Supplementary Information

Pain without Nociceptors: Nav1.7-independent pain mechanisms

Michael S. Minett, Sarah Falk, Sonia Santana-Varela, Yury D. Bogdanov, Mohammed A. Nassar, Anne-Marie Heegaard and John N. Wood

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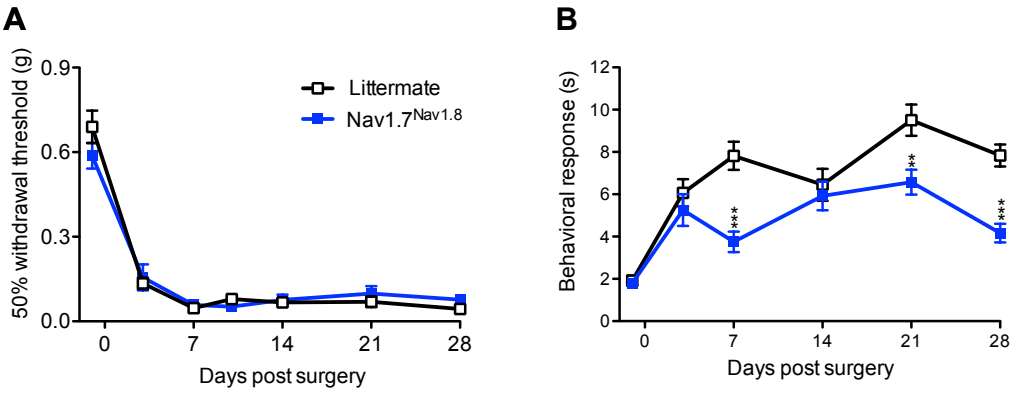

Figure S1: **Nav1.7$^{Nav1.8}$ mice show normal mechanical allodynia but attenuated cold allodynia following partial nerve ligation surgery.** Nav1.7$^{Nav1.8}$ mice (blue squares, n=10) develop partial nerve ligation induced mechanical allodynia (A) and but not cold allodynia (B) in comparison to littermate mice (white squares, n=13). Data analysed by two-way analysis of variance followed by the Bonferroni post hoc test. Results are presented as mean ± S.E.M. ** $P < 0.01$ and *** $P < 0.001$ (individual points). Related to Figure 1.

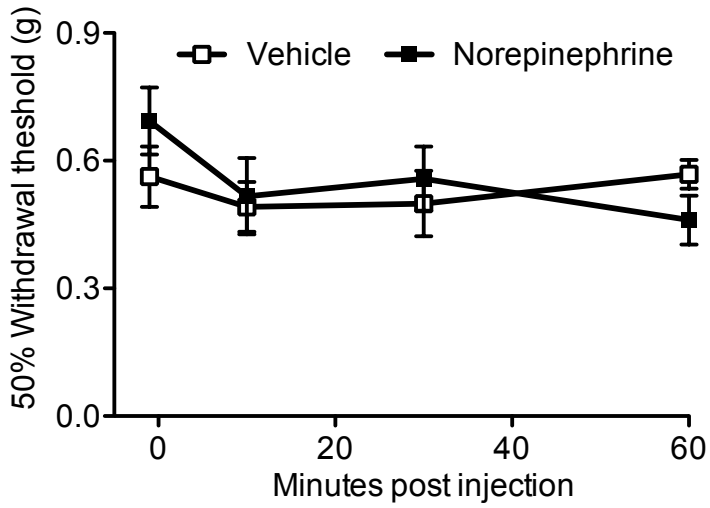

Figure S2: **Intraplantar injection of norepinephrine does not induce mechanical allodynia in naïve mice.** Behavioral responses of C57/black6 mice to von Frey hairs applied to the plantar surface of the hindpaw are not altered following intraplantar injection of 200ng norepinephrine (n=12) when compared to vehicle alone (n=12). Data analysed by two-way analysis of variance followed by the Bonferroni post hoc test. Results are presented as mean ± S.E.M. Related to Figure 2.

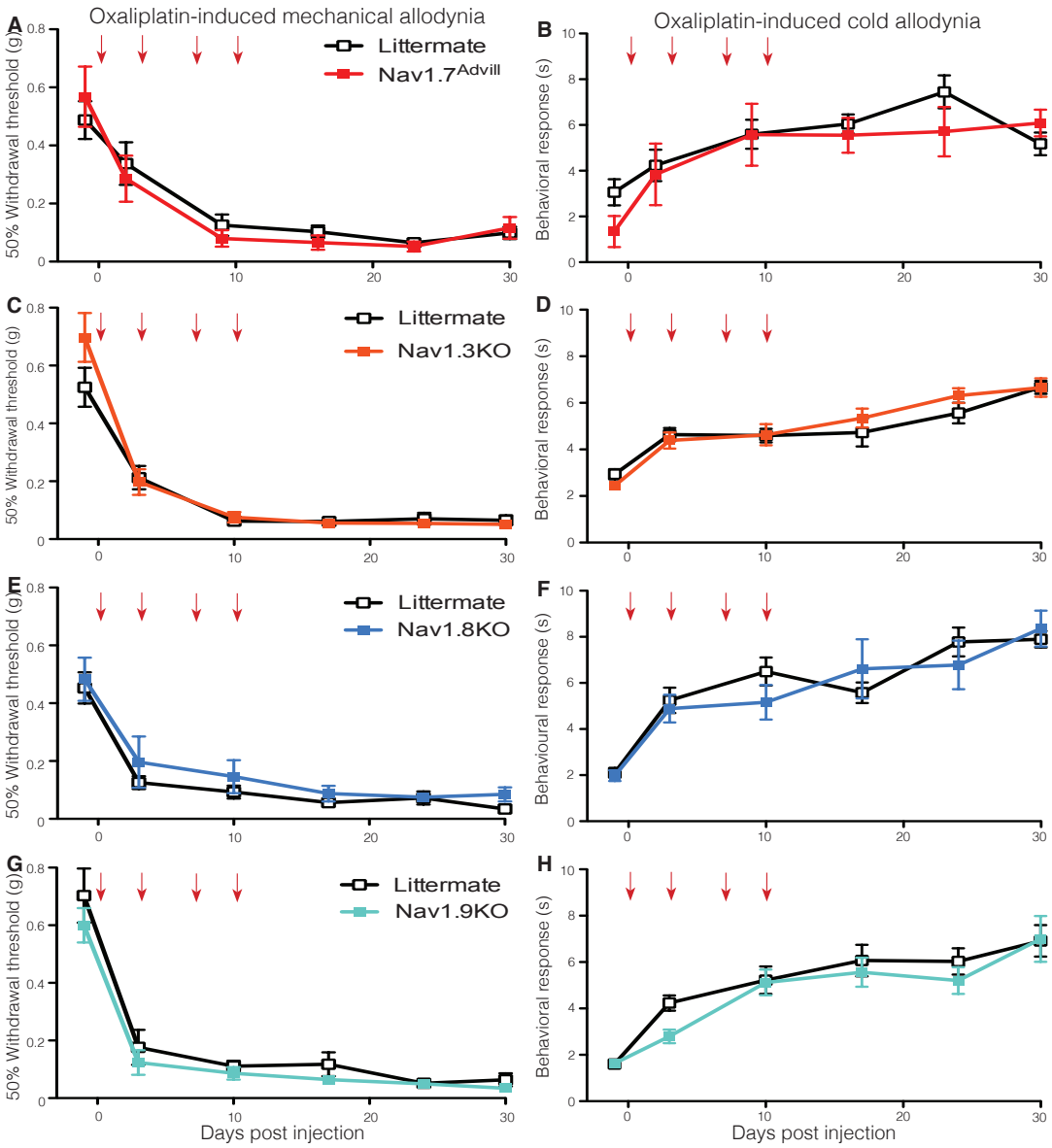

Figure S3: **Oxaliplatin-induced mechanical and cold allodynia does not require Nav1.7, Nav1.3, Nav1.8 or Nav1.9 expression, whilst mice do not develop mechanical or cold allodynia following induction of bone cancer.** Mice treated twice weekly (red arrows) with 3.5 mg/kg oxaliplatin (i.v.) develop both mechanical and cold allodynia. (A&B) Nav1.7^{Advill} mice (red squares, n=6) and littermate mice (white squares, n=12). (C&D) Nav1.3KO mice (orange squares, n=12) and littermate mice (white squares, n=12). (E&F) Nav1.8KO mice (blue squares, n=7) and littermate mice (white squares, n=9). (G&H) Nav1.9KO (turquoise squares, n=9) and littermate mice (white squares, n=6). Data analysed by two-way analysis of variance followed by the Bonferroni post hoc test. Results are presented as mean ± S.E.M. Related to Figure 4.

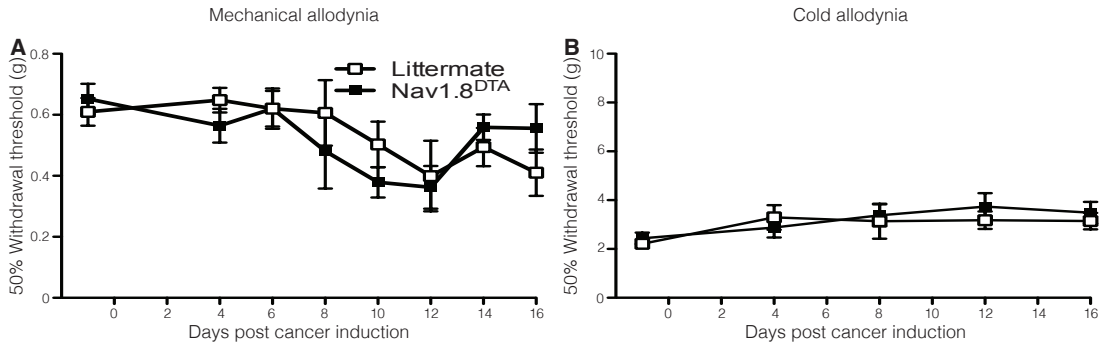

Figure S4: **Mice do not develop mechanical or cold allodynia following induction of bone cancer.** Behavioral responses of Nav1.8^{DTA} mice (black squares, n=9) and littermate mice (white squares, n=8) show that, in line with existing literature, neither the von Frey test (A) or the acetone test (B) are useful measures of cancer-induced bone pain. Data analysed by two-way analysis of variance followed by the Bonferroni post hoc test. Results are presented as mean ± S.E.M. Related to Figure 4.

Extended Experimental Procedures

Animals

All tests were approved by the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Experiments were conducted using both male and female wildtype littermate and knockout mice, all of which were at least 6 weeks old when tested. Observers who performed behavioral experiments were blind to the genotype of the animals. The production of the following transgenic mice was documented, respectively, in following articles; Nav1.8-Cre mice (Stirling et al., 2005), Advillin-Cre mice (Minett et al., 2012; Zhou et al., 2010), Wnt1-Cre mice (Danielian et al., 1998), Nav1.8^{DTA} mice (Abrahamsen et al., 2008), Nav1.3 global knockout mice (Nassar et al., 2006), floxed Nav1.7 mice (Nassar et al., 2004), Nav1.8 global knockout mice (Akopian et al., 1999) and Nav1.9 global knockout mice (Östman et al., 2008).

Genotyping

Genomic DNA was isolated from ear or dorsal root ganglia. The genotyping was then determined using Polymerase Chain Reactions (PCR). Reactions consisted of 1ng of template DNA, 0.4µM of each primer, 11µl DreamTaq (Fermentas) and 11µl H_2O. The following PCR conditions were used:

- Initial denaturing step: 94°C – 2 minutes

1) Cycling denaturing step: 94°C – 30 seconds
2) Cycling annealing step: 65°C (adjusted where necessary) – 30 seconds
3) Cycling extension step: 72°C – 60 seconds

- Steps 1, 2 & 3 cycled 30 times (adjusted where necessary)
- Final extension: 72°C – 2 minutes

Reactions were carried out in a PCT-220 DNA Dyad (MJ research) machine and resolved on a 1% agarose (Invitrogen) gel. Primer sequences can be found in Table 1. DNA modifications to were detected with the following primer pairs and expected band sizes:

Cre recombines

Nav1.8-Cre

- Wildtype fragment (460bp): primers Nav1.8 Cre 12a & 13s
- Cre fragment (420bp): primers Nav1.8 Cre 5a & 13s

Advillin-Cre/Advillin-CreERT2

- Advillin wildtype fragment (480bp): primers *Advillin* 1 & 2
- Advillin-Cre fragment (180bp): primers *Advillin* 1 & 3

Wnt1-Cre

- Wnt1-Cre fragment (629bp): primers *Wnt1* Forward & *Wnt1* Reverse

Voltage Gated Sodium Channels
Nav1.7 floxed

- Wildtype (317bp) and floxed fragment (461bp): primers *SCN9A* Ex.16 & 17
- Knockout fragment (395bp): primers *SCN9A* Ex.16 & 18

Nav1.3 KO

- Wildtype fragment (212bp): primers Seq26a & Seq27s
- Knockout fragment (572bp): primers Seq26a & Seq28s

Nav1.8 KO

- Wildtype fragment (1100bp): primers G_G3 & MEX 4
- Knockout fragment (900bp): primers G_G3 & Neo_SL

Nav1.9 KO

- Wildtype fragment (450bp): primers Nav1.9s & Nav1.9a
- Knockout fragment (600bp): primers Nav1.9s & Nav1.9neo

Table 1: **Genomic primer sequences**

Primer name	Sequence (5'-3')	Description
Advillin Cre - 1	CCCTGTTCACTGTGAGTAGG	Wildtype forward
Advillin Cre - 2	AGTATCTGGTAGGTGCTTCCAG	Wildtype reverse
Advillin Cre - 3	GCGATCCCTGAACATGTCCATC	Cre reverse
DTA - R26R1	AAAGTCGCTCTGAGTTGTTAT	Rosa forward
DTA - R26R2	GCGAAGAGTTTGTCCTCAACC	DTA reverse
DTA - R26R3	GGAGCGGGAGAAATGGATATG	Rosa reverse
Nav1.3 – Seq. 26	GAGAGAAAGACACTTAAATGCAGACAT	Wildtype forward
Nav1.3 - Seq. 27	GCTTTTTGTTCAAGTCTATCATATTCAA	Wildtype reverse
Nav1.3 - Seq. 28	AAGGATGGCATCACCCACAAG	Knockout reverse
Nav1.7 - Seq. 16	CAGAGATTTCTGCATTAGAATTTGTTC	*SCN9A* forward
Nav1.7 - Seq. 17	AGTCTTTGTGGCACACGTTACCTC	Floxed reverse
Nav1.7 - Seq. 18	GTTCCTCTCTTTGAATGCTGGGCA	Knockout reverse
Nav1.8 - G_G3	GAGTGATGCATATGATGTCAT	Wildtype forward
Nav1.8 - MEX4	GCCTTCACTGTTGTTTACACCT	Wildtype reverse
Nav1.8 - NEO_SL	GCAGCGCATCGCCTTCTATC	Knockout reverse
Nav1.8 Cre - 12a	TTACCCGGTGTGTGCTGTAGAAAG	Wildtype reverse
Nav1.8 Cre - 13s	TGTAGATGGACTGCAGAGGATGGA	Wildtype forward
Nav1.8 Cre - 5a	AAATGTTGCTGGATAGTTTTTACTGCC	Cre reverse
Nav1.9 - Antisense	AACAGTCTTACGCTGTTCCGATG	Wildtype reverse
Nav1.9 - Neo	CTCGTCGTGACCCATGGCGAT	Knockout reverse
Nav1.9 - Sense	ATGTGGCACTGGGCTTGAACTC	Wildtype forward
Wnt1 Cre - Forward	ATCCGAAAAGAAAACGTTGA	Wnt1 forward
Wnt1 Cre - Reverse	ATCCAGGTTACGGATATAGT	Wnt1 reverse

Tissue dissection and preparation

Adult mice were terminally anaesthetised via intraperitoneal (i.p.) injection of sodium pentobarbitone (150mg/kg) (Rhône Mérieux). Once the animal became unresponsive to painful stimuli, the thoracic cavity was opened. The right atrium was punctured, and the animal perfused, via the left ventricle, with 10ml heparinized saline (0.9% w/v NaCl) followed by 20mls of ice-cold 4% paraformaldehyde (BDH) in PBS (pH 7.4). The DRG were post-fixed in the same fixative solution for 2 hours at 4°C and then cryoprotected overnight in 30% w/v sucrose containing 0.02% sodium azide in 0.1M PB at 4°C. These perfused DRG were embedded in OCT embedding compound (BDH) and set on dry ice, then stored at -80°C ready for sectioning.

Tissue sectioning

Tissues were sectioned using a cryostat (Bright). 10µm DRG and 20µm spinal cord sections were mounted on electrostatically charged slides (Superfrost Plus, BDH) and left to dry for around 30 min at room temperature. For non-fixed sections, the slides were placed in ice-cold 4% PFA for 5-10 minutes.

Immunocytochemistry

Tissues were simply washed in PBS + 0.3% Trition X (PBST). Slides were then blocked in PBST +10% goat serum for 1 hour at room temperature, then incubated in the primary antibody, diluted (Table 2) in the PBST +10% goat serum, overnight at 4°C. After washing in PBST, bound primary antibodies were detected by incubating with the secondary antibody (Table 3) at room temperature for 2 hours. The slides were then washed in PBST and mounted using aqueous mounting solution.

Table 2: **Primary antibodies**

Antibody	Description	Dilution
N52	IgG mouse monoclonal anti-neurofilament 200 (Sigma, UK, N0142)	1:1000
Tyrosine Hydroxylase	IgG roabbit ploYclonal anti- Tyrosine Hydroxylase (Millipore, UK, AB152)	1:500

Table 3: **Secondary antibodies**

Antibody	Description	Dilution
Alexa594	Monoclonal goat anti rabbit IgG (Invitrogen, UK, 11037	1:1000
Alexa488	Monoclonal goat anti mouse IgG (Invitrogen, UK, A11017)	1:1000

Quantification of sympathetic sprouting

Tissue samples were visualised using a Leica DMRB microscope, a Hamamatsu ORCA-R^2 digital camera and HCImage 2.0.1.16 software. Images were captured in monochrome and pseudo-coloured using HCImage. ImageJ64 1.47a analysis software (NIH) was used to quantify sympathetic axons. To generate innervation density data, the total area of DRG cell layer, excluding axonal tracts, was measured. Following this, the length of TH-positive axons within the marked area was measured. A reference image with known grid size was used to calculate units.

Chemical Sympathectomy

6-OHDA (Sigma) was dissolved in sterile saline containing 0.01% (w/v) ascorbic acid (vehicle) and was injected i.p. at a concentration of 200mg/kg. Control mice received an equivalent volume of vehicle alone. To verify the effectiveness of this treatment in depleting norepinephrine, spleens were collected and analyzed for the presence of norepinephrine by liquid chromatography–mass spectrometry.

Intraplantar norepinephrine injections

Norepinephrine (Sigma) was dissolved in sterile saline containing 0.01% (w/v) ascorbic acid (vehicle) and 200ng was injected i.p. Control mice received an equivalent volume of vehicle alone.

Mouse pain behavioural tests

Mechanical allodynia was measured by applying von Frey hairs to the plantar surface of the hindpaw using the up-down method to obtain a 50% threshold (Chaplan et al., 1994). Cold allodynia was measured using the acetone test were behavioral responses are timed following application of ~200µl to the plantar surface of the hindpaw (Bautista et al., 2006).

Spinal nerve transection of the 5th lumbar vertebrae

Animals were anaesthetised with isoflurane before a midline incision was made in the skin of the back at the L2–S2 levels and the left paraspinal muscles separated from the spinal processes, facet joints and transverse processes at the L4–S1 levels. A 2mm section was removed from the left L5 spinal nerve. The muscle and skin were closed in layers, using 3-0 sutures and surgical staples (Mabuchi et al., 2003; 2004; Minett et al., 2011). Thermal and mechanical thresholds were then monitored up to 28 days post-surgery (Malmberg and Basbaum, 1998; Minett et al., 2011).

Chronic constriction injury

Animals were anaesthetised with isofluorene before an incision was made in the skin in line with the left femur. The left sciatic nerve was exposed at mid-thigh level through blunt dissection. Three loose ligatures using 8-0 sutures (Ethicon) were made around the sciatic nerve (Bennett and Xie, 1988; Minett et al., 2011). The muscle and skin were closed in layers, using 3-0 sutures and surgical staples. Thermal and mechanical thresholds were then monitored up to 28 days post-surgery (Malmberg and Basbaum, 1998; Minett et al., 2011).

Partial Nerve Ligation

Animals were anaesthetised with isoflurane before an incision was made in the skin in line with the left femur. The left sciatic nerve was exposed at mid-thigh level through blunt dissection. A tight ligature around approximately 1/3 to 1/2 the diameter of the sciatic nerve was tied using 8-0 sutures (Ethicon) (Seltzer et al., 1990; Minett et al., 2011). The muscle and skin were closed in layers, using 3-0 sutures and surgical staples. Thermal and mechanical thresholds were recorded at baseline and up to 28 days post surgery (Minett et al., 2011).

Oxaliplatin

Oxaliplatin (Sigma) was dissolved and diluted in 5% glucose solution (vehicle) and was administered intravenously by tail vein injection (3.5 mg/kg). Mice received a total of 4 injections separated by 3 then 4 days (Renn et al., 2011; Stirling et al., 2005). Thermal and

mechanical thresholds were then measured periodically throughout the treatment period (Minett et al., 2011; 2012; Zhou et al., 2010).

Metastatic bone cancer

Cell line

Syngeneic LL/2 Lewis lung carcinoma cells (Sigma) were cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, 1% Penicillin-Streptomycin. Cancer cells were split two days prior to surgery and on the day of surgery harvested with 0.25% trypsin-EDTA and resuspended in DMEM medium to a final concentration of $2x10^7$ cells/ml. All cancer cells were kept on ice until use.

Bone cancer surgery:

The bone cancer was introduced as previously described (Clohisy et al., 1996) with a few modifications. Briefly, Animals were anaesthetised with isoflurane and an incision was made in the skin overlying the right patella. The lateral site of the patella tendon and lateral retinaculum tendon were loosened and the patella pushed aside to expose the distal femoral epiphysis (Falk et al., 2013). A 30-gauge needle was used to drill a hole into the medullary cavity through which $2x10^5$ carcinoma cells in 10µl DMEM medium were inoculated with a 0.5 ml insulin syringe. The hole was closed with dental cement (IRM Intermediate Restorative Material, Dentsply) and the wound thoroughly irrigated with sterile saline. The skin was sutured with 8-0 suture (Ethicon), and Xylocaine spray (2% w/v) applied to the wound. Sham-operated control mice underwent the same surgery, but were inoculated with DMEM medium alone.

Spontaneous and movement-evoked nociception:

Limb use: The mouse was allowed to move freely around in a transparent standard cage without bedding (280mm × 350mm × 185mm). Following 10 min of acclimation, the animal was observed for 2 min and a limb use score from 4 to 0 was assigned to the gait of the operated hind limb as follows: 4: normal use of hind limb, 3: insignificant limping, 2: significant limping, 1: significant limping and partial lack of limb use and 0: total lack of limb use.

Weight bearing: Weight-bearing deficit was measured using an Incapacitance Meter (IITC Life Science Inc.). The mouse was placed with the hind legs on two separate scales and the individual load of each hind limb was measured for 10s. The test was performed in triplicate, and the mouse forced to change position before each measurement. An average weight-bearing ratio was calculated as the weight placed on the right hind limb divided by total weight on hind limbs and this average ratio was subjected to data analysis.

Tissue preparation for x-ray analysis

On day 16 mice were terminally anaesthetised with isoflurane followed by cervical dislocation. The femur and the proximal part of the tibia were removed, drop fixed in 4%

paraformaldehyde (PFA) for seven days and subsequently stored in phosphate buffered saline (PBS) with 0.1% PFA and 0.1% NaN3 at 4°C until scanned.

Statistics

Data were analysed using the GraphPad Prism 5. Student's t-test (two-tailed) was used for comparison of difference between two distributions. Multiple groups were compared using one-way or two-way analysis of variance with the Bonferroni post hoc test.

Supplementary reference

Abrahamsen, B., Zhao, J., Asante, C.O., Cendan, C.M., Marsh, S., Martinez-Barbera, J.P., Nassar, M.A., Dickenson, A.H., and Wood, J.N. (2008). The cell and molecular basis of mechanical, cold, and inflammatory pain. Science 321, 702–705.

Akopian, A.N., Souslova, V., England, S., Okuse, K., Ogata, N., Ure, J., Smith, A., Kerr, B.J., McMahon, S.B., Boyce, S., et al. (1999). The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat Neurosci *2*, 541–548.

Bautista, D.M., Jordt, S.-E., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E.N., Basbaum, A.I., and Julius, D. (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell 124, 1269–1282.

Bennett, G.J., and Xie, Y.K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

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

Clohisy, D.R., Palkert, D., Ramnaraine, M.L., Pekurovsky, I., and Oursler, M.J. (1996). Human breast cancer induces osteoclast activation and increases the number of osteoclasts at sites of tumor osteolysis. J. Orthop. Res. 14, 396–402.

Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K., and McMahon, A.P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr. Biol. 8, 1323–1326.

Falk, S., Uldall, M., Appel, C., Ding, M., and Heegaard, A.-M. (2013). Influence of sex differences on the progression of cancer-induced bone pain. Anticancer Res. 33, 1963–1969.

Mabuchi, T., Matsumura, S., Okuda-Ashitaka, E., Kitano, T., Kojima, H., Nagano, T., Minami, T., and Ito, S. (2003). Attenuation of neuropathic pain by the nociceptin/orphanin FQ antagonist JTC-801 is mediated by inhibition of nitric oxide production. Eur J Neurosci *17*, 1384–1392.

Mabuchi, T., Shintani, N., Matsumura, S., Okuda-Ashitaka, E., Hashimoto, H., Muratani, T., Minami, T., Baba, A., and Ito, S. (2004). Pituitary adenylate cyclase-activating polypeptide is required for the development of spinal sensitization and induction of neuropathic pain. J Neurosci *24*, 7283–7291.

Malmberg, A.B., and Basbaum, A.I. (1998). Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain *76*, 215–222.

Minett, M. S., Quick, K. & Wood, J. N. Current Protocols in Mouse Biology Vol. 1 383–412 (Hoboken, NJ, USA, 2011).

Nassar, M.A., Stirling, L.C., Forlani, G., Baker, M.D., Matthews, E.A., Dickenson, A.H., and Wood, J.N. (2004). Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. Proc. Natl Acad. Sci. USA 101, 12706–12711.

Nassar MA, Baker MD, Levato A, Ingram R, Mallucci G, McMahon SB, Wood JN. (2006) Nerve injury induces robust allodynia and ectopic discharges in Nav1.3 null mutant mice. Mol. Pain 2, 33.

Renn, C.L., Carozzi, V.A., Rhee, P., Gallop, D., Dorsey, S.G., and Cavaletti, G. (2011). Multimodal assessment of painful peripheral neuropathy induced by chronic oxaliplatin-based chemotherapy in mice. Mol. Pain 7, 29.

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(2), 205–218.

Stirling, L.C., Forlani, G., Baker, M.D., Wood, J.N., Matthews, E.A., Dickenson, A.H., and Nassar, M.A. (2005). Nociceptor-specific gene deletion using heterozygous NaV1.8-Cre recombinase mice. Pain *113*, 27–36.

Zhou, X., Wang, L., Hasegawa, H., Amin, P., Han, B.-X., Kaneko, S., He, Y., and Wang, F. (2010). Deletion of PIK3C3/Vps34 in sensory neurons causes rapid neurodegeneration by disrupting the endosomal but not the autophagic pathway. Proc. Natl Acad. Sci. USA 107, 9424–9429.

Ötman JA, Nassar MA, Wood JN, Baker MD. (2008). GTP up-regulated persistent Na+ current and enhanced nociceptor excitability require NaV1.9. J Physiol. 586(4), 1077-1087