Inhibition of neuronal FLT3 receptor tyrosine kinase alleviates peripheral neuropathic pain in mice

Cyril Rivat1,2, Chamroeun Sar1,2, Ilana Mechaly1,2, Jean-Philippe Leyris1,3, Lucie Diouloufet1, Corinne Sonrier1,3, Yann Philipson4, Olivier Lucas1, Sylvie Mallié1,2, Antoine Jouvenel1,2, Adrien Tassou1,2, Henri Haton1,2, Stéphanie Venteo1, Jean-Philippe Pin5, Eric Trinquet6, Fabienne Charrier-Savournin6, Alexandre Mezghrani1, Willy Joly1, Julie Mion1, Martine Schmitt4, Alexandre Pattyn1, Frédéric Marmigère1, Pierre Sokoloff3, Patrick Carroll1, Didier Rognan4 & Jean Valmier1,2

Peripheral neuropathic pain (PNP) is a debilitating and intractable chronic disease, for which sensitization of somatosensory neurons present in dorsal root ganglia that project to the dorsal spinal cord is a key physiopathological process. Here, we show that hematopoietic cells present at the nerve injury site express the cytokine FL, the ligand of fms-like tyrosine kinase 3 receptor (FLT3). FLT3 activation by intra-sciatic nerve injection of FL is sufficient to produce pain hypersensitivity, activate PNP-associated gene expression and generate short-term and long-term sensitization of sensory neurons. Nerve injury-induced PNP symptoms and associated-molecular changes were strongly altered in Flt3-deficient mice or reversed after neuronal FLT3 downregulation in wild-type mice. A first-in-class FLT3 negative allosteric modulator, discovered by structure-based in silico screening, strongly reduced nerve injury-induced sensory hypersensitivity, but had no effect on nociception in non-injured animals. Collectively, our data suggest a new and specific therapeutic approach for PNP.

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1 Institute for Neurosciences of Montpellier, INSERM, Institut National de la Santé et de la Recherche Médicale, UMR1051, Hôpital Saint-Eloi, Montpellier 34000, France. 2 Université de Montpellier, Montpellier 34000, France. 3 Biodol Therapeutics, Cap Alpha, Clapiers 34830, France. 4 Laboratoire d’Innovation Thérapeutique, UMR7200, CNRS-Université de Strasbourg, Illkirch 67400, France. 5 Institut de Génomique Fonctionnelle, CNRS, INSERM, Univ. Montpellier, 34094 Montpellier, France. 6 Cisbio Bioassays, Parc Marcel Boiteux, BP8417530200 Codolet, France. These authors contributed equally: Cyril Rivat, Chamroeun Sar, Ilana Mechaly, Jean-Philippe Leyris. Correspondence and requests for materials should be addressed to D.R. (email: rognan@unistra.fr) or to J.V. (email: jean.valmier@umontpellier.fr)
Peripheral neuropathic pain (PNP), for which specific and effective therapies are lacking, is a broad public health problem particularly due to its high prevalence (estimated to be 6.9–10% in the general population\(^1\)), debilitating effects, and its high social cost\(^2,3\). Currently, PNP treatments consist essentially in repurposed anti-depressant drugs (e.g., tricyclic anti-depressants and serotonin-noradrenaline uptake inhibitors) and anti-epileptic drugs of the class of \(\alpha_2\)-d-voltage gated calcium channels blockers, both having poor efficacy and producing several side effects\(^4\). There is a crucial need for specific PNP medications, which requires the identification of new specific targets implicated in the initiation and maintenance of PNP.

PNP arises from aberrant functioning of somatosensory neurons present in dorsal root ganglia (DRG) that project to the dorsal spinal cord (DSC)\(^5,6\). Environmental stimuli are converted into voltage changes in somatosensory neurons by ionic transducer channels that respond to specific thermal, mechanical, and chemical stimuli and activate sodium channels that generate and propagate action potentials to the DSC. Nerve injury rapidly induces peripheral sensitization due to reduced thresholds of both transducers and voltage-activated channels, increasing responsiveness to stimuli, and axonal hyperexcitability\(^7,8\). Within hours, multiple adaptive modifications occur in the DRG, including gene expression changes, post-translational protein alterations, and modifications of protein trafficking. For example, after nerve damage, TRPV1, a member of the TRP transducer family\(^9\) with a well-established role in inflammatory pain, is upregulated in different models of PNP both at DRG peripheral and central synapses. This upregulation is correlated with the development and the maintenance of thermal hypersensitivity\(^10\). In addition, decreasing TRPV1 levels or inhibiting its activity reduces part of the neuropathic hypersensitivity\(^11,12\). Peripheral sensitization leads to central sensitization in the DSC that is the cornerstone of PNP chronification. Currently, how peripheral sensitization develops and persists is incompletely understood.

Neuro-immune interactions are key regulators of local peripheral sensitization\(^13,14\). They are mediated by immune cells, which invade the lesion site after nerve blood barrier permeabilization, secrete sensitizers (cytokines, chemokines, growth factors) that contribute to the development of the nerve injury-induced hypersensitivity and the maintenance of PNP\(^13,14\). Cytokines and their receptors have been identified as important actors in these interactions\(^15\). Among them, with the notable exception of the cytokine FL\(^16,17\) and its cognate receptor FLT3\(^18\), all the members of the class III receptor tyrosine kinase (RTK) family, which comprises stem cell factor (SCF) receptor (c-Kit), colony-stimulating factor type-I (CSF1) receptor (CSF1R), and platelet-derived growth factor (PDGF) receptors (PDGFR) have been shown to be involved in normal nociception and/or pain\(^19–22\). For example, nerve injury induces de novo neuronal expression of CSF1, which is transported to the DSC where it targets CSF1R expressed by microglia cells\(^19\). Microglia activation is considered as a major factor in central PNP chronification\(^13,14\). However, the mechanism leading to CSF1 induction is unidentified.

FLT3 is expressed in most hematopoietic organs, such as spleen, thymus, peripheral blood and bone marrow, and its gain-of-function mutation promotes hematopoietic cells proliferation that is targeted for therapy of hematologic cancers\(^23\). However, Flt3\(^{KO}\) mice have a normal number of mature hematopoietic cells in all hematopoietic organs, even though some discrete populations of cell progenitors, but not more mature cells, are reduced\(^24\).

**Fig. 1** Infiltration of CCI-injured nerve by CD45/FL-positive cells and effects of intrathecal FL injections on pain sensitivity and PNP-related biomarkers. **a** CD45- and FL-positive cells at the site of nerve injury in the sciatic nerve, delineated by dotted lines, 10 days post-CCI. Scale bars = 50 µm. **b** Enlargement of the dotted square areas in **a**. Bars = 20 µm. **c** Mechanical pain sensitivity, measured as the percentage of withdrawal to application of von Frey filament, 5 h (5 h) to 5 days (D5) after a single intrathecal saline or different doses of FL injection (concentrations in ng/10 µl). Bsl basal score before injection. **d** Cold and heat thermal sensitivity 24 h after intrathecal saline or FL injection (500 ng). **e** Mechanical sensitivity after a single intrathecal injection of FL (500 ng/10 µl) in wild-type (WT) or Flt3-deficient (Flt3\(^{KO}\)) mice. **f** Mechanical pain sensitivity after repeated intrathecal injections of saline or FL at the dose of (500 ng/10 µl), measured 1–10 days after FL injections performed on days 1, 2, 4, 7, and 9. Control animals received no injections. **g** Normalized gene expressions of PNP biomarkers in the DRG and DSC measured by q-PCR 24 h after a single saline or FL injection (500 ng/10 µl) and 10 days after repeated injections as in **f**. Results are means ± s.e.m. of data from 8 animals **c**-**f** or 4 animals (g). Two-way ANOVA and Dunnett’s test (c, e, f). Unpaired Student’s t-test (d, g). *P < 0.05; **P < 0.01 vs. saline or WT or no injection.
neurons from wild-type (WT) or TRPV1 activator, or capsaicin combined with FL (0.56 nM) on \([\text{Ca}^{2+}]_i\) levels measured by real-time \([\text{Ca}^{2+}]_i\) cell imaging in cultured DRG neurons. Ratio F340/F380 is the ratio of fluorescence signals measured at 340 and 380 nm excitation wavelengths. b Effects of capsaicin (Cap, 2.5 µM) or FL at the indicated concentrations, either alone or in combinations on \([\text{Ca}^{2+}]_i\) levels, measured at the fourth application of capsaicin as in a. Results are expressed as response amplitudes normalized to capsaicin alone (Δf peak). c Effects of FL on capsaicin-induced increased \([\text{Ca}^{2+}]_i\) levels, measured as in a, in DRG neurons from wild-type (WT) or Flt3KO mice. d Potentiation by FL (0.56 nM) of increase in \([\text{Ca}^{2+}]_i\) levels induced by capsaicin (Cap, 2.5 µM), cinnamaldehyde, Cina (50 µM), and menthol (1 µM). e Traces of voltage-clamp whole-cell recording of capsaicin (Cap, 2.5 µM)-induced TRPV1 currents and their potentiation by FL (10 nM). f Quantification of FL effects measured as in e. g In vivo potentiation by FL of capsaicin-induced pain-related behaviors. Animals received either 4 repeated identical injections of capsaicin (15 ng) or a combination of Cap and FL (2 ng) followed by 3 repeated capsaicin injections in the paw, at the indicated times. Results are means ± s.e.m. of data from the number of cells indicated in columns (b–d, f) or data from 8 animals (g). Unpaired Student t-test. NS non-significant; *P < 0.05; **P < 0.01; ***P < 0.001

**Results**

**Flt3 transcripts are expressed in various regions of the nervous system, in particular in human and mice DRGs.** Flt3 modulates the in vitro survival of embryonic mouse DRG neurons but its role in the adult nervous system is not known. Here we demonstrate that neuronal Flt3 in DRG is a critical actor for PNP initiation and maintenance in different mouse models. In addition, we show that PNP can be alleviated by specific Flt3 inhibition using a new chemical entity, thus identifying a novel therapeutic strategy for PNP treatment.

**FL induces PNP-related behavioral and molecular changes.** We used the chronic constriction injury (CCI) model in mice as a model of persistent PNP, consisting in three chronic ligatures tied loosely around the sciatic nerve. On longitudinal sections of sciatic nerve 10 days after nerve injury, intense FL immunoreactivity was present in the nerve at the site of injury in CCI mice, whereas it was absent in normal, sham-operated nerve. All of the FL-positive cells also expressed CD45, a marker of the immune hematopoietic lineage (Fig. 1a, b; Supplementary Fig. 1a). Further analysis showed that 60% of the FL-positive cell population express CD11b, a marker of myeloid cells, but not CD68, a marker of macrophages (Supplementary Fig. 1b, c). No colocalization was found with CD3, a marker of T lymphocytes (Supplementary Fig. 1d). Therefore, FL-expressing cells that penetrate the nerve at the lesion site can be identified as monocytes, neutrophils, and/or natural killer cells but not macrophages or T lymphocytes.

To assess whether FL could participate in the generation of PNP symptoms, uninjured mice were injected intrathecally with recombinant FL and their sensitivity to mechanical stimulation of the hind-paw was measured. A single FL injection induced a dose-dependent increase in the percentage of paw withdrawal to a calibrated von Frey filament (Fig. 1c), i.e., mechanical hypersensitivity, a hallmark of PNP, which was present 5 h post-injection and persisted for at least 2 days. In a separate experiment, the onset of FL-induced mechanical pain hypersensitivity was determined to be 120 min: the percentage of paw withdrawal after an intrathecal injection of 500 ng of FL were (means ± s.e.m., n = 8) 31.3 ± 4.5, 18.8 ± 2.6 and 51.3 ± 3.2% at 30, 90 and 120 min post-FL injection, whereas those after saline injection were 30.0 ± 3.7, 21.3 ± 3.4, and 21.3 ± 3.9% (P < 0.0001 for comparison of FL vs. saline at 120 min by two-way ANOVA, not significant at other time points).

FL-injected mice also displayed thermal hypersensitivity in response to cold and hot stimuli (Fig. 1d). Mice with a homozygous deletion of Flt3 (Flt3KO mice) failed to develop mechanical hypersensitivity following FL injection (Fig. 1e), whereas they displayed normal proportions of sensory neurons expressing typical molecular markers, motor behaviors, and pain responses to noxious stimuli (Supplementary Fig. 2a, b). This indicates that FL-induced hypersensitivity resulted from activation of its cognate Flt3 receptor. Repeated injections of FL every 2 days during 10 days maintained mechanical hypersensitivity that persisted as long as the treatment continued (Fig. 1f).
We next examined changes in nerve injury-associated genes in DRG and DSC. Twenty-four hours after a single FL injection, expression levels in DRG of the stress-induced gene transcript Atf3 and several important neuronal pain-related gene transcripts, e.g., neuropeptide Y (NpY) and transient receptor potentials TrpVI and TrpAl were increased (Fig. 1g). Expression levels of PNP-associated gene transcripts in the DSC at this time point (24 h) showed no change compared to saline-injected animals (Fig. 1g). In contrast, repeated injections of FL over 10 days caused striking changes in the DSC expression of genes associated with the process of central pain sensitization, notably the activated microglia markers Iba1 and Cd11b and the activated astrocyte marker Gfap (Fig. 1g). Thus, FLT3 activation causes, in the short-term, upregulation of PNP-related genes in the DRG, and in the long-term, molecular changes in the DSC typical of those occurring during chronicization of PNP.

In cultured DRG neurons, application of capsaicin, a specific TRPV1 agonist, increased intracellular Ca$^{2+}$ levels ([Ca$^{2+}$]i) and repeated capsaicin applications attenuated TRPV1 responses, which reflects receptor desensitization (Fig. 2a) 39. FL alone had no effect on basal [Ca$^{2+}$]i (Fig. 2b), but markedly potentiated, in a concentration-dependent manner, the TRPV1 response to repeated capsaicin applications (Fig. 2b). Although the capsaicin response was normal in cultures established from Flt3KO mice, the potentiating effect of FL was completely abolished (Fig. 2c). Similarly to its effect on TRPV1 function, FL potentiated both the [Ca$^{2+}$]i response to cinna motif and menthol, specific activators of TRPA1 and of TRPM8 channels, respectively (Fig. 2d). Voltage-clamp whole-cell recording confirmed that capsaicin-induced TRPV1 currents were effectively potentiated by FLT3 activation (Fig. 2e, f). Furthermore, hind-paw FL injection also potentiated capsaicin-induced spontaneous pain-related behaviors (Fig. 2g).

Fig. 3 FLT3 is critical for the development of pain hypersensitivity. a--f Flt3-knockout (Flt3KO), compared to wild-type (WT) animals, showed marked reductions of hypersensitivity to mechanical nociceptive stimuli as measured by the von Frey test (a), thermal stimuli as measured via the Hargreaves test 14 days post-injury (b) and punctate tactile stimuli (c), and of conditioned place preference induced by clonidine-evoked analgesia 14 days post-injury (d). Flt3KO mice also showed decrease of mechanical pain hypersensitivity in the spared nerve injury (SNI) model (e) and in the oxaliplatin model of generalized peripheral neuropathic pain (g). All the values are means ± s.e.m. (n = 8 except in d, n = 11). Two-way ANOVA and Dunnett’s test (a–c, f; Student’s t-test (d). Two-way ANOVA and Bonferroni’s test (e, g). *P < 0.05; ***P < 0.001 vs. Sham WT, Sham CCI, CCI Flt3KO, or vehicle paired.

Thus, acute and chronic in vivo FLT3 activation by FL recapitulates in mice some of the molecular and functional changes in sensory neurons and behavioral alterations that are normally induced by peripheral nerve injury. Altogether, these data strongly suggest that FL directly acts on primary sensory neurons. Indeed, injection of FL directly into the sciatic nerve, but not systemic FL augmentation by intravenous injection (Supplementary Fig. 3a, b), caused mechanical hypersensitivity, showing that systemic peripheral activation of FLT3 is not involved in pain behavior. This indicates that FLT3 triggering PNP-like symptoms is present in the nerve and/or DRG. In agreement with this observation, Western blot detected FLT3 in DRG tissue from WT, but not Flt3KO mice, (Supplementary Fig. 3c) and Flt3 mRNA was visualized in DRG neurons by in situ hybridization (Supplementary Fig. 3d).

Neuronal FLT3 controls PNP development and maintenance. Considering the similarities in the effects of FLT3 activation and those induced by peripheral nerve injury, we then asked whether downregulation of FLT3 functioning could influence the molecular, cellular, and behavioral responses to nerve injury. In the CCI model, injury of the sciatic nerve produced mechanical hypersensitivity that lasted for more than 2 months in WT mice, whereas Flt3KO mice failed to develop pain-related behavior over the same period (Fig. 3a). Note that the repetition of painful mechanical stimulus applications is unlikely to produce sensitization or tolerance since the maximal change in paw withdrawal threshold was achieved after 2 weeks and was maintained over time until the end of the experiment. The heat hypersensitivity and mechanical allodynia observed in WT mice after CCI were also absent in Flt3KO mice (Fig. 3b, c). The conditioned place preference paradigm has been used to reveal the presence of non-
FLT3 is critical for upregulation of markers associated with peripheral nerve injury. a Changes in PNP-related biomarkers mRNA expression, measured by q-PCR 3 days after sham or CCI surgery in wild-type (WT) or Flt3-knockout (Flt3KO) mice. Values are means ± s.e.m of data from 4 animals. One-way ANOVA and Bonferroni’s test, *P < 0.05; **P < 0.01 vs. respective Sham, ###P < 0.001 vs. WT. b ATP3, NPY, and GFAP immunoreactivity in DRG from WT and Flt3KO CCI mice, 14 days post-CCI. Scale bars = 100 µm except for GFAP. 50 µm. c Quantification of experiments as in (b). d GFAP and IBA1 immunoreactivity in DSC from WT and Flt3KO CCI mice. Scale bars = 100 µm. e Quantification of experiments as in (d). f Normalized gene expressions of Csfl and CsflR measured by q-PCR in DRG and DSC from WT and Flt3KO sham or 3 days post-CCI mice. Means ± s.e.m. of data from 4 animals. One-way ANOVA and Bonferroni’s test, *P < 0.05; **P < 0.01 vs. respective Sham, ###P < 0.001 vs. WT. g Csfl and ATF3 mRNA expression in DRG from WT and Flt3KO CCI mice at 3 days post-injury. Bars = 100 µm. h Quantification of experiments as in (g). i Csfl-induced mechanical pain hypersensitivity in WT and Flt3KO mice. Means ± s.e.m. of data from 8 animals. c, e, f, h, i NS non-significant; unpaired Mann-Whitney t-test (c, d, f, h, i). Two-way ANOVA and Bonferroni’s test (d), *P < 0.05; **P < 0.01 vs. saline

Evoked ongoing pain in nonverbal animals. Administration of the non-rewarding and rapidly-acting analgesic drug clonidine, referred to as clonidine-induced analgesia, produced a place preference in wild-type CCI animals, which was totally abolished in Flt3KO CCI mice (Fig. 3d), suggesting a loss of non-evoked ongoing pain in Flt3KO mice. Furthermore, the results on nerve injury-induced mechanical hypersensitivity could be extended to different PNP models, the spared nerve injury (SNI) and spinal nerve ligation (SNL) models, and also to the oxaliplatin model of chemotherapy-induced generalized PNP (Fig. 3e–g). Nevertheless, Flt3KO mice have normal responses to chemically-induced noceception in the formalin test and in the complete Freund’s adjuvant (CFA) chronic inflammatory pain model (Supplementary Fig. 4a, b).

We next assessed whether the deletion of Flt3 could modulate the expression of pain-related factors induced by nerve injury involved in peripheral and central sensitization responsible for PNP chronification. The injury-induced increases in transcripts of Atf3, Npy, Trpv1, and Glap in DRG and Iba1, C11l1b in DSC were abrogated or diminished after Flt3 deletion, at 3 days post-CCI (Fig. 4a). These variations were confirmed by immunohistochemistry analysis, showing that, in DRG, Flt3 deletion inhibited the injury-induced increases in ATF3, NPY, and GFAP, seen in WT mice at 14 days post-CCI (Fig. 4b, c). In DSC, Flt3 deletion also diminished nerve injury-induced changes of astrocyte (GFAP) and a similar trend was seen for microglial (IBA1) activation markers (Fig. 4d, e). Flt3 deletion in CCI mice attenuated the upregulation of Csfl ligand transcript in DRG and CsflR in DSC (Fig. 4f). These results were confirmed by the quantification of reduced numbers of DRG Csfl-positive cells using immunohistochemistry (Fig. 4g, h). Finally, intrathecal Csfl injection induced mechanical hypersensitivity in both WT and Flt3KO mice (Fig. 4i), suggesting that Flt3 acts upstream of Csfl/CsflR, a major signaling pathway in neuropathic pain chronification.

Downregulation of Flt3 expression reverses established PNP. To determine whether PNP-like symptoms, once established, could be reversed by Flt3 downregulation, we injected...
injury. Altogether, these results show that PNP symptoms produced by either FL injection or nerve (Fig. 5g). Thus, inhibition of largely reduced percentage of withdrawal in CCI mice, whereas an behaviors (Fig. 5f), thus showing that the effect of FL is exerted as increase pain score, which takes into account pain-related directly via neuronal FLT3. Furthermore, Flt3 produce evoked mechanical pain hypersensitivity (Fig. 5e), as well (Supplementary Fig. 6c, d), intrathecal FL injections failed to treatment with the virus vector co-expressing an shRNA. g Quantification of experiments as in b. Means ± s.e.m. of data from 4 animals. Mann-Whitney t-test, *P < 0.05. d Localization of the AAV9 virus expressing anti-Flt3 shRNA and GFP in neurons (TUJ1-positive cells), but not in CD45- or S100β-positive cells in DRG of WT mice. Scale bars = 100 µm. e, f Effects of Flt3-shRNA on FL-induced mechanical pain hypersensitivity (e), and on pain-related behaviors (f). FL (500 ng/10 µl) was injected intrathecally 17 days after the virus. Means ± s.e.m. of data from 8 animals. One-way ANOVA and Dunnett’s test; *P < 0.05; **P < 0.01; ***P < 0.001 vs. Bsl; two-way ANOVA with repeated measures and Dunnett’s test; #P < 0.05 vs. non-targeting shRNA. g Effects of Flt3-shRNA on mechanical pain hypersensitivity in CCI mice. The virus was injected intrathecally 48 h before CCI. Means ± s.e.m. of data from 8 animals. Two-way ANOVA with repeated measures and Dunnett’s test; *P < 0.05 vs. non-targeting shRNA

BTD001, an FLT3 inhibitor, reverses PNP symptoms. The X-ray structure of human FL-FLT3 complex (Fig. 6a) was used to design a pharmacophore recapitulating all molecular interactions between the FLT3 D3 domain and the N-terminal part (H8-S13) of the FL ligand. Owing to the surprisingly compact FL-binding epitope (FL-FLT3 interface area of 749 Å²), the FL–FLT3 interaction pharmacophore (Fig. 6b) is simple enough to be fitted by small molecular weight compounds. In silico screening of ca. 3 million commercially available compounds afforded 221 unique compounds fulfilling the pharmacophore, out of which 28 chemically representative hits (Fig. 6c; Supplementary Table 1) were selected for purchase.

One compound (compound Hit #3, Fig. 6d, e) effectively prevented extracellular FL binding to FLT3, as measured by time-resolved fluorescence resonance energy transfer (trFRET, see Supplementary Fig. 7a, b for binding assay validation). Among the 21 commercial and close structural analogs of compound 3 (Supplementary Table 2), seven compounds were more potent than parent compound 3 in the competition binding assay (Supplementary Fig. 8a). Notably compounds 66 and 75 inhibited FL binding to FLT3 with IC₅₀ values of 11 and 17 µM, respectively (Supplementary Fig. 8a). Binding affinities of the 21 analogs permitted to establish preliminary structure–activity relationships (SAR) on the chemical series (Supplementary Fig. 8b). After in-house synthesis (Supplementary Fig. 9a),
compound 66 (hereafter referred to as BDT001, Fig. 6e) was confirmed as a true FLT3 inhibitor, which inhibited FL binding in a non-competitive manner and disrupted positive cooperativity of FL binding (Fig. 6f). BDT001 inhibited FL-induced FLT3 phosphorylation in leukemia-derived RS4+11 cells, also measured by trFRET (see Supplementary Fig. 7a, b for phosphorylation assay validation) with an IC50 of 18–24 µM that was almost unchanged with increasing FL concentrations (Fig. 6g). These results suggest that BDT001 is an FLT3 negative allosteric modulator.

In primary cultures of adult DRG neurons, BDT001 affected neither capsaicin-induced TRPV1 activation (Fig. 7a) nor basal [Ca2+]i (Fig. 7b), but reversed in a dose-dependent manner the potentiation by FL, with a maximal effect at 1 µM, and a partial effect at 0.1 µM (Fig. 7b). Thus, the effective BDT001 concentration required for functional inhibition of FLT3 in neurons was significantly lower than that required for inhibiting FL binding and FL-induced FLT3 auto-phosphorylation in RS4+11 cells. Among the rare examples of extracellular RTK inhibitors, two similar situations have already been encountered (Supplementary Note 1). The effect of BDT001 was FLT3-dependent, as the compound at 10 µM had no effect when TRPV1 potentiation was abrogated (Fig. 7d). In the CCI model, a single injection of BDT001 (5 mg/kg i.p.) reversed mechanical hypersensitivity for 2 days (Fig. 8a). As compared to pregabalin, a standard of care for PNP, BDT001 produced longer effects when considering either paw withdrawal thresholds (Fig. 8b) or pain-related behaviors (Fig. 8c). Repeated injections of BDT001 every day during 3 days fully reversed mechanical allodynia as long as the treatment continued (Fig. 8d). In agreement with the data on Flt3KO mice, BDT001 did not change CFA-induced inflammatory mechanical pain hypersensitivity (Fig. 8e). Neither sensory–motor functions in naive mice (Fig. 8f) nor body weight in nerve-injured mice (Fig. 8g) were affected by single and repeated BDT001 (5 mg/kg i.p.) injections during 4 days, respectively. In a preliminary pharmacokinetics study, the BDT001 plasma level reached 0.19 ± 0.06 µM (right). In agreement with the data on Flt3KO mice, BDT001 did not change CFA-induced inflammatory mechanical pain hypersensitivity (Fig. 8e). 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that FLT3 signaling modulates the expression of the cytokine CSF1, known to activate microglial cells in the DSC, a key process in central sensitization thought to underlie PNP maintenance. Hence, FLT3 in sensory neurons appears as a key upstream trigger and controller of PNP.

FLT3 activation by FL indeed requires peripheral nerve lesion, rupture of the blood–nerve barrier and presence of FL-expressing cells. Thus, our data demonstrate that FLT3 is involved in nerve injury-induced hyperalgesia and pain sensitization, but not in normal nociception nor in inflammatory pain, which does not involve nerve lesion. We have therefore discovered a specific therapeutic target for PNP.

Many high-affinity inhibitors targeting the intracellular ATP-binding site of the FLT3 kinase domain have been developed and are currently under clinical evaluations for the treatment of FLT3-mutated acute myeloid leukemia. However, even the most potent and selective FLT3 inhibitor to date (quizartinib) still inhibits several other receptor tyrosine kinases (e.g., c-KIT, RET, PDGFRB, CSF1R) at single digit nanomolar concentrations. Severe side effects associated with the therapeutic use of existing FLT3 inhibitors are acceptable in oncology patients, but not in PNP patients in the perspective of a long-lasting treatment. Using a structure-based virtual screening approach, we targeted the unique extracellular FLT–FLT3 interface. This strategy of extracellular RTK inhibition by low-molecular weight compounds was shown to be successful in at least two examples in which potent TrkR48 and FGFR8, extracellular negative allosteric modulators were discovered. We have identified a low-molecular weight compound (BDT001) as an FLT3 negative allosteric modulator that specifically inhibits FL binding and blocks FL-induced FLT3 receptor phosphorylation with moderate potency (ca. 10–20 µM) in artificial in vitro assays. However, BDT001 exhibits a much higher potency for inhibiting FL-induced effects in DRG neurons, e.g., TRPV1 potentiation compared to inhibition of binding and phosphorylation in RS4–11 cells. The concentrations of BDT001 needed to inhibit TRPV1 potentiation (0.1 µM) are similar to those reached in plasma after systemic administration at a dose that also produces robust anti-hyperalgesic and anti-allodynic effects in an PNP model in mice. At this low concentration, BDT001 does not affect any kinase binding (23 receptor tyrosine kinases tested) nor functional activity (49 kinases tested) and therefore seems to display the needed pharmacological selectivity. Indeed, BDT001 completely inhibits FL-induced mechanical hypersensitivity, an effect shown here to be dependent on FLT3 activation. This may suggest that BDT001 is a potent agent against PNP, due to the particular environment of neuronal FLT3 not present in the myeloid cell line RS4–11, or to the high sensitivity of PNP mechanisms to FLT3 inhibition. Thus, although the precise molecular mode of interaction of BDT001 with FLT3 remains to be elucidated, this compound represents a prototypical selective FLT3 inhibitor. Remarkably, BDT001 has anti-hyperalgesic effects without altering sensory–motor functions, such as nociceptive sensitivity or motor balance and coordination, which supports the selectivity of the compound. Furthermore, BDT001 does not affect inflammatory pain hypersensitivity, which suggests that BDT001 or an analog may be a specific treatment for PNP. Moreover, the effects of BDT001 last 48 h after a single administration. Such a long-lasting effect, in spite of rapid decrease in plasma drug levels, may be due to the compound remaining in a somatosensory compartment or locked in an FLT3 inactive conformation after its elimination from the systemic circulation. Another more likely hypothesis is that this long-lasting effect, may be due to prolonged inhibition of FLT3 phosphorylation, as was shown in the case of in vivo administration of the small-molecule FLT3 inhibitor sunitinib in a tumor xenograft model.

By specifically targeting the upstream trigger and controller of PNP, the novel FLT3-based therapy may be more efficacious than the selectivity of the compound. Furthermore, BDT001 does not affect inflammatory pain hypersensitivity, which suggests that BDT001 or an analog may be a specific treatment for PNP. Moreover, the effects of BDT001 last 48 h after a single administration. Such a long-lasting effect, in spite of rapid decrease in plasma drug levels, may be due to the compound remaining in a somatosensory compartment or locked in an FLT3 inactive conformation after its elimination from the systemic circulation. Another more likely hypothesis is that this long-lasting effect, may be due to prolonged inhibition of FLT3 phosphorylation, as was shown in the case of in vivo administration of the small-molecule FLT3 inhibitor sunitinib in a tumor xenograft model.

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current therapies targeting either non-specific PNP mechanisms (e.g., anti-epileptics or antidepressants), or mechanisms operating on narrow aspects of PNP, such as excitability of sensory neurons (e.g., voltage-dependent sodium channel blockers) or inflammation (e.g., interleukins or chemokines inhibitors).

In conclusion, our data support peripheral sensory neuron FLT3 as an innovative and specific target for PNP management. BDT001, a rationally designed FLT3 negative allosteric modulator, is consequently a novel prototypic therapeutic tool to alleviate PNP symptoms.

Methods

Animals. Experiments were performed in C57BL/6 naive mice (Janvier, France) or mice carrying a homozygous deletion of Flt3 (Flt3<sup>fl</sup>KO) mice<sup>24</sup> and their littermates (WT) weighing 25–30 g. All the procedures were approved by the French Ministry of Research (authorization #1006). Animals were maintained in a climate-controlled room on a 12 h light/dark cycle and allowed access to food and water ad libitum. Male and female mice were first considered separately in behavioral procedures. Both sexes showed mechanical hypersensitivity of same intensity after intracheal FL injection and nerve injury and were similarly affected by Flt3 deletion (ANOVA followed by Bonferroni’s test, n = 8 for both sexes and genotypes for each experiment). Thereafter, experiments were performed only on male mice.

Chronic pain models. Four different models of peripheral neuropathic pain and one model of chronic inflammatory pain were used. All surgical procedures were performed under deep isoflurane anesthesia.

The CCI model was performed as described previously<sup>29</sup> and adapted for mice.<sup>31</sup> Briefly, skin was incised and the sciatic nerve was exposed unilaterally at the mid-high level by dissecting through the biceps femoris. Three ligations (catgut 6.0) were loosely tied around the sciatic nerve with about 1 mm spacing to reduce blood flow. The skin was then closed with staples. In sham-operated animals the sciatic nerve was exposed without ligation.

The SNI procedure was performed as already described<sup>41</sup>. Briefly, the three terminal branches of the sciatic nerve were exposed after incision of the lateral surface of the thigh through the biceps femoris muscle. Then, the common peroneal and the tibial nerves were tightly ligated with 6.0 silk thread and sectioned distal to the ligation. The sural nerve was left intact.

The SNL procedure was performed as described previously<sup>42</sup>. Briefly, the L6 traversing process was removed to expose the L4 and L5 spinal nerves. The L5 spinal nerve was then isolated and tightly ligated with 6.0 silk thread. For sham operations, the L5 spinal nerve was exposed but not ligated.

The induction of oxaliplatin-induced peripheral neuropathy was performed according to Descouer et al.<sup>53</sup> Oxaliplatin (3 mg/kg) was dissolved in saline and intraplantar injection (20 µl) of a solution of 1 mg of mycobacterium tuberculosis (Sigma-Aldrich) per ml was performed in the left hind-paw of wild-type or Flt3<sup>fl</sup>KO animals.

Behavioral testing. Before testing, mice were acclimatized for 60 min in the temperature and light-controlled testing room within a plastic cylinder or on wire mesh. Experimenters were blinded to the genotype or the drug administered.

Three different types of tests were performed to evaluate mechanical sensitivity. A 0.6 g von Frey filament was used to test hind-paw mechanical hypersensitivity. Sharp withdrawal of the stimulated hind-paw was considered as a positive response. The procedure was applied 10 times and the percentage of positive responses was calculated. According to Ducourneau et al.<sup>24</sup> we quantified pain-related behaviors in response to mechanical stimulation by recording a pain score as followed: 0; no withdrawal, 1; movements of the toes without withdrawal, 2; slow withdrawal, 3; sharp withdrawal, 4; withdrawal with nociceptive behaviors such as flinching, shaking/or licking. Tactile withdrawal threshold was also determined in response to probing of the hind-paw with eight calibrated von Frey filament.
filaments (Stoeling, Wood Dale, IL, USA) in logarithmically spaced increments ranging from 0.4 to 15 g (4–150 mN). Filaments were applied perpendicularly to the plantar surface of the paw. The 50% paw withdrawal threshold was determined in grams by the Dixon nonparametric test57. The protocol was repeated until three changes in behavior occurred. In the dynamic von Frey procedure, animals were placed on a metal mesh surface in an enclosed area. A stainless steel filament (0.5 mm diameter) was automatically exerted an increasing force to the plantar surface until paw withdrawal. The latency until withdrawal, in seconds, and the force, in grams, at which the paw was withdrawn were recorded.

For assessing cold sensitivity, acetone (60 µl) was applied first on the left hind-paw for all animals then on the right hind-paw. The time spent licking or biting the paw was recorded with a stopwatch and reported as the cumulative time of licking/biting for the two hindpaws. A cut-off time of 45 s was used in each trial.

For assessing heat sensitivity, a radiant heat source (plantar test Apparatus, IITC Life Science, Woodland Hills, USA) was focused onto the plantar surface of the paw. The paw was observed for 3 min, and behavioral changes associated with intrathecally saline or FL (500 ng/10 µl), capsaicin injection was recorded. As soon as either paw withdrawal or licking of the paw was observed for 20 min and spontaneous pain-related behaviors were evaluated.

The model of formalin-induced pain56,57 was used to assess acute inflammatory pain. For FL, IGF-1 or saline injection, mice were restrained in a transparent acrylic chamber (20 × 18 × 25 cm) interconnected by a rectangular corridor (20 × 7 × 25 cm) diameter) was automatically applied to the hind-paw. The latency until paw withdrawal was recorded. Data are expressed as duration of paw withdrawal latency or pain score calculated by dividing the paw withdrawal latency by the score of nociceptive behavior measured for each animal.

For assessing nociceptive behavior, a laser beam (Life Science, Woodland Hills, USA) was focused onto the plantar surface of the paw. The time spent shaking and licking the injected hind-paw. Data are expressed as the duration of responses every 3 min.

In the Rotarod test, the speed was set at 10 rpm for 60 s and subsequently accelerated to 80 rpm over 5 min. The time taken for mice to fall after the beginning of the acceleration was recorded.

Conditioned place preference (CPP) was performed as follows. Tonic-aversive state in neuropathic pain can be unmasked by the administration of non-rewarding and rapidly-acting analgesic drugs such as clonidine. All experiments were conducted using the standard CPP protocol as described previously for rodents.58,59 CPP apparatus (Bioseb, Vitrolles, France) consists of two equally sized chambers (20 × 18 × 25 cm) interconnected by a rectangular corridor (20 × 7 × 25 cm). The chambers are differentiated by the wall pattern (dotes versus stripes) and color (different shades of grey versus black). Fourteen days after CCI surgery, animals were tested in a CPP apparatus in the morning for 20 min and on the afternoon for 20 min. In day 3, a pre-conditioning bias test was performed to determine whether a preexisting chamber bias existed. In this test, mice were placed into the middle chamber and allowed to explore open field with access to all chambers for 15 min. No animal spending more than 80% or <20% of the total time in one of the chambers was tested. The data are expressed as duration of response in each chamber.

For assessing heat sensitivity, a radiant heat source (plantar test Apparatus, IITC Life Science, Woodland Hills, USA) was focused onto the plantar surface of the paw. For assessing acetylcholine injection, a laser beam (Life Science, Woodland Hills, USA) was focused onto the plantar surface of the paw. The time spent shaking and licking the injected hind-paw. Data are expressed as duration of responses every 3 min.

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incubated in a humidified 95% air–5% CO₂ atmosphere at 37 °C. We used 6 and 21 animals for path-clamp and calcium imaging experiments, respectively.

Whole-cell patch-clamp recordings from isolated DRG neurons. Whole-cell voltage-clamp was performed on small and medium somatic diameter (15–25 μm) DRG neurons after one day in vitro (DIV) at 37 °C. Currents were recorded with an RK-400 amplifier (Biologic) and computed with a Digidata 1322 A analogue interface (Axon Instruments) and the pClamp software (Clampex 8.02; Axon Instruments). Signals were filtered at 3 and sampled at 5 kHz. Series resistances were corrected to 20 MΩ. The membrane voltage was held at −60 mV. Glass electrodes (2.5–3.5 MΩ) were made from capillary glass, using a Narishige puller, and coated with parafilm wax to minimize pipette capacitance. The Standard External Solution (SES) contained: 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM glucose (pH adjusted to 7.4 with NaOH and osmolality between 300 and 310 mOsm). The solutions were filtered to obtain a number of different conditions (high and low pH combined). Whole-cell recordings were performed on DRG neurons, respectively, as identified by transgenic fluorescence imaging. The following solution: 145 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 10 mM HEPES (pH adjusted to 7.4 with KOH and osmolality between 300 and 310 mOsm). Capacitance (500-1 mN-m) and FL (100 nM) were dissolved in extracellular solution. Sequential applications were: SES (1 min)—capacitin (1 μg)—SES (3 min)—capacin 2 ± FL (15 s)—SES (3 min)—capacin 3 ± FL (15 s)—SES (1 min).

Calium imaging. For calcium imaging video microscopy (Ca²⁺) fluorescence imaging, DRG neurons were loaded with fluorescent dye 2.5 μM Fura-2 AM (Invitrogen, Carlsbad, CA) for 30 min at 37 °C in standard external solution containing: 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM glucose (pH adjusted to 7.4 with NaOH and osmolality between 300 and 310 mOsm). The following protocol was used: the number of identified neurons in DRG and DSC corresponded to the published data, for which an abundant literature exists. For instance, it was checked that immunoreactivity against commonly used glial markers (GFAP, IBA1) and neurons (TUJ1, NeuN, NPY, CSF1) is restricted to glial cells and neurons, respectively, as identified by morphometric features. Most of these antibodies have been previously used in our lab conditions. In addition, the anti–FLT3 antibody, which has not been extensively used, was validated by using FliKOKO mice (Supplementary Figure 3c).

Antibodies. The primary antibodies that were used in the study are listed below, together with manufacturer, catalog number, dilutions used and references to external publications using the antibodies. Each antibody was validated in the specific condition and tissue applied in the study by routine controls (omission of primary or secondary antibody) and by checking that the pattern of immunolabelling distribution of the antibody of interest overlaps with that of a specific molecular marker (e.g., Fig.4d). A minimum of six sections from lumbar DRG were counted from at least 4 animals. All neurochemical markers of sensory neurons (subtypes) was determined by counting cells in which the neuronal cell body was encircled at least two-thirds by GFAP staining. The total number of neuronal cells in the DRG were counted to determine a percentage of area relative to the total area of the spinal dorsal horn of the section was obtained for each animal across the different tissue sections. Four animals have been excluded from the immunohistochemistry analysis (Fig. 3c, i) because of poor quality of slices.

Western Blot. Mice received an intraperitoneal injection of phenobarbital and transcardially perfused with PBS. After isolation, tissues were mechanically homogenized at 4 °C in NP40 buffer (1% NP40, 150 mM NaCl, 50 mM Tris-HCl, pH = 7.8 and protease inhibitor). Lysates were clarified for 10 min at 4 °C at 10,000 × g. A protein concentration was determined with the NTerminal Protein Assay Kit (Pierce).

Immunohistochemistry. Mice were transcardially perfused with PBS followed by 4% formaldehyde for all experiments except for the AVV9-GFP ShFLT3 experiment where mice were perfused with 1% saline buffer. DRG (L4–L6), spinal cord (lumbar segment), and sciatic nerves were collected and post-fixed in 4% paraformaldehyde between 10 min and 2 h depending on the antibody and tissue before being cryoprotected in 30% sucrose in PBS overnight before being frozen in OCT compound (Sakura Finetek). Sections (12 μm) were prepared using the Cysteïque Leica CM2800E. For immunostaining, frozen sections were blocked and permeabilized in Ca²⁺/Mg²⁺-free PBS (PBS 1×/–/) containing 10% donkey serum and 0.1% or 0.3% Triton X-100 for 10 min at 30 min. The sections were then incubated with primary antibodies, at 4 °C overnight in PBS 1×/–/ containing 1% BSA. Sections were then rinsed in PBS 1×/–/ containing 0.03% Triton X-100. After extensive wash in PBS 1×/–/ (3 times for 10 min minimum each) sections were incubated with appropriate secondary antibody conjugated to AlexaFluor and Hoechst (Sigma 1μg/ml), in the same buffer as the primary antibodies, at room temperature for 1 h and then washing (three times for 15 min each) before mounting with DABCO. Images were acquired under a Zeiss LSM 510 and 700 Confocal microscope with ZEN software (Carl Zeiss Microscopy).
was performed using chemiluminescent reagent (SuperSignal West Femto-ECL, Sigma). After incubation with primary and secondary antibodies, immunodetection lysates were run on SDS-PAGE and transferred to nitrocellulose membrane.

Production of human recombinant FL (rh-FLT3-L)

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