrin-4–mutant (*Tp/Tp*; B) rats. Results are representative from three independent experiments. (C–E) Netrin-4 expression in the DRG, sciatic nerve, and thalamus. LacZ staining showed that $LacZ^+$ cells were not present in the DRG (C) or sciatic nerve (D). (E) In the thalamus, a Netrin-4 signal was observed in the ventral part of the lateral geniculate nucleus, but not in the sensory nuclei, including the ventrobasal complex (VB), or dorsal lateral geniculate nucleus (dLGN). vLGN, ventrolateral geniculate nucleus. The dashed lines represent the boundaries of the thalamic nuclei. Bars: (A and B) 0.2 mm; (C) 50 µm; (D) 100 µm; (E) 0.5 mm. Results are representative from two independent experiments.

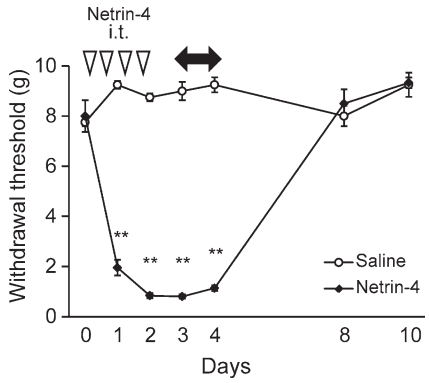

Figure S3. **The procedure for the electrophysiological analyses after Netrin-4 administration.** A Netrin-4 (◆) or saline (○) solution was intrathecally (i.t.) injected twice daily during the first 2 d (open arrowheads), and the development of tactile allodynia was assessed with the von Frey filament test. Netrin-4–induced allodynia was sustained for 2 d after the final injection (1, 2, 3, and 4 d after the first injection; **, $P < 0.01$; Tukey-Kramer test) and then recovered (8 and 10 d; $P > 0.05$). Spinal cord slices were generated between 1 and 2 d after the final administration (arrow) and used for the electrophysiological analyses. This experiment was performed three times.

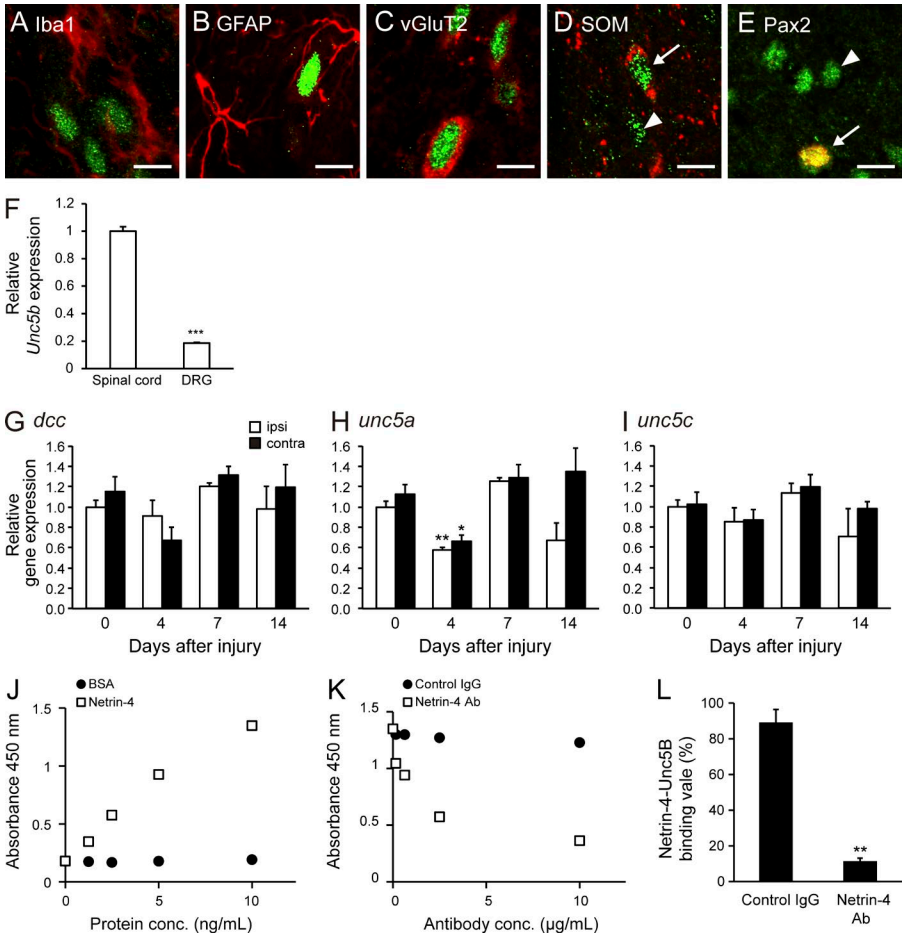

Figure S4. **Spinal cord neurons express Unc5B, which binds to Netrin-4.** (A–E) Double labeling of Unc5B and cell-type markers in the dorsal horn under pathological conditions. (A) Double-immunofluorescence labeling for Unc5B (green) and the microglial marker Iba1 (red). (B) Double-immunofluorescence labeling for Unc5B (green) and the astrocytic marker GFAP (red). Unc5B immunoreactivity was not observed in Iba1- or GFAP-expressing cells, indicating that Unc5B is not expressed in microglia or astrocytes. (C, D, and E) Some Unc5B signals (green) were observed in SOM (red, C)-expressing cells, vGluT2-expressing excitatory neurons (D), and Pax2-expressing inhibitory neurons (E). Arrows indicate colocalization with markers, and arrowheads indicate no colocalization. (A–E) Results are representative from two independent experiments. Bars, 10 µm. (F) Unc5B gene expression in the spinal cord and the DRG. Gene expression of Unc5B in the spinal cord was significantly higher than in the DRG. ***, $P < 0.001$; Student's *t* test. $n = 6$ for each group. (G–I) Gene expression of other Netrin receptors after peripheral nerve injury. The graphs show the time course of the relative gene expression levels. Gene expression of DCC, Unc5A, and Unc5C in the spinal cord was examined. There was no significant up-regulation of gene expression in the ipsilateral (ipsi; unshaded) and contralateral (contra; shaded) sides relative to the injury at 4–14 d after PSL injury. *Unc5A* was significantly decreased at 4 d after injury in both sides. *, $P < 0.05$; **, $P < 0.01$ vs. day 0; Tukey-Kramer test. $n = 3$ for each group. (J–L) Blockade of the Netrin-4–Unc5B interaction using a Netrin-4 antibody (Ab). Binding between Netrin-4 and Unc5B was assessed using an ELISA. The Netrin-4–His protein was applied to Unc5B-coated dishes, and the immunoreactivity of the His-tag was examined using absorbance. (J) Netrin-4 binds to Unc5B in a dose-dependent manner. The decision coefficient of the regression line was 0.9759. (K) The inhibitory effect of the Netrin-4 antibody on Netrin-4–Unc5B binding. Addition of the Netrin-4 antibody, which was used in Figs. 6 and 8, reduced the absorbance in the presence of 10 ng/ml Netrin-4. This result suggested that the Netrin-4 antibody prevented the interaction between Netrin-4 and Unc5B. (L) Value of the interaction between Netrin-4 and Unc5B. The values were calculated from the slope and the intercept of the regression line in J. $n = 3$. **, $P < 0.01$; Student's *t* test. conc., concentration. The data are presented as the mean ± SEM.

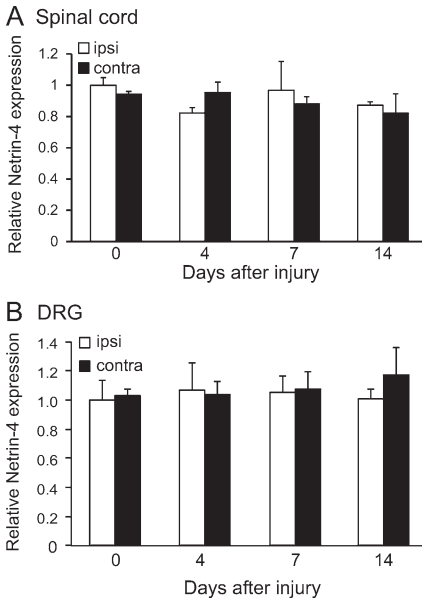

Figure S5. **Netrin-4 expression after peripheral nerve injury.** (A) Gene expression of Netrin-4 in the spinal cord after peripheral nerve injury. Note that there was no significant difference between the ipsilateral (ipsi; unshaded) and contralateral (contra; shaded) sides relative to the injury at 4–14 d after PSL injury. n = 4–6 for each group. (B) Gene expression of Netrin-4 in the DRG tissue after peripheral nerve injury. A Tukey-Kramer test was used. n = 3–6 for each experiment. The data are presented as the mean ± SEM.