Identification of spinal circuits involved in touch-evoked dynamic mechanical pain

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Mechanical hypersensitivity is a debilitating symptom for millions of chronic pain patients. It exists in distinct forms, including brush-evoked dynamic and filament-evoked punctate hypersensitivities. We reduced dynamic mechanical hypersensitivity induced by nerve injury or inflammation in mice by ablating a group of adult spinal neurons defined by developmental co-expression of VGLUT3 and Lbx1 (VT3Lbx1 neurons): the mice lost brush-evoked nocifensive responses and conditional place aversion. Electrophysiological recordings show that VT3Lbx1 neurons form morphine-resistant polysynaptic pathways relaying inputs from low-threshold Aβ mechanoreceptors to lamina I output neurons. The subset of somatostatin-lineage neurons preserved in VT3Lbx1-neuron-ablated mice is largely sufficient to mediate morphine-sensitive and morphine-resistant forms of von Frey filament-evoked punctate mechanical hypersensitivity. Furthermore, acute silencing of VT3Lbx1 neurons attenuated pre-established dynamic mechanical hypersensitivity induced by nerve injury, suggesting that these neurons may be a cellular target for treating this form of neuropathic pain.

Clinical observations showing that pain can be evoked by innocuous mechanical stimuli, called mechanical allodynia, have contributed to the development of the gate-control theory of pain1–3. According to this theory, spinal pain transmission neurons receive inputs from both nociceptors and low threshold mechanoreceptors (LTMRs); LTMR inputs are gated by feedforward inhibition4–8. In chronic pain conditions caused by nerve lesions or inflammation, attenuation of this feedforward inhibition plus sensitization of primary and relay neurons opens the gate, allowing LTMR inputs to activate pain transmission neurons and causing allodynia9–12. Allodynia exists in multiple forms in humans, including dynamic, static and punctate. Dynamic allodynia, one of most distressing and prevalent forms of chronic pain, is evoked by stimulation as gentle as touching the skin with garments, running water or even wind13. Static allodynia is evoked by pressure generated by a large probe (1-cm diameter in human studies)14,15, which is probably equivalent to the Randall-Selitto assay used for animal studies. Punctate allodynia, sometimes wrongly termed ‘static’ allodynia in animal studies5,16,17, is evoked by von Frey filament stimulation. In human psychophysical studies, dynamic allodynia is associated with shooting, lancing, burning and sore sensations, and static allodynia is associated with burning sensations13–15,18,19. Percepts associated with punctate allodynia may have not yet been carefully documented.

Several studies indicate that different neural substrates mediate distinct forms of allodynia or mechanical hypersensitivity (in this paper, we use ‘allodynia’ to mean pain evoked by innocuous stimuli, whereas ‘mechanical hypersensitivity’ is used to describe enhanced nocifensive motor behaviors in response to innocuous stimuli without actual measurement of emotional and/or cognitive aspects of pain). For primary afferents, myelinated LTMRs are required to mediate dynamic allodynia in humans14,15,18–22, whereas unmyelinated C fibers can sufficiently transmit pressure-evoked static allodynia14,15. Animal studies show that punctate mechanical hypersensitivity may be transmitted via both A and C fibers23–25. However, it remains unknown whether or not there are spinal circuits differentially involved in distinct forms of allodynia and/or mechanical hypersensitivity. Glycinergic neurons gate dynamic hypersensitivity at hindbrain levels16 but also the punctate form at spinal levels4,6. Similarly, spinal interneurons expressing the gamma isoform of protein kinase C (PKCγ) may mediate both dynamic16 and punctate4,26 mechanical hypersensitivity. Spinal neurons marked by somatostatin16–20 (SOMCre20) and spinal circuits whose maturation is influenced by transient developmental expression of the vesicular glutamate transporter 3 (VGLUT3) are involved with both forms of hypersensitivity as well20.

Here we selectively ablated or silenced adult spinal neurons that are marked by co-expression of the Cre and Flpo DNA recombinases driven from the Vglut3 (Slc17a8) gene locus (VT3Cre) and the Lbx1 gene locus (Lbx1Flpo), respectively. We referred to these neurons as VT3Lbx1 neurons. Using behavioral and electrophysiological studies,
we show that VT3\textsuperscript{Lbx1} neurons form a morphine-resistant microcircuit necessary for the transmission of brush-evoked dynamic mechanical allodynia. We further characterized multiple morphine-sensitive and morphine-resistant spinal circuits that are eliminated in SOM\textsuperscript{Lbx1}-ablated mice but preserved in VT3\textsuperscript{Lbx1}-ablated mice and which could potentially mediate von Frey filament-evoked punctate hypersensitivity.

**RESULTS**

**Spinal neurons marked by VT3\textsuperscript{Cre} expression**

To mark VGLUT3 lineage neurons, we generated the Vglut3-\textit{IRES-Cre} (referred to as VT3\textsuperscript{Cre}) mice, in which a cassette containing the Cre recombinase gene was inserted into the Vglut3 gene locus, 3’ to the stop codon sequence (Supplementary Fig. 1a). By crossing VT3\textsuperscript{Cre} mice with Ai14-tdTomato reporter mice\textsuperscript{28}, spinal neurons that developmentally express VT3\textsuperscript{Cre} were labeled with the red fluorescent tdTomato protein (referred to as VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons; Fig. 1a-c). At postnatal day 4 (P4), most neurons with detectable VGLUT3 mRNA (92%, 304 of 330) co-expressed tdTomato (Fig. 1a). The percentage of tdTomato\textsuperscript{+} neurons with detectable VGLUT3 mRNA declined from 34% (304 of 889) at P4 to 21% (288 of 1,344) at P7 and to near none at adult stages, consistent with transient VGLUT3 expression\textsuperscript{22}.

We next performed double-staining immunohistochemistry with lamina-specific markers. The neurokinin (NK1) receptor marks a major subset of ascending projection neurons in lamina I\textsuperscript{23}. VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons are located ventral to neurons expressing NK1 receptors, with little overlap (2 of 195 neurons expressed both; Fig. 1b).

They are intermingled with IB4\textsuperscript{+} terminals that label nonpeptidergic primary afferent nociceptors terminating in the inner layer of dorsal lamina II (\textit{II}i)\textsuperscript{28} and are also found in the outer layer of lamina II (\textit{II}o), which receives inputs from peptidergic nociceptor afferents (Fig. 1b)\textsuperscript{29}. PKC\textgamma\textsuperscript{+} interneurons, which have been implicated in the transmission of neuropathic pain\textsuperscript{4,7,26}, are located in the inner layer of ventral lamina II (\textit{II}v) and in the most dorsal area of lamina III (\textit{III}), an area receiving inputs from LTMRs\textsuperscript{30,31} and which is referred to as the II–III border area (or, \textit{II}v–\textit{III}). VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons were observed in both \textit{II}v–\textit{III} and more ventral lamina III (Fig. 1b), and 42% (234 of 567) of PKC\textgamma\textsuperscript{+} neurons co-expressed tdTomato.

Thus, VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons were scattered throughout laminae II and III. The vast majority of adult VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons were excitatory, with 96% (540 of 563) expressing VGLUT2 (Supplementary Fig. 1c) and only very few tdTomato\textsuperscript{+} cells express inhibitory neuron markers (Supplementary Fig. 1b). VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} cells accounted for 32% (540 of 1,671) of total VGLUT2 glutamatergic neurons within laminae II and III (Fig. 1c).

Spinal neurons marked by SOM\textsuperscript{Cre} transmit acute mechanical pain and mediate chronic mechanical hypersensitivity\textsuperscript{5}. In \textit{II}v–\textit{III}, 38% (126 of 328) of VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons co-expressed SOM, and 28% (126 of 455) of SOM\textsuperscript{+} neurons co-expressed tdTomato (Supplementary Fig. 1c). Partial overlap between VT3\textsuperscript{Cre}-tdTomato

![Figure 1](link-to-figure1.png)

Figure 1 Characterization of VT3\textsuperscript{Cre}-tdTomato neurons and punctuate sensitivity in VT3\textsuperscript{Lbx1}-ablated mice. (a-c) Spinal sections from (a) P4 mice and (b,c) adult mice showing tdTomato signals (red) and VGLUT3, NK1 receptors (NK1R), IB4 (a marker for cells mainly expressing MrgrpD), PKC\textgamma for VGLUT2 (green). Right panels in a represent higher magnification of the boxed areas. Arrows indicate co-localization, and arrowhead in a indicates a cell expressing VGLUT3 but not tdTomato. (d) Genetic strategy for driving DTR expression in spinal VT3\textsuperscript{Lbx1} neurons. Tau, the Mapt gene locus; LoxP and FRT, recognition sequences for Cre and Flpo DNA recombinases, respectively. (e) Ablation of 86% of VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} neurons in adult lumbar cord (control; 96 ± 11 cells per hemisection, ablated (VT3\textsuperscript{Cre}-Abl): 14 ± 4 cells; n = 3 mice per group; two-tailed Student’s unpaired t test; \textit{t} = 6.885, *** \textit{P} = 0.002). Large arrowhead, remaining cells; small arrowhead, processes likely from VT3\textsuperscript{Cre}-tdTomato\textsuperscript{+} primary afferents. TMT, tdTomato\textsuperscript{+} neurons. (f) Increased withdrawal thresholds to von Frey filament stimulations (\textit{n} = 17, control; \textit{n} = 15, ablated; two-tailed Student’s unpaired \textit{t} test; \textit{t} = 4.4107, ** \textit{P} < 0.01). No detectable changes in pinprick responses (\textit{n} = 10, control; \textit{n} = 12, ablated; unpaired \textit{t} test, \textit{t} = 1.180, \textit{P} = 0.2519), pinch (\textit{n} = 10, control; \textit{n} = 12, ablated; two-tailed Student’s unpaired \textit{t} test, \textit{t} = 0.7881, \textit{P} = 0.4399) or Randall-Selitto tests (\textit{n} = 11 in each group; two-tailed Student’s unpaired \textit{t} test, \textit{t} = 1.618, \textit{P} = 0.1213). Scale bars, 50 μm in all images. Data are presented as mean ± s.e.m.; n.s., nonsignificant.
and SOM was also observed in I–II, and in III–IV, albeit to lower degrees than in I, II, and III (Supplementary Fig. 1c). We also found that 45% (369 of 827) of Calb2+ (calretinin Brasil) cells were marked by VT3Cre::tdTomato (Supplementary Fig. 1d), indicating that spinal neurons marked by our knock-in VT3Cre were not identical to those marked by the transgenic VT3::Cre, which marks only 8% of adult Calb2+ neurons.

**Impaired transmission of light punctate mechanical information following ablation of VT3Cre-marked neurons**

To assess the function of the VT3Cre-derived neurons, we used an intersectional genetic strategy5,32 to express the diphtheria toxin (DTX) receptor (DTR) in these neurons (Fig. 1d). To do this, VT3Cre mice and Lbx1Fpoe mice5 were crossed with intersectional Tau-LSL-FSF-DTR mice carrying the Cre-dependent ROSA26-LSL-tdTomato reporter allele. In the resulting TauDTR/+;ROSAtdTomato+/+;Lbx1Fpoe/+;VT3Cre/+ quadruple heterozygous mice, DTR expression was restricted to dorsal spinal and hindbrain neurons that co-expressed VT3Cre and Lbx1Fpoe, while all neurons with developmental VT3Cre expression were marked with tdTomato. One month after DTX injections into adult mice, 86% of VT3Cre::tdTomato+ neurons were eliminated in the dorsal spinal cord (Fig. 1e) and hindbrain trigeminal nuclei (Supplementary Fig. 2). No ablation was observed in other parts of the nervous system (Supplementary Fig. 2). We refer to these mice as VT3Lbx1-ablated (VT3Lbx1-Abl). The 14% of VT3Cre::tdTomato+ neurons preserved in VT3Lbx1-ablated mice (Fig. 1e) potentially represented cells that also lacked Lbx1Fpoe expression, and the dense tdTomato+ processes in I (Fig. 1e) likely corresponded to the central terminals of nonablated VT3Cre-derived dorsal root ganglia (DRG) neurons.

Behavioral analyses were performed at least 1 month after the first DTX treatment, when transient activation of glia cells induced by cell ablation had resolved to baseline levels (data not shown). Littermates lacking DTR expression but receiving the same DTX injection served as controls. VT3Lbx1-ablated mice showed reduced sensitivity to gentle von Frey filament-evoked punctate stimulations (Fig. 1f) but did not display significant changes in their responses to intense noxious mechanical stimuli, such as pinpricking, pinching or intense pressure evoked by the Randall-Selitto apparatus (Fig. 1f). VT3Lbx1-ablated mice also showed normal locomotor coordination, touch and thermal sensitivity, as well as scratching responses as evoked by various pruritogens (Supplementary Fig. 3a–i).

**Impact of VT3Lbx1 ablation on mechanical hypersensitivity**

We then examined two forms of mechanical hypersensitivity induced by nerve injury or inflammation: brush-evoked dynamic and filament-evoked punctate hypersensitivity (Fig. 2a). We first used the spared nerve injury (SNI) model to assess neuropathic mechanical hypersensitivity. Both male and female VT3Lbx1-ablated mice displayed a marked attenuation of brush-evoked dynamic hypersensitivity following SNI (Fig. 2b and Supplementary Fig. 4a). No significant change in filament-evoked punctate hypersensitivity (Fig. 2b) or cold allodynia (Supplementary Fig. 3j) was detected. In two inflammatory pain models induced by intraplantar injection of the complete Freund’s adjuvant (CFA; 3.0 mg) or carrageenan (Supplementary Fig. 3a), we found that 45% (369 of 827) of Calb2+ (calretinin+) cells were marked by VT3Cre::tdTomato, which was used to express the diphtheria toxin (DTX) receptor (DTR) in these neurons (Fig. 1d). To do this, VT3Cre mice and Lbx1Fpoe mice were crossed with intersectional Tau-LSL-FSF-DTR mice carrying the Cre-dependent ROSA26-LSL-tdTomato reporter allele. In the resulting TauDTR/+;ROSAtdTomato+/+;Lbx1Fpoe/+;VT3Cre/+ quadruple heterozygous mice, DTR expression was restricted to dorsal spinal and hindbrain neurons that co-expressed VT3Cre and Lbx1Fpoe, while all neurons with developmental VT3Cre expression were marked with tdTomato. One month after DTX injections into adult mice, 86% of VT3Cre::tdTomato+ neurons were eliminated in the dorsal spinal cord (Fig. 1e) and hindbrain trigeminal nuclei (Supplementary Fig. 2). No ablation was observed in other parts of the nervous system (Supplementary Fig. 2). We refer to these mice as VT3Lbx1-ablated (VT3Lbx1-Abl). The 14% of VT3Cre::tdTomato+ neurons preserved in VT3Lbx1-ablated mice (Fig. 1e) potentially represented cells that also lacked Lbx1Fpoe expression, and the dense tdTomato+ processes in I (Fig. 1e) likely corresponded to the central terminals of nonablated VT3Cre-derived dorsal root ganglia (DRG) neurons.

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we again observed a marked attenuation of dynamic, but not punctate, hypersensitivity. We next tested a lower dosage of carrageenan (0.5%) and found that the VT3Lbx1-ablated mice could be grouped into two response clusters (Fig. 2d). The major cluster (10 of 16 mice) developed robust hypersensitivity, with withdrawal thresholds (0.96 ± 0.89 g) comparable to those of VT3Lbx1-ablated mice without inflammation (1.56 ± 0.80 g, P = 0.14; Fig. 2d). Thus, under weak inflammatory conditions, VT3Lbx1 neurons contributed to the induction or expression of punctate hypersensitivity (see “Discussion” section, below). However, these neurons are not required for this form of hypersensitivity under strong inflammatory conditions or following nerve lesion.

Mechanical hypersensitivity induced by nerve injury or inflammation is caused by both neuronal sensitization and disinhibition9–12. To assess whether the VT3Lbx1 neurons mediated mechanical hypersensitivity induced purely by disinhibition, we gave mice intrathecal injections of bicuculline and strychnine to inhibit GABA A and glycine receptors, respectively. Once again, we detected a reduction in brush-evoked dynamic, but not filament-evoked punctate, mechanical hypersensitivity in VT3Lbx1-ablated mice (Fig. 2e). In contrast, both dynamic and punctate mechanical hypersensitivities induced by bicuculline and strychnine were eliminated in SOMLbx1-ablated mice (Supplementary Fig. 4b), consistent with reported loss of both forms of hypersensitivity induced by SNI or complete Freund’s adjuvant in these ablation mice. Microglia activation following SNI, critical for the development of neuropathic pain33, however, appeared unaffected in SOMLbx1-ablated mice (Supplementary Fig. 4c).

To further assess the requirement of VT3Lbx1 neurons for transmission of dynamic mechanical information, we examined brush-evoked expression of c-Fos in the dorsal spinal cord. In control littermates following SNI, brushing led to c-Fos expression in dorsal horn laminae I–V, and the numbers of c-Fos+ neurons were greatly reduced in VT3Lbx1-ablated mice (Fig. 3a). Brushing naive control mice without SNI resulted in minimal c-Fos
expression (Fig. 3a). Taken together, these results indicate that VT3Lbx1 spinal neurons transmitted sensory information related to brush-evoked dynamic mechanical hypersensitivity induced by nerve injury or strong inflammation.

Silencing VT3Lbx1 neurons attenuates pre-established dynamic mechanical hypersensitivity

To determine whether VT3Lbx1 neurons maintain a neuropathic mechanical hypersensitive state, we acutely silenced VT3Lbx1 neurons using the inhibitory G-protein-coupled receptor hM4Di (Fig. 3b) 34,35. To restrict hM4Di expression to spinal VT3Lbx1 neurons, we crossed ROSA26-LSL-FSF-hM4Di mice with VT3Cre and Lbx1Flpo mice, resulting in triple heterozygous mice, hereafter referred to as VT3Lbx1-hM4Di mice (Fig. 3b). Seven and 30 days after SNI, we acutely silenced VT3Lbx1 neurons after activating hM4Di via intrathecal injection of clozapine-N-oxide (CNO) 34 and observed attenuated brush-evoked dynamic hypersensitivity (Fig. 3c, d) without detectable changes in filament-evoked punctate sensitivity (Fig. 3c, d). CNO injections in control littermates that lacked hM4Di expression did not have any effect (Fig. 3c, d). Thus, acute silencing of VT3Lbx1 neurons reproduces phenotypes caused by VT3Lbx1 neuron ablation (Fig. 2b). We have previously reported that chemical silencing also recaptures the same phenotypes caused by neuronal ablation for neuropeptide Y (NPY Cre)-marked spinal inhibitory neurons that gate mechanical itch 35. Thus, behavioral deficits observed in VT3Lbx1-ablated mice were unlikely to be due to injury responses associated with neuronal cell ablation. Crucially, these findings indicate that VT3Lbx1 neurons were necessary for the expression of pre-established neuropathic mechanical hypersensitivity, suggesting that they may be a valid cellular target for treating this form of pain.

Loss of brush-evoked conditional place aversion following nerve lesions in VT3Lbx1-ablated mice

To assess the emotional and cognitive aspects of dynamic mechanical hypersensitivity, we developed a conditional place aversion (CPA) assay (Fig. 4a). We measured the amounts of time mice stayed in the dark chamber on preconditioning day 1 (t1) and on postconditioning day 6 (t2), and the difference (Δt = t1 − t2) was used to assess the degree of CPA. We found that brushing evoked robust CPA in control littermates with SNI, with Δt reaching 339 ± 38 s, compared to 19 ± 35 s in non-SNI mice (Fig. 4b,c). From this assay we conclude that the brushing from heel to toe in mice with SNI generated an unpleasant

**Figure 5** Characterization of type 1 and type 2 VT3Cre-tomato+ neurons. (a, b) Low-threshold Aβ intensity stimulation-induced inputs and outputs in type 1 and type 2 VT3Cre-tomato+ neurons (a) before and (b) after treatment with bicuculline (10 μM) plus strychnine (2 μM). Light gray traces are higher magnifications of the portions of the top black traces marked with the rectangular bar (only shown in the first panel). For type 1 neurons, in the presence of Bic+stry Aβ intensity stimulation evoked both fast-onset (red arrows) and slow-onset (red arrowheads) currents with AP firing. Type 2 neurons (right panel) received slow Aβ inputs only with bicuculline and strychnine. ACSF, artificial cerebrospinal fluid. (c) Bath application of NMDAR antagonist APV (50 μM) blocked slow-onset long-lasting Aβ-evoked currents and AP firing induced by Bic+stry. Black arrows, stimulation artifacts; red vertical dashed lines, the 10-ms timepoint following stimulation; red horizontal dashed lines, baseline. (d) Predominant AP firing patterns for type 1 and type 2 VT3Cre-tomato+ neurons. All recordings are composed of three repeated traces.
aversive feeling, and mice learned to associate it with and avoid the stimulation chamber. This brush-evoked CPA following SNI was largely abolished in VT3Lbx1-ablated mice, with ∆t decreased to 56 ± 40 s, compared to 339 ± 38 s in control littermates (P < 0.001; Fig. 4b,c). Thus, spinal VT3Lbx1 neurons were part of the circuit(s) transmitting and processing the affective and/or cognitive aspects of dynamic neuropathic mechanical pain, not just mediating nocifensive motor responses.

**Afferent inputs to VT3Cre-tdTomato+ neurons**

We next assessed afferent inputs to VT3Cre-tdTomato+ neurons. In humans, brush-evoked dynamic allodynia is transmitted via myelinated Aβ (and possibly Aδ) mechanoreceptors14,15,19,20. Whole-cell recordings were performed following dorsal root stimulation, first at intensities sufficient to activate Aβ fibers but not Aδ or C fibers (25 µA; Supplementary Fig. 5a,b). Whether an Aβ input was monosynaptic or not was determined by high-frequency stimulations (Supplementary Fig. 5c–e). The recordings revealed two types of tdTomato+ neurons (Fig. 5a), based on whether they produced detectable (type 1) or nondetectable (type 2) Aβ-evoked excitatory postsynaptic currents (EPSCs) under normal recording conditions containing artificial cerebrospinal fluid. Type 1 neurons were dominant (65%, 24 of 37 cells) in the dorsal and medial lamina III (dIII–mIII), whereas type 2 cells were dominant (82%, 27 of 33) in the dorsal and medial part of lamina II (IIα–IIβ). The two types were more mixed in the “IIα–dIII border area, with types 1 and 2 representing 56% (48 of 86) and 41% (38 of 86) of VT3Cre-tdTomato+ neurons, respectively. As described in Supplementary Figure 1c, 38% of VT3Cre-tdTomato+ neurons in IIα–dIII co-expressed SOM. We have previously reported findings that all SOM lineage neurons in this area receive Aβ-evoked EPSCs under normal conditions5, which matches the definition of type 1 cells. Thus, a majority of type 1 VT3Cre-tdTomato+ neurons in IIα–dIII (38% + 56% = 68%) must have been co-expressing SOM. Furthermore, based on the lack of Aβ inputs under normal conditions, type 2 cells are not SOM lineage neurons. The latencies of Aβ-evoked EPSCs in type 1 cells were in the range of 1.6–7.8 ms. At least 56% (48 of 86) and 79% (27 of 34) of type 1 cells in IIα–dIII and in mIII–III, respectively, received monosynaptic Aβ inputs, based on their ability to follow high frequency stimulations (20 Hz, latency variation < 0.5 ms) (Supplementary Fig. 5c–e). By holding the membrane potential at −45 mV to facilitate detecting evoked inhibitory postsynaptic currents (IPSCs), we also found that most type 1 neurons received Aβ-evoked IPSCs (Supplementary Fig. 6a–c), indicating feedforward inhibition.

We then used current-clamp recording to measure excitatory postsynaptic potentials. Stimulation Aβ fibers was insufficient to evoke action potential (AP) firing in 91–98% of VT3Cre-tdTomato+ neurons (Fig. 5a and Supplementary Fig. 6c). To determine whether the lack of AP firing was due to feedforward inhibition, we recorded under disinhibition conditions with the presence of bicuculline and strychnine to block GABA A and glycine receptors, respectively. We found that type 1 neurons now generated Aβ-evoked AP firing (Fig. 5b and Supplementary Fig. 6d), while a majority of type 2 neurons received a different form of Aβ-evoked EPSCs than they had without disinhibition: these had slow onset (latencies > 10 ms) and long durations (> 50 ms), resulting in prolonged AP firing (Fig. 5b and Supplementary Fig. 6d). Such slow Aβ currents also emerged in most type 1 cells under disinhibition conditions (Fig. 5b and Supplementary Fig. 6d).

We next performed pharmacological studies to assess the nature of fast and slow Aβ inputs. Most primary afferents, including Aβ fibers, use glutamate for fast synaptic transmission29. We found that application of 10 µM of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an antagonist for non-NMDA glutamate receptors, eliminated both fast and slow Aβ inputs in randomly selected neurons from lamina III to lamina I (data not shown), indicating that transmission of Aβ inputs required the activation of non-NMDA receptors. We then found that application of 50 µM of APV (2-amino-5-phosphonopentanoic acid), an antagonist of NMDA receptors, failed to block fast Aβ-evoked EPSCs in type 1 cells but did prevent them from firing APs under disinhibition conditions (Fig. 5c and Supplementary Fig. 6d). Furthermore, APV treatment led to a complete loss of slow-onset, long-lasting Aβ inputs in both type 1 and type 2 VT3Cre-tdTomato+ neurons (Fig. 5c and Supplementary Fig. 6d), as well as in randomly selected neurons in laminae II–III (data not shown), consistent with previous reports36,37.

Collectively, the above recordings indicate that under normal conditions, type 1 VT3Cre-tdTomato+ neurons received fast Aβ inputs dominated by non-NMDAR-mediated currents and that these inputs were insufficient to activate NMDARs or to fire APs due to feedforward inhibition (Supplementary Fig. 6e). Under disinhibition conditions, Aβ inputs evoked both non-NMDAR-mediated and NMDAR-mediated currents, which collectively drove AP firing. Type 2 cells received Aβ inputs only under disinhibition conditions, potentially via type 1 cells (Supplementary Fig. 6e). Since NMDA receptors exhibit slow kinetics during both activation and decay phases38, latencies of the first Aβ-evoked AP in 92% (22 of 24) of type 1 cells were > 20 ms (Fig. 5b), which could explain the slow-onset but long-lasting Aβ inputs seen in disinhibited type 2 cells (Fig. 5a,b) if they received inputs from type 1 or type 1-like neurons (Supplementary Fig. 6e). Type 1 and type 2 cells also displayed distinct firing patterns following current injection, with type 1 and type 2 cells dominated by phasic and delayed firing, respectively (Fig. 5d and Supplementary Fig. 6f). Furthermore, both types of cells could be divided into two subtypes...
 Based on differential responses to stimulations at intensities sufficient to activate Aδ and/or C fibers (Supplementary Fig. 7). It should be noted that type 2 cells with delayed firing were not observed in spinal cord neurons marked by the transgenic VTS3::Cre products.20.

Nerve-injury-induced Aβ inputs to I–IIo neurons affected by ablation of VT3lbx1 or SOMlbx1 neurons

We next examined how neuronal ablation affected nerve-injury-induced Aβ inputs to neurons in laminae I and IIo, where a subset of pain output neurons is located.29 To do this, mice were given SNI at P18–21, and slice recordings were performed at P27–31. In control mice without SNI, 37% (17 of 46) of neurons in laminae I and IIo received fast, Aβ inputs, most of which did not result in APs (Fig. 6a and Supplementary Fig. 8). Following SNI, neurons receiving Aβ inputs increased to 80% (57 of 71, χ² test, P < 0.001), and of these, 46% (31 of 68) generated APs, an increase from the 9% (4 of 46) of cells that fired APs in naive mice (χ² test, P < 0.001; Fig. 6a and Supplementary Fig. 8). Aβ evoked fast-onset (latency < 8 ms) EPSCs, long-duration slow-onset (latency > 10 ms, duration > 50 ms) EPSCs or both types from the same cell (Fig. 6a and Supplementary Fig. 8). In VT3lbx1-ablated mice with SNI, the percentages of I–IIo neurons displaying fast-onset Aβ-evoked EPSCs (59%, 42 of 71) or AP firing (27%, 19 of 71) were not different from those in control mice with SNI (EPSCs: 59%, 42 of 71; χ² test, P = 1.000; APs: 29%, 20 of 68; χ² test, P = 0.728). In contrast, there was a significant loss of neurons with slow-onset Aβ-evoked EPSCs, from 63% (45 of 71) in control mice to 11% (8 of 71, χ² test, P < 0.001) in ablated mice (Fig. 6a,b and Supplementary Fig. 8). Furthermore, the few neurons retaining slow Aβ inputs failed to fire APs. As a result, only fast-onset Aβ-evoked AP firing induced by SNI was observed in VT3lbx1-ablated mice (Fig. 6b and Supplementary Fig. 8). Consistent with the requirement of VT3lbx1 neurons for transmission of slow Aβ inputs to most superficial dorsal horn laminae, VT3Crev-tdTomato+ neurons generated Aβ-evoked AP firing following nerve injury (Supplementary Fig. 9). Similarly, when disinhibited by the presence of bicuculline and strychnine, the percentages of neurons in laminae I and IIo, respectively, that generated slow-onset Aβ-evoked AP firing were again selectively reduced in VT3lbx1-ablated mice (Supplementary Fig. 10). In contrast, SOMlbx1-ablated mice showed a loss of both fast and slow components induced by nerve injury (Fig. 6c and Supplementary Fig. 8).

The above recordings revealed two pathways linking Aβ inputs to neurons in laminae I and IIo (Supplementary Fig. 11). The fast pathway, which was eliminated in SOMlbx1-ablated mice but preserved in VT3lbx1-ablated mice, likely reflected direct (not necessarily monosynaptic) Aβ inputs onto vertical cells whose dendrites were present in lamina III. The slow polysynaptic Aβ pathway was via type 1 and/or type 2 VT3Crev-tdTomato+ neurons (Supplementary Fig. 11), with a majority of type 1 cells in I–III receiving monosynaptic inputs. The convergence of VT3lbx1-dependent slow inputs to a subset of vertical cells in I–IIo (Supplementary Fig. 11) was indicated by the observation that while the percentage of neurons that contained fast Aβ components did not change between control and VT3lbx1-ablated mice, neurons containing dual fast and slow components with AP firing were no longer detected in VT3lbx1-ablated mice (Fig. 6b and Supplementary Fig. 8), indicating their conversion to fast-only neurons. As described above, many type 1 VT3Crev-tdTomato+ cells co-expressed SOM (Fig. 1) and vertical cells include SOM lineage neurons.5

Morphine resistance of VT3lbx1-neuron-dependent spinal pathways

Neuropathic pain patients respond inconsistently to morphine treatment.39 We therefore asked whether VT3lbx1-neuron-dependent and -independent spinal pathways likewise respond differentially to morphine. To test this, we first examined morphine effects on mechanical hypersensitivity induced by SNI. In control littermates, intrathecal injection of 1 nmol of morphine at day 7 after SNI inhibited filament-evoked punctate hypersensitivity (Fig. 7a). This morphine sensitivity was no longer observed at day 30 following SNI (Fig. 7a), analogously to what is seen in rats.40 In VT3lbx1-ablated mice, both morphine-sensitive and morphine-resistant punctate hypersensitivity remained intact (Fig. 7a). Dynamic hypersensitivity at either day 7 or day 30 was unaffected by intrathecal injection of 1 nmol (Fig. 7b) or 5 nmol morphine (data not shown) in control mice, consistent with the resistance to intrathecal morphine treatment observed in other models.16,17,41,42 In VT3lbx1-ablated mice, morphine-resistant dynamic hypersensitivity was greatly attenuated at both time points (Fig. 7b).

Spinal slice recordings showed that nerve injury-induced slow-onset and fast-onset Aβ inputs to lamina I–IIo neurons were resistant to morphine (Fig. 7c,d and Supplementary Fig. 12a). We also assessed Aβ inputs resulting from bicuculline and strychnine disinhibition. We reported previously that only slow-onset, possibly polysynaptic Aβ inputs with AP firing increase under these disinhibition conditions.5 These slow Aβ inputs, which were largely dependent on VT3lbx1 neurons (Supplementary Fig. 10), were again resistant to morphine treatment (Fig. 7c and Supplementary Fig. 12a) at a dosage (25 μM) sufficient to suppress other spinal pathways (see below). Accordingly, removal of these slow inputs in VT3lbx1-ablated mice and in SOMlbx1-ablated mice likely caused the loss of morphine-resistant dynamic allodynia.

Gated C-fiber inputs to I–IIo neurons and their transmission via SOMlbx1 neurons

The preservation of morphine-resistant fast Aβ inputs to I–IIo neurons (Fig. 7c) might be a candidate for mediating the morphine-resistant punctate hypersensitivity preserved in VT3lbx1-ablated mice. To search for candidate pathways mediating morphine-sensitive punctate hypersensitivity preserved in VT3lbx1-ablated mice, we next recorded C-fiber inputs to neurons located in lamina IIo. This region is innervated by C-fiber LTMRs expressing tyrosine hydroxylase and is also adjacent to central terminals originating from nociceptors expressing the G-protein-coupled receptor MrgrpD, both of which are involved in punctate mechanical hypersensitivity.23,31,43 Characterization of C-fiber inputs to I–IIo neurons is difficult, however, due to masking by fast and slow Aβ inputs, particularly under disinhibition conditions. To overcome this, we developed our own slice preparation that eliminated electrically low-threshold Aβ inputs, by moving the second cut to a more lateral position (Fig. 7d), taking advantage of the fact that low-threshold Aβ fibers enter the dorsal horn via the dorsal funiculus.31 In this preparation, stimulations at the Aβ intensity (25 μA) failed to generate EPSCs in neurons in laminae I–III, even with the presence of bicuculline and strychnine (Fig. 7d). C intensity stimulations (500 μA, 0.1 ms) evoked detectable EPSCs in 47% (14 of 30) of VT3Cre-negative neurons (tdTomato+ cells in VT3Cre-tdTomato mice) in I–IIo, but these inputs failed to evoke AP firing under our recording conditions (Fig. 7d). In the presence of bicuculline and strychnine, 72% (23 of 32) of these neurons fired C-fiber-evoked APs (Fig. 7d and Supplementary Fig. 12b), and a subset of them received high-threshold Aβ inputs (based on latencies < 6 ms, see Supplementary Fig. 5) without AP firing (see below, Fig. 8). Among VT3Cre-negative neurons firing C-evoked APs, 68% (13 of 19) were inhibited by 25 μM morphine (Fig. 7d and Supplementary Fig. 12b). Bath application of 25 μM morphine caused direct hyperpolarization only in a small subset of dorsal horn neurons (Supplementary Fig. 12c).
Figure 7 Differential morphine sensitivity of spinal pathways. (a,b) Effects of intrathecal morphine or saline injections on punctate and dynamic hypersensitivity (n = 6 in each group). (a) Morphine attenuated punctate hypersensitivity at day 7 after SNI in both control and VT3Lbx1-Abl mice (two-way ANOVA, F₁,₁₀ = 47, P < 0.001) and the degrees of inhibition were not significantly different (two-way ANOVA, F₁,₁₀ = 0.0691, P = 0.798). Morphine had no inhibitory effect at day 30 (two-way ANOVA, F₁,₁₀ = 0.237, P = 0.637). (b) Morphine did not affect dynamic hypersensitivity in either wild-type control or VT3Lbx1-Abl mice (two-way ANOVA, day 7: F₁,₁₀ = 1.403, P = 0.264; day 30: F₁,₁₀ = 0.366, P = 0.558). Note that dynamic hypersensitivity was attenuated in VT3Lbx1-ablated mice in comparison with control at either timepoint (two-way ANOVA; Day 7: F₁,₁₀ = 81.824, P < 0.001; day 30: F₁,₁₀ = 60.324, P < 0.001). Data are presented as mean ± s.e.m.; n.s., nonsignificant. (c) Left: schematic illustration of sagittal spinal cord slice preparations to preserve electrically low-threshold Aβ-fiber inputs. Right: morphine resistance of Aβ-evoked AP outputs in slices from mice with SNI or with Bic+stry. Red lines, the 10-ms timepoint following stimulation; black arrows, stimulation artifacts; red arrows and arrowheads, fast-onset and slow-onset Aβ-evoked APs, respectively. (d) Left: in this C-input preparation, electrically low-threshold Aβ inputs to VT3Cre-tdTomato-negative jI neurons were absent (top two traces). Bottom trace shows C-intensity stimulation-evoked EPSPs and IPSPs without APs. Right: C-intensity stimulation-generated APs outputs with Bic+stry (control) in VT3Cre-tdTomato-negative neurons in jI, which were either sensitive or resistant to morphine treatment. 1, the lateral cutting site; 2 and 2′, medial cutting sites during sagittal slice preparation, with 2′ being more lateral. All recordings are composed of three repeated traces.
suggesting that morphine-mediated inhibition might operate mainly on primary afferents. Furthermore, following nerve lesions (SNI), 50% (5 of 10) of neurons in \( \text{II} \) generated C-fiber-evoked AP firing (Supplementary Fig. 13), in comparison with none (0 of 30, \( \chi^2 \) test, \( P < 0.001 \)) in slices from naive mice without SNI under our recording conditions (Fig. 7d). Thus, VT3\text{Cre}-negative neurons in \( \text{II} \) received C-fiber inputs with feedforward inhibition, and these gated C pathways became sensitized following nerve injury.

We next asked how C-fiber inputs to \( \text{II} \) neurons in SOM\text{Lbx1}-ablated mice are affected in VT3\text{Lbx1}-ablated and SOM\text{Lbx1}-ablated mice, again using the slice preparation removing low-threshold A\( \beta \) inputs (Fig. 8a) and recording in the presence of bicuculline and strychnine (Fig. 8c,d). No significant change in the percentage of \( \text{II} \) neurons with C-fiber-evoked AP firing was detected between control mice (64%, 23 of 36) and VT3\text{Lbx1}-ablated mice (57%, 13 of 23; \( \chi^2 \) test, \( P > 0.05 \); Fig. 8b,c). However, in SOM\text{Lbx1}-ablated mice, the percentage of neurons with C-fiber-evoked AP firing was reduced to 10% (2 of 20), and C-fiber inputs to the remaining 90% of \( \text{II} \) neurons were either lost or reduced to levels that were incapable of firing APs under our recording conditions (Fig. 8d). All together, we identified three gated spinal pathways
mechanical hypersensitivity preserved in VT3 Lbx1-ablated mice, and laminae I/II o and C-fiber inputs to VT3 Cre-negative neurons in vIIi from low-threshold mechanoreceptors (A-fiber or C-fiber LTMRs) following nerve injury or inflammation, central sensitization and/or from those transmitting chronic punctate mechanical information. Following nerve injury or inflammation, direct Aβ neurons expressing NPY2R44 innervating δ inputs, morphine-sensitive C inputs and morphine-resistant C inputs that were eliminated in SOMLbx1-ablated mice but preserved in VT3Lbx1-ablated mice, and these pathways could serve as candidates for mediating morphine-sensitive and morphine-resistant punctate hypersensitivity (Supplementary Fig. 14).

**DISCUSSION**

**Spinal substrates transmit acute or chronic punctate mechanical information**

Our studies reveal a difference in the spinal substrates transmitting light versus intense acute punctate mechanical information. VT3Lbx1 neurons were involved in transmitting light, acute punctate mechanical information evoked by von Frey filaments but were not required for transmission of superthreshold punctate stimuli evoked by pinpricking or for other forms of intense mechanical pain (Fig. 1). Other studies show that primary afferents expressing MrgrpD, which innervate δIIo, are required to sense light punctate force23, whereas pinprick-evoked mechanical pain is mediated via thinly myelinated Aδ afferents expressing NPY2R44. We consistently found that type 2a VT3Lbx1 neurons in IIo−δIIo received inputs from C fibers but not from Aδ fibers (Supplementary Fig. 7b).

We also found a dissociation of spinal substrates transmitting acute from those transmitting chronic punctate mechanical information. Following nerve injury or inflammation, central sensitization and/or disinhibition opens the gate. This opening allows subthreshold inputs from low-threshold mechanoreceptors (A-fiber or C-fiber LTMRs) to induce AP firing in laminae I–IIo output neurons10–12,43, causing a drastic drop in withdrawal thresholds. This recruitment of LTMR-mediated new pain pathways could then explain why VT3Lbx1 neurons are largely dispensable for transmitting punctate hypersensitivity following nerve lesion or inflammation, despite being required for transmission of light acute punctate mechanical stimuli. Both acute and chronic forms of punctate mechanical sensitivity are abolished in SOMLbx1-ablated mice, suggesting that chronic punctate mechanosensitive circuits can be transmitted via a subset of SOMLbx1 neurons preserved in VT3Lbx1-ablated mice. Our data, however, do not rule out a redundant role of VT3Lbx1 neurons in mediating punctate hypersensitivity (Supplementary Fig. 14).

**Do distinct spinal substrates transmit dynamic versus punctate mechanical hypersensitivity?**

Despite preserving punctate mechanical hypersensitivity, ablating spinal VT3Lbx1 neurons led to a marked deficit in brush-evoked dynamic mechanical hypersensitivity induced by nerve lesions, inflammation or central blockade of GABAA or glycine receptors. Using a robust CPA assay, we further showed that spinal VT3Lbx1 neurons were required for transmitting the affective and/or cognitive aspect of dynamic neuropathic mechanical pain46. Electrophysiological recordings then revealed that VT3Cre neurons formed morphine-resistant polysynaptic pathways that relayed Aβ inputs from lamina III to lamina I (Supplementary Fig. 14). Our studies also revealed multiple VT3Lbx1-neuron-independent gated spinal pathways that become available following nerve injury, including direct Aβ inputs to vertical neurons in laminae I/IIo and C-fiber inputs to VT3Cre-negative neurons in IIo (Supplementary Fig. 14). These pathways could mediate punctate hypersensitivity preserved in VT3Lbx1-ablated mice, and all of them were eliminated in SOMLbx1-ablated mice (Supplementary Fig. 14), potentially explaining the loss of both dynamic and punctate hypersensitivity in SOMLbx1-ablated mice.

Two models could account for the loss of dynamic mechanical hypersensitivity but the preservation of punctate mechanical hypersensitivity in VT3Lbx1-ablated mice. The first model proposes the existence of spinal circuits transmitting distinct forms of mechanical hypersensitivity, with VT3Lbx1 neurons forming circuits involved selectively in brush (touch)-evoked dynamic allodynia. Recently, a class of Aβ field-LTMRs forming circumferential endings around hair follicles was shown to respond to gentle stroking across the skin, but not to light punctate mechanical stimuli47, making them an attractive candidate mediating dynamic allodynia. Alternatively, brushing a large skin area activates a spectrum of LTMRs, and temporal and/or spatial summation of these LTMR inputs might be required to activate VT3Lbx1-neuron-dependent circuits to mediate dynamic allodynia. Future in vivo or ex vivo recordings are needed to test this possibility. The second model postulates that the VT3Lbx1-neuron-dependent polysynaptic circuit is one of several redundant spinal pathways that transmit mechanical allodynia, and brushing might represent a lower-force stimulus compared with von Frey filament stimulation. Accordingly, the stronger von Frey filament stimulation might produce a level of VT3Lbx1-neuron-independent pathway activity sufficient for generating withdrawal responses in ablated mice. In contrast, the weaker brushing stimulus might require a summation of inputs from both VT3Lbx1-neuron-dependent and -independent pathways to drive nocifensive behaviors, leading to impaired dynamic allodynia in VT3Lbx1-ablated mice.

The morphine experiments done at day 7 following nerve injury lend support for the first model, revealing the existence of a morphine-sensitive spinal pathway necessary for punctate, but not dynamic, hypersensitivity. The second model appears to be supported by the finding that when the concentration of carragenan was reduced to 0.5%, only two-thirds of the VT3Lbx1-ablated mice developed punctate mechanical hypersensitivity (Fig. 2). Weak inflammation might drive suboptimal sensitization of the VT3Lbx1-neuron-dependent and -independent pathways, making them both necessary for the full expression of punctate hypersensitivity. However, this data does not necessarily argue against the first model. VT3Lbx1 neurons could play a minor role in the induction of punctate hypersensitivity, but once allodynia is fully induced via VT3Lbx1-neuron-independent mechanisms, the VT3Lbx1 neurons may become dispensable for the expression of punctate allodynia.

**Implications for preclinical and clinical studies**

Regardless of which model is correct, our studies show that VT3Lbx1 neurons were necessary for the expression of brush (touch)-evoked dynamic allodynia. Furthermore, acute silencing of VT3Lbx1 neurons attenuated pre-established dynamic neuropathic mechanical pain, indicating that these neurons are a valid cellular target for treating this form of pain. Our analyses also suggest that the von Frey filament assay lacked the resolution to reveal selective loss of this form of nerve lesion- or inflammation-induced pain. As such, our findings highlight a major paradox in current preclinical pain studies that predominantly measure von Frey filament-evoked punctate mechanical allodynia, even though touch-evoked dynamic allodynia is the major issue for neuropathic pain patients13. The lack of sufficient attention to measuring clinically more relevant dynamic allodynia could contribute to failures in translating preclinical successes into new pain medications18,49. Furthermore, the existence of multiple gated spinal pathways with differential morphine sensitivities might explain why different cohorts of patients show inconsistent responses to morphine treatment39. Thus, it becomes critical to develop mechanism- and phenotype-based clinical trials and treatments for different cohorts of chronic pain patients50.
METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
L.C., B.D., T.H., Q.M. and Y.W. designed the study. L.C. and Y.Z. performed electrophysiological experiments and analyzed the recording data. B.D., T.H., X.R. and Y.C. performed histochemical and behavioral experiments and analyzed the data. O.R., L.C.-C. and M.G. provided interosseous ablation mouse lines before publication. L.V. and B.B.L. generated the TV3Cre mice. Q.M. and Y.W. supervised the whole study. Q.M., L.C., B.D., T.H., M.G. and Y.W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Experimental animals. All animal experiments, including behavioral tests, were performed with protocols approved by the Institutional Animal Care and Use Committee at Dana-Farber Cancer Institute and followed NIH guidelines. Mice were housed at room temperature with a 12-h/12-h light/dark cycle and ad libitum access to standard lab mouse pellet food and water.

To generate Vglut3-IREs-Cre (VT3Cre) knock-in mice, a cassette containing the Cre recombinase gene preceded by an internal ribosomal entry sequence (IRES) was targeted just distal to the stop codon of the endogenous Vglut3 (Sk17a8) allele so that the endogenous Vglut3 promoter drove Cre-recombinase expression. In brief, a 129 BAC genomic clone containing the Vglut3 gene was used to target a cassette containing the Cre recombinase gene preceded by an internal ribosomal entry sequence (IRES-Cre-FKFP)\(^{51}\) just distal to the stop codon. A PCR amplicon containing the IRES-Cre-FKFP cassette was amplified with a forward primer, containing 50 bases of Vglut3 homology sequence including the stop codon and 20 bases of IRES-Cre-FKFP sequence beginning at the IRES sequence, and a reverse primer, containing 50 bases of Vglut3 homology sequence starting three bases after the stop codon and 20 bases corresponding to the 3’ end of the IRES-Cre-FKFP cassette. The PCR amplicon was then transformed into electroporocompetent EL250 bacteria containing the aforementioned Vglut3 BAC. Homologous recombination in EL250 cells was induced as previously described\(^{25,52}\) to insert the IRES-Cre-FKFP cassette into the Vglut3 BAC three bases downstream from the stop codon. The Cre coding sequence was confirmed by DNA sequencing. The Vglut3-IREs-Cre-FKFP targeting construct was prepared using a commercially available kit (Qiagen, Valencia, CA), linearized and electroporated into W4 ES cells. Targeted clones were injected into blastocysts. Chimeras were obtained and bred for germline transmission of the Vglut3-IREs-Cre-FKFP targeted allele. Offspring carrying the Vglut3-IREs-Cre-FKFP allele were subsequently bred to F10p3 recombinase mice\(^{26}\) to remove the FKFP cassette and are referred to throughout this paper as VT3Cre mice.

The generation of mice carrying SOM-IREs-Cre (SOMcre)\(^{56,57}\) (ref. 55), ROSA26-LSL-tdTomato (All14-tdTomato reporter mice)\(^{58}\), Lbx1Flpo (refs. 5, 32), Tau-LSL-FSF-DTR (refs. 5, 32) and ROSA26-LSL-FSL-mH4DI (ref. 34) was done as described previously.

To ablate DTR-expressing neurons for behavioral and histochemical studies, 6–10-week-old male and female mice were intraperitoneally injected with diphertheria toxin (DNT, 50 µg/kg; Sigma-Aldrich, D10646) at day 1 and then again at day 4. Behavioral and histochemical experiments were performed 4 weeks after DTX injection. For characterization of VT3Cre-tdTomato neurons, spinal cords from 2 or 3 mice at P4, P7 and P60 were used. For quantitative histochemical studies comparing control and VT3Cre-ablated mice, three pairs of 2–3-month-old control and ablated mice were used. For each behavioral analysis, 5–17 pairs of 10–14-week-old ablated and control littersmates, including males and females, were used. To ablate DTR-expressing neurons for electrophysiological recording, mice (P18–P21) were intraperitoneally injected with diphertheria toxin (50 µg/kg) at day 1 and then again at day 4. Recordings were performed 9–12 d after the first DTX injection (P27–P31). Animals were assigned to treatment groups in a blinded, randomized fashion, and pain responses were measured in a blinded manner.

In situ hybridization and inuromohistochemistry. In situ hybridization (ISH) procedures were conducted as described previously\(^{56,57}\). Immunohistochemistry on spinal cord sections was performed using rabbit anti-NK1R (1:1,000, Sigma-Aldrich, S8030)\(^{5}\), rabbit anti-PKCy (1:400, Santa Cruz, sc-211)\(^{26}\), IB4-biotin (10 µg/ml, Sigma-Aldrich, L2140)\(^{40}\) and goat anti-Iba1 (1:1,000, Abcam, ab5076)\(^{26}\) diluted in 0.2% Triton X-100 plus 10% normal goat or donkey serum in PBS. ISH/tdTomato double-staining was performed as previously described\(^{25,56,57}\). Both fluorescent and ISH signals were collected using a fluorescent microscope. The tdTomato fluorescent signal was photographed first, followed by ISH. Pseudofluorescent ISH signals (for VGLUT2, VGLUT3, SOM, GAD1 and GlyT2) were converted from brightfield images and then merged with the tdTomato images using Photoshop. Quantitative experiments were done in at least 3 animals each. For each animal, at least 3 different spinal section images were taken. ImageJ (Fujifilm) software was used to measure the average fluorescence intensity, to evaluate Iba1 expression in the whole dorsal horn, prepared from mice 7 d after SNI.

Surgery, hindpaw injection of inflammatory reagents and intrathecal injection of morphine or bicculline plus strychnine. Surgery to establish the SNI model of neuropathic pain was performed as described previously\(^{55–58}\). For behavioral tests, adult control and ablated male and female mice (10–14 weeks old) underwent surgery and were subjected to testing 1–3 d after nerve injury in the lateral plantar region of the left hindpaw, which remained innervated by the undamaged sural nerve. For whole-cell patch-clamp recordings, control and ablated mice at P18–P21 received SNI surgery and were killed for analysis 9–12 days later. For CFA or carrageenan-induced inflammation, mice were briefly anesthetized with isoflurane (3–5 min at 2%), and 20 µl of complete Freund’s adjuvant (CFA, Sigma-Aldrich, F5881) or either 3% or 0.5% carrageenan (Sigma-Aldrich, C1013) was injected into the plantar surface of the left hindpaw. Behavioral testing was performed 1 d and 3 d after CFA or 24 h after carrageenan treatment. For alldynia induction by in vivo blockage of GABA\(_A\) receptors and glycine receptors, 10 µl saline containing bicculline (Sigma-Aldrich, 14340) and strychnine (Sigma-Aldrich, S0532) was intrathecally injected into the spinal cord. To avoid excessive, prolonged, acute seizure-like behaviors, including biting, vocalization and scratching responses, we first titrated the dose of bicculline and strychnine and found that 0.02 µg bicculline and 0.05 µg strychnine only caused short-term (< 5 min) of biting and scratching at the flank and low abdomen regions. We then started to measure mechanical allodynia 10 min after injection. Morphine (Patterson Veterinary Supply, 1 nmol or 5 nmol, 10 µl with saline) or saline control (10 µl) was injected intrathecally and behavioral tests were performed following injection. Both punctate and dynamic forms of mechanical hypersensitivity were measured for 60 min after morphine injection, and the peak analgesic effect of morphine for punctate mechanical hypersensitivity was 30 min after injection. Pilot injection of 10 nmol appeared to cause motor deficits and scratching (data not shown), and we did not further test this dosage.

Behavioral testing. For all experiments, the investigator performing behavioral tests was blinded to genotypes and treatments. All data points were included for subsequent statistical analyses. All control animals were littersmates of VT3Cre, ablated mice, and thus all had the same B6/129 mixed genetic background.

The following behavioral assays were performed as described previously\(^5\): rotarod, light touch, sticky tape, acetone, cold plate, hot plate, Hargreaves, von Frey, pinprick, pinch, punctate and dynamic mechanical hypersensitivity and itsch behavioral tests. For behavioral tests, we habituated mice for 30 min/for 3 consecutive days before testing. We performed most acute behavioral tests in 4 d in order: rotarod, light touch and sticky tape (day 1); von Frey and Hargreaves (day 2), hot plate and cold plate (day 3); and acetone, pinprick, pinch and Randall-Selitto (day 4). The interval between different tests was at least 2 h. Following acute behavioral tests, different cohorts of mice were used to test inflammatory pain, neuropathic pain (punctate and dynamic mechanical hypersensitivity were measured on the same day at 23 min intervals) or itch behaviors (compound 48/80 was tested first and chloroquine was tested on subsequent days).

Tail mechanical sensitivity was measured by the Randall-Selitto device (ITTC, USA). Mice were placed in a restraining plastic tube and allowed 5 min to acclimatize. Slowly increasing pressure was then applied to a point midway along the tail. The withdrawal threshold was determined by the force at which the animal showed a clear sign of discomfort or tried to escape. The test was repeated six times with 5-min intertrial intervals.

For cold allodynia test, a drop of acetone solution was gently placed on the lateral plantar surface of the paw, using a blunt needle connected to a syringe and without touching the skin. The total duration of the withdrawal, flinching and licking responses was recorded.

Dynamic mechanical hypersensitivity was measured by light stroking (velocity: ~2 cm/s) of the external lateral side of the injured hind paw from heel to toe using a paintbrush (5/0, Princeton Art & Brush Co.). The typical response of naive mice to the dynamic mechanical stimulation is a very fast movement, lifting the stimulated paw for less than 1 s (score 0). However, after nerve injury or inflammation, several pain-suggestive responses can be observed, such as sustained lifting (more than 2 s) of the stimulated paw toward the body or a single gentle flinching of the stimulated paw (score 1); one strong lateral paw lift, above the level of the body (a lateral kick, more resembling an exaggerated hindpaw withdrawal than a flinch) or a startle-like jump (score 2); and multiple flinching responses or licking of the affected paw (score 3). We repeated the stimulation three times at intervals of at least 3 min and obtained an average score for each mouse. The paintbrush was prepared by trimming the tip and making it blunt. The total length of brush was about 5 mm. We also removed the outer layer of hairs. We prepared a series of

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paintbrushes and tested mice with and without SNI, and we considered a brush suitable if it produced an average score of –0 for naive mice without SNI but >1.5 (or preferably –2.0) in mice with SNI.

To measure the negative valence effect associated with brush-evoked dynamic mechanical hypersensitivity, we used a biased compartment–assignment procedure, in which we measured the influence of V3**δ** neurons on the time of mice spending in the dark compartment receiving conditional stimulations. The CPA apparatus consisted of two chambers (10 × 10 × 15 cm per compartment), one dark (A) and one bright (B), with a metal mesh floor (i.e., the chambers, which had no floor, were placed onto a metal mesh used as the floor). The center was an inserted black (facing compartment A) and white (facing compartment B) plastic wall with a rectangular hole in the bottom hole (4 cm × 8 cm; Fig. 4a). Mouse movement was recorded by a Sony camcorder. The amount of time a mouse spent in chamber A was evaluated by the experimenter after recording. On day 1, each mouse was placed in the bright compartment (B) and allowed to freely explore between chambers A and B for 15 min (pre-test). With this apparatus design, most if not all naive mice showed an initial preference for the dark chamber. Mice were conditioned over a 4-d period. On days 2 and 4, the hole in the center wall was blocked with dark film on the A compartment side. The mouse was put in the bright chamber for 20 min. On days 3 and 5, the hole in the center wall was blocked with dark film on the B compartment side. The mouse was then placed in chamber A, and the injured hindpaw was brushed with the paintbrush from heel to toe for 20 min at ~2-s intervals. On day 6 the hole in the central wall was unblocked. The mice were tested for their compartment preference by placing them in the bright compartment first and allowing them to freely explore the entire apparatus for 15 min (post-test). The aversion score was measured as the difference in time (in s) spent in the dark compartment during pre-test versus during post-test (i.e., aversion score = (pre-test time in dark chamber) − (post-test time in dark chamber)).

**Acute silencing.** For the silencing experiments, V**T3****C**-**Lbx1**-**f**β**h**-**hMAD4** (V**T3****C**-**hMAD4) and V**T3****C**-**hMAD4** (V**T3****C**-**hMAD4) mice and their control littermates were used. Before or after nerve injury, mice were acclimatized in a chamber with a mesh floor for 30 min/d for 2 d before the experiment. On the day of the experiment, mice were acclimated for 30 min and then briefly removed from the chamber for intraperi- tional injection of doxapine-N-oxide (CNO, 10 μg/g in stock, Sigma-Aldrich, C0832, 5 mg/kg). Mice were then returned to the chamber. Brush-evoked dynamic mechanical hypersensitivity and filament-evoked post-activation mechan- ical hypersensitivity were evaluated at 10-min intervals after CNO injection for 90 min. At every ti- mepoint, the external lateral side of the injured hindpaw received punctate mechanical stimuli via von Frey filaments, and the thresholds causing withdrawal responses were measured using Dixon's up–down method. Then, at 3 min intervals, we stroked the external lateral side of the injured hind paw with a paintbrush, from heel to toe three times at ~10-s intervals. The average score of responses was evaluated to calculate the dynamic score. To measure cold allodynia after silencing V**T3****δ** neurons, control and V**T3****δ**-**hMAD4** mice were intraperitoneally injected with CNO 7 d after SNI. Cold allodynia (see above) was induced by acetone drops was measured 40 min after CNO injection.

c-Fos induction. To induce c-Fos expression by dynamic brush stimulations in V**T3****δ**-ablated and control mice, each mouse was put in a bright chamber (10 × 10 × 15 cm) with a metal mesh floor. A 5/0 paintbrush with trimmed tip was used to stroke the external lateral side of the injured hindpaw from heel to toe ~600 times in a 20-min period (~2-s intervals). Two hours later, mice were killed for analysis and the lumbar spinal cord was dissected, fixed, embedded and then sectioned into sagittal slices for further c-Fos immunostaining using rabbit anti-c-Fos (1:500, Millipore, AB14577).

Spinal cord slice preparation. Preparation of spinal cord slices with the full length of the dorsal root and DRG attached was done as described previously. Briefly, mice (P24–P31) were deeply anesthetized with isoflurane and decapitated, and the lumbar spinal cord was quickly removed to ice-cold modified artificial cerebrospinal fluid (ACSF), containing (in mM) 80 NaCl, 2.5 KCl, 1.25 NaHPO\(_4\), 0.5 CaCl\(_2\), 3.5 MgCl\(_2\), 25 NaHCO\(_3\), 75 sucrose, 1.3 sodium ascorbate and 3.5 sodium pyruvate, with pH 7.4 and osmolality 310–320 mOsm. The spinal cord, with full-length dorsal roots and DRG attached, was cut on a VT1000S vibratome (Leica, Germany), as illustrated in Figure 7c. To eliminate low-threshold Aβ-fiber inputs to dorsal horn neurons in some experiments, slices were prepared as illustrated in Figure 7d. Slices were incubated for about 1 h at 35 °C in a solution containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 1.25 NaHPO\(_4\), 26 NaHCO\(_3\), 25 d-glucose, 1.3 sodium ascorbate and 3.0 sodium pyruvate, with pH 7.2 and measured osmolality 310–320 mOsm, oxygenated with 95% O\(_2\) and 5% CO\(_2\). Slices were then transferred into a recording chamber and perfused with oxygenated recording solution at 5 mL/min before electrophysiological recordings at room temperature (23–25 °C).

**Patch-clamp recordings and dorsal root stimulation.** Whole-cell recording experiments were conducted as described previously. We used an internal solution containing (in mM) 130 potassium gluconate, 5 KCl, 4 Na\(_2\)ATP, 0.5 NaGTP, 20 HEPES and 0.5 EGTA, pH 7.28 with KOH and measured osmolality at 310–320 mOsm. Data were acquired with pClamp 10.0 software (Molecular Devices, USA) using a MultiClamp 700B patch-clamp amplifier and Digidata 1440A (Molecular Devices, USA). Responses were low-pass filtered online at 2 kHz and digitized at 5 kHz.

The stimulus threshold ranges for Aβ, Aδ and C fibers are 12–16 μA, 30–35 μA and 100–300 μA, respectively, as determined previously. Accordingly, the intensity ranges used in this study for different fibers were 525 μA for Aβ, 30–50 μA for Aδ and 100–500 μA for C fibers. We therefore used 25 μA, 50 μA and 500 μA to screen for Aβ, Aδ and C-fiber-mediated synaptic inputs/outputs in the spinal dorsal horn (pulse widths 0.1 ms or 0.5 ms).

As previously described, we recorded under three different conditions to test primary afferent inputs to dorsal horn neurons. First, by holding membrane potential at −70 mV (sometimes −80 to −85 mV), evoked inhibitory postsynaptic currents (eIPSC) were minimized so even small evoked excitatory postsynaptic currents (eEPSC) could be detected. This recording condition was used to study whether a neuron received inputs directly (mono-eEPSC) or indirectly (poly-eEPSC) from Aβ, Aδ or C fibers. See Supplementary Figure 5 for parameters used to identify synaptic inputs from Aβ, Aδ and C fibers. In this recording condition, the distance from the tip of the suction electrode (stimulation electrode) to the entrance of the attached dorsal root was ~8 mm. Under the disinhibition or nerve injury conditions, dorsal root stimulations at the low-threshold Aβ intensity range induced two types of inputs in dorsal horn neurons: (i) fast-onset inputs, with latency usually less than 10 ms, and (ii) slow-onset inputs, with latency usually more than 10 ms and duration usually more than 50 ms (some poten- tially electrically low-threshold C fiber inputs, with latency more than 10 ms but duration less than 50 ms, were also included, but were excluded in our previous study, which may explain the difference between these two studies in the percentages of vIIi–dIII neurons receiving slow-onset inputs under normal conditions). Fast-onset Aβ inputs include both mono- and polysynaptic inputs. Second, by holding membrane potential at −45 mV, both eEPSCs and eIPSCs could be detected simultaneously. Third, current-clamp recording at the resting membrane potential (or slightly hyperpolarized to block the generation of spontaneous AP firings) was used to record dorsal-root stimulation-evoked APs (Aβ, Aδ and C-evoked APs), in either the normal recording solution or under disinhibition conditions with the presence of both bicuculline (10 μM) and strychnine (2 μM). AP firing patterns following current injections were determined based on holding the membrane potentials around −85 mV.

Series resistances for all neurons recorded in this study were within 30 MΩ, and the liquid junction potentials were ~15 mV. When we randomly selected neurons, we chose neurons in a given lamina (from control or ablated mice) under a brightfield microscope, without considering their sizes or morphologies. All recordings were performed at room temperature. Drugs were bath applied by exchanging a perfusion solution containing a known drug concentration and without altering the perfusion rate.

Statistical analyses. Results are expressed as mean ± s.e.m. Statistical analyses were done using GraphPad Prism. For locomotion coordination, touch, itch and acute pain assessment, data were subjected to Student’s t tests. For CFA-induced inflammatory pain, SNI-induced neuropathic pain and pain induced by intrath- ceral injection of bicuculline and strychnine, time-course measurements were assessed by Bonferroni’s post hoc ANOVA. For statistical analysis of incidence of electrophysiological results, data were analyzed with chi-squared (χ\(^2\)) tests (see Supplementary Methods Checklist). All data sets were tested for normality.
using SigmaStat 3.5 software; if the criteria of normality and equal variance were not met, we used Mann-Whitney U tests, and results were expressed as median ± quartiles. A threshold of \( P < 0.05 \) was accepted as statistically different. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications\(^4\). 

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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