Research Paper

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# Presynaptic inhibition of optogenetically identified VGluT3$^+$ sensory fibres by opioids and baclofen

Silke D. Honsek[a], Rebecca P. Seal[b], Jürgen Sandkühler[a,*]

**Abstract**

Distinct subsets of sensory nerve fibres are involved in mediating mechanical and thermal pain hypersensitivity. They may also differentially respond to analgesics. Heat-sensitive C-fibres, for example, are thought to respond to μ-opioid receptor (MOR) activation while mechanoreceptive fibres are supposedly sensitive to δ-opioid receptor (DOR) or $GABA_B$ receptor ($GABA_BR$) activation. The suggested differential distribution of inhibitory neurotransmitter receptors on different subsets of sensory fibres is, however, heavily debated. In this study, we quantitatively compared the degree of presynaptic inhibition exerted by opioids and the $GABA_BR$ agonist baclofen on (1) vesicular glutamate transporter subtype 3–positive (VGluT3$^+$) non-nociceptive primary afferent fibres and (2) putative nociceptive C-fibres. To investigate VGluT3$^+$ sensory fibres, we evoked excitatory postsynaptic currents with blue light at the level of the dorsal root ganglion (DRG) in spinal cord slices of mice, expressing channelrhodopsin-2. Putative nociceptive C-fibres were explored in VGluT3-knockout mice through electrical stimulation. The MOR agonist DAMGO strongly inhibited both VGluT3$^+$ and VGluT3$^-$ C-fibres innervating lamina I neurons but generally had less influence on fibres innervating lamina II neurons. The DOR agonist SNC80 did not have any pronounced effect on synaptic transmission in any fibre type tested. Baclofen, in striking contrast, powerfully inhibited all fibre populations investigated. In summary, we report optogenetic stimulation of DRG neurons in spinal slices as a capable approach for the subtype-selective investigation of primary afferent nerve fibres. Overall, pharmacological accessibility of different subtypes of sensory fibres considerably overlaps, indicating that MOR, DOR, and $GABA_BR$ expressions are not substantially segregated between heat and mechanosensitive fibres.

**Keywords:** ChR2, Opioid, $GABA_B$, Primary afferent, Analgesic, Spinal

## 1. Introduction

Pain is frequently associated with enhanced or ongoing input from sensory nerve fibres to spinal dorsal horn neurons.[19,35,41] Depending on the subtypes of sensory fibres involved, this effect can lead to diverse symptoms such as mechanical and thermal pain hypersensitivity.[24] For example, vesicular glutamate transporter 3–positive (VGluT3$^+$) sensory fibres do not mediate pain in naive animals or heat hypersensitivity after injury, but they are involved in mechanical and cold hypersensitivity in some animal models of neuropathic and inflammatory pain.[13,44] It has been suggested that different populations of sensory fibres, and consequently, different modalities of pain, are differentially targeted by pharmaceuticals. Although μ-opioid receptor (MOR) agonists are suggested to inhibit heat pain, δ-opioid receptor (DOR) agonists and $GABA_B$ receptor ($GABA_BR$) agonists supposedly inhibit acute mechanical pain, as well as mechanical hypersensitivity after tissue or nerve injury.[11,42] There is, however, a considerable controversy regarding the modality-specific distribution of presynaptic neurotransmitter receptors on primary afferent nerve terminals.[6,9,46,48] Although some investigators report a clear segregation of MORs and DORs on peptidergic and nonpeptidergic DRG neurons,[42] others found that MOR and DOR mRNAs are co-localized in the same neurons.[48] Similarly, the distribution of $GABA_BR$s on synaptic terminals of sensory nerve fibres is unclear. Despite having been proposed to preferentially inhibit high-threshold C-fibres,[32] $GABA_BR$ agonists have been implicated in alleviating mechanical allodynia mediated by low-mechanical threshold afferents.[28] In any case, it would be advantageous for pharmacological therapies if drugs differentially influenced neurotransmitter release from non-nociceptive vs nociceptive fibres.

However, evidence for the presence of functional opioid or $GABA_BR$s at synaptic terminals of distinct fibre populations is scarce, as most studies to date have focused on somatic receptor expression in dissociated dorsal root ganglion (DRG) neurons.[33,48] In electrophysiological recordings, an approach well suited to study synaptic transmission, subpopulations of A- and C-fibres cannot readily be distinguished, unless genetic modifications are introduced.[10,47] We used an optogenetic approach to specifically investigate subpopulations of VGluT3$^+$ sensory fibres. Mice that express Cre recombinase in VGluT3$^+$ neurons (VGluT3-cre) were crossed to cre-dependent channelrhodopsin-2 (ChR2) mice, (Ai27 or Ai32), and blue light was applied to specifically activate VGluT3-positive sensory fibres. The degree of presynaptic inhibition exerted by the MOR agonist DAMGO, the DOR agonist SNC80, and the $GABA_BR$ agonist baclofen on VGluT3$^+$ A- and C-fibres innervating spinal

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lamina I and II neurons was compared with the presynaptic inhibition of putative nociceptive C-fibres activated by electrical stimulation in VGluT3$^{-/-}$ mice. μ-Opioid receptor agonists more strongly depressed synaptic transmission of C-fibres innervating lamina I than lamina II neurons. δ-Opioid receptor activation had only minor effects, whereas $GABA_BR$ activation powerfully depressed synaptic transmission of VGluT3$^{+}$ and VGluT3$^{-}$ fibres.

## 2. Materials and methods

### 2.1. Animals and genotyping

Experiments were performed in male Ai27 or Ai32 mice[31] crossed to VGluT3-cre mice,[20] and VGluT3-knockout (VGluT3$^{-/-}$) mice.[44] Genotyping was performed for hChop in Ai27 mice and for enhanced yellow fluorescent protein (eYFP) in Ai32 mice. Genotyping in VGluT3-cre mice was performed on an irregular basis as homozygous mice were used for breeding. Primers used were as follows: (1) hChop forward: GTC CGT CCT GGT CCC TGA GGA T; hChop reverse: TGT GTT CGC GCC ATA GCA CAA T; (2) eYFP forward: AGC TGA CCC TGA AGT TCA TCT G; eYFP reverse: ACT CCA GCA GGA CCA TGT GAT; (3) iCre forward: CAT CAG AAA CCT GGA CTC TG; Mvglut3 sq 2A reverse: AGG CTC CAG AAA CAG TCT AAC G. All procedures were performed in accordance with European directives on the use of animals for scientific purposes and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP).

### 2.2. Dissection procedure

For pharmacological recordings, acute spinal cord slices with dorsal roots and dorsal root ganglia attached were obtained from 3- to 4-week-old male mice after a dorsal laminectomy. As axonal photostimulations were reported to be more difficult in younger mice,[36] 5- to 7-week-old mice were used for stimulating axons at the level of the spinal dorsal horn. Dissection, in either case, was performed in chilled artificial cerebrospinal fluid (ACSF) (310-320 mOsm $kg^{-1}$) consisting of the following (in mM): 95 NaCl, 1.8 KCl, 1.2 $KH_2PO_4$, 0.5 $CaCl_2$, 7 $MgSO_4$, 26 $NaHCO_3$, 15 glucose, and 50 sucrose, oxygenated with 95% $O_2$ and 5% $CO_2$, resulting in a pH of 7.4. Slices (~600 μm thick) were cut from the lumbar spinal cord with a vibrating microslicer (DTK-1000, Dosaka, Kyoto, Japan) at an angle of 30 to 40 degrees to the transverse plane and were kept at 34 °C for 20 to 30 minutes, before they were allowed to rest at room temperature until use. Dorsal root ganglion patch-clamp recordings were performed after coarsely removing the meninges and incubating DRG in ACSF supplemented with 10 mg/mL collagenase IV (Sigma-Aldrich) for 60 to 120 minutes at 36 °C.[25]

### 2.3. Patch-clamp recordings

Whole-cell patch-clamp recordings were performed at room temperature with an Axopatch 200B amplifier coupled to a Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Signals were amplified 2 to 5-fold and recorded at 10 kHz using the pClamp 9 software package (Molecular Devices). To visualize neurons under the 20x objective (Leica HCX APO, NA 1.0) of a Leica DM6000CFS microscope (Leica Microsystems, Wetzlar, Germany), infrared illumination was performed from above the tissue with a 860 nm light-emitting diode (LED) (Thorlabs, Munich, Germany) coupled to an optical fibre (BFL37-300, Thorlabs, Munich, Germany). In slice patch-clamp recordings, neurons whose somata were within 25 μm of the dorsal white matter border, or those situated clearly within the dorsal eYFP band in VGluT3-cre × Ai32 mice, were considered lamina I neurons. Neurons with somata at a distance of 25 to 125 μm to the dorsal white matter border were considered lamina II neurons[17] (see also Ref. 26). During recordings, ACSF was identical to that during incubation with the exception of (in mM): 127 NaCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 0 sucrose. Pipettes were pulled on a horizontal puller (P-87, Sutter Instruments, Novato, CA), and resistances were 2-5 MΩ with an intracellular solution consisting of the following (in mM): 120 K-$MeSO_3$, 20 KCl, 2 $MgCl_2$, 20 HEPES, 0.5 EGTA, 2 $Na_2ATP$; pH 7.28 with KOH. During pharmacological recordings, 0.5 mM GDPβS and 100 μM AlexaFluor 568 or 594 were added to the intracellular solution. Drugs were washed in no earlier than 15 minutes after breaking in, or 15 minutes after wash-out of the first drug, when cells were probed with more than 1 drug consecutively. Cells were held at holding potentials of −70 mV, and liquid junction potentials were not corrected. Series resistances were monitored by a 50 ms 5 mV hyperpolarizing pulse at the beginning of each recording. Recordings were discarded if the series resistance during application of the drug deviated more than 30% from control. Enhanced YFP and tdTomato fluorescence were assessed in DRG and slices on the same microscope using laser scanning microscopy. TdTomato was assessed with confocal microscopy at an excitation wavelength of 543 nm, while eYFP was assessed using multiphoton microscopy to avoid activating ChR2. To this end, a Ti-sapphire laser (Chameleon-XR, Coherent, Germany) at 960 nm was used.

### 2.4. Primary afferent stimulation

Channelrhodopsin-2 activation was performed at the level of the DRG with an optical fibre (BFL48-400 or BFL48-1000) coupled to a 470 nm LED (Thorlabs, Munich, Germany), unless stated otherwise. Light-intensities, measured with a hand-held power meter (Lasercheck; Coherent, Dieburg, Germany), were 60 to 80 $mW/mm^2$. In VGluT3$^{-/-}$ mice, dorsal root stimulation was performed by a square current pulse of 0.1 ms duration and at least twice the response threshold, delivered with a suction electrode coupled to a constant current stimulator (A320, WPI, Sarasota, FL), as described previously.[27] Paired-pulse stimulation was performed every 30 seconds at paired-pulse intervals of 300 to 500 ms.

### 2.5. Data analysis

Electrophysiological data were analysed off-line using Clampfit (Molecular Devices), IGORPro (Wavemetrics, Lake Oswego, OR), and Microsoft Excel. For illustrations, data were filtered at 1 kHz. In box and whisker plots, boxes range from the 25th to 75th percentile, and the median is indicated. Whiskers correspond to minimum and maximum values. In addition to the 95% confidence interval of the population median (CI), mean ± SEM are given in the text. Statistical analyses were performed with SigmaPlot 12.0 (Systat Software Inc, San Jose, CA). Student *t* test was used for comparing 2 unpaired groups that were normally distributed. For paired groups, the Wilcoxon signed-rank test was used because the Shapiro-Wilk test for normal distribution failed for some groups. Repeated-measures analysis of variance plus Dunnett method for multiple comparisons was used if more than 2 paired groups were to be compared. The level of statistical significance was set at 0.05.

### 2.6. Chemicals

Chemicals were obtained from Sigma-Aldrich, Invitrogen, and Tocris. SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide), DAMGO ([D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin), and baclofen were prepared in stock solutions, stored at −20°C, and were diluted to final concentration in ACSF before use.

## 3. Results

### 3.1. Validation of the animal model

We used a BAC transgenic VGluT3-cre mouse line[20] to target ChR2 to a particular subset of primary sensory neurons. Our previous immunohistochemical analysis revealed cre expression in 19% of DRG neurons dividing into the following subpopulations[13]: (1) neurofilament 200 (NF200)-positive A-fibres most likely transiently expressing VGluT3 and innervating Merkel cells, (2) $NF200^-$/tyrosine hydroxylase (TH)-positive low-threshold mechanoreceptive C-fibres (C-LTMRs), and (3) $NF200^-/TH^-$ C-fibres, largely coexpressing transient receptor potential cation channel, subfamily M, member 8 (TRPM8) mRNAs. In our initial electrophysiological recordings in spinal cord slices, VGluT3-cre mice were crossed to the cre-dependent ChR2 mouse line Ai27. This mouse line contains ChR2 in frame with the red fluorescent tdTomato downstream of a floxed stop codon (Madisen et al., in 2012). In VGluT3-cre × Ai27 mice, we observed red fluorescence in small- and large-diameter DRG neurons (**Fig. 1A**), corresponding to $VGluT3^+$ C- and A-fibres, respectively. In the spinal dorsal horn, we detected tdTomato fluorescence in lamina I and lamina II inner, consistent with the termination pattern of $VGluT3^+$ C-fibres,[44] as well as in deeper laminae of the spinal dorsal horn, the latter likely reflecting ChR2 expressing A-fibres (**Fig. 1B**). We did not observe neuronal profiles expressing the ChR2-tdTomato construct in lamina I and II of the spinal dorsal horn (see also higher magnification image in **Fig. 1C**).

To test whether neurotransmitter release from axons of primary afferent nerve fibres can reliably be evoked by light in VGluT3-cre × Ai27 mice, we next recorded light-evoked postsynaptic responses with the whole-cell patch-clamp technique in spinal dorsal horn neurons, which themselves did not express the ChR2-tdTomato construct. Flashes of blue light (1 ms 488 nm), delivered through the microscope objective and illuminating the entire field of view, elicited excitatory postsynaptic currents (EPSCs) in 20/37 dorsal horn neurons. Mean amplitudes in responding neurons were 109 ± 48 pA (range, 18-417 pA, n = 20, **Fig. 1F**), and light-evoked EPSCs were completely blocked by 0.5 to 1 μM TTX (n = 3), confirming that they were not due to direct light-evoked depolarizations. Upon repetitive stimulation every 30 seconds, a small fraction of neurons (11%, 4/35 cells) responded reliably, ie, without failures, and with a trial-to-trial jitter of less than 1 ms (see also Ref. 36). Interestingly, when directly assessing light-evoked depolarizations in ChR2 expressing neurons, 0/10 tdTomato-positive DRG neurons were depolarized to action potential threshold, whereas 3/4 tdTomato-expressing cortical neurons fired light-evoked action potentials. Because our measured light intensities (60-80 mW/mm$^2$) were comparable with

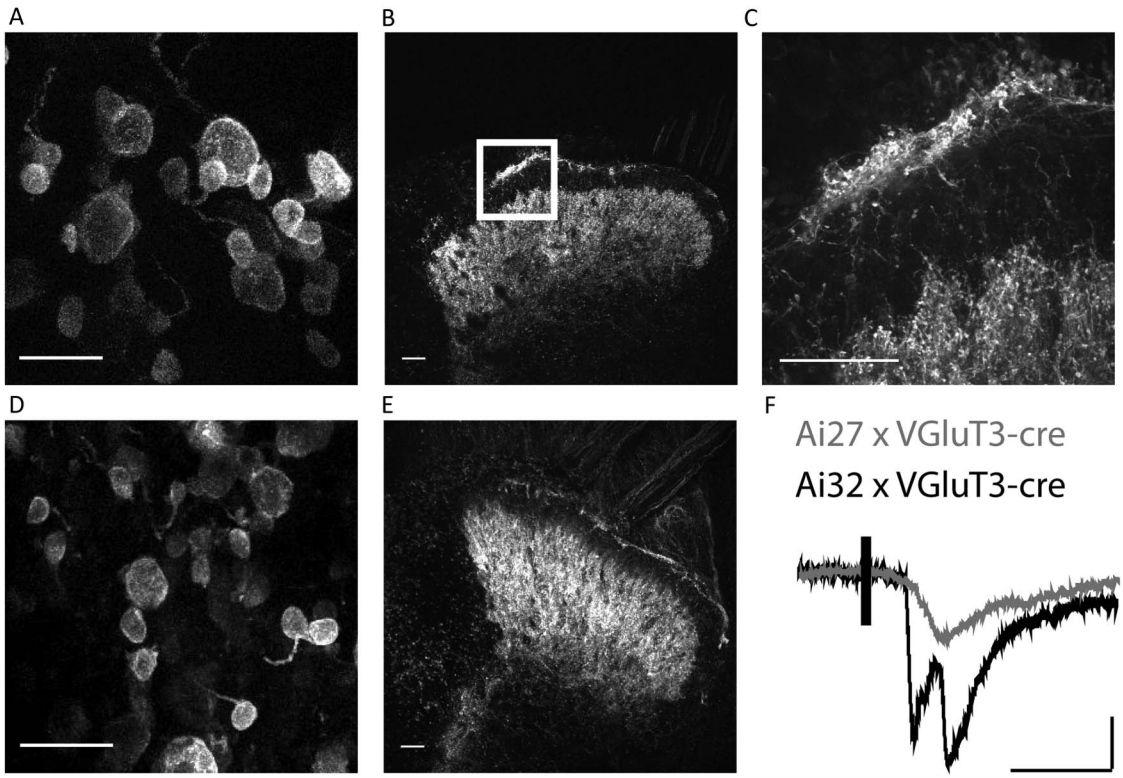


**Figure 1.** Comparison of Ai27 and Ai32 mice. (A-C) Maximal intensity z-projection of laser scanning microscopy stack through DRG (A) and spinal cord (B and inset C) of an Ai27 × VGluT3-cre mouse. TdTomato fluorescence, and correspondingly ChR2 expression, is restricted to a subpopulation of small- and large-diameter DRG neurons, and the spinal dorsal horn. (D and E) Maximal intensity z-projections of laser scanning microscopy stacks through DRG (D) and spinal cord (E) of an Ai32 × VGluT3-cre mouse. Enhanced yellow fluorescent protein fluorescence, indicating ChR2 expression, closely resembled tdTomato fluorescence of Ai27 mice. (F) Stimulation of the spinal cord with blue light (1 ms, 488 nm, intensity: ~1.7 mW), generated with a monochromator (Poly V, TILLvisION), and delivered through the microscope objective (Leica HCX APO, ×20, NA 1.0), revealed larger postsynaptic responses in Ai32 × VGluT3-cre mice (black) than in Ai27 × VGluT3-cre mice (grey). The figure illustrates typical examples of photostimulated currents. Vertical black bar indicates time point of stimulation. Scale bars in A-E correspond to 50 μm; in F, scale bars indicate 20 ms and 50 pA.

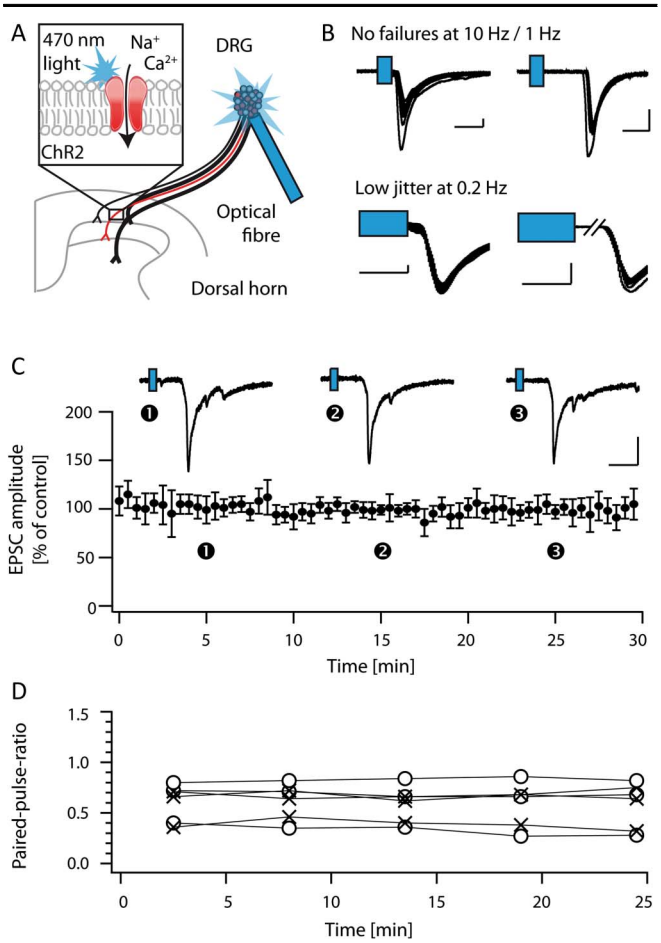


**Figure 2.** Light activation of VGluT3$^+$ fibres. (A) Scheme depicting experimental approach: EPSCs were evoked with blue light (470 nm) at the level of the DRG in spinal cord slices with dorsal roots and DRG attached. (B) Excitatory postsynaptic currents were considered of monosynaptic origin, if they (1) did not exhibit failures upon repetitive stimulation (top left and right: sample traces of A- and C-fibres [distinguished by their conduction velocities], respectively), and (2) if they exhibited a low jitter upon stimulation at 0.2 Hz (bottom, see also Table 1). Scale bars indicate 200 pA, and 10 or 5 ms, for top and bottom traces, respectively. (C) Control recordings revealed that light-evoked EPSCs recorded in lamina I and II were stable over the entire recording period (mean value +/− CI; n = 7; top: sample traces recorded at the 3 indicated time points). (D) Paired-pulse ratios recorded at paired-pulse intervals of either 300 ms (o) or 500 ms (×) were stable over the entire recording period (n = 6).

those reported in the literature,[7] we concluded that Ai27 mice were not well suited to reliably evoke action potentials in axons or somata of VGluT3$^+$ DRG neurons by light.

A similarly designed cre-dependent ChR2 mouse line, Ai32, has been shown to exhibit larger light-evoked currents than Ai27 mice.[31] We therefore tested whether these mice are better suited for activating VGluT3$^+$ sensory neurons. Ai32 mice contain the eYFP in place of tdTomato, but are otherwise constructed identically to the Ai27. On a gross level, the distribution of eYFP fluorescence in the DRG and spinal cord of VGluT3-cre × Ai32 mice resembled that seen with the tdTomato fluorescence in the VGluT3-cre × Ai27 mice (**Fig. 1D, E**). In VGluT3-cre × Ai32 mice, 17/19 eYFP-ChR2-negative spinal neurons (89%) responded to the axonal light stimulation, which is significantly more than in VGluT3-cre × Ai27 mice, where only 54% of cells had responded ($P = 0.02$). Mean amplitudes of evoked EPSCs in responding neurons amounted to 140 ± 51 pA, ranging from 27 to 353 pA, n = 17 (**Fig. 1F**). When stimulated repetitively, every 30 seconds,

**Table 1**

**Parameters for identifying light-evoked monosynaptic A- and C-fibre–mediated responses.**

| DRG recordings* | Large-diameter neurons | Small-diameter neurons |
|---|---|---|
| Action potential delay after onset of light pulse | 2-4 ms | 2-8 ms |
| Mean action potential jitter at 0.2 Hz | ~1 ms | ~1 ms |
| **Postsynaptic EPSCs†** | **A-fibre–mediated responses** | **C-fibre–mediated responses** |
| Conduction velocity (as determined from response delay) | ≥1.5 m/s | ≤1.0 m/s |
| Failures upon repetitive stimulation | No failures at 10 Hz | No failures at 1 Hz |
| Jitter upon 0.2 Hz stimulation | Jitter minus 1 ms <10% of EPSC latency | |

* Action potential delay and jitter upon photo-stimulation in A- and C-fibres, as measured in large- and small-diameter eYFP-positive DRG neurons, respectively.

† Criteria used for classifying light-evoked EPSCs as monosynaptic A- or C-fibre–mediated responses.

DRG, dorsal root ganglion; EPSCs, excitatory postsynaptic currents; eYFP, enhanced yellow fluorescent protein.

7/15 neurons (47%) responded with a trial-to-trial jitter of less than 1 ms. The proportion of neurons responding reliably to repetitive stimulations was, thus, significantly higher when making use of the Ai32 rather than the Ai27 mouse line ($P = 0.017$). Additional DRG neuron recordings revealed that 20/27 DRG neurons fired action potentials when directly stimulated with a flash of blue light. As controls, eYFP was not expressed in DRG neurons of cre-negative littermates (not shown), and electrophysiological experiments confirmed that neither DRG neuron depolarization nor postsynaptic responses in spinal lamina I and II neurons were elicited by 470 nm light in cre-negative mice (n = 4 and 10, respectively; not shown). Taken together, these results demonstrate that Ai32 mice crossed to VGluT3-cre mice are a suitable animal model for studying VGluT3$^+$ primary afferents.

### *3.2. Pharmacological profiles of different sensory fibre populations*

We then studied the modulation of synaptic transmission at synapses of VGluT3$^+$ primary afferent fibres and spinal dorsal horn neurons in spinal cord slices with dorsal roots and DRG attached. ChR2-positive fibres were activated at the level of the DRG with a light guide coupled to a high-power 470 nm LED (pulse duration 5 ms). This approach allowed us to activate VGluT3$^+$ fibres at the level of the DRG, ie, at a distance of at least 4 mm from the site of neurotransmitter release in the superficial spinal dorsal horn (depicted in **Fig. 2A**). Postsynaptic neurons were classified as lamina I or II neurons, as judged from the distance of their cell bodies to the dorsal boundaries of the slice (see Methods for details). We confirmed the monosynaptic origin of evoked EPSCs by means of repetitive light stimulations. To account for the increased delay and jitter of light-evoked action potentials as compared with electrical stimulations (see also Ref. 43), we first performed patch-clamp recordings in the presynaptic DRG neurons to quantitatively assess these parameters under our experimental conditions (**Table 1**). The observed action potential jitter in DRG neurons was approximately 1 ms when stimulated at 0.2 Hz, and increased with increasing stimulation frequencies. Although not all DRG neurons followed a 1-Hz or 10-Hz stimulation without failures, only spinal dorsal horn recordings of cells not exhibiting failures upon stimulation at

1 Hz (C-fibres, conduction velocity ≤1 m/s) or 10 Hz (A-fibres, conduction velocity ≥ 1.5 m/s) were included in the analysis. The jitter of postsynaptic responses recorded in the spinal dorsal horn was determined at a frequency of 0.2 Hz, and recordings with a high trial-to-trial jitter were discarded (**Fig. 2B**; see **Table 1** for details on classification of neurons). These stringent criteria likely resulted in the exclusion of a number of monosynaptic responses; however, they allowed us to be reasonably sure that the cells we recorded from received indeed monosynaptic input from VGluT3$^+$ primary afferents. In all experiments, GDPβS (0.5 mM) was included in the recording pipette to block postsynaptic G-protein–mediated responses.[2] We additionally assessed the paired-pulse ratio as an indication for presynaptic drug effects, and further control experiments confirmed that light-evoked EPSCs could reliably be evoked every 30 seconds over a period of at least 30 minutes (n = 7, **Fig. 2C**). Average run-down was 2% per 10 minutes, as determined by a linear fit to the mean data. Paired-pulse ratios did not change significantly during the recording period (n = 6, **Fig. 2D**).

### 3.2.1. VGluT3$^+$ C-fibres

First, we assessed the pharmacological profile of VGluT3$^+$ C-fibres. The MOR agonist DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin, 0.5 μM) reversibly reduced light-evoked C-fibre–mediated EPCS recorded in lamina I neurons to 32.1 ± 6.4% (CI: 13.9-52.2%) of control (n = 11, $P < 0.001$, **Fig. 3A, B**). DAMGO reversibly inhibited only 2/8 lamina II neurons to less than 50% of control. The average EPSC amplitudes in this group were, however, not significantly reduced (EPSC amplitude in DAMGO 81.3 ± 13.7% of control [CI: 21.9-114.2%], n = 8, $P = 0.461$). Taken together, the inhibition exerted by DAMGO was much stronger in lamina I neurons than in lamina II neurons ($P = 0.02$, **Fig. 3B**), and was associated with an increase in the paired-pulse ratio (n = 13 lamina I and II neurons, **Fig. 3C**). The nonpeptide DOR agonist SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; 50 μM) did not significantly influence VGluT3$^+$ C-fibre–mediated EPSCs in either lamina I or lamina II (mean amplitude in SNC80 [5 minutes] 100.3 ± 4.0% [CI: 90.5-106.1%] and 98.4 ± 3.0% [CI: 90.7-105.3%] of control for lamina I and lamina II neurons, respectively; n = 7, $P = 0.938$ and 0.688; **Fig. 3B**). In line with the lack of effect on EPSC amplitudes, paired-pulse ratios remained unaffected in the presence of SNC80 (n = 11; **Fig. 3C**). The $GABA_BR$ agonist baclofen (1 μM) virtually abolished light-evoked, C-fibre–mediated EPSCs both in lamina I and lamina II neurons (inhibition to 8.4 ± 3.2% [CI: 0-15.8%] and 5.9 ± 3.2% [CI: 0-16.4%] of control for lamina I and II neurons, respectively; n = 7, $P = 0.016$). In the presence of baclofen, paired-pulse ratios were significantly increased, consistent with a presynaptic inhibition (**Fig. 3C**; calculated from 5/14 neurons, as evoked EPSCs of the remainder were completely blocked).

### 3.2.2. A-fibres transiently expressing VGluT3

Next, we assessed postsynaptic responses, evoked by A-fibres, transiently expressing VGluT3 during development. In this group of recordings, cells were only included if they followed a 10-Hz stimulation without failures. The response jitter was determined by stimulating at 0.2 Hz, as described above (see also **Fig. 2B** and **Table 1**). On average, DAMGO (0.5 μM) reduced evoked EPSCs in lamina I to 63.1 ± 17.2% of control (CI: 19.6-100.3%, n = 7, $P = 0.109$, **Fig. 4A**). Interestingly, in roughly half of the neurons (3/7 cells), DAMGO almost completely blocked A-fibre–evoked

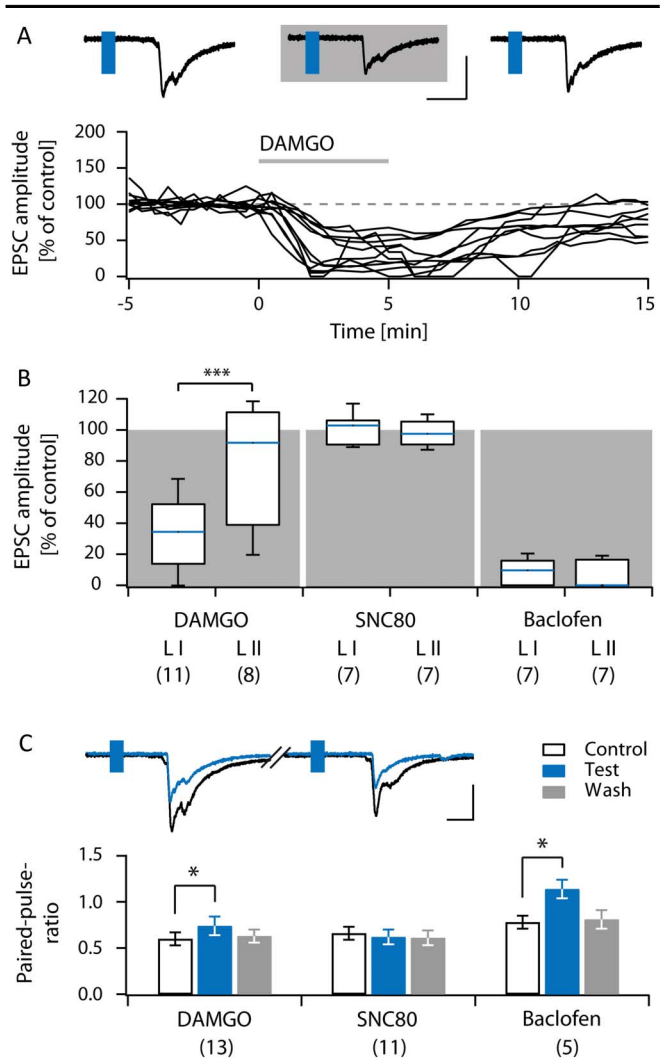


**Figure 3.** Pharmacology of VGluT3$^+$ C-fibres in lamina I and II. (A) Sample traces of light-evoked EPSCs recorded in lamina I before, during (grey shaded area), and after the application of DAMGO (top). Normalized EPSC amplitudes of 11 lamina I neurons plotted over time, grey bar indicates DAMGO application (bottom). (B) Box and whisker diagram showing normalized amplitudes of light-evoked C-fibre–mediated EPSCs recorded in lamina I and II in the presence of DAMGO (0.5 μM), SNC80 (50 μM), and Baclofen (1 μM). The number of experiments is given in brackets below each group. (C) Sample paired-pulse recording in control conditions and during application of DAMGO (top). Quantification of paired-pulse ratios of lamina I and II recordings during control conditions (black outline), application of test substances (blue) and wash (grey, bottom panel). The number of experiments is given in brackets below each group. Scale bars in A and C indicate 10 milliseconds or 200 pA. Blue rectangles in sample traces indicate flash of 470 nm light.

EPSCs (inhibited to ~20% or less), whereas responses in the other half of neurons remained unaffected (4/7 cells). In lamina II, responses of most neurons were only slightly affected, and in only 2/10 neurons A-fibre–evoked EPSCs were inhibited to ~50% or less, together resulting in a statistically nonsignificant group effect (normalized mean amplitude in DAMGO 84.8 ± 9.4% [CI: 75.6-104.7%], n = 10, $P = 0.193$, **Fig. 4A**). Application of DAMGO significantly increased mean paired-pulse ratios (n = 12; **Fig. 4B**). Mean EPSC amplitudes in the presence of the DOR agonist SNC80 (50 μM) were 93.7 ± 4.3% of control (CI: 91.7-102.6%) in lamina I (n = 6, $P = 0.219$), and 87.3 ± 3.4% of control (CI: 79.9-98.5%) in lamina II (n = 7; $P = 0.016$; **Fig. 4A**). SNC80 did not significantly affect paired-pulse ratios (n = 11; **Fig. 4B**). A-fibre–evoked responses were virtually abolished by baclofen

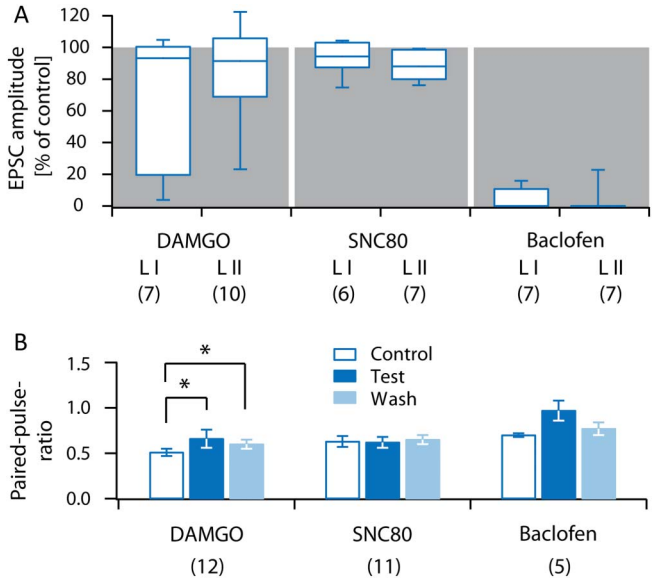


**Figure 4.** Pharmacology of VGluT3$^{+}$ A-fibres in lamina I and II. (A) Box and whisker diagram showing normalized EPSC amplitudes of light-evoked A-fibre–mediated EPSCs recorded in lamina I and II in the presence of DAMGO (0.5 μM), SNC80 (50 μM), and Baclofen (1 μM). The number of experiments is given in brackets below each group. (B) Quantification of paired-pulse ratios of lamina I and II recordings during control conditions (blue outline), application of test substances (dark blue), and wash (light blue). The number of experiments is given in brackets below each group.

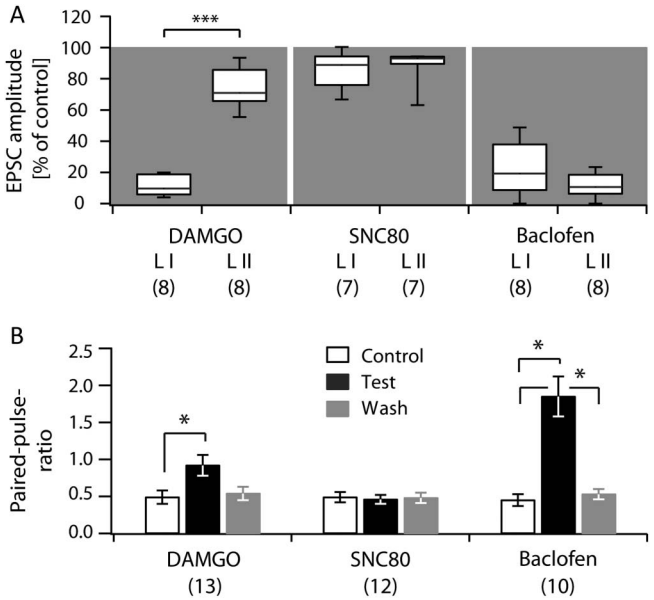


**Figure 5.** Pharmacology of putative nociceptive C-fibres in lamina I and II. (A) Box and whisker diagram showing normalized EPSC amplitudes of electrically evoked C-fibre–mediated EPSCs recorded in lamina I and II of VGluT3$^{-/-}$ mice in the presence of DAMGO (0.5 μM), SNC80 (50 μM), and Baclofen (1 μM). The number of experiments is given in brackets below each group. (B) Quantification of paired-pulse ratios of lamina I and II recordings during control conditions (black outline), application of test substances (black), and wash (grey). The number of experiments is given in brackets below each group.

(1 μM; inhibition to 5.3 ± 2.6% [CI: 0-10.7%] and 3.3 ± 3.2% [CI: 0-0%] of control in lamina I and II, respectively; n = 7 each, $P$ = 0.016, **Fig. 4A**). In 5/14 neurons, EPSC amplitudes remained at sufficient size to calculate paired-pulse ratios, resulting in a statistically nonsignificant increase, likely b lack of statistical power (**Fig. 4B**).

### *3.2.3. Putative nociceptive C-fibres*

We then assessed the pharmacological profile of putative nociceptive C-fibres in VGluT3$^{-/-}$ mice (**Fig. 5**). The lack of VGluT3 leads to reduced loading of glutamate into presynaptic vesicles of sensory fibres normally expressing VGluT3 (10-15% of all C-fibres), resulting in disrupted neurotransmitter release from these fibres. Electrically evoked C-fibre–mediated responses in VGluT3$^{-/-}$ mice therefore predominantly result from activation of VGluT3-negative peptidergic (ie, CGRP-positive) and nonpeptidergic (ie, IB-4-positive) C-fibres. DAMGO (0.5 μM) inhibited electrically evoked C-fibre–mediated responses in lamina I and II of VGluT3$^{-/-}$ mice to 11.4 ± 2.3% (CI: 5.7-19 4%) and 73.8 ± 4.4% (CI: 65.5-87.8%) of control, respectively (n = 8 each, $P$ = 0.008, **Fig. 5A**). Excitatory postsynaptic currents of all lamina I neurons were strongly inhibited (less than 20% of control), whereas in none of the lamina II neurons, EPSCs were reduced to less than 50% of control by DAMGO. The decrease in EPSC amplitudes was associated with an increase in paired-pulse ratios (**Fig. 5B**). Bath application of SNC80 slightly reduced EPSC amplitudes in lamina I and II to 85.3 ± 4.6% (CI: 75.9-94.3%) and 88.5 ± 4.3% (CI: 89.6-94.2%) of control, respectively (n = 7 each, $P$ = 0.031 and $P$ = 0.016, **Fig. 5A**). Paired-pulse ratios were not significantly altered (**Fig. 5B**). Baclofen strongly inhibited all responses recorded in lamina I and II neurons, and increased paired-pulse ratios (EPSC amplitudes reduced to 31.6 ± 11.0% [CI: 8.1-38%] and 19.5 ± 6.3% [CI: 5.6-19.3%] of control after a 5 minute wash-in period, respectively, n = 8 each, $P$ = 0.008, **Fig. 5A, B**).

## 4. Discussion

This study shows that Ai32 mice are better suited than Ai27 mice for subtype-specific optogenetic stimulation of sensory fibres. We demonstrate that MOR agonists inhibit all groups of C-fibres innervating lamina I neurons considerably stronger than those innervating lamina II neurons. δ-Opioid receptor agonists did not have any pronounced presynaptic effects in any fibre type studied. In contrast, $GABA_BR$ agonists strongly depressed evoked EPSCs in all fibre types tested.

### *4.1. Experimental approach*

The expression of neurotransmitter receptors by different subclasses of primary afferent fibres has previously been probed on dissociated DRG neurons through binding studies, in situ hybridization, or immunohistochemical stainings.[33,48] Somatic expression, however, is rather indirect evidence for the expression of functional receptors at synaptic terminals. Behavioural studies, however, are useful for probing the influence of analgesics on the different modalities of pain, but do not reveal the precise site of action. The presently used optogenetic approach allowed us to directly probe the degree of inhibition exerted by selective receptor agonists on different classes of primary afferent fibres. Our VGluT3-cre mouse expressed cre recombinase in the expected subpopulations of primary afferent fibres. In agreement with a previous study in the brain,[31] we found that Ai32 mice are better suited than Ai27 mice for optogenetic stimulation of sensory fibres. The reason is unclear but might be due to interference of the tdTomato tag with the ChR2 channel.[1,31] In comparison with previous attempts to activate

distinct subsets of primary afferent fibres by light,[47] the present approach yielded more reliable monosynaptic responses, as judged from repetitive stimulations at 0.2 Hz.

A major concern in optogenetic studies are the slow kinetics of ChR2, resulting in calcium-dependent afterdepolarizations, and increased release probabilities when directly stimulating presynaptic terminals.[43,52] We circumvented this drawback by stimulating at a distance of at least 4 mm from the site of neurotransmitter release. This approach not only allowed us to rule out confounding effects on neurotransmitter release,[51] but also enabled us to clearly distinguish A- and C-fibre–mediated responses by their different latencies. Our experimental approach can be adapted to virtually any genetically identified type of sensory fibre, provided a mouse line, expressing cre recombinase in the desired type of neuron exists.

### 4.2. Presynaptic opioid receptors

Virtually all clinically used opioids target MORs. In animal models, MOR agonists have been reported to preferentially inhibit heat pain[42] or to reduce withdrawal latencies independent of pain modality, Refs. 8, 23, 34 and others. We found that nociceptive C-fibres innervating lamina I neurons were inhibited more strongly by the MOR agonist DAMGO than fibres innervating lamina II neurons. The strong inhibition, observed in lamina I, is in line with MOR expression on peptidergic, CGRP-positive fibres, terminating in lamina I. Thus, our results are compatible with a MOR-dependent inhibition of primary afferents triggering heat pain, as suggested previously.[42] μ-Opioid receptor expression is, however, by no means restricted to this fibre population, as significant inhibition was also observed in VGluT3$^+$ C-fibres innervating lamina I (but not lamina II) neurons. The preferential inhibition of VGluT3$^+$ C-fibres innervating lamina I vs lamina II neurons could be explained by the 2 subpopulations of VGluT3$^+$ C-fibres described to date. Small-diameter DRG neurons coexpressing VGluT3 and TH innervate the lanceolate endings of hairy skin, and most likely function as low-threshold mechanoreceptive C-fibres.[29,30,36] The TH-negative subpopulation of VGluT3$^+$ small-diameter neurons is less well characterized. They seem to express the TRPM8 channel[13] and could be muscle afferents (R.P. Seal, unpublished observations) or epidermal free nerve endings.[30] Interestingly, TH-positive DRG neurons have recently been shown not to express MORs.[4] Considering the lack of inhibition by DAMGO observed in most VGluT3$^+$ C-fibres innervating lamina II neurons (this study), this suggests that the population of VGluT3$^+$ C-fibres innervating lamina II neurons could be C-LTMRs. Provided that the termination pattern of TH-positive and TH-negative VGluT3-positive C-fibres in the spinal dorsal horn is indeed segregated, TH-negative neurons might be the ones innervating lamina I neurons and being inhibited by MOR activation.

In particular, VGluT3$^+$ C-fibres innervating lamina II neurons as well as VGluT3$^+$ A-fibres displayed a high degree of variability in their responses to DAMGO. This higher variability, as compared with nociceptive C-fibres, was unexpected, as we hypothesised that VGluT3$^+$ fibres would be a more homogeneous group than the large populations of peptidergic and nonpeptidergic C-fibres that were electrically stimulated in VGluT3$^{-/-}$ mice. However, a recent study also reported heterogeneous responses in putative C-LTMRs upon application of hypo-osmotic solution or a TRPA1-receptor agonist.[12] The response variability observed by us and others may reflect functional heterogeneity or the existence of further not yet identified subpopulations of VGluT3$^+$ A- and C-fibres.

Functional DORs have recently been found on mechanosensitive Aβ fibres innervating Merkel cells and terminating in lamina III/IV of the spinal dorsal horn,[4] which might transiently express VGluT3 during development.[30] It is, however, still unclear whether DORs are differentially expressed in mechanical vs heat-sensitive primary afferent fibres terminating in the superficial layers I and II of the spinal dorsal horn. Depending on the experimental approach, DOR receptors have been found largely in peptidergic fibres,[3,21,48] in a small subset of nonpeptidergic C-fibres and myelinated A-fibres,[4,42] or not at all at DRG neuron membranes of naive animals.[6,9] Our functional approach now revealed that none of the investigated fibre types was considerably inhibited by saturating concentrations of the DOR agonist SNC80. These results indicate that, at most, a small number of DRG neurons innervating the most superficial layers of the spinal dorsal horn express functional DOR receptors in naive animals. The minor inhibition we observed in putative nociceptive fibres might have resulted from an effect on the TRPA1- or MrgprD-positive subpopulations of primary afferent neurons.[4,49] These findings are in line with behavioural studies, in which significant antinociceptive effects were also not observed upon application of SNC80 in naive mice.[16]

### 4.3. Presynaptic $GABA_B$ receptors

The $GABA_B$R agonist baclofen has been suggested to inhibit C-fibres more efficiently than A-fibres,[2,32] and to inhibit pinch-evoked EPSCs more robustly than touch-evoked EPSCs.[15] These findings point to a preferential inhibition of nociceptive vs low-mechanical threshold primary afferent fibres. However, baclofen has been found to be useful for the treatment of mechanical allodynia in animal models.[28,40] Our present results provide an explanation for this apparent discrepancy: Baclofen, at much lower concentrations than reported in previous studies (1 μM vs 10 μM), strongly inhibited synaptic transmission by VGluT3$^+$ C-LTMRs, which have been attributed an aetiology-dependent role in mechanical and cold allodynia after tissue or nerve injury.[13,44] This presynaptic inhibition by baclofen could underlie the suggested antiallodynic effects. In addition, the high sensitivity of VGluT3$^+$ fibres to $GABA_B$R activation suggests that these fibres, similar to nociceptive fibres, are under strong presynaptic GABAergic inhibition under physiological conditions.[50]

### 4.4. Implications for pain therapy

It has been suggested that pain modality rather than aetiology would be the better predictor for treatment outcome in conditions of chronic pain.[22,38] Our study now suggests that the degree of MOR-dependent inhibition depends more on laminar location of the postsynaptic neuron than on the types of afferent fibres involved. In contrast to MOR activation, DOR and $GABA_B$R activation exhibited uniform effects on all fibres investigated. However, neurotransmitter receptor expression or function might be altered in conditions of chronic pain, and intrathecal DOR agonists may prove useful therapeutic agents for the treatment of pain, if chronic injury or inflammation leads to an increased functional coupling of DORs to voltage-gated calcium channels,[37] or to an enhanced plasma membrane expression in distinct fibre populations[3,5,18] (see also Ref. 45). Given that DOR activation was largely ineffective in inhibiting synaptic transmission in naive animals, such a treatment would most likely result in reduced adverse effects as compared with pharmaceuticals acting on a large number of fibres in the naive state.

Our finding that virtually all classes of primary afferent fibres were strongly inhibited by baclofen, including those that were unresponsive to MOR activation, suggests that the activation of presynaptic $GABA_BR$ may be advantageous for the treatment of pain states that either result from the activity of a number of different fibre populations, or from activity in fibres not responsive to MOR agonists. And indeed, there is evidence from case studies in humans that intrathecal baclofen can be beneficial for relieving severe tactile allodynia or burning pain, refractory to treatment with morphine.[53] As $VGluT3^+$ primary afferent are implied in chemotherapy (oxaliplatin)-induced pain,[13] and baclofen strongly inhibited all classes of $VGluT3^+$ primary afferents (this study), baclofen may prove a beneficial agent for the treatment of oxaliplatin-induced mechanical and cold allodynia. Our finding that near-complete inhibition of neurotransmission can be achieved through activation of presynaptic $GABA_BR$, likely containing the $GABA_B1a$ subunit,[14,39,46] lets us further speculate that the development of $GABA_B1a$ subunit-specific agonists for intrathecal injection may prove a highly beneficial therapeutic strategy for limiting the side effects of pharmacological therapy.

## Conflict of interest statement

The authors have no conflicts of interest to declare.

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