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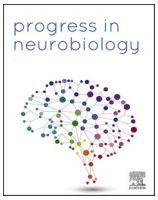

Activation of neuronal FLT3 promotes exaggerated sensorial and emotional pain-related behaviors facilitating the transition from acute to chronic pain

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ABSTRACT

Acute pain has been associated with persistent pain sensitization of nociceptive pathways increasing the risk of transition from acute to chronic pain. We demonstrated the critical role of the FLT3- tyrosine kinase receptor, expressed in sensory neurons, in pain chronification after peripheral nerve injury. However, it is unclear whether injury-induced pain sensitization can also promote long-term mood disorders. Here, we evaluated the emotional and sensorial components of pain after a single (SI) or double paw incision (DI) and the implication of FLT3. DI mice showed an anxiodepressive-like phenotype associated with extended mechanical pain hypersensitivity and spontaneous pain when compared to SI mice. Behavioral exaggeration was associated with peripheral and spinal changes including increased microglia activation after DI versus SI. Intrathecal microglial inhibitors not only eliminated the exaggerated pain hypersensitivity produced by DI but also prevented anxiodepressive-related behaviors. Behavioral and cellular changes produced by DI were blocked in *Flt3* knock-out animals and recapitulated by repeated intrathecal FL injections in naive animals. Finally, humanized antibodies against FLT3 reduced DI-induced behavioral and microglia changes. Altogether our results show that the repetition of peripheral lesions facilitate not only exaggerated nociceptive behaviors but also induced anxiodepressive disorders supported by spinal central changes that can be blocked by targeting peripheral FLT3.

1. Introduction

Understanding the transition from acute to chronic pain remains an important challenge to better manage pain in patients (Price and Gold, 2018). This is specifically true in the context of surgery where chronic post-surgical pain (CPSP) is significantly debilitating, under-evaluated and affecting several millions of people each year in the world (Fregoso et al., 2019; Macrae, 2008; Montes et al., 2015; Richebé et al., 2018). Pre-existing pain is a major risk factor as it profoundly modifies patients internal homeostasis altering the degree of pain sensitization (Aasvang et al., 2010; Kehlet et al., 2006; McGreevy et al., 2011). Hence, sensitization after sensorial trauma appears to be a critical event in the development of chronic pain by amplifying signaling in previously affected nociceptive pathways (Glare et al., 2019). Animal models called

Abbreviations: CCI, Chronic constriction injury; CPSP, Chronic post-surgical pain; DI, double incision; CPP, Conditioned place preference; DRG, Dorsal root ganglia; FLT3, Fms-like receptor tyrosine kinase 3; FL, FLT3 ligand; FST, Forced swim test; HTRF, Homogeneous time resolved fluorescence; NSF, Novelty suppressed feeding test; PFA, paraformaldehyde; SI, Single incision; ST, Splash test.

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"hyperalgesic priming" are classically used to study the neuroplasticity underlying persistent pain sensitization (Asiedu et al., 2011; Bogen et al., 2012; Dina et al., 2003; Kim et al., 2016, 2015; Melemedjian et al., 2010; Moy et al., 2017; Parada et al., 2005; Reichling and Levine, 2009). These models consist in performing an initial nociceptive stimulation that induces a prolonged period of susceptibility to exaggerated pain behavior after a subsequent stimulation. This phenomenon has been named latent pain sensitization (Rivat et al., 2007) and its mechanisms have been further studied (Custodio-Patsey et al., 2020; Marvizon et al., 2015; Taylor et al., 2019). Furthermore, the clinical observation that chronic pain incidence after injury is largely related to pre-injury pain status (McGreevy et al., 2011) suggests that the maintenance of latent sensitization may facilitate the transition from acute to persistent pain. Mood disorders such as depression and anxiety are frequently observed in patients suffering from chronic pain. It has been reported that at least 50 % of chronic pain patients experience a major depressive disorder (Maletic and Raison, 2009), making depression and chronic pain together a widespread comorbidity (Bair et al., 2003; McWilliams et al., 2004; Simon et al., 1999). Similarly, persistence of mood disorders is a maladaptive process maintained by neuropathological mechanisms that can aggravate the sensory abnormalities of chronic pain (Arntz et al., 1994; Ploghaus et al., 2001). Although this comorbidity is clinically well established, the underlying pathophysiological mechanisms remain poorly understood. It has been proposed that depression is a consequence of the presence of chronic pain (Dworkin and Gitlin, 1991; Sheng et al., 2017) and that peripheral nociceptive inputs may act as a trigger for central mechanisms that leave predisposed individuals at increased risk of anxiodepressive disorders. Thus, we hypothesize that peripheral nociceptive inputs may generate sustained neurochemical alterations (i.e. latent sensitization) leading to affective disorders after subsequent re-exposure to the same nociceptive stimulus as shown with exaggerated pain hypersensitivity in hyperalgesic priming models. To date, few studies examined the affective dimension in animal models of hyperalgesic priming (Baptista-de-Souza et al., 2020) and this incomplete characterization has impeded the understanding of the role of peripheral nociceptive inputs in the development of affective disorders. Consequently, we characterized a model of repeated hindpaw injuries where the animals undergo two hind paw surgery 7 days apart, by studying both sensory and affective alterations.

Recently, we reported the expression of the fms-like tyrosine kinase 3 receptor (FLT3) in the peripheral nervous system (Rivat et al., 2018) and its implication in the development and maintenance of chronic neuropathic pain *via* its ligand, the cytokine FL. Originally identified as part of the hematopoietic system, we showed that FLT3, expressed in neurons of the dorsal root ganglia (DRG), induces long-term molecular modifications, leading to neuronal hyperexcitability giving rise to neuropathic pain symptoms. In contrast, the genetic and pharmacological inhibition of FLT3 prevents, but also reverses, hyperexcitability and pain-related behaviors associated with nerve injury. These results strongly highlight the key role of FLT3 in the development of long-term neuropathic pain sensitization. Although its role in the sensorial component of neuropathic pain is now established, we are still lacking evidence about the extent to which peripheral FLT3 can be involved in the central consequences of latent sensitization, especially in the affective dimension of pain. Hence, in a second objective, we revealed a FLT3 dependent peripheral sensitization that triggers both exaggerated nociceptive and affective disorders after repeated surgical injury.

2. Methods and materials

2.1. Animals

Experiments were performed in C57BL/6 naive mice (Janvier, France), mice carrying a homozygous deletion of *Flt3* (*Flt3*$^{-/-}$ mice) (Mackarehtschian et al., 1995) or *CX3CR1*^{EGFP} mice and their littermates (Wild-type, 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 intrathecal FL injection and after surgery 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.

2.2. Surgeries

Paw incision models: Mice C57Bl6/J were anesthetized under isoflurane (3 % vol/vol). For the single incision (SI) model (Brennan et al., 1996), a 0.7 cm incision was applied with a number 20 blade on the skin and fascia of the left hindpaw plantar surface. Subcutaneous plantaris muscle was then isolated, exposed and longitudinally incised. After hemostasis, the wound was sutured with two 6.0 absorbable sutures and the mice were finally placed in recovery cages. For the double incision (DI) model (Cabañero et al., 2009), the same procedure was repeated on the opposite hindpaw 1 week after the first incision. Control animals underwent a Sham procedure, which consisted in isoflurane (3 % vol/-vol) anesthesia alone.

Chronic constriction injury (CCI) model: CCI of the sciatic nerve was performed as described previously (Bennett and Xie, 1988) and adapted for mice (Costa et al., 2008). Briefly, skin was incised and the sciatic nerve was exposed unilaterally at the mild-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.

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

Recombinant FL was produced in the E. coli Rosetta (DE3) strain (Novagen) in our laboratory using the pET15b-rhFL plasmid according to the protocol described (Verstraete et al., 2009)with some minor modifications. The rh-FL was checked for endotoxin content using the Pyrogen Recombinant Factor C endotoxin detection assay from LONZA (Walkersville MD, USA) and was found free of endotoxins.

2.4. Endogenous FL measurements

After collection of ipsilateral skin and dorsal horn of the spinal cord from Sham, DI and SI animals, tissues were snap-frozen in liquid nitrogen and stored at −80 °C until processing for ELISA. Skin and dorsal of the spinal cord were homogenized in the MagNA Lyser Instrument (Roche; twice for the speed of 6000 for 30 s) with ceramic spheres (Lysing Matrix D, MP Biomedicals, #116913100) in lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 % Triton X100, 1 mM EDTA, 1 mM EGTA, DTT 1 mM) supplemented with complete protease inhibitor cocktail (Sigma-Aldrich #P8340). Then, centrifugation (10 000 rpm for 15 min at 4 °C) was performed. Supernatants were collected and FL levels were measured using the Quantikine Mouse Flt-3 Ligand Immunoassay by R&D Systems (#MFK00) according to manufacturer recommendations.

2.5. Injections

FL injections: To study FL induced microgliosis, human FL or vehicle was intrathecally injected daily for 3 days, spinal cords were then collected for immunohistochemistry. To know whether FL can induce anxiodepressive-like disorders, mice underwent FL or vehicle intrathecal injection (50 ng/5 μl) every 3 days during 15 days and were subjected to anxiodepression testing 24 h after the last injection. Curative minocycline was intrathecally injected 3 days post-incision or sham

at a dose of 300 μg/5 μl/mouse. Preventive GW2580 was intrathecally injected during surgery or sham at a dose of 1 μg/5 μl/mouse. Preventive administration of mAbA3 consisted in a single intraperitoneal injection (200 μg in 200 μl) during the first incision. Curative administration of mAbA3 consisted in 2 intraperitoneal injections (200 μg in 200 μl), one just before the second incision and one 4 h after. Repeated injections consisted in 1 injection every 2 days during the first incision phase for a total of 4 injections for the evaluation of anxiodepressive-like behaviors 2 weeks post-injury.

2.6. Anti-FLT3 antibody development and production

Three anti-FLT3 human and murine cross-reacting scFv antibodies were selected by phage display from the human scFv synthetic library HuscI (Philibert et al., 2007; Robin and Martineau, 2012) after sequential panning against recombinant human-FLT3-hFc and murine FLT3-hFc (R&D systems). The human scFv were reformatted as a chimeric human/murine IgG2a with Fc N297A mutation to block FcgR binding and thus antibody-mediated immune cells and complement recruitment. Recombinant mAb was produced using HEK-293 T cells after transfection with polyethylenimine PEI (Polyscience) and purified using protein A chromatography. Antibody was eluted at acidic pH (glycine.HCl pH 2.7), and the solution immediately neutralized with Tris.HCl buffer, pH 9.0. Antibody was concentrated and dialyzed against PBS using an ultrafiltration centrifugal device with a cut-off of 50 kDa, sterilized by 0.2 μm filtration and stored at 4 °C. A test for the presence of endotoxins was performed and was lower than 0.25 EU/mg.

2.7. Affinity measurements of anti-FLT3 antibody for human and murine FLT3

The surface plasmon resonance (SPR) experiments were performed on a T200 apparatus at 25 °C on CM5S dextran sensor chip in HBS-EP+ buffer (20 mM Hepes Buffer pH7.4 containing 150 mM of NaCl and 0.05 % P20 surfactant). Recombinant human and murine FLT3 hFc Chimeric (R&D systems) were captured on anti-human Fc using Ab human Capture Kit (Cytiva) according to the manufacturer's instructions (Cytiva) and increasing concentrations (from 0.75 to 100 mM) of anti-FLT3 were injected at 50 μl/min. A pulse of 3 M MgCl2 was used as regenerant between cycles. The kinetic constants were evaluated from the sensorgrams after double-blank subtraction with T200 Evaluation software (Cytiva) using a bivalent fitting model.

2.8. Behavioral testing

2.8.1. Mechanical nociception assay

Tactile withdrawal threshold was determined in response to probing of the hindpaw with eight calibrated von Frey filaments (Stoeling, Wood Dale, IL, USA in logarithmically spaced increments ranging from 0.04 to 8 g. Filaments were applied perpendicularly to the plantar surface of the paw. The 50 % paw withdrawal threshold was determined in grams by the Dixon nonparametric test (Dixon, 1980). The protocol was repeated until four changes in behaviour occurred.

2.8.2. Heat test

A radiant heat source (plantar test Apparatus, IITC Life Science, Woodland Hills, USA) was focused onto the plantar surface of the paw. The paw withdrawal latency was recorded 3 times per paw. A maximal cut-off of 20 s was used to prevent tissue damage.

2.8.3. Anxiodepression testing

Splash test (ST), Novelty suppressed feeding test (NSF) and Forced swim test (FST) were conducted as previously described (Barthas et al., 2015; Porsolt et al., 1977; Santarelli et al., 2003).

2.8.4. 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.

2.8.5. Conditioned place preference (CPP)

Pain-induced tonic aversive state can be unmasked by the administration of non-rewarding and rapidly acting analgesic drugs such as clonidine. All experiments were conducted by using the single trial CPP protocol as described previously for rodents (King et al., 2009). The apparatus (bioseb) consists of 2 chambers (size 20 cm x 18 cm x 25 cm) distinguished by the texture of the floor and by the wall patterns connected to each other by a central chamber (size 20 cm × 7 cm x 25 cm). First, animals went through a 3-day pre-conditioning period with full access to all chambers for 20 min. Mice underwent surgery (SI or DI) the first day of this period. At this step, mice with a spontaneous preference up to 75 % were removed from the experiment. Day 4 is the conditioning day. Briefly, mice received saline intrathecal injection (5 μl) and were restricted for 15 min to one chamber. 4 h later, mice received clonidine intrathecal injection (1 μg/5 μl) and were restricted for 15 min to the opposite chamber. On the test day (d5), 20 h after the afternoon pairing and 4 days after surgery, mice were placed in the middle chamber of the CPP box with all doors open so animals could have free access to all chambers. The time spent in each chamber was recorded for 20 min for analysis of chamber preference.

2.9. Retrograde labeling

10 μl of 1 % Fluorogold was applied on the open wound incision during surgery. DRGs were then dissected 7 days after in PBS and post-fixed for 15 min in 4 % paraformaldehyde (PFA). Tissues were rinsed twice in PBS before immersion overnight in 25 % sucrose/PBS at 4 °C and were finally frozen. Frozen DRGs were then subjected to cryosection and imaging. Images were collected using a Carl Zeiss LSM 700 confocal microscope and were processed with ZEN software.

2.10. Immunohistochemistry

Mice were transcardially perfused with PBS followed by 4 % formaldehyde for all experiments. DRG (L4-L6), spinal cord (lumbar segment), and brains were collected and post-fixed in 4 % paraformaldehyde between 10 min and 12 h depending on the antibody and tissue before being cryoprotected in 30 % sucrose in PBS. Tissues were then frozen in O.C.T (Sakura Finetek). Sections (DRG: 12 μm; Spinal cord: 14 μm) were prepared using the Cryostat Leica CM2800E. Brain sections were prepared using a Vibratome. For immunostaining, frozen sections were blocked and permeabilized in $Ca^{2+/}Mg^{2+}$-free PBS (PBS 1 × −/−) containing 10 % donkey serum and 0.1 % or 0.3 % Triton x-100 during 30 min. The sections were then incubated with primary antibodies, at 4 °C overnight in PBS 1 × −/− containing 1 % donkey serum and 0.1 %, 0.01, or 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 1 μg/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 Mowiol.

Immunostainings were performed with the following antibodies (see Table S1): ATF3 (Santa Cruz), CSF1 (R&D), Iba1 (Wako), GFAP (abcam), CGRP (abcam), NeuN (Millipore), BrdU (Abcam), and fluorophore coupled secondary antibodies (Alexa Fluor 488, 555, 594, 647) (Invitrogen). Images were collected with a Carl Zeiss LSM 700 microscope or a Zeiss Axioscan slides scanner and were processed with Fiji/ImageJ (NIH). Corresponding images (e.g. ipsilateral vs. contralateral; FL vs vehicle; wt vs. mutant) were processed in an identical manner. Each experiment was performed in at least 3 animals. For proliferation assays, BrdU (Sigma) was injected intraperitoneally into adult mice (2 mg/

animal), 24 hr before sacrifice. For BrdU detection, slides were incubated in 10 mM sodium citrate buffer (pH 6.0) at 95 °C for 15 min, then at room temperature (RT) for 15 min and subjected to immunostaining.

2.11. RNA isolation and real time polymerase chain reaction

L4 DRGs and dorsal spinal cords were dissected, ipsilateral and contralateral parts were separated and tissues were stored at −80 °C until RNA extraction with the TRI reagent/ chloroform technique following the protocol of the manufacturer (sigma). After extraction, samples were treated with DNAse (M610A, promega) and reverse transcribed. Q-PCR reactions were made in 96 well plates with a final volume of 10 μl composed of 3 μl cDNA (final dilution 1: 90), 0.5 μM of forward and reverse primers and 5 μl of SybrGreen I master mix (Roche life Sciences). Measures were realized with a LightCycler 480 (Roche). The relative amounts of specifically amplified cDNAs were normalized with the geometric mean of RNA polymerase II polypeptide J (*Polr2j*) and DEAD box polypeptide 48 (*Ddx48*) as stable control genes for L4 DRGs or *Ddx48,* Ubiquitin Conjugating Enzyme E2 E3 (*Ube2e3)* and 14–3–3 protein zeta/delta*(Ywhaz)* for dorsal spinal cord. Sequences of the primer pairs used are listed in table S2.

2.12. In situ hybridization

Sense and antisense digoxigenin (DIG)-labelled RNA probes were generated from a mouse Flt3 cDNA clone (IRAMp995N1310Q, GenomeCUBE Source Bioscience) in a 20 μl reaction containing 1 μg of linearized plasmid (digested with *Hin*dIII and *Nhe*I respectively) using the DIG RNA labelling mix (Roche Diagnostics) and T7 and Sp6 RNA polymerases respectively (Promega) following the manufacturer's instructions. DIG-labelled RNA probes were purified on MicroSpin G50 columns (GE Healthcare). Naive or DI-injured WT and $CX3CR1^{EGFP}$ mice were euthanized by Euthasol vet injection (25 μl). Spinal cords were dissected in phosphate-buffered saline (PBS) and fixed for 30 min in 4 % PFA at room temperature. Tissues were rinsed twice in PBS before immersion overnight in 25 % sucrose/PBS at 4 °C. *In situ* hybridization was performed with standard procedures on transverse sections of 14 μm as described (Ventéo et al., 2012). Image acquisition was done using an Axioscan slides scanner ZEISS.

2.13. HTRF binding assay

Tag-lite binding assays were performed 24 h after transfection on fresh cells in 96-well plates. Cells were incubated with 0.5 nM Red-FL in the presence of increasing concentrations of anti-FLT3 mAb to be tested and incubated for 1 h at Room temperature prior to the addition of Red-FL. Plates were then incubated at room temperature overnight before signal detection.

2.14. HTRF autophosphorylation assay

RS4–11 cells were used to carry out this assay in suspension in 384-well small volume plates to determine the degree of phosphorylation of FLT3 receptor. In the plates containing RS4–11 cells, anti-FLT3 mAb to be tested were added and incubated for 90 min at room temperature prior to the addition of rh-FL at 10 or 1 nM for 10 min at room temperature. After incubation, cells were lysed and a mix of anti-FLT3 and anti-TYR-969 FLT3 antibodies labeled with Lumi4-Tb and d2 respectively were added to the cell lysate. Plates were then incubated at room temperature for 2 h before signal detection. Autophosphorylation measured in the absence of FL represents FLT3 constitutive activity whereas that measured in the presence of FL represents FL-induced activity.

2.15. Statistical analysis

Data were analyzed with graphpad prism 9. Area Under Curve (AUC) was calculated based on the trapezoidal rule to have the volume of area above or under the baseline across different time points until full recovery. Data are expressed as the mean ± S.E.M. All sample sizes were chosen based on our previous studies except for animal studies for which sample size has been estimated *via* a power analysis using the G-power software. The power of all target values was 80 % with an alpha level of 0.05 to detect a difference of 50 %. Statistical significance was determined by analysis of variance (ANOVA one-way or two-way for repeated measures, over time). In all experiments in which a significant result was obtained, the test was followed by Holms-Sidak post-hoc test for multiple comparisons, as appropriate. In case of two experimental groups, unpaired two-tailed t-test was applied. For cell counting, and area quantification, statistical analyses were performed using Mann-Whitney test. The applied statistical tests are specified in each figure legend.

3. Results

3.1. Characterization of anxiodepressive-like behaviors after repeated hindpaw injuries

SI (Single Incision, one incision on the left hindpaw) (Brennan et al., 1996) and DI (Double Incision, first incision on the left hindpaw and second one on the right hindpaw one week later) models were compared and tested at three different time points after surgery: the following days after surgery (1st week post-incision), just after nociceptive recovery of the second incision (2nd week post-incision) and 1 month after incision (Fig. 1A). The first week after surgery, SI and DI mice responded to the NSF with an increased latency to eat compared to Sham mice while only DI mice responded to ST and FST with a decreased grooming behavior and immobility, respectively (Fig. 1B-D). The second week after surgery, we found no difference between Sham mice and SI mice in all the tests while DI mice showed sustained changes in the FST, ST and NSF tests compared to the Sham group (Fig. 1E-G). One month after surgery, all the experimental groups returned to Sham values (Fig. 1H-J). These results are resumed in Supplementary Fig. 1A. Because these behaviors require motor functions, motor coordination was assessed on the first week post-injury and found unchanged (Supplementary Fig. 1B). Decreased neurogenesis at the level of the dentate gyrus has been associated with depression in human (Eisch and Petrik, 2012) and animal (Blugeot et al., 2011; Bouvier et al., 2017) studies. We therefore hypothesized that DI-induced changes in ST, FST and NSF could be associated with a decrease in the rate of newborn neurons in this region as evaluated with Brdu staining. Brdu stained neurons (Brdu/NeuN colocalization) were substantially decreased in DI mice 7 days after the surgery compared to SI and Sham mice (Fig. 1K-L) supporting the development of mood disorder in DI mice.

3.2. Characterization of pain-related behaviors after repeated hindpaw injuries

We tested the effect of SI vs. DI on sensory thresholds and ongoing pain. Six hours after the first surgery, mice mechanical thresholds as measured by the VF test strongly decreased up to ten-fold in ipsilateral hindpaw (left hindpaw) indicating the appearance of mechanical allodynia (Fig. 2A). Mice recovered a normal mechanical threshold after 7 days, and mechanical threshold was unchanged in the SI contralateral hindpaw (right hindpaw). By contrast to the first incision, the second incision induced a robust decrease of mice withdrawal thresholds at the ipsilateral side (right hindpaw) and at the contralateral side (left hindpaw; Fig. 2A). As reported in a previous work (Cabañero et al., 2009), recovery to a normal threshold of both hindpaws was delayed by 7 days compared to the first incision, with a full recovery at day 22. Area Under Curve (AUC) that represents the period of mechanical hypersensitivity

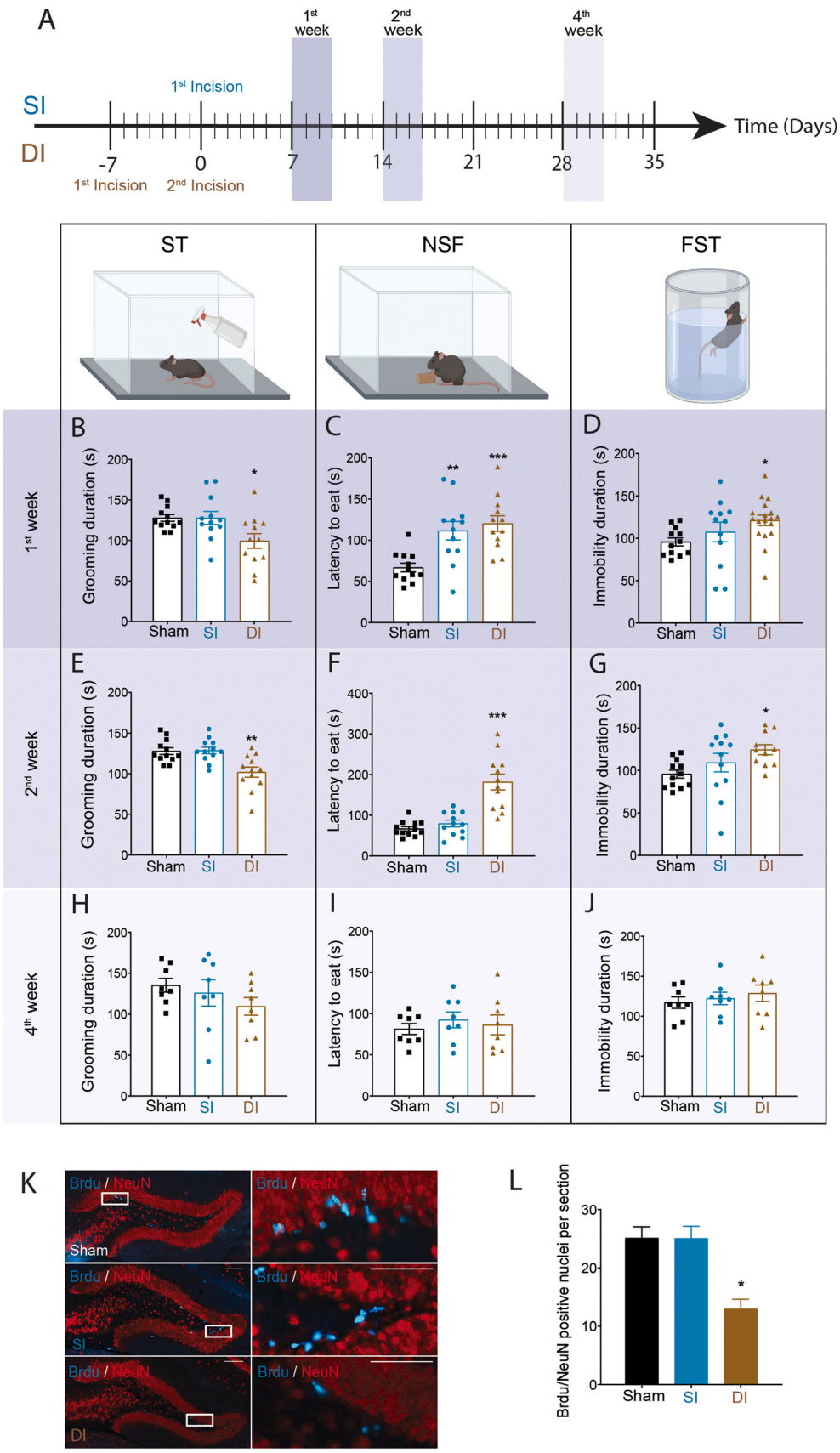

Fig. 1. Influence of single (SI) or double incision (DI) on anxiodepressive-related behaviors and neurogenesis. In the first week post-injury (Sham, SI, DI), (A) decreased grooming behavior is found after DI in the splash test (ST), (B) increased latency to eat is found after SI and DI in the novelty suppressed feeding test (NSF) and (C) increased immobility duration in the forced swim test (FST) is observed in DI compared to control mice. In the second week, (D) Decreased grooming behavior in the splash test, (E) increased latency to eat in the novelty suppressed feeding test and (F) increased immobility duration in the forced swim test is observed only in DI mice compared to control mice. In the fourth week, (G) no significant changes were found in the splash test, (H) novelty suppressed feeding test and (I) forced swim test in DI mice compared with control mice. (J) Brdu/NeuN positive nuclei per section in the dentate gyrus is significantly reduced only in DI mice 7 days post-injury (scale bars = 200 μm) and (K) related quantification. All the values are means ± s.e.m. ($n = 12$ except in J, K, $n = 4$). One-way ANOVA and Holm-Sidak's test (A-I) or Kruskal-Wallis test (K); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. Sham.

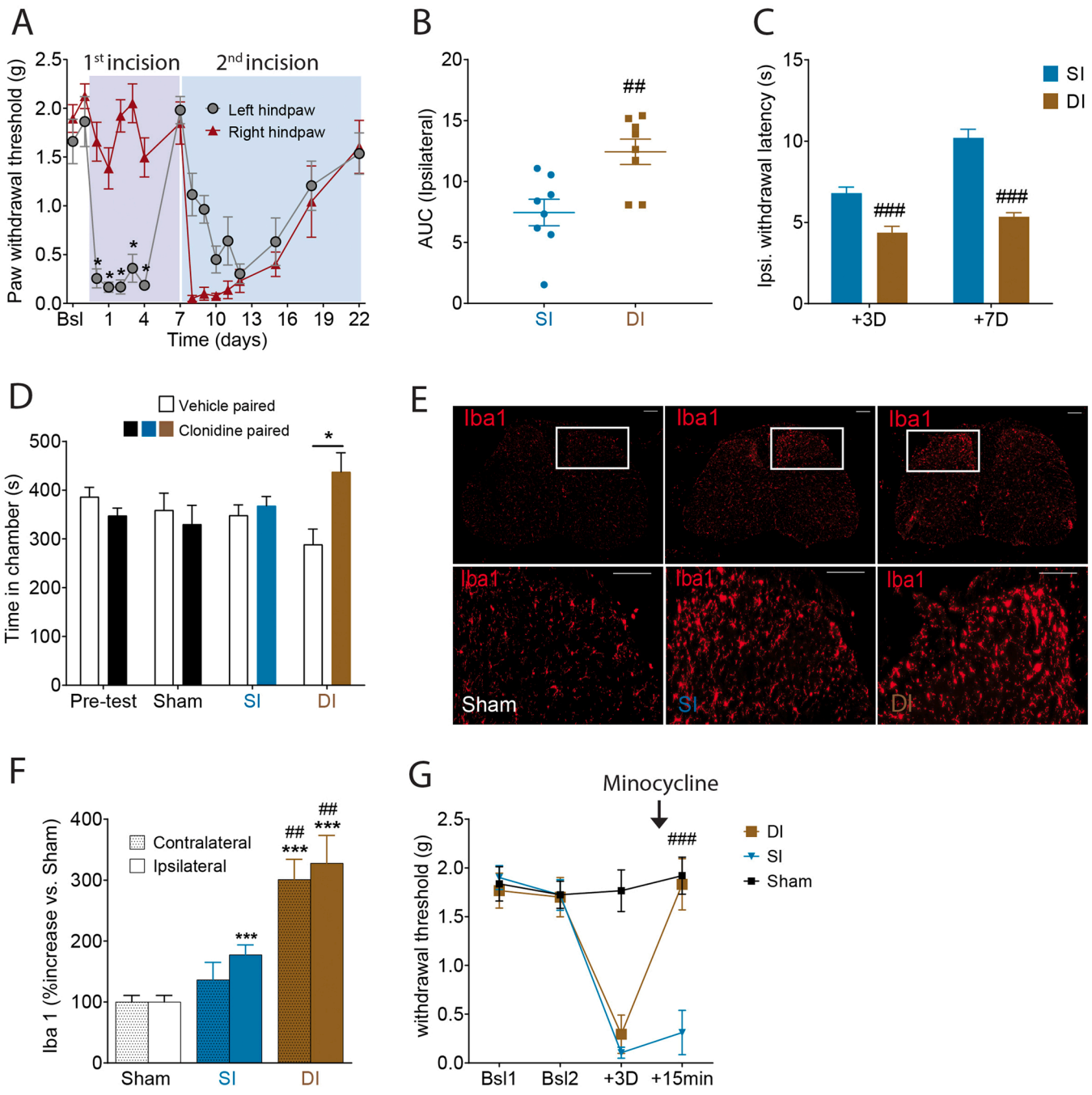

Fig. 2. : Influence of single (SI) or double (DI) incision on sensorial behaviors and microgliosis. (A) Mechanical hypersensitivity after incisions as measured by the Von Frey test and (B) cumulative area under curve representation show increased mechanical hypersensitivity after DI. (C) Ipsilateral withdrawal latency of Hargreave's test shows increased thermal hypersensitivity after DI at D3 and D7. (D) Conditioned place preference induced by clonidine-evoked analgesia 4 days post-Sham, SI or DI showing a place preference only after DI. (E) Iba1 immunoreactivity in the whole spinal cord (left panel) and zoomed dorsal horn (right panel) 7 days after Sham, SI or DI (scale bars = 200 μm) and (F) related quantification shows increased Iba1 staining density ipsilateraly after SI compared to sham, further increased after DI compared to SI. (G) Mechanical threshold of Sham, SI and DI animals before (3 days post-procedure) and 15 min after intrathecal injection of minocycline (300 μg/mouse) show antinociceptive effect of minocycline only after DI. All the values are means ± s.e.m. (n = 8 except in D, n = 10 and F-G, n = 4). Two-way ANOVA and Holm-Sidak's test (A); Student's t-test (B-C); One-way ANOVA and Holm-Sidak's test (D-G); *P < 0.05; * *P < 0.01; * **P < 0.001 vs. Sham, baseline or vehicle paired; #P < 0.05; ##P < 0.01; ###P < 0.001 vs. SI.

observed the days after incision on the ipsilateral paw to the first or the second incision further confirmed that DI mice exhibited exaggerated mechanical pain hypersensitivity compared to SI mice (Fig. 2B). Mice also displayed prolonged thermal pain hypersensitivity after the second incision compared to the first one, as evaluated with the HG test (Fig. 2C). To evaluate spontaneous pain we used CPP experiments 3 days after surgery (SI or DI) or sham (King et al., 2009). Only the DI experimental group developed a place preference for the clonidine-paired chamber (Fig. 2D) indicative of significant ongoing pain after DI.

Next, to characterize the peripheral nervous system alterations induced by the procedures, we first topically applied Fluorogold to retrogradely label the DRG neurons affected by incision. At Day 7 (D7), most of the Fluorogold positive cells were found in L4 (DI: 27 %; SI: 22

%), few in L5 (DI, SI: ~3 %) and none in L6 DRGs (Supplementary Fig. 2A-B). We also evaluated the expression of ATF3 and CSF1, two important molecular factors specifically expressed after neuronal stress or injury (Guan et al., 2015; Obata et al., 2003), (Supplementary Fig. 2C-E). Similarly, at D7, most of ATF3 and CSF1 positive neurons were found in L4 (10–15 %), few in L5 (~3 %) and 0 % in L6 DRGs. Using real time quantitative PCR (RT-qPCR), we then looked at the mRNA expression of additional neuronal (*Atf3, Trpv1)* and immune markers (*Iba1*, *Il1-β*, *Il6*, *Tnf-α*), known to be involved in peripheral sensitization. On the ipsilateral side, most of the markers were upregulated after SI or DI at D3 compared to Sham animals, except for *Trpv1*, which was found upregulated only after DI (Supplementary Fig. 2F). On the contralateral side, markers were upregulated only on the side of the paw subjected to the first incision after DI (Supplementary Fig. 2G). We next examined the expression of different molecular and cellular markers involved in pain sensitization in the dorsal horn of the spinal cord. Interestingly, the nerve injury factors *Atf3* and *Sprr1a* mRNA in ipsilateral dorsal horn spinal cord were strongly increased after incision, with a potentiation in DI as compared to SI (Supplementary Fig. 3A). We further quantified markers associated with microglial activation. Levels of *Cd11b*, *Iba1*, *Csf1* and *Csf1r* mRNA were all found higher after incision with no differences between SI and DI conditions. We also looked at alterations of different spinal processes and cell-types through the labeling of peptidergic afferents (CGRP) and glial cells (Iba1 for microglia and GFAP for astrocytes). We found increased ipsilateral CGRP staining in dorsal horns of SI and DI compared to Sham mice at D3, without any difference between SI and DI mice (Supplementary Fig. 3B and C). CGRP was not increased at D7 (Supplementary Fig. 3D). While we found no difference in GFAP staining at D3 and D7 compared to the control group (Supplementary Fig. 3E-G), Iba1 staining density was found increased compared to Sham at D7 (SI vs. Sham: x 1.8) and potentiated in DI compared to SI mice (DI vs. Sham: x 3.2; DI vs. SI: x 1.6) (Fig. 2E-F). To better understand the implication of microglia in the exaggeration of nociceptive behaviors, we then treated Sham, SI and DI mice with a single intrathecal injection of microglial inhibitors (Kobayashi et al., 2013), minocycline (300 μg/5 μl/animal) at D3, when incision-induced pain hypersensitivity was still observed. Unexpectedly, minocycline restored mechanical thresholds to normal values only after DI and had no effect in Sham or SI mice (Fig. 2G). To test for a potential sex-related difference in microglia-mediated pain hypersensitivity as previously described in many reports (Inyang et al., 2019; Sorge et al., 2015; Tansley et al., 2022), we repeated the experiment in both male and female mice after DI (Supplementary Fig. 4). We observed a strong long lasting antinociceptive effect of minocycline in males still present 3 h after injection. Yet, minocycline produced a weak and short-term antinociceptive effect in females (Supplementary Fig. 4A). Note however that Iba1 staining density was still increased on the ipsilateral side (right hindpaw) in females after DI (Supplementary Fig. 4B-C). To evaluate the effects of microglia proliferation in male animals, we used GW2580 (1 μg/5 μl/animal), which blocks microglia proliferation (Conway et al., 2005). Similar results were obtained when intrathecally administered at the time of the surgeries (Supplementary Fig. 5A-B) without affecting motor coordination (Supplementary Fig. 5C). Strikingly, mice treated with GW2580 displayed enhanced grooming behaviors during the ST, reduced latency to eat during the NSF and immobility duration during the FST ($P = 0.0570$) compared to vehicle-treated mice indicating that the inhibition of spinal microgliosis also has a role in the development of mood disorders (Supplementary Fig. 5D-F). Collectively we show here differentially expressed neuronal injury genes as well as increased microglia activation in the spinal cord after repeated injury bringing evidence of an increased central sensitization responsible for prolonged mechanical hypersensitivity and mood disorder comorbidity.

3.3. Knocking-out FLT3 expression prevents both sensorial and anxiodepressive sensitization following DI

The overexpression of neuronal stress-related molecular markers (*Sprr1a* and *Atf3*) after DI also suggests a higher neuropathic component after DI compared to SI. We recently showed that peripheral FLT3 inhibition prevents the development of neuropathic pain (Rivat et al., 2018). Hence, we questioned the implication of FLT3 receptors in the development of exaggerated pain-related behaviors by assessing both sensorial- and anxiodepressive-like behaviors after DI in *Flt3* Knock-out (*Flt3*$^{-/-}$) mice. In the VF test, the basal mechanical threshold was not affected by FLT3 silencing. After surgery, *Flt3*$^{-/-}$ male mice displayed a faster recovery (Fig. 3A-C) along with reduced mechanical pain hypersensitivity to both the first and the second incision compared to male *Flt3* wild type (*Flt3*$^{+/+}$) mice. Similar results were observed in female *Flt3*$^{-/-}$ mice (Supplementary Fig. 6A-B). CPP experiments also confirm the presence of a place preference for the clonidine-paired chamber in the DI *Flt3*$^{+/+}$ group. This place preference was totally prevented in DI *Flt3*$^{-/-}$ animals (Fig. 3D). To check for a potential role of FLT3 in NSF, ST and FST in basal conditions, we compared behaviors of *Flt3*$^{-/-}$ with *Flt3*$^{+/+}$ animals in naive condition (Supplementary Fig. 6C-E). We did not observe differences between *Flt3*$^{-/-}$ and *Flt3*$^{+/+}$ mice in the ST and NSF but *Flt3*$^{-/-}$ already displayed decreased immobility duration in the FST. In addition, we evaluated the effects of FLT3 silencing on NSF, ST and FST. Locomotor activity and motor coordination were assessed in all tested animals (Fig. 3E; Supplementary Fig. 6F) and were found unchanged. *Flt3*$^{-/-}$ animals showed no significant differences with WT mice in NSF but were significantly less immobile in the FST and presented increased grooming in the ST (Fig. 3G-I, Supplementary Fig. G-I). To understand what could be the cellular alterations possibly responsible for the behavioral modifications after FLT3 inhibition, we thought to look at microglia activation. Iba1 staining in the dorsal horn of WT animals was increased while no change was found in *Flt3*$^{-/-}$ animals after DI (Fig. 3I-L).

3.4. FL injection produces pain hypersensitivity and anxiodepressive-like behaviors

To evaluate whether local FLT3 activation alone can induce both sensorial and alterations in anxiodepressive-like behaviors as displayed in the DI model, mice received intrathecal injections of FL (50 ng/5 μl/animal) and were tested. As previously shown, a single injection led to decreased mechanical threshold for at least 2 days (Fig. 4A). Repeated intrathecal FL (2 weeks, 1inj/3days) failed to impair motor coordination but decreased grooming behavior, increased latency to eat and immobility duration on the ST, NSF and FST compared to vehicle-treated animals, respectively (Fig. 4B-E). All behaviors were significantly modified in all tests after repeated FL treatment, mimicking features of the DI model. At the cellular level, we previously reported that FL produced the activation in DRG of the stress-induced gene transcript Atf3 and several important neuronal pain-related gene transcripts (Rivat et al., 2018). Here, we show that Iba1 staining was found quantitatively increased after repeated FL injections (Fig. 4F-G), supporting a role for FLT3 in central changes. To rule out a possible implication of FLT3 expression in microglia cells after surgery, we performed Flt3 *in situ* hybridization of spinal cord from the CX3CR1^{EGFP} mouse line, in which EGFP is expressed in CX3CR1 + cells, known as macrophages (including microglia). Our data revealed an absence of colocalization between *Flt3* mRNA and CX3CR1 (Supplementary Fig. 7). Then, we evaluated the level of the FLT3 ligand FL after SI or DI in the skin and the dorsal horn of the spinal cord on the ipsilateral side compared to sham animals. Whereas no significant difference was observed between Sham and SI, DI seems to produce an increase in FL concentration in the skin of the ipsilateral hindpaw in comparison with Sham at D3 (Supplementary Fig. 8). No difference was observed in the dorsal horn of the spinal cord. These data suggest that neuronal FLT3 which induces spinal microglial

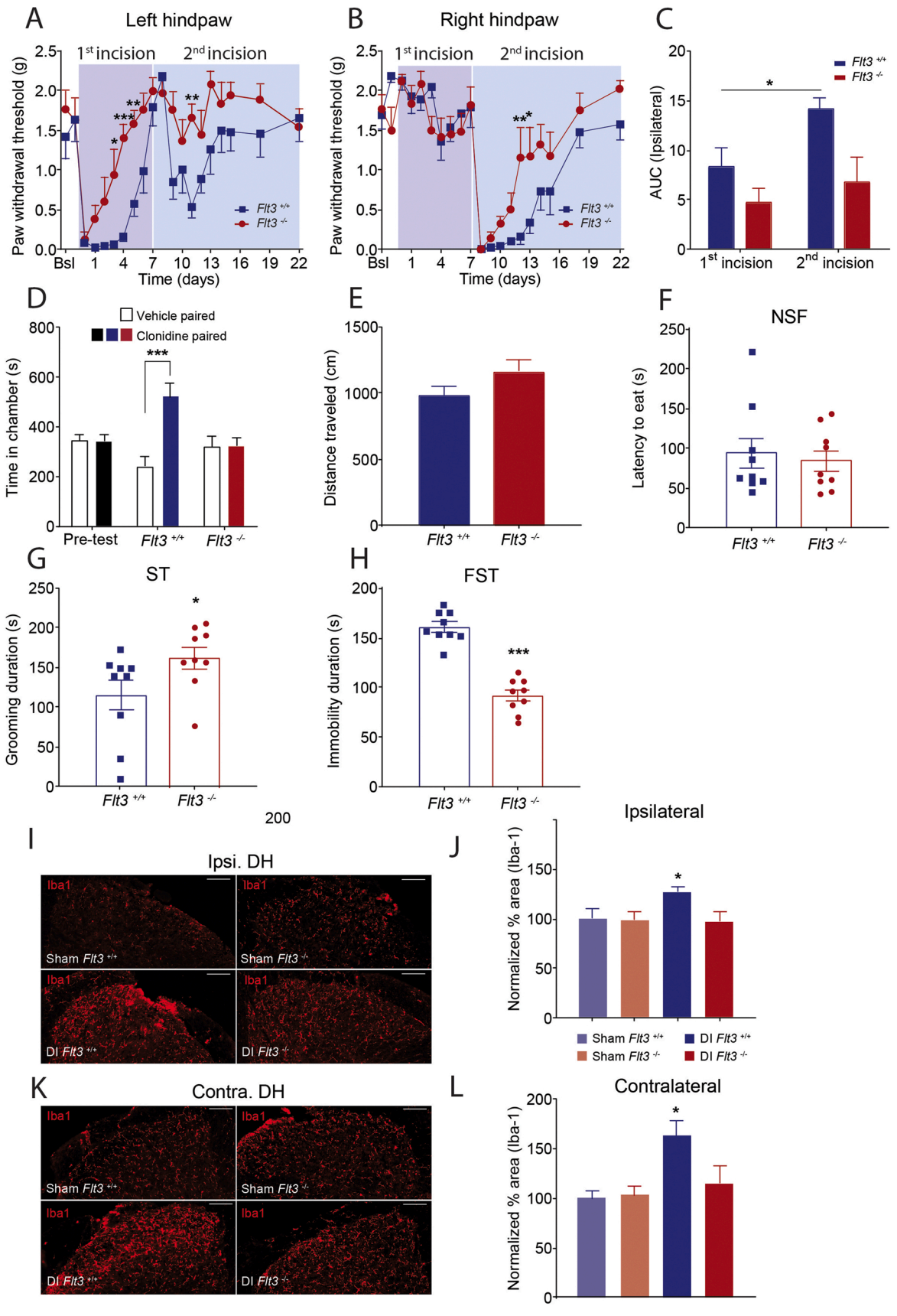

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Fig. 3. : Silencing *Flt3* expression blocks DI-induced behavioral and molecular sensitization. (A, B) Left and right hindpaw mechanical hypersensitivity after incisions on $Flt3^{+/+}$ or $Flt3^{-/-}$ mice as measured by the Von Frey test and (C) cumulative area under curve representation highlight the absence of mechanical hypersensitivity exaggeration after DI in $Flt3^{-/-}$. (D) Conditioned place preference induced by clonidine-evoked analgesia 4 days post-injury is totally prevented in $Flt3^{-/-}$. Silencing *Flt3* expression does not change (E) locomotor activity and (F) latency to eat in novelty suppressed feeding test, but increases (G) grooming duration in splash test and decreases (H) immobility duration in forced swim test compared to $Flt3^{+/+}$ mice. (I) Iba1 immunoreactivity of spinal cord ipsilateral dorsal horn dissected from Sham or DI ($Flt3^{-/-}$ or control) mice and (J) related quantification show significantly higher labeling density in DI $Flt3^{+/+}$ mice compared to Sham $Flt3^{+/+}$. (K) Iba1 immunoreactivity of spinal cord contralateral dorsal horn dissected from Sham or DI ($Flt3^{-/-}$ or control) mice and (L) related quantification show significantly higher labeling density in DI $Flt3^{+/+}$ mice compared to Sham $Flt3^{+/+}$. All the values are means ± s.e.m. (n = 8/9 except in I-L, n = 6/7). Two-way ANOVA and Holm-Sidak's test (A-B); Student's t-test (E-H); One-way ANOVA and Holm-Sidak's test (C, D, J, L); *P < 0.05; * *P < 0.01; * **P < 0.001 vs. $Flt3^{+/+}$ or Sham $Flt3^{+/+}$ or Sham $Flt3^{-/-}$.

activation through its activation by peripheral FL, promotes central pain sensitization and is associated with emotional and sensorial alterations.

3.5. Humanized antibodies against both human and mice FLT3 as a tool for conditional inhibition

Lastly, we developed cross-reacting antibodies specifically directed against both human and mice FLT3. Functional antibodies have the interesting property to not cross the blood brain barrier and could be an advantageous tool for confirming peripheral FLT3 implication, and potentially treat postoperative pain in humans without CNS secondary effects. We produced the mAbA3 antibody that was found to present high affinity for both receptors (Fig. 5A-B; Supplementary Fig. 9). Moreover, Homogeneous Time Resolved Fluorescence (HTRF) signals of FLT3-expressing RS4–11 cells in the absence or presence of FL were strongly reduced in a dose dependent manner after exposure to mAbA3 (Fig. 5C-E). Because we know the therapeutic benefits of inhibiting FLT3 in neuropathic pain models, we validated the *in vivo* efficacy of the antibody in the Chronic Constriction Injury (CCI) of the sciatic nerve model of neuropathic pain. Single or repeated (Fig. 6A, C) systemic injections of mAbA3 totally blocked mechanical pain hypersensitivity evaluated on the injured hindpaw. Inhibition of mechanical hypersensitivity started at a dose of 50 μg/animal of mAbA3 (Fig. 6B). We next tested the efficacy of mAbA3 in animals that underwent repeated incisions. A single preventive systemic injection of 200 μg of mAbA3 during the first incision strongly accelerated recovery to a baseline mechanical threshold after both the first and the second incision on the ipsilateral hindpaw (Supplementary Fig. 10A and B) without affecting either motor coordination (Supplementary Fig. 10C) or DI-induced changes in NSF, ST and FST (Supplementary Fig. 10D-F). Similarly, curative mAbA3 treatment was able to slightly reduce mechanical pain hypersensitivity on the VF test (Supplementary Fig. 10G-H) but failed to prevent the development of anxiodepressive-like disorders as no differences were found between vehicle-treated and mAbA3-treated mice on the ST, NSF and FST (Supplementary Fig. 10J-L). We then determined whether repeated administration of mAbA3 was effective in reducing both sensory and anxiodepressive-related behaviors. Repeating preventive injections of mAbA3 (1 injection every 2 days starting from the day of the first incision until the second incision) not only reduced pain hypersensitivity but also reduced the increase in NSF and FST, and the decrease in ST (Fig. 6D-H). At the cellular level, repeating preventive injections of mAbA3 also blocked the increase in Iba1 immunoreactivity on both sides of the spinal cord 7 days after DI (Fig. 6I-J), further confirming the role of peripheral neuronal FLT3 as the starter of pain-related behaviors *via* spinal activated microglia.

4. Discussion

Our results show that the repetition of surgical incision (DI) leads to sustained depressive-like behaviors along with the exaggeration of nociceptive behaviors and the appearance of spontaneous pain recapitulating the different features of chronic pain in humans (Mills et al., 2019). The altered behaviors are associated with the increased expression of neuropathic-related molecular markers such as *Trpv1* in the DRG, *Atf3* and *Sppr1a* in the spinal cord, a potentiation of spinal microglia activation as well as decreased neurogenesis in the hippocampus supporting central alterations after DI. The DI model thus constitutes an interesting model to study pain chronification. We also report the implication of FLT3 in sustained pain sensitization produced by repeated injury. Strikingly, FLT3 activation alone is sufficient to trigger microglial activation, pain and anxiodepressive-like behaviors. Conversely, FLT3 deletion leads to the complete prevention of DI-related behavioral and molecular modifications. We thus developed an innovative therapeutic tool, the FLT3 human antibody (mAbA3), targeting both human and murine FLT3. Treatment with FLT3 antibodies not only blocked spinal microglial activation and exaggerated pain behaviors produced by repeated surgical procedure but also the subsequent depressive-like behaviors.

To better examine the mechanisms involved in pain chronification after acute injury, we used an experimental procedure that consists of repeating a surgical incision on the opposite hindpaw of the same animals 7 days apart (Cabañero et al., 2009). Here, our main objective was to determine whether repeated acute injury would produce pain sensitization peripherally and centrally. To address this question, we compared the behavioral and molecular alterations after SI and DI. As previously reported, the pain hypersensitivity produced by the second surgery is largely enhanced compared to the one produced by the first surgery. After SI, 7 days were required for the pain hypersensitivity to resolve, compared to 14 days after DI. This observation suggests that surgery, like inflammation, produces a sustained sensitized state leading to increased vulnerability to develop persistent pain when sensitized individuals are challenged with subsequent stimuli leading to exaggerated nociceptive behaviors (Reichling and Levine, 2009; Rivat et al., 2002). However, the impact of such a repetition on the development of emotional disorders has not been evaluated yet. Our data revealed that DI not only produces exaggerated pain behaviors but also leads to anxiodepressive-like behaviors starting after the second injury and lasting 3 weeks post-injury. Behavioral alterations are associated with reduced hippocampal neurogenesis which is consistent with the depressive phenotype described in both rodents and humans (Bremner et al., 2000; Duman and Monteggia, 2006; Pittenger and Duman, 2008; Sheline et al., 1999). Interestingly, depressive-like behaviors were already reported in other pre-clinical models of pain but usually develop after 5–7 weeks following nerve injury (Gonçalves et al., 2008; Suzuki et al., 2007; Yalcin et al., 2011). These models allow us to identify the neuronal circuits supporting comorbidity of persistent pain and mood disorders (Becker et al., 2020; Zhou et al., 2019). Therefore, the DI could become an outstanding model to study chronic pain but also mood-related comorbidities due to the fast development of pain and depression.

As already proposed, the mechanical pain hypersensitivity exaggeration produced by the second surgery on the previously non-operated hindpaw (right hindpaw) strongly suggests that pain sensitization is maintained mainly through central nervous system mechanisms (Rivat et al., 2002), without excluding a contribution of the peripheral nervous system in the onset of such sensitization as observed with the increased expression of *Trpv1* in DI animals compared to SI. The central dimension is also supported by the pain hypersensitivity triggered in the contralateral hindpaw (left hindpaw) to the second surgery. The behavioral modifications after DI could result in the summation of peripheral

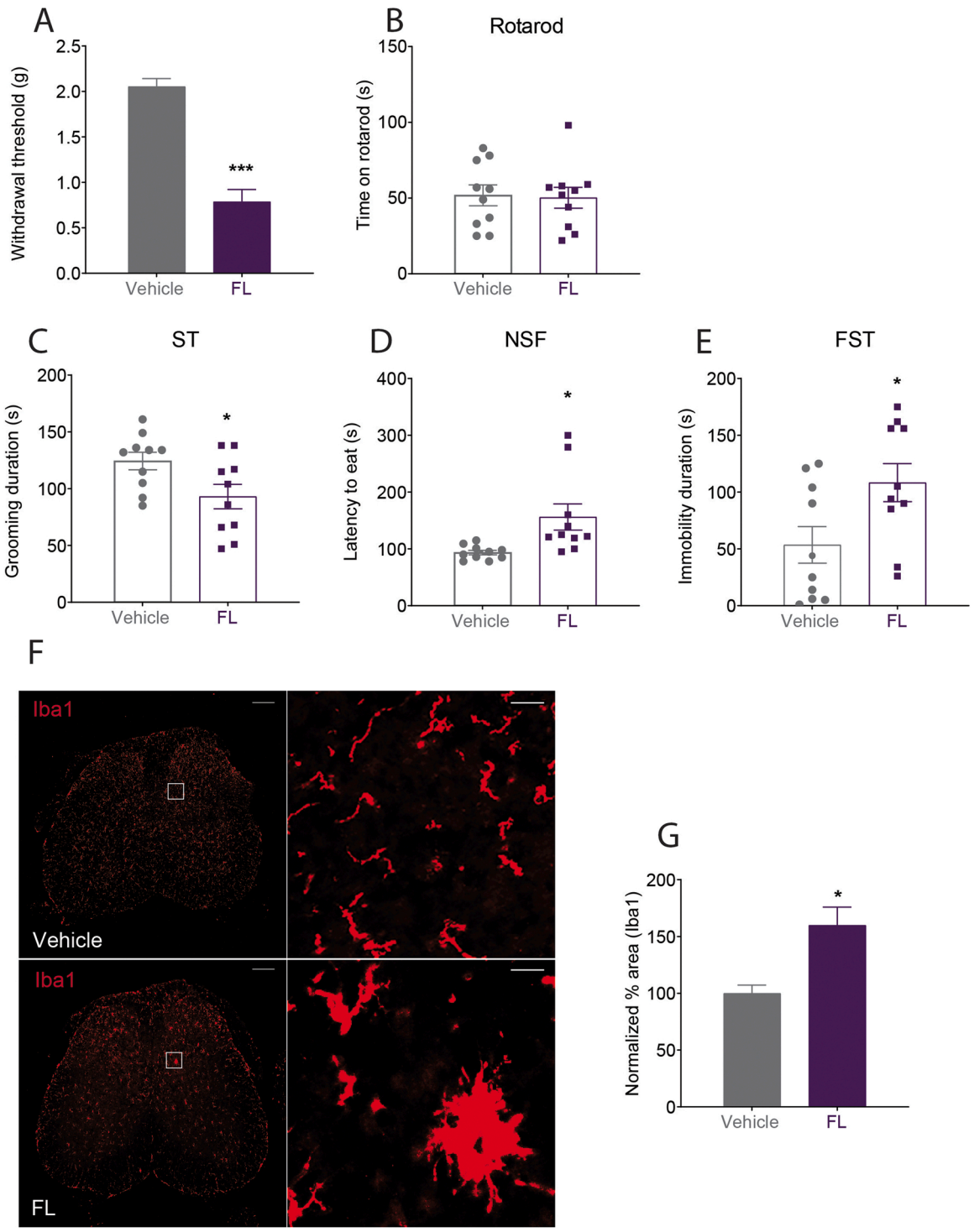


Fig. 4. FL administration mimics DI induced behavioral and molecular alterations. (A) Mechanical hypersensitivity measured with Von Frey filaments after a single intrathecal injection of FL (50 ng/5 μl) or saline shows decreased withdrawal thresholds after FL. (B) Repeated intrathecal injections of FL (50 ng/5 μl, 5 injections delivered across 15 days) fail to affect performance on rotarod, (C) decrease grooming duration in splash test, (D) increase latency to eat in novelty suppressed feeding test and (E) immobility duration in forced swim test compared to control injections. (F) Iba1 immunoreactivity of whole spinal cord dissected from vehicle injected and FL injected mice (50 ng/5 μl, daily injection for 3 days) 24 h after the last injection (left scale bars = 200 μm; right scale bars = 20 μm) and (G) related quantification shows increased Iba1 staining density after FL. All the values are means ± s.e.m. (n = 10 except in F, G, n = 4). Student's t-test (A-E); unpaired Mann-Whitney t-test (G); *P < 0.05; * **P < 0.001 vs. I.t. Vehicle.

nociceptive inputs from both sides reaching the spinal cord leading to increased central changes. We thus evaluated potential modifications in the spinal cord of these animals. We showed that microglia activation is significantly enhanced after DI compared to SI or control. Note however that this was only observed at the protein level and that transcriptional changes related to microglia activation were found unchanged. An explanation could be that once microglia is activated, transcriptional changes are no longer required to potentiate microglia reactivity. Instead, post-transcriptional modifications would occur and regulate microglia. This aspect remains to be clarified. In males, the curative

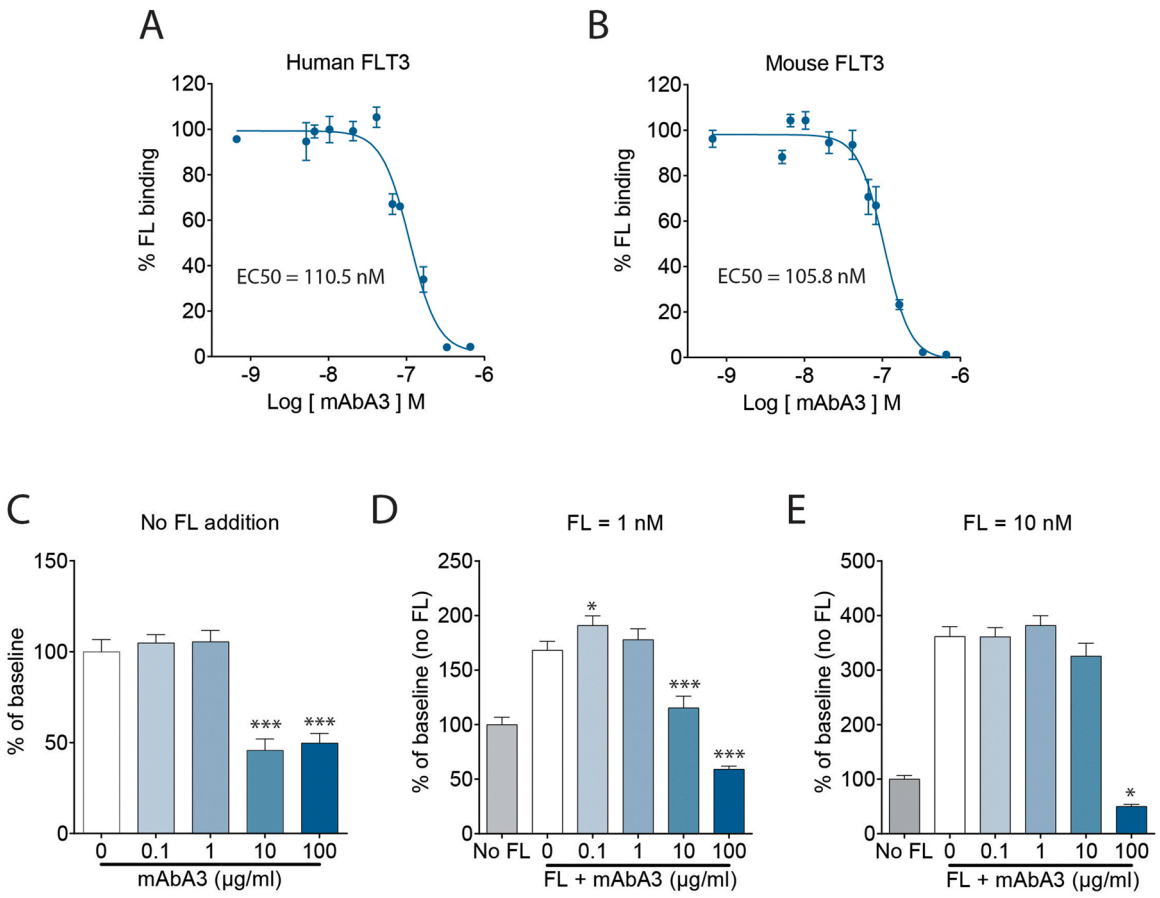
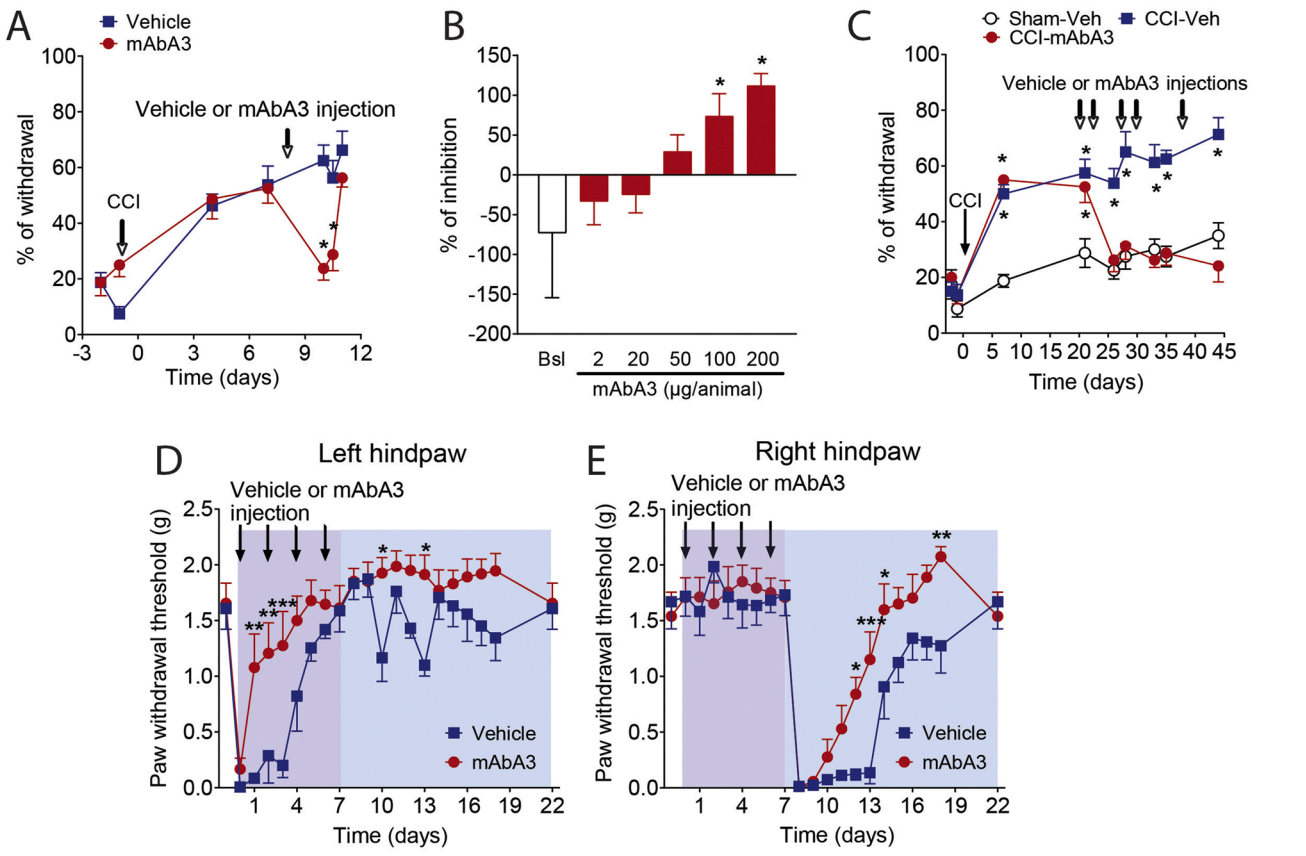


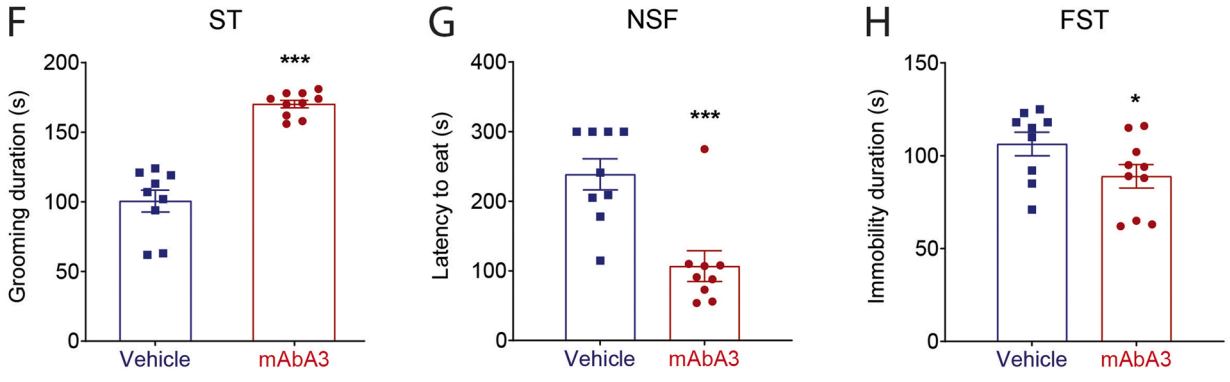
Fig. 5. : MAbA3 is a high affinity functional antibody against both human and murine FLT3 receptors. Binding curves of FL on human (**A**) and murine FLT3 (**B**) in presence of increasing doses of mAbA3. MAbA3 dose dependently reduces HTRF signal reporting mouse FLT3 constitutive activity (**C**) and FL-induced increased FLT3 activity after FL application at 1 nM (**D**) and 10 nM (**E**). All the values are means ± s.e.m. (n = 4); *P < 0.05; * *P < 0.01; * **P < 0.001 vs. Baseline.

inhibition of microglial activation *via* minocycline or GW2580 leads to a quick recovery of pain sensitivity only after DI. Our observation agrees with previous data reporting that primed spinal microglia by neonatal incision is involved in the persistent pain in re-incised adult rats (Schwaller et al., 2015). Interestingly, the microglial inhibition *via* GW2580 also reduces anxiodepressive-related behaviors, indicating that spinal microglial overactivation produced by repeated incisions may be responsible for both DI-induced sensorial and emotional alterations. In females, microglia is also activated after DI but, in agreement with previous reports (Inyang et al., 2019; Sorge et al., 2015; Tansley et al., 2022), microglia inhibition has a less pronounced curative effect on mechanical hypersensitivity. These data suggest that microglia in female animals is unlikely responsible for the maintenance of exaggerated mechanical nociceptive behaviors after DI. Further studies will be necessary to examine whether spinal T lymphocytes may be involved as already described in females after nerve injury (Sorge et al., 2015). Concerning astroglial activation, GFAP expression was not different in the spinal cord after SI or DI, as opposed to what has been reported in rats (Obata et al., 2006). We can arguably propose that astrogliosis potentially appears later than the investigated timeframe. Altogether, our results show the involvement of central pain sensitization mechanisms in the exaggeration of pain and depressive-like behaviors, in accordance with other chronic pain models (Zhou et al., 2019).

In neuropathic pain models, central sensitization is partly mediated by CSF1 release from sensory neurons of the DRG (Guan et al., 2015) that then reaches the spinal cord to activate microglial CSF1R. Several observations across the study are supporting a major neuropathic component after incision, especially after DI. First, we discovered a peripheral activation of CSF1 in both SI and DI, which is absent in an inflammatory pain model (Okubo et al., 2016). Secondly, this activation was associated with an activation of ATF3 expression in the majority of CSF1+ sensory neurons. Likewise, we also observed the spinal expression of *Atf3* and *Sprr1a* mRNAs, two markers of nerve injury (Méchaly et al., 2006; Obata et al., 2003). More importantly, FLT3 inhibition has been shown to induce therapeutic effects specifically in neuropathic pain rather than inflammatory pain models. The observation of an improved post-surgical recovery after FLT3 inhibition brings more evidence of a neuropathic component inherent to our models. Finally, the increased expression of the spinal markers in DI vs. SI suggests a stronger neuropathic component in DI compared to SI supporting the clinical observation that persistent post-surgical pain is partially neuropathic in nature (Haroutiunian et al., 2013).

In the continuity of our previous work showing the involvement of FLT3 in neuropathic pain and considering the important neuropathic contribution in these models, we then evaluated the effects of FLT3 inhibition and activation on incision-induced central sensitization. Absence of *Flt3* expression in *Flt3*$^{-/-}$ mutant mice leads to faster recovery of pain hypersensitivity after incision, and to a total prevention of spontaneous pain and anxiodepressive-like behaviors. Spinal microgliosis activation is also reduced in *Flt3*$^{-/-}$ male animals. Notably, beneficial behavioral effects of FLT3 silencing are common to both males and females. As a difference between males and females has been observed, the sexual dimorphism suggests that different FLT3-dependent mechanisms are responsible for the exaggerated pain-related behaviors observed after DI in female animals. Further studies will be necessary to elude how FLT3 exaggerates nociceptive behaviors in female animals. A major finding of the study is that beside the effects on DRG previously reported by our team (Rivat et al., 2018), FL alone





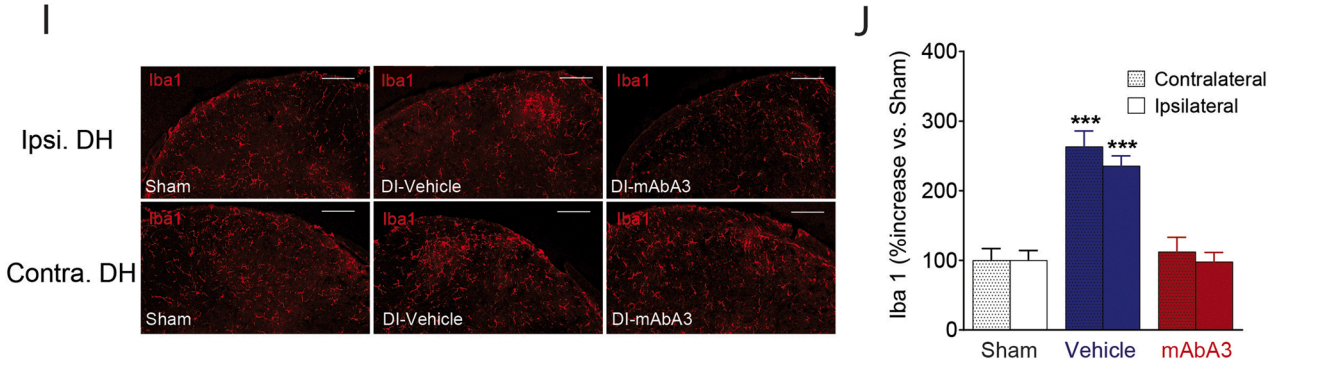


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Fig. 6. : MAbA3 treatment against FLT3 prevents the development of CPSP. (A) A single injection of mAbA3 (100 μg/mouse, intraperitoneal) reduces CCI-related (9 days after chronic constriction injury of the sciatic nerve) mechanical hypersensitivity as measured 1, 1.5 and 2 days after injection in the Von Frey test. (B) Experiments were performed as in A, with doses of mAbA3 ranging from 2 to 200 μg/animal. Data represent the maximum percentage of pain hypersensitivity inhibition. (C) Intraperitoneal vehicle or mAbA3 injections (200 μg/animal) were initiated 25 days after CCI of the sciatic nerve and repeated at times indicated by arrows. Mechanical hypersensitivity was recorded 26, 28, 33, 35 and 44 days after CCI. (D-E) Left and right hindpaw mechanical hypersensitivity after incisions in either vehicle or mAbA3 treated (arrows= 200 μg/animal, intraperitoneal) mice as measured by the Von Frey test. Repeated intraperitoneal injections of mAbA3 (200 μg/animal, 1 injection each 2 days during the first incision phase for a total of 4 injections) increases grooming duration in splash test (F), decreases latency to eat in novelty suppressed feeding test (G) and immobility duration in forced swim test (H) in treated mice compared to control mice. (I) Iba1 immunoreactivity of spinal cord dissected from sham, vehicle-DI or mAbA3-DI mice at D7 (scale bars = 200 μm) and related quantification shows decreased Iba1 immunoreactivity after mAbA3 treatment (J). Two-way ANOVA and Holm Sidak's test (A-E), Student's t-test (F-H); (J) One-way ANOVA and Holm-Sidak's test. *P < 0.05; * *P < 0.01; * **P < 0.001 vs. Sham-Veh or Vehicle.

can also activate microglia in the dorsal horn of the spinal cord and recapitulate all the aspects of emotional and pain sensitization exhibited in DI. Interestingly, the effect of FL on FLT3 seems to be independent of microglia itself because *Flt3* mRNA is absent in CX3CR1 positive cells. Moreover, since *Flt3* mRNA is poorly expressed in the superficial dorsal horn layers of the spinal cord, we can arguably speculate that the intrathecal injection of FL leads to the activation of peripheral FLT3, which then trigger the activation of spinal microglia through an indirect mechanism. In addition, we previously reported that FLT3 may regulate the expression of CSF1 after peripheral nerve injury (Rivat et al., 2018). Here, even if no difference is observed in the increased expression of CSF1 in the DRGs and the spinal cord between SI and DI, one may speculate that the release of CSF1 by sensory neurons after SI may prime spinal microglia activation. After DI, additional CSF1 release on sensitized spinal cord may produce a sustained general neuroinflammation, as observed with the microglial activation on the ipsilateral and contralateral side after DI, responsible for exaggerated sensory and emotional behaviors. This is supported by the fact that CSF1R inhibitor GW2580 reduces exaggerated pain-related behaviors after DI. Future studies will aim at better understanding the neuro-immune interactions responsible for this mechanism. To test for a possible role of peripheral FLT3 in central sensitization leading to comorbidities, we then developed functional antibodies (mAbA3) to reach high affinity and specificity for both mouse and human FLT3 using phage-display and a scFv synthetic library (Robin et al., 2014; Robin and Martineau, 2012). The preventive use of mAbA3 largely decreases the time of recovery and totally hinders the development of anxiodepressive disorders and related microglial activation. These results clearly support the role of peripheral neuronal FLT3 in the development of pain sensitization after repeated injury, the crossing of functional antibodies into the central nervous system being impeded by the blood-brain barrier.

In conclusion, we demonstrate that repeated peripheral incisions lead to sustained sensorial and emotional alterations through central pain sensitization. Increased expression of axonal regeneration genes and markers of neuropathy unveils the strong neuropathic component in our models, particularly in DI. Our study supports peripheral FLT3 as an important upstream neuronal modulator of central pain sensitization leading to exaggerated pain-related behaviors and anxiodepressive disorders. Targeting FLT3 *via* functional inhibitory antibodies efficiently alleviates sensitization, opening new avenues for the management and the prevention of pain chronification and related mood disorders.

CRediT authorship contribution statement

A.T., M.T., J.B. and C.S. performed behavioral pharmacology. A.T., D.G., L.D. and M.T. performed histology. J.P.L. performed *in vitro* studies. A.T. and I.M. performed RNA transcriptomic studies. A.J. provided critical input on study design and interpretation. S.M. performed mouse genotyping. C.R., J.V. and A.T. designed studies and wrote the manuscript. M.C., M.N., B.R., P.M. generated mAbA3. C.R. and J.V. conceived the project and X.C., C.R. supervised all experiments. All the authors have contributed to data analysis, interpretation and editing of the manuscript.

Conflict of interest statement

C.S., L.D., and J.P.L. were full-time employees at Biodol Therapeutics. J.V. is inventor of patents claiming the use of FLT3 inhibitors for the treatment of neuropathic pain and is co-founder of Biodol Therapeutics. The other authors declare no conflict of interests.

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pneurobio.2023.102405.

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