Involvement of Wnt/β-catenin signaling in the development of neuropathic pain

Takahide Itokazu[1,2], Yasufumi Hayano[1], Ryosuke Takahashi[2], Toshihide Yamashita[1]

[1]Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

[2]Department of Neurology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Address correspondence to: Toshihide Yamashita

Department of Molecular Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Phone: + 81-6-6879-3661; Fax: + 81-6-6879-3669

E-mail: yamashita@molneu.med.osaka-u.ac.jp

Keywords: neuropathic pain, Wnt signaling, microglia, brain-derived neurotrophic factor

Abstract

Despite tremendous research effort in the field, our current understanding of the molecular mechanisms underlying neuropathic pain is still incomplete. In the present study, our objective was to elucidate the involvement of the Wnt/β-catenin signaling pathway in the development of neuropathic pain. We showed that Wnt/β-catenin signaling is activated in the spinal cord dorsal horn after partial sciatic nerve ligation (PSL). Expression of Wnt3a, a prototypic Wnt ligand that activates the Wnt/β-catenin pathway, was also upregulated in the dorsal horn. We then tested the effect of intrathecal administration of XAV939, a Wnt/β-catenin signaling inhibitor, and found that this treatment effectively attenuated the induction of neuropathic pain. Conversely, intrathecal administration of Wnt3a to the lumbar spinal cord of naïve animals triggered the development of allodynia. These results suggest a critical involvement of the Wnt/β-catenin pathway in the development of neuropathic pain. Moreover, we also found that PSL-induced microglial activation was significantly suppressed by intrathecal administration of XAV939 treatment. Because it was revealed that Wnt3a treatment triggered brain-derived neurotrophic factor (BDNF) release from microglial cells *in vitro*, it is possible that Wnt3a upregulation in the dorsal horn leads to the activation of microglial cells, then triggers BDNF secretion that is responsible for the establishment of neuropathic pain. Further studies will be needed for the comprehensive understanding of the roles of Wnt/β-catenin signaling in the development of neuropathic pain.

1. Introduction

Clinical management of neuropathic pain is currently aimed at achieving relief from symptoms of this condition; unfortunately, in many cases such efforts fall short of this goal. Therefore, efforts to develop disease-modifying therapeutics that effectively treat neuropathic pain are of great importance. Previous studies have revealed that numerous processes are involved in the development of neuropathic pain, including structural changes in neural circuits in the spinal cord dorsal horn (Woolf et al., 1992; Navarro et al., 2007) and abnormal excitability due to alterations of neurotransmitter release (Basbaum et al., 2009). Moreover, recent studies provide increasing evidence for the role of inflammatory processes, via the actions of immune cells and glial cells, in the development of neuropathic pain (Scholz et al., 2007; Ellis et al., 2013). Nevertheless, our knowledge of the molecular mechanisms underlying these processes is very limited.

Wnt family proteins play important roles in many aspects of central nervous system (CNS) development: neural induction, cell proliferation, neuronal migration, axon guidance, dendritic arborization, and synaptogenesis (Ciani et al., 2005; Salinas, 2012). At least three Wnt signaling pathways have been demonstrated; these include the canonical Wnt/β-catenin pathway, planar cell polarity pathway, and Wnt/Ca2+ pathway. Among them, the Wnt/β-catenin pathway is the best understood, and previous studies have suggested its possible contributions to the pathogenesis of several CNS disorders, including cerebral ischemia (Zhang et al., 2008; Mastroiacovo et al., 2009), psychiatric disorders (Okerlund et al., 2011), Parkinson's disease (L'Episcopo et al., 2011), Alzheimer's disease (Shruster et al., 2011; Halleskog et al., 2011), and epilepsy (Busceti et al., 2007). Moreover, recent studies suggest the involvement of Wnt signaling in generating inflammatory responses in the CNS through glial cell modifications (Marchetti et al., 2012). This suggests the possible involvement of Wnt signaling in various aspects of the development of neuropathic pain.

In the Wnt/β-catenin pathway, Wnt ligands bind to Frizzled (Fzd) and LRP5/6. Subsequent binding of Fzd to the scaffold protein Disheveled (Dvl) leads to the inhibition of the destruction complex, a group of proteins which include Dvl, axin, glycogen synthase kinase-3β (GSK-3β), casein kinase 1, and adenomatous polyposis coli (APC) protein. In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds and phosphorylates β-catenin; the phosphorylated β-catenin is then ubiquitinated and destroyed in the proteasome. Thus, Wnt signaling allows β-catenin accumulation in the cytoplasm. β-catenin then enters the nucleus to interact with T cell/lymphoid enhancer factor (TCF/LEF) transcription factors and regulate the transcription of target genes (Clevers et al., 2012; Kikuchi et al., 2011).

In the present study, we showed the activation of Wnt/β-catenin signaling and upregulation of Wnt3a in the spinal cord dorsal horn after sciatic nerve injury. Then, we tested the effect of intrathecal administration of a Wnt/β-catenin signaling inhibitor and found that the treatment effectively attenuated the induction of neuropathic pain. We also confirmed that recombinant Wnt3a application was enough to trigger tactile allodynia. Moreover, using microglial cell lines, we found that Wnt3a treatment upregulated BDNF release from microglial cells *in vitro*. These findings suggest the possibility of a novel mechanism that may be involved in the development of neuropathic pain. Specifically, our results indicate that injury-induced Wnt3a upregulation in the dorsal horn triggers microglial activation, the stimulation of BDNF release, and enduring changes in the properties of dorsal horn neurons that initiate central sensitization.

2. Materials and methods

2.1. Animals

Adult male C57BL/6J mice (8−9 weeks old, 20–25 g; SLC, Shizuoka, JP, for western

blotting and immunohistochemistry experiments) and male Wistar rats (8−10 weeks old, 200–250 g; SLC, Shizuoka, JP, for intrathecal administration experiments) were group-housed under a 12-h light/dark cycle and received food and water *ad libitum*. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Osaka University.

2.2. Surgical procedures

Neuropathic pain was induced by partial sciatic nerve ligation (PSL), as previously described (Seltzer et al., 1990). Briefly, under anesthesia with 2% isoflurane (Takeda Yakuhin Kougyou, JP), the left sciatic nerve was carefully isolated at the high-thigh level, after which 4−0 nylon suture was inserted into the nerve, and tightly ligated so that the dorsal one-third to half of the nerve thickness was trapped in the ligation. The wound was closed with 4−0 nylon suture.

For intrathecal administration, a modified catheter-through-a-needle technique was applied, as per the methods detailed in previous reports (Maeda et al., 2009; Milligan et al., 1999). Briefly, under 2% isoflurane anesthesia, polyethylene tubing (PE-10, Clay-Adams, Becton-Dickinson and Co., Sparks, MD, USA) was inserted between lumbar vertebrae 5 (L5) and 6 (L6) in rats. To verify the catheter placement, 25 µl of 2% lidocaine followed by 10 µl saline flush was injected. Hind paw paralysis indicated successful catheterization. Animals showing neurological deficits after catheter implantation were excluded from subsequent drug/protein administration or behavioral testing. For continuous administration, a mini-osmotic pump (Alzet, Cupertino, CA) was connected to the end of the implanted catheter and placed in the subcutaneous space. Filled solutions and delivery rates were as follows: for XAV939 administration, XAV939 (Stemgent, Cambridge, MA) in 10% dimethyl sulfoxide (DMSO)/ phosphate-buffered saline (PBS) at 0.5 nmol/hour, and 10% DMSO/PBS as vehicle

control; for Wnt3a administration, recombinant Wnt3a (R&D Systems, Minneapolis, MN) in 0.01% bovine serum albumin (BSA)/PBS at 1.25 ng/hour, and 0.01% BSA/PBS as vehicle control.

2.3. Assessment of mechanical allodynia

Rats receiving PSL treatment were tested for tactile allodynia prior to surgery and at various time points post-injury. For assessing tactile sensitivity, von Frey filaments were used, according to previously reported methods (Chaplan et al., 1994). Briefly, after a 10-minute period of habituation on a wire mesh, a calibrated plantar filament (Semmes-Weinstein von Frey anesthesiometer; Muromachi Kikai, Tokyo, JP) was applied to the plantar surface of the hind paw, and the 50% paw withdrawal threshold was determined using the up-down method.

2.4. Western blot analysis

Deeply anesthetized mice were transcardially perfused with 30 ml PBS. Immediately, the lumbar spinal cord was exposed by laminectomy, and the L4−L6 segment was excised. On dry ice, the right and left dorsal halves of the spinal tissue were carefully separated under a dissecting microscope and stored at −80°C. The corrected tissues were homogenized in radio immunoprecipitation assay lysis buffer with a protease inhibitor cocktail (Roche Diagnostics, Basel, CH), NaF, and Na_3VO_4. Equal amounts of protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then immunoblotted to detect active β-catenin, Wnt3a, and β-actin. The following antibodies were used: mouse anti-active β-catenin (clone 8E7; 1:1000; Millipore, Massachusetts, US), anti-Wnt3a (1:1000; Millipore, Massachusetts, US), anti-β-actin (13E5; 1:1000; Cell Signaling Technology, Danvers, MA), and appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). Protein

bands were visualized using an enhanced chemiluminescence system (GE Healthcare) with an LAS-3000 image analyzer (Fuji Film, Tokyo, JP). Densitometry was conducted for each band by using ImageJ software (NIH), and the ratios of active β-catenin and Wnt3a to β-actin band density were calculated.

2.5. Immunohistochemistry

The animals were deeply anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Following perfusion, lumbar spinal cords were post-fixed in the same fixative solution at 4°C overnight and subsequently transferred to 30% sucrose in PBS at 4°C overnight. The segments of spinal cord containing the L4−L6 level were cut into 20-μm-thick axial sections using a cryostat and placed on MAS-coated glass slides (Matsunami, Osaka, JP). The sections were washed in PBS thrice and blocked with 5% BSA/0.1–0.3% Triton X-100/PBS for 1.5 h. Next, the sections were incubated with primary antibodies overnight at 4°C. Mouse anti-NeuN (1:100; Millipore), rabbit anti-Wnt3a (1:200; Millipore, Massachusetts, US), mouse anti-MAP2 (1:500; Millipore, Massachusetts, US), rabbit anti-Iba1 (1:1000; Wako, Osaka, JP), and mouse anti-CD68 (clone ED1, 1:150; LifeSpan BioSciences, Seattle, US) were used as primary antibodies. The sections were then washed in PBS with Tween 20 three times, followed by incubation with appropriate secondary antibodies conjugated with Alexa 488 or 568 (1:500; Invitrogen, California, US) for 1 hour at room temperature in the dark. All images were acquired with a fluorescence microscope (Keyence, Biorevo BZ-9000, Osaka, JP) or a confocal laser-scanning microscope (Olympus FluoView FV1000; Olympus, Tokyo, JP). For quantitative analysis of Iba1 and CD68 staining, we made serial sections of spinal cord, and injury center was determined based on the microglial activation (mostly activated section was chosen). Then confocal images were obtained at the same exposure time (5 sections/ animal

around the injury center, magnification; ×400). The fluorescence intensity was normalized to background, then using ImageJ software (NIH), immunofluorescence positive area in the dorsal horn of both sides were calculated. Obtained data were averaged per animal, and used for statistical analysis.

2.6. Cell culture

To investigate the microglial response to Wnt3a, we used a mouse microglial cell line (MG5). Both MG5 microglial and A1 astrocyte-like cell lines were originally established from p53-deficient mice (Ohsawa et al., 1997). In this study, MG5 cells were cultured in 35-mm dishes in A1 conditioned medium. A1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, California, US) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen, California, US). MG5 cells were treated for 3 hours with either recombinant Wnt3a (100 ng/ml in 0.1% BSA/PBS; R&D Systems, Minneapolis, MN) or an equal amount of 0.1% BSA/PBS as a control.

2.7. BDNF enzyme-linked immunosorbent assay (ELISA)

The extracellular concentrations of mature BDNF in the supernatant of MG5 cells (obtained as described in section *2.6.*) or dorsal horn lysate (obtained as described in section *2.4.*) were measured using the BDNF Emax Immunoassay System (Promega, Madison, US) according to the manufacturer's instructions. Briefly, 96-well microplates (Thermo Fisher Scientific, Waltham, MA, USA) were incubated overnight at 4°C with anti-BDNF monoclonal antibody in coating carbonate buffer. After washing with TBS-T buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.05% (v/v) Tween-20), plates were blocked with Block and Sample 1× Buffer (1× BSB) for 1 hour at room temperature. After TBS-T wash, sample supernatant (1×) or lysate (50×) were applied in triplicate to the plates (100 µl/well) and incubated for 2

hours at room temperature with shaking. After washing (TBS-T buffer), anti-human BDNF polyclonal antibody (1:500, 100 µl/well) was added and incubated for 2 hours at room temperature with shaking. After additional washing with TBS-T, anti-IgY HRP (1:200, 100 µl/well) was then added and incubated for 1 hour at room temperature with shaking, and wells were washed with TBS-T again. Finally, TMB One solution (100 µl/well) was added and incubated at room temperature with shaking for 15 minutes, after which the color reaction was stopped by 1 M HCl, and the absorbance at 450 nm was measured. BDNF concentrations were reported in units of pg/mL by normalization using BDNF standard curves obtained in each experiment, and relative concentrations compared to the control were calculated in every experiment.

3. Results

3.1. Activation of Wnt/β-catenin signaling in the spinal cord after sciatic nerve injury

We first addressed whether Wnt/β-catenin signaling in the spinal cord was altered by using a rodent model of neuropathic pain. We employed a widely accepted neuropathic pain model, the partial sciatic nerve ligation (PSL) model, and we confirmed that PSL successfully and reproducibly induced tactile allodynia. We observed that PSL caused microglial activation (Iba-1 staining) and C-fiber terminal loss (IB4 staining) in the injured side, confirming two typical histological features of this model (data not shown). We focused on dephosphorylated active β-catenin, a key downstream effector of the Wnt/β-catenin signaling pathway (Fig. 1A), and determined the expression of active β-catenin in the spinal cord on the second day following PSL treatment. This time point was chosen because PSL induced profound allodynia 1 day after treatment and symptoms were maximal on the third day. Western blotting showed significant upregulation of active β-catenin at the level of L4−L6 in the dorsal spinal cord of the injured side compared to the contralateral side (Fig. 1B, C).

Having shown that Wnt/β-catenin signaling was activated in the PSL model mice, we next examined whether Wnt3a, a prototypic Wnt ligand that activates the Wnt/β-catenin pathway, was upregulated in the spinal cord. Western blotting showed significant upregulation of Wnt3a in the dorsal spinal cord of the injured side compared to the contralateral side (Fig. 2A, B). This upregulation was also confirmed by immunohistochemistry (Fig. 2C). Wnt3a was mainly expressed by neurons (neuronal somata and dendrites) in the dorsal horn (Fig. 2C) and rarely by astrocytes and microglia (data not shown). Wnt3a was also upregulated in the ventral horn, and microglial activation (confirmed by Iba1 staining) was also detected in the same area (data not shown). Sciatic nerve ligation causes injury not only to afferent fibers but to efferent fibers, and cell bodies of these injured efferent fibers exist in the ventral horn, thus we thought it possible that Wnt3a upregulation and microglial activation in this area were the responses to the injury to their axons.

3.2. Wnt/β-catenin signaling is involved in the development of neuropathic pain

These results prompted us to examine whether Wnt/β-catenin signaling played a role in the development of neuropathic pain in this model. To address this, we performed intrathecal administration of the Wnt inhibitor XAV939 at the level of L3–5 to PSL model rats. XAV939 is a low molecular compound that selectively inhibits the Wnt/β-catenin pathway by stabilizing axin and promoting β-catenin phosphorylation/degradation through the inhibition of tankyrase (Fig. 1A) (Huang et al., 2009). Moreover, one recent study has provided evidence that XAV939 successfully inhibits Wnt/β-catenin signaling *in vivo* (Fancy et al., 2011). In sciatic nerve-ligated rats, continuous injection of the inhibitor (time-line is presented in Fig. 3A) resulted in drastic attenuation of the development of allodynia, as confirmed by the von Frey filament test (Fig. 3B). Importantly, XAV939 had no effect on the withdrawal threshold of the contralateral side, indicating that symptom relief was not due to

inappropriate desensitization. These results strongly support the hypothesis that enhanced Wnt/β-catenin signaling in the dorsal horn contributes to the development of neuropathic pain.

Next, we tried to assess whether the activation of Wnt/β-catenin signaling in the spinal cord would be enough to induce a neuropathic pain phenotype in the absence of nerve injury. For this purpose, we performed continuous intrathecal administration of recombinant Wnt3a protein to the intact rat at the level of L3–5 (time-line is presented in Fig. 4A). Soon after initiation of Wnt3a administration, animals began to develop allodynia; after 2 days, the extent of hypersensitivity reached almost the same level as observed in PSL model animals on the corresponding days (Fig. 4B).

3.3. Possible mechanism of neuropathic pain induced by Wnt/β-catenin signaling activation: Involvement of BDNF released from microglia

Next, we investigated the mechanism underlying pain development triggered by the activation of Wnt signaling. A growing body of evidence indicates that interactions between neurons and glia are critical in establishing neuropathic pain. In particular, microglia are considered to be a key player in these phenomena. Moreover, BDNF from spinal microglia has been shown to be critically involved in the pathogenesis of neuropathic pain. Consistent with previous reports, microglial activation was confirmed by immunohistochemistry (Fig. 5A). We also confirmed that BDNF concentrations in the dorsal horn were significantly increased in the affected side compared to the contralateral side of PSL spinal cords (Fig. 5B). Therefore, we were motivated to assess whether upregulated Wnt3a induces microglia to release BDNF. To address this question using *in vitro* methods, we applied recombinant Wnt3a protein to cultured MG5 cells, a microglial cell line established from the p53-KO mouse (Ohsawa et al., 1997). Consistent with previous reports showing Wnt receptor expression in microglial cells,

Wnt3a treatment induced active β-catenin accumulation in the nuclei of MG5 cells, and induced drastic morphological changes, suggesting microglial activation (Fig. 5C). These morphological changes were completed within 3 hours of treatment. Next, we tried to test the influence of Wnt3a on BDNF secretion from microglia. Three hours after addition of 0.1% BSA/PBS (control) or recombinant Wnt3a to the medium, the supernatant was collected, and the concentration of mature BDNF was evaluated by ELISA. As shown in Fig. 5D, BDNF secreted in the medium was significantly increased in the Wnt3a-stimulated group. These results suggest that Wnt3a triggers microglial activation and BDNF release from microglia, raising the possibility that the same signaling cascade plays a pivotal role in the development of neuropathic pain. To assess whether Wnt/β-catenin signaling is actually involved in the pathologic activation of microglia *in vivo*, we tested the effect of intrathecal administration of XAV939 to PSL animals. PSL surgery and XAV939 administration were performed in the same way as in Fig.3. At 3 days post injury, after confirming the effect of XAV939 treatment (attenuation of allodynia), animals were sacrificed for further analysis. As we expected, immunohistochemical analysis revealed that XAV939 treatment partially but significantly suppressed the activation of microglia in the dorsal horn (Fig. 5E, F, G and H).

4. Discussion

In the present study, we attempted to identify a new molecular target for developing of a disease-modifying treatment for neuropathic pain. We found that active β-catenin was upregulated in the dorsal horn in the PSL model of neuropathic pain; this suggested activation of Wnt/β-catenin pathway signaling. Moreover, Wnt3a, a prototypic ligand that activates this pathway, was also found to be upregulated in the dorsal horn in this model, as indicated by both western blotting and immunohistochemistry results. Very recently, other groups have confirmed upregulation of β-catenin and Wnt3a in the dorsal horn using different neuropathic pain models. Findings from the L5 spinal nerve ligation (SNL) model (Shi et al., 2012) and chronic constriction injury (CCI) model (Zhang et al., 2013) suggest that the activation of Wnt/β-catenin pathway in the spinal cord is a universal feature of neuropathic pain pathophysiology. Involvement of this pathway is substantiated by our finding that selective Wnt/β-catenin pathway inhibitor XAV939 (Huang et al., 2009) had an obvious inhibitory effect on the development of allodynia. Conversely, we observed that intrathecal administration of recombinant Wnt3a to the lumbar spinal cord was sufficient to induce allodynia. These results strongly support the notion that peripheral nerve injury-induced activation of Wnt signaling in the spinal cord may be critically involved in the pathophysiology of neuropathic pain.

Based on these observations, our next objective was to reveal the mechanism underlying the development of pain triggered by Wnt signaling activation. Although neuron-to-neuron signaling has long been considered the crucial interaction responsible for the pathophysiological changes underlying neuropathic pain, it is now widely accepted that within the spinal cord, local glia-neuron interactions also play key roles (Beggs et al., 2012; Scholz et al., 2007); in particular, the influence of microglia appears to be crucial (Tsuda et al., 2005; Trang et al., 2011). A recent report (Halleskog et al., 2011) have shown that various

Wnt receptor proteins are expressed by microglial cells (N13 cells and primary mouse microglia), and another work (Kilander et al., 2011) has demonstrated that Wnt proteins can activate β-catenin-dependent (and also β-catenin-independent) signaling in N13 cells, suggesting a possible interaction between Wnt signaling and microglia-mediated neuroinflammation. In fact, we confirmed that intrathecal administration of the Wnt/β-catenin pathway inhibitor significantly attenuated the pathological activation of microglia triggered by PSL as well as allodynia development, suggesting the interaction between Wnt signaling and the activation of microglial cells under pathological condition of neuropathic pain. Importantly, Wnt3a stimulation of primary microglial cells facilitated release of proinflammatory cytokines IL-6, IL-12, and TNFα (Halleskog et al., 2011). Since inflammatory mediators (e.g., IL-6, TNFα, and IL-1β) have been shown to contribute to the generation of neuropathic pain (Ellis et al., 2013), it is reasonable to assume that microglial activation by Wnt3a is responsible for the exacerbation of pain symptoms. Indeed, a recent work has revealed that CCI treatment or Wnt/β-catenin activation (by a Wnt agonist) increased proinflammatory cytokine expression in the spinal cord (Zhang et al., 2013). To advance our understanding of the interaction between microglia and Wnt/β-catenin signaling in the development of neuropathic pain, we focused on BDNF, which is a member of the neurotrophin family and is involved in various aspects of CNS development, maintenance, and plasticity. BDNF level in the spinal cord dorsal horn is increased in various nerve injury-induced neuropathic pain models, including the CCI model (Ha et al., 2001; Lu et al., 2009), SNL model (Ha et al., 2001; Geng et al., 2010), and sciatic nerve crush injury model (Cho et al., 1998). Moreover, microglial BDNF mediates central pathophysiological effects in the development of neuropathic pain (Tsuda et al., 2005; Trang et al., 2011; Scholz et al., 2007; Siniscalco et al., 2011). Most strikingly, intrathecal delivery of recombinant BDNF to the lumbar spinal cord rapidly induces allodynia by producing a depolarizing shift in the anion

reversal potential of spinal neurons (Coull et al., 2005), and on the other hand, it has also been reported that, by using PSL model, the induction of the pain-like behavior was drastically attenuated by intrathecal administration of specific anti-BDNF antibody or anti-TrkB antibody (Yajima et al., 2002). Consistent with previous findings, our results confirmed microglial activation and BDNF upregulation in the injured side of the dorsal horn in our model. Based on these evidences, we next assessed the effect of Wnt3a stimulation on microglial cells *in vitro* and showed that microglial cells were rapidly activated by Wnt3a stimulation. Moreover, we also revealed that Wnt3a application triggers BDNF release from microglial cells. Taken together, it is possible that Wnt3a upregulation in the dorsal horn leads to the activation of microglial cells, then triggers BDNF secretion that is responsible for the development of the neuropathic pain phenotype. Nevertheless, further studies will be needed for the comprehensive understanding of the roles of Wnt/β-catenin signaling in the development of neuropathic pain. Given that Wnt/β-catenin signaling is under intense investigation because of its critical involvement in the prognosis of various malignant neoplasms, it is expected that Wnt/β-catenin pathway inhibitors will be clinically available. Therefore, we believe that our findings will have practical implications for the therapeutic approach to neuropathic pain in the near future.

Figure legends

Figure 1. Activation of Wnt/β-catenin signaling in the spinal cord dorsal horn after PSL

(A) Schematic illustration of Wnt/β-catenin signaling. Wnt signaling allows β-catenin accumulation in the cytoplasm. XAV939, the Wnt signaling inhibitor used in this report, works through axin stabilization. (B) Representative active β-catenin immunoblots of spinal dorsal horn lysate prepared from a PSL model mouse. (C) Quantitative analysis of the blot. Band density of active β-catenin was normalized with that of the β-actin control band, and relative density in the injured side was compared to that in the contralateral side. Active β-catenin was significantly upregulated in the injured side. *$p < 0.05$, Welch's *t* test, $n = 4$.

Figure 2. Upregulated expression of Wnt3a in the spinal cord dorsal horn after PSL

(A) Representative Wnt3a immunoblots of spinal dorsal horn lysate prepared from a PSL model mouse. (B) Quantitative analysis of the blot. Band density of Wnt3a was normalized with that of the β-actin control band, and relative density compared to the contralateral side was assessed. Wnt3a was significantly upregulated in the ipsilateral side. *$p < 0.05$, Welch's *t* test, $n = 5$. (C) (a) Immunofluorescent staining of Wnt3a showing increased expression in the injured side of the dorsal spinal cord. Scale bar, 200 μm. (a') Higher power image of dashed line frame in (a). (b), (b') and (c), (c') Wnt3a signal is mainly overlaps with NeuN and MAP2. Scale bar, 10 μm.

Figure 3. Intrathecal administration of Wnt/β-catenin signaling inhibitor attenuates mechanical allodynia

(A) Schematic time-course for catheter insertion, PSL surgery, osmotic pump implantation, and behavioral testing. (B) Hind paw withdrawal threshold for mechanical stimulation by von Frey filaments measured before (pre) and 2, 3, 4, and 5 days after PSL treatment (n = 7, vehicle group; n = 11, XAV939 group). Data are represented as mean ± SEM. *$p < 0.05$ versus vehicle group at corresponding time point, $^{\#}p < 0.01$ versus vehicle group (two-way ANOVA followed by Tukey–Kramer test).

Figure 4. Intrathecal administration of Wnt3a induces allodynia

(A) Schematic time-course for catheter insertion, osmotic pump implantation, and behavioral testing. (B) Hind paw withdrawal threshold for mechanical stimulation by von Frey filaments measured before (pre) and 12, 24, and 48 hours after beginning of the intrathecal administration (n = 5, control group; n = 4, Wnt3a group). Data are represented as mean ± SEM *$p < 0.05$, **$p < 0.01$ versus vehicle group at corresponding time point, $^{\#}p < 0.01$ versus vehicle group (two-way ANOVA followed by Tukey–Kramer test).

Figure 5. Wnt3a stimulation triggers BDNF secretion from microglia

(A) Transverse section of the lumbar spinal cord after PSL injury showing microglial activation in the injured side. Scale bar, 100 μm. (B) Increase in the concentration of BDNF (detected by ELISA) in the injured side of the dorsal horn after PSL. **$p < 0.01$, student's *t* test, n = 6. (C) Confocal images of MG5 cells treated with Wnt3a (100 ng/ml) or 0.1% BSA for 3 hours. Wnt3a stimulation induced morphological changes suggesting microglial activation (upper, original magnification; ×100) and induced β-catenin stabilization and its accumulation to the nucleus (lower, original magnification; ×400). (D) After Wnt3a

stimulation (100 ng/ml, 3 hours), BDNF concentration in the supernatant of MG5 cells was evaluated by ELISA. BDNF levels were increased significantly by Wnt3a stimulation (n = 3), *p < 0.05 (student's *t* test). (E–H) Immunofluorescent staining of Iba1 (E) and CD68 (G) showing the inhibitory effect of XAV939 on the activation of microglia induced by PSL. Scale bar, 30 μm. (F) Quantitative analysis of the immunofluorescence of Iba1 staining shown in (E). Data are represented as mean ± SEM *p < 0.05, student's t test, n = 3. (H) Quantitative analysis of the immunofluorescence of CD68 staining shown in (G). Data are represented as mean ± SEM **p < 0.01, student's t test, n = 3.

Acknowledgments

We thank Jin X for material contributions to this work (MG5 and A1 cells). We are also grateful to Dr. M. Fujitani and Dr. A. Kikuchi at Osaka University and M. Ueno at Cincinnati Children's Hospital for their helpful comments. This work was supported by a Grant-in-Aid for Scientific Research (S) from JSPS (25221309) to T.Y.

References

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D., 2009. Cellular and molecular mechanisms of pain. Cell 139, 267-284

Beggs, S., Trang, T., Salter, M.W., 2012. P2X4R+ microglia drive neuropathic pain. Nat. Neurosci. 15, 1068-1073

Busceti, C.L., Biagioni, F., Aronica, E., Riozzi, B., Storto, M., Battaglia, G., Giorgi, F.S., Gradini, R., Fornai, F., Caricasole, A., Nicoletti, F., Bruno, V., 2007. Induction of the Wnt inhibitor, Dickkopf-1, is associated with neurodegeneration related to temporal lobe epilepsy. Epilepsia 48, 694-705

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55-63

Cho, H.J., Kim, J.K., Park, H.C., Kim, J.K., Kim, D.S., Ha, S.O., Hong, H.S., 1998. Changes in brain-derived neurotrophic factor immunoreactivity in rat dorsal root ganglia, spinal cord, and gracile nuclei following cut or crush injuries. Exp. Neurol. 154, 224-230

Ciani, L., Salinas, P.C., 2005. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat. Rev. Neurosci. 6, 351-362

Clevers, H., Nusse, R., 2012. Wnt/β-catenin signaling and disease. Cell 149, 1192-1205

Coull, J.A., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M.W., De, Koninck, Y., 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438, 1017-1021

Ellis, A., Bennett, D.L., 2013. Neuroinflammation and the generation of neuropathic pain. Br.

J. Anaesth. 111, 26-37

Fancy, SP., Harrington, E.P., Yuen, T.J., Silbereis, J.C., Zhao, C., Baranzini, S.E., Bruce, C.C., Otero, J.J., Huang, E.J., Nusse, R., Franklin, R.J., Rowitch, D.H., 2011. Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination. Nat. Neurosci. 14, 1009-1016

Geng, S.J., Liao, F.F., Dang, W.H., Ding, X., Liu, X.D., Cai, J., Han, J.S., Wan, Y., Xing, G.G., 2010. Contribution of the spinal cord BDNF to the development of neuropathic pain by activation of the NR2B-containing NMDA receptors in rats with spinal nerve ligation. Exp. Neurol. 222, 256-266

Ha, S.O., Kim, J.K., Hong, H.S., Kim, D.S., Cho, H.J., 2001. Expression of brain-derived neurotrophic factor in rat dorsal root ganglia, spinal cord and gracile nuclei in experimental models of neuropathic pain. Neuroscience 107, 301-309

Halleskog, C., Mulder, J., Dahlström, J., Mackie, K., Hortobágyi, T., Tanila, H., Kumar, Puli. L., Färber, K., Harkany, T., Schulte, G., 2011. WNT signaling in activated microglia is proinflammatory. Glia 59, 119-131

Huang, S.M., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C.J., Mickanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H., Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M.W., Lengauer, C., Finan, P.M., Tallarico, J.A., Bouwmeester, T., Porter, J.A., Bauer, A., Cong, F., 2009. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461, 614-620

Kikuchi, A., Yamamoto, H., Sato, A., Matsumoto, S., 2011. New insights into the mechanism of Wnt signaling pathway activation. Int. Rev. Cell Mol. Biol. 291, 21-71

Kilander, M.B., Halleskog, C., Schulte, G., 2011. Recombinant WNTs differentially activate β-catenin-dependent and -independent signalling in mouse microglia-like cells. Acta Physiol. 203, 363-372

L'Episcopo, F., Tirolo, C., Testa, N., Caniglia, S., Morale, M.C., Cossetti, C., D'Adamo, P., Zardini, E., Andreoni, L., Ihekwaba, A.E., Serra, P.A., Franciotta, D., Martino, G., Pluchino, S., Marchetti, B., 2011. Reactive astrocytes and Wnt/β-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. Neurobiol. Dis. 41, 508-527

Lu, V.B., Biggs, J.E., Stebbing, M.J., Balasubramanyan, S., Todd, K.G., Lai, A.Y., Colmers, W.F., Dawbarn, D., Ballanyi, K., Smith, P.A., 2009. Brain-derived neurotrophic factor drives the changes in excitatory synaptic transmission in the rat superficial dorsal horn that follow sciatic nerve injury. J. Physiol. 587, 1013-1032

Maeda, Y., Aoki, Y., Sekiguchi, F., Matsunami, M., Takahashi, T., Nishikawa, H., Kawabata, A., 2009. Hyperalgesia induced by spinal and peripheral hydrogen sulfide: evidence for involvement of Cav3.2 T-type calcium channels. Pain 142, 127-132

Marchetti, B., Pluchino, S., 2013. Wnt your brain be inflamed? Yes, it Wnt! Trends Mol. Med. 19, 144-156

Mastroiacovo, F., Busceti, C.L., Biagioni, F., Moyanova, S.G., Meisler, M.H., Battaglia, G., Caricasole, A., Bruno, V., Nicoletti, F., 2009. Induction of the Wnt antagonist, Dickkopf-1, contributes to the development of neuronal death in models of brain focal ischemia. J. Cereb. Blood Flow Metab. 29, 264-276

Milligan, E.D., Hinde, J.L., Mehmert, K.K., Maier, S.F., Watkins, L.R., 1999. A method for increasing the viability of the external portion of lumbar catheters placed in the spinal

subarachnoid space of rats. J. Neurosci. Methods 90, 81-86

Navarro, X., Vivó, M., Valero-Cabré, A., 2007. Neural plasticity after peripheral nerve injury and regeneration. Prog. Neurobiol. 82, 163-201

Ohsawa, K., Imai, Y., Nakajima, K., Kohsaka, S., 1997. Generation and characterization of a microglial cell line, MG5, derived from a p53-deficient mouse. Glia 21, 285-298

Okerlund, N.D., Cheyette, B.N., 2011. Synaptic Wnt signaling - a contributor to major psychiatric disorders? J. Neurodev. Disord. 3, 162-174

Park, M., Shen, K., 2012. WNTs in synapse formation and neuronal circuitry. EMBO J. 31, 2697-2704

Salinas, P.C., 2012. Wnt signaling in the vertebrate central nervous system: from axon guidance to synaptic function. Cold Spring Harb. Perspect. Biol. 4, a008003

Scholz, J., Woolf, C.J., 2007. The neuropathic pain triad: neurons, immune cells and glia. Nat. Neurosci. 10, 1361-1368

Seltzer, Z., Dubner, R., Shir, Y., 1990. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 43, 205-218

Shi, Y., Yuan, S., Li, B., Wang, J., Carlton, S.M., Chung, K., Chung, J.M., Tang, S.J., 2012. Regulation of Wnt signaling by nociceptive input in animal models. Mol. Pain 8, 47

Shruster, A., Eldar-Finkelman, H., Melamed, E., Offen, D., 2011. Wnt signaling pathway overcomes the disruption of neuronal differentiation of neural progenitor cells induced by oligomeric amyloid β-peptide. J. Neurochem. 116, 522-529

Siniscalco, D., Giordano, C., Rossi, F., Maione, S., de Novellis, V., 2011. Role of

neurotrophins in neuropathic pain. Curr. Neuropharmacol. 9, 523-529

Trang, T., Beggs, S., Salter, M.W., 2011. Brain-derived neurotrophic factor from microglia: a molecular substrate for neuropathic pain. Neuron Glia Biol. 7, 99-108

Tsuda, M., Inoue, K., Salter, M.W., 2005. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. Trends Neurosci. 28, 101-107

Woolf, C.J., Shortland, P., Coggeshall, R.E., 1992. Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355, 75-78

Yajima, Y., Narita, M., Narita, M., Matsumoto, N., Suzuki, T., 2002. Involvement of a spinal brain-derived neurotrophic factor/full-length TrkB pathway in the development of nerve injury-induced thermal hyperalgesia in mice. Brain Res. 958, 338-346

Zhang, Q.G., Wang, R., Khan, M., Mahesh, V., Brann, D.W., 2008. Role of Dickkopf-1, an antagonist of the Wnt/beta-catenin signaling pathway, in estrogen-induced neuroprotection and attenuation of tau phosphorylation. J. Neurosci. 28, 8430-8441

Zhang, Y.K., Huang, Z.J., Liu, S., Liu, Y.P., Song, A.A., Song, X.J., 2013. WNT signaling underlies the pathogenesis of neuropathic pain in rodents. J. Clin. Invest. 123, 2268-22

Figure 1

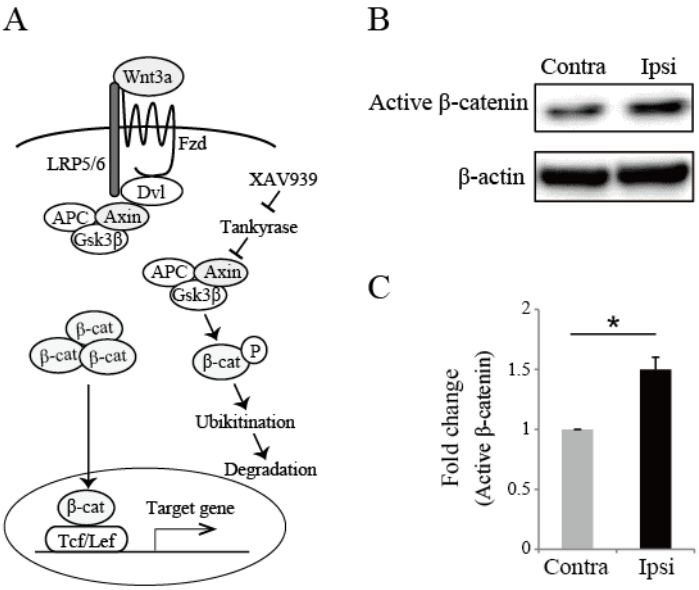

A
Wnt3a
Fzd
LRP5/6
Dvl
XAV939
APC
Axin
Gsk3β
Tankyrase
β-cat
P
Ubikitination
Degradation
Target gene
Tcf/Lef
B
Contra
Ipsi
Active β-catenin
β-actin
C
Fold change
(Active β-catenin)
2
1.5
1
0.5
0
*
Contra
Ipsi

Figure 2

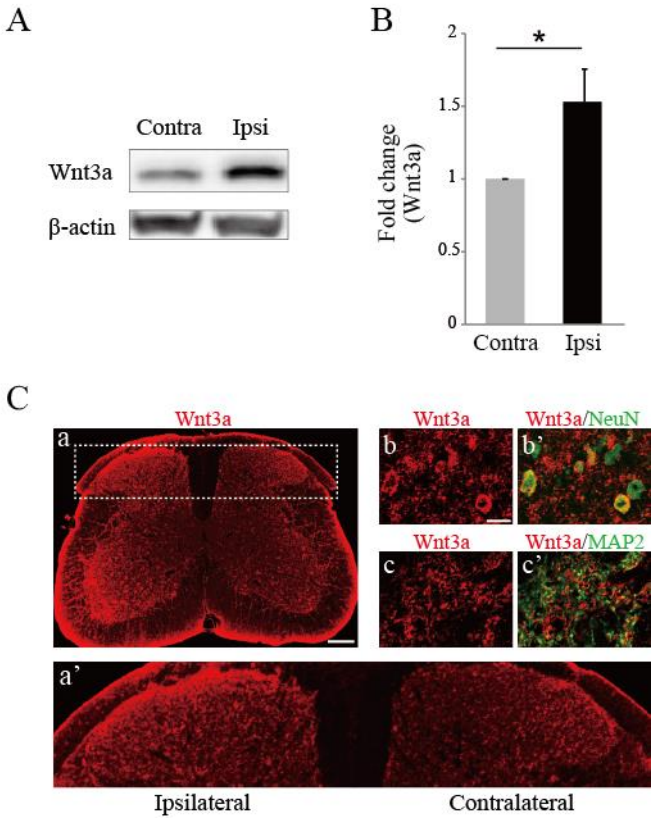

A
Contra
Ipsi
Wnt3a
β-actin
B
*
Fold change (Wnt3a)
2
1.5
1
0.5
0
Contra
Ipsi
C
Wnt3a
a
Wnt3a
Wnt3a/NeuN
b
b'
Wnt3a
Wnt3a/MAP2
c
c'
a'
Ipsilateral
Contralateral

Figure 3

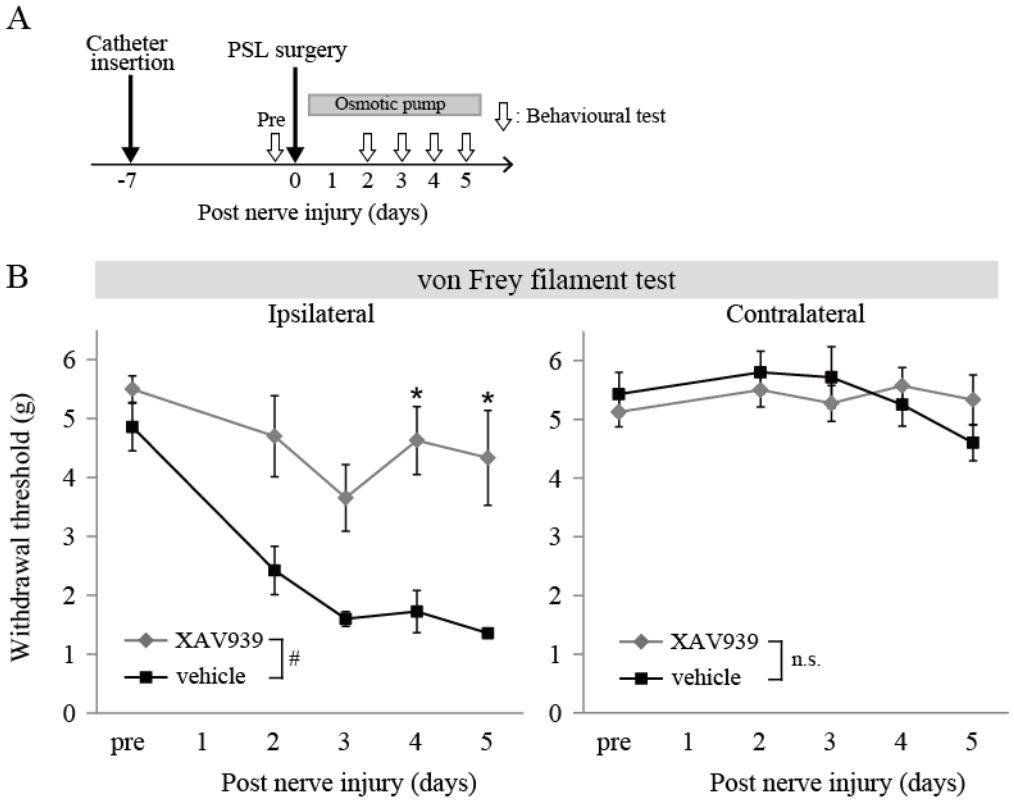

A
Catheter insertion
PSL surgery
Pre
Osmotic pump
: Behavioural test
-7
0 1 2 3 4 5
Post nerve injury (days)
B
von Frey filament test
Ipsilateral
Contralateral
Withdrawal threshold (g)
XAV939
vehicle
#
n.s.
*
*
pre 1 2 3 4 5
Post nerve injury (days)

Figure 4

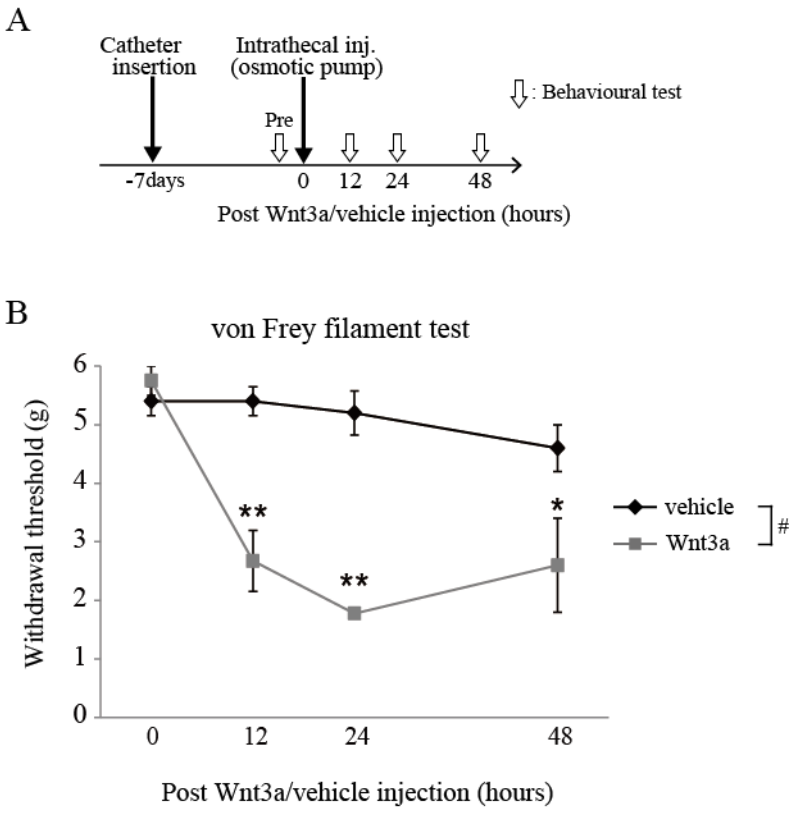
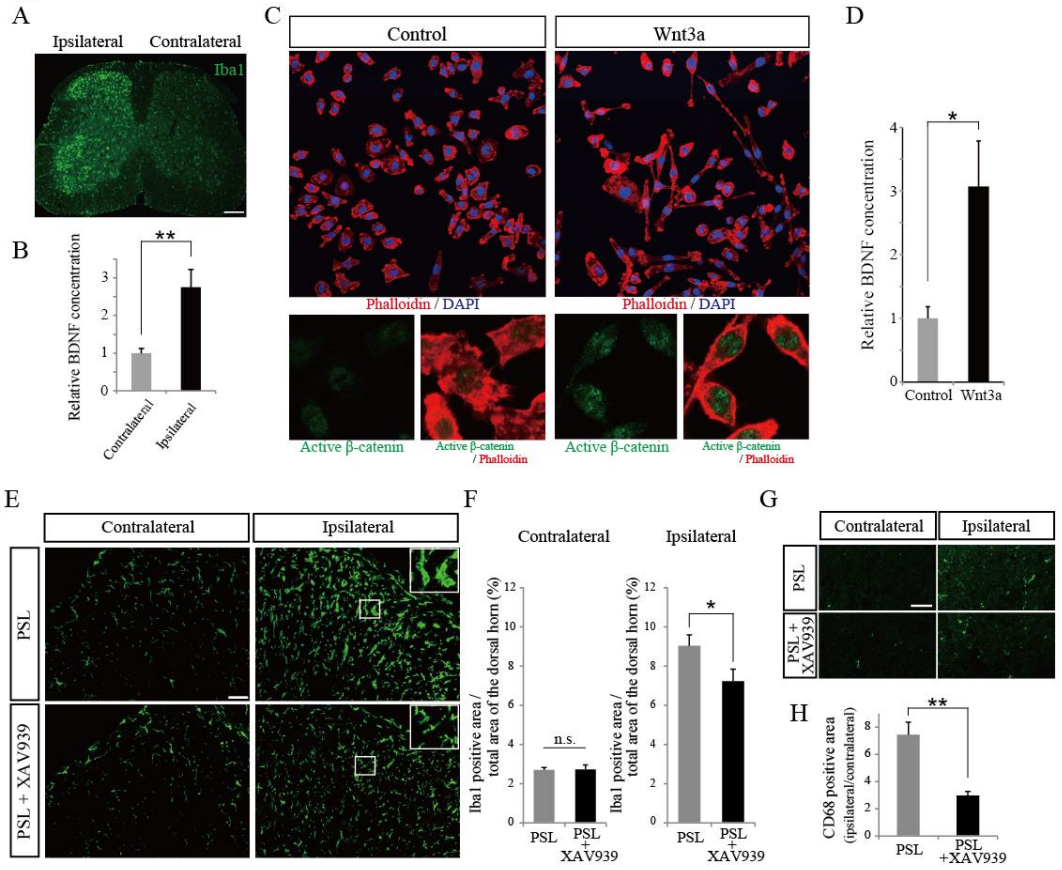

A
Catheter insertion
Intrathecal inj. (osmotic pump)
: Behavioural test
Pre
-7days
0
12
24
48
Post Wnt3a/vehicle injection (hours)
B
von Frey filament test
Withdrawal threshold (g)
6
5
4
3
2
1
0
**
**
*
vehicle
Wnt3a
#
0
12
24
48
Post Wnt3a/vehicle injection (hours)

Figure 5


A
Ipsilateral
Contralateral
Iba1
B
Relative BDNF concentration
**
Contralateral
Ipsilateral
C
Control
Wnt3a
Phalloidin / DAPI
Active β-catenin
Active β-catenin / Phalloidin
D
Relative BDNF concentration
*
Control
Wnt3a
E
Contralateral
Ipsilateral
PSL
PSL + XAV939
F
Iba1 positive area / total area of the dorsal horn (%)
n.s.
*
PSL
PSL + XAV939
G
PSL
PSL + XAV939
H
CD68 positive area (ipsilateral/contralateral)
**
PSL
PSL +XAV939