



Substance P-Botulinum Mediates Long-term Silencing of Pain Pathways that can be Re-instated with a Second Injection of the Construct in Mice

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Abstract: Chronic pain presents an enormous personal and economic burden and there is an urgent need for effective treatments. In a mouse model of chronic neuropathic pain, selective silencing of key neurons in spinal pain signalling networks with botulinum constructs resulted in a reduction of pain behaviours associated with the peripheral nerve. However, to establish clinical relevance it was important to know how long this silencing period lasted. Now, we show that neuronal silencing and the concomitant reduction of neuropathic mechanical and thermal hypersensitivity lasts for up to 120d following a single injection of botulinum construct. Crucially, we show that silencing and analgesia can then be reinstated with a second injection of the botulinum conjugate. Here we demonstrate that single doses of botulinum-toxin conjugates are a powerful new way of providing long-term neuronal silencing and pain relief.

Perspective: This research demonstrates that botulinum-toxin conjugates are a powerful new way of providing long-term neuronal silencing without toxicity following a single injection of the conjugate and have the potential for repeated dosing when silencing reverses.

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Key words: Pain, Botulinum-based construct, Analgesia, Neuronal silencing

Noxious stimuli of sufficient intensity to induce tissue damage lead to increased excitability of peripheral and central neuronal circuits that heightens pain experience and serves to protect damaged tissue from further trauma.^{1,2} In some cases,

ongoing disease or the failure of potentiated pain signalling networks to return to pre-injury levels leads to chronic pain conditions.³ Chronic or persistent pain is highly prevalent and extremely difficult to treat with currently available drugs, often having significant unwanted side effects. Although research into developing new analgesic drug therapies has been intense, translating knowledge from preclinical observations in animal models to new therapies in the clinic has been challenging.⁴

Previous research has identified neuronal networks within the dorsal horn and pathways from the dorsal horn to the brain as important for the regulation of

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persistent pain states.⁵ Recently the contribution of ascending pathways from the superficial dorsal horn of the spinal cord to the brain has also been shown to be essential for the maintenance of persistent pain states.^{6–11} Many of these superficial spinal projection neurons express the substance-P (SP) preferring neurokinin 1 receptor (NK1R)¹² and can be ablated with intrathecal (i.t.) application of substance P-saporin (SP-SAP) conjugates^{5,7} resulting in normal acute thermal and mechanical stimulus detection but a loss of mechanical and thermal hypersensitivity in inflammatory and neuropathic pain models. Unfortunately, while SP-SAP treatment was shown to alleviate bone cancer pain in companion dogs,¹³ clinicians have been cautious about taking the approach forward to the clinic. We therefore, designed alternative constructs using botulinum neurotoxin serotoxin A (BoNT/A) which silenced but did not damage neurons.^{14–16} Once internalised within the neuron, the light chain of BoNT/A has the capacity to silence neurons via the specific proteolytic cleavage of synaptosomal-associated protein 25 (SNAP25), a protein essential for synaptic release.¹⁷ By exploiting a recently introduced protein ‘stapling’ method we linked the light-chain/translocation domain (LcTd) of botulinum neurotoxin type A (BOT) to neurotransmitter ligand SP to target key pain-processing neurons in the dorsal horn. A single intrathecal injection of the neuropeptide substance P conjugate (SP-BOT) was shown to alleviate inflammatory and neuropathic pain states through the silencing of nociceptive pathways for up to 30 days.¹⁸ We also showed that SP-BOT was internalised only by NK1R-expressing neurons and we confirmed a loss of activity in the ascending pathway to the brain in SP-BOT treated- and stimulated- mice, directly implicating spinal to parabrachial NK1R+ projection neurons as targets for the construct.¹⁸

These studies were, however, all carried out within the relatively short time frame of 30 days. The present study was therefore designed to examine 1) the length of time over which a single injection of SP-BOT was effective in silencing the ascending pain pathways, 2) if affective pain behaviours as well as ‘reflex’ or signal detection behaviours, were also affected by SP-BOT treatment, 3) if neuronal silencing could be reinstated by a second intrathecal injection of SP-BOT, 4) if silencing of NK1R+ neurons in naïve mice was the same as silencing neurons in the presence of a pain state. Long-term silencing of neurons after establishing a pain state could lead to a permanent rearrangement of nociceptive networks.

Methods

Experimental Design

This study was designated to evaluate the longevity of SP-BOT-induced silencing and its subsequent effect on pain sensitivity. The experimenter was always blind to treatment. In all experiments, mice were randomly assigned into treatment group. The numbers of mice in each group are designated in the individual figures. For

Fig 1, $n = 6$ mice per group were used. Using our previous experiments,¹⁸ we estimated the average effect size to be .6 for a standard deviation between (.35–.5). Therefore with a Type 1 error set at 5%, a Power at 80%, and a 2-tailed assumption, $n = 6$ was adequate. Please note that in the saline group, 1 mouse was culled due to fighting wounds. For Fig. 2–6 the effect size could not have been predicted, Mead’s Resource equation was used. It considers n of 8 to 12 adequate for comparing 2 groups. Therefore 8 mice were used in each group. For Fig 6, 2 mice were excluded from the study because of body damage from fighting with cage mates. Although the effect size of drug effects was not predictable, sample sizes in this study are consistent with the norm in the field.¹⁹

Animals

Animal use for pain studies complies with the ARRIVE guidelines.²⁰ All efforts were made to minimise animal suffering (UK Animal Act, 1986) and to reduce the number of mice used (3Rs). Experiments were designed to use the minimum number of animals to provide sufficient statistical power based on our previous experience with the behavioural assays. Existing data in the literature have shown no sex differences when NK1R+ neurons were ablated using SP-SAP,^{21,22} in experimental investigations of NK1R+ projection neurons^{9,23} and in our ongoing experiment with SP-BOT (in preparation). Moreover, no difference in effect size has been observed between male and female patients undergoing treatment with BoNT/A.²⁴ We therefore only used male mice in this project.

Subjects in all experiments were adult male C56BL/6J mice (8–12 weeks old) purchased from Charles River (UK) and housed to acclimatise for 1 week prior to experiments.

All mice were kept in their home cage in a temperature-controlled ($20^{\circ} \pm 1^{\circ}\text{C}$) environment, with a light-dark cycle of 12 hours (lights on at 7:30 AM). Food and water were provided ad libitum.

This study was conducted under a Project Licence granted from the Home Office to Maria Maiarú.

Design and Purification of Botulinum Constructs

Each BoNT/A consists of three domains: the binding domain, the translocation domain, and the catalytic light-chain domain, a zinc metallopeptidase. We used a protein stapling technique to produce LcTd conjugated to SP. The synthesis that has been described previously for SP within vitro controls for specificity is detailed in ref.¹⁵ Briefly, to synthesise the constructs, first, a fusion protein consisting of the LcTd of the botulinum type A1 strain was fused to SNAP25 (LcTd-S25) and was prepared as previously described in ref.^{14,25} The chemically synthesised syntaxin-SP peptide had the sequence Ac-EIKLENSIRELHDMFMMDMAMLVESQGEMIDR-IEYNVEHAVDYVE-Ahx-Ahx-RPKPQQFFGLMNH₂, where Ahx stands for aminohexanoic acid. Second, the protein “staple” was prepared recombinantly from the rat

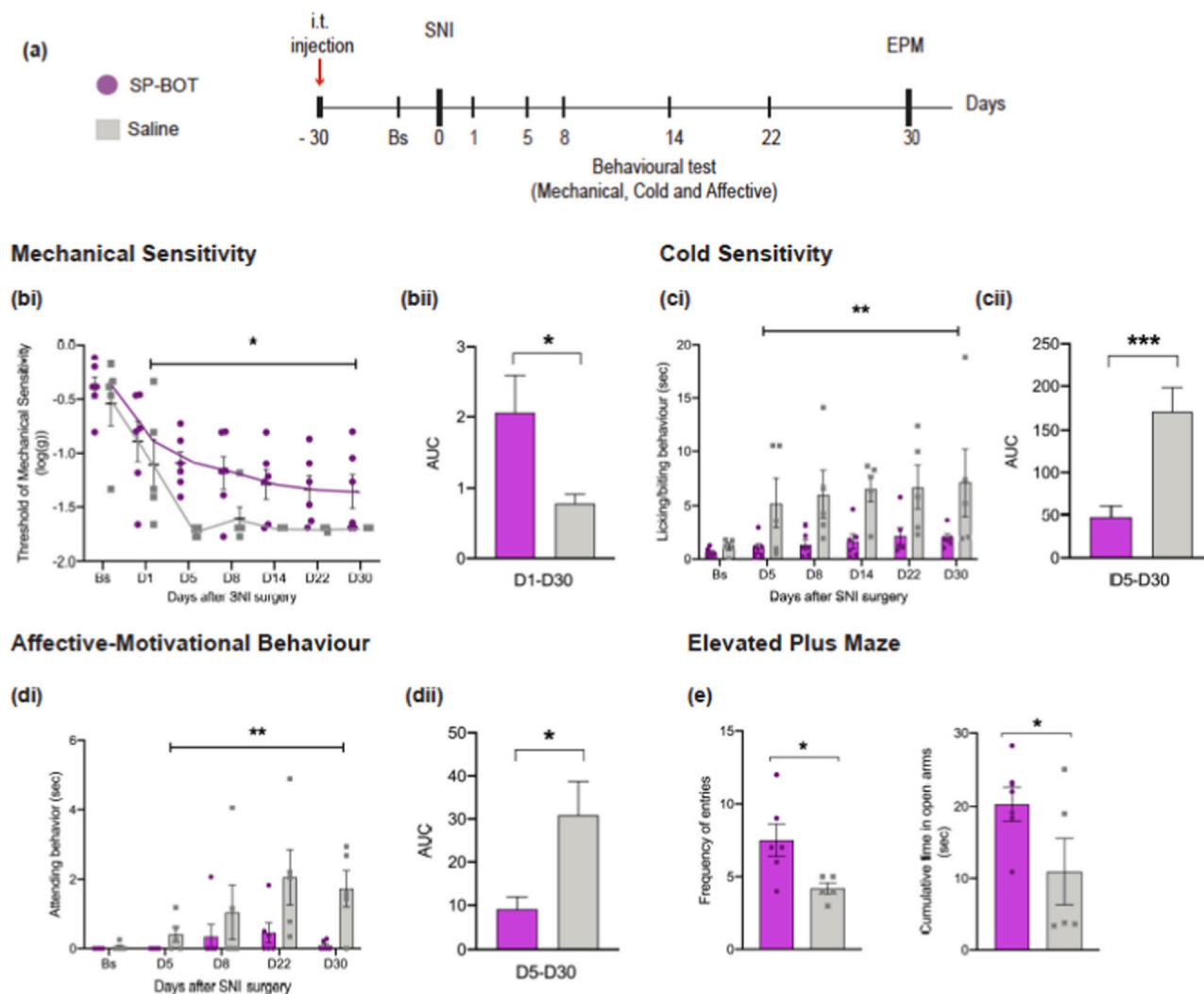


Figure 1. Intrathecal (i.t.) injection of SP-BOT 30 days before spared nerve injury (SNI) was effective in reducing pain behaviour. **(a)** SNI surgery was performed 30 days after intrathecal injection of SP-BOT (100 ng/3 μ l) or saline vehicle (3 μ l). Behavioural testing was carried out before and after SNI. **(bi)** Scatter plot representing mechanical threshold of individual mice assessed using calibrated von Frey filament before (Bs, Baseline) and after SNI surgery (D1–D30); **(bii)** Mechanical sensitivity threshold presented as area under curve (AUC); **(c)** Cold allodynia assessed using acetone drop applied to the hindpaw ipsilateral to the injury (left) before (Bs) and after SNI surgery; **(cii)** Cold allodynia presented as area under curve (AUC); **(di)** Time course of attending behaviour in response to a one-second von Frey stimulation (.02 g) before (Bs) and after SNI surgery and **(dii)** the data presented as area under curve (AUC). **(e)** Anxiety behaviour measured using elevated plus maze (EPM) represented by the frequency of entries to the open arm and time spent in the open arm 30 days after SNI surgery; Student's t-test. SP-BOT, n = 6; Saline, n = 5. Data show means \pm SEM. * P < .05, ** P < .01, *** P < .001. For bi, ci, and di, * represent results of repeated measure mixed model ANOVA. Abbreviation: ANOVA, Analysis of Variance.

vesicle-associated membrane protein 2 (VAMP2) sequence (amino acids 3–84) inserted into the *Xho*I site of pGEX-KG. Oriented attachment of peptides to protein was achieved by the SNARE assembly reaction. LcTd-S25, VAMP2 (3–84), and syntaxin-SP were mixed at a molar ratio of 1:1:1 in 100 mM NaCl (sodium chloride), 20 mM HEPES, and 0.4% n-octylglucoside at pH 7.4 (buffer A). Reactions were left at 20 $^{\circ}$ C for 1 hour to allow formation of the SNARE ternary complex. SDS-resistant and irreversibly assembled protein conjugates were visualised using Novex NuPAGE 12% bis-tris SDS-PAGE (polyacrylamide gel electrophoresis) gels (Invitrogen) run at 4 $^{\circ}$ C in a NuPAGE MES SDS running buffer (Invitrogen). All recombinant proteins were expressed in the BL21-Gold (DE3)pLysy strain of *Escherichia coli* (Agilent) in pGEX-KG vectors as

glutathione S-transferase C-terminal fusion proteins cleavable by thrombin. Glutathione S-transferase fusion constructs were purified by glutathione affinity chromatography and cleaved by thrombin. Synthetic peptides were made by Peptide Synthetics Ltd.

Intrathecal Injections

Intrathecal injections were performed in naïve mice or SNI-operated mice under isoflurane anaesthesia.²⁶ Mice were held firmly but gently by the pelvic girdle using thumb and forefinger of the nondominant hand. The skin above the iliac crest was pulled tautly to create a horizontal plane where the needle was inserted. Using the other hand, the experimenter traced the spinal column of the mouse, rounding or curving the column

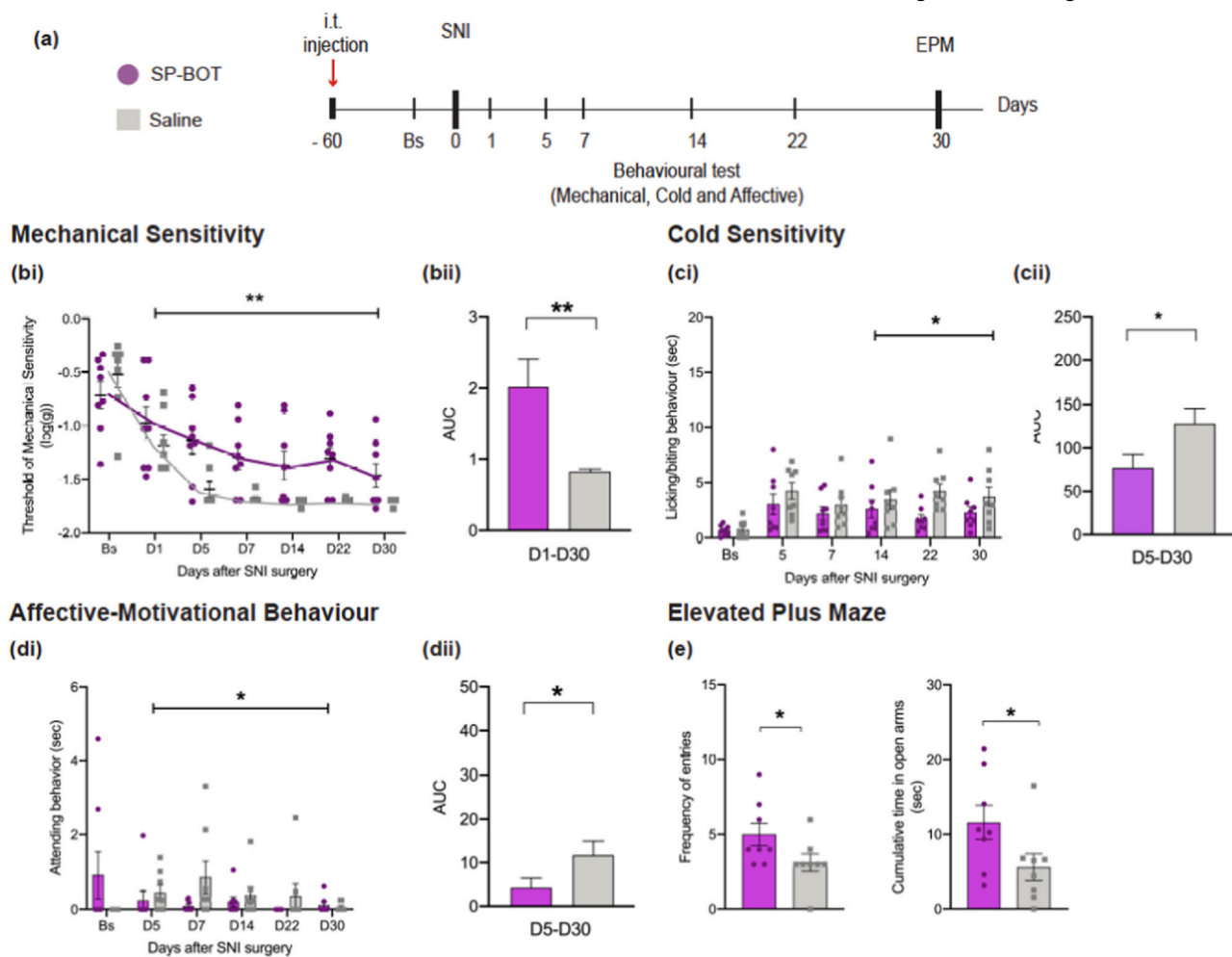


Figure 2. Intrathecal (i.t.) injection of SP-BOT 60-days before spared nerve injury (SNI) was effective in reducing pain behaviour. **(a)** SNI surgery was performed 60-days after intrathecal injection of SP-BOT (100 ng/3 μ l) or saline vehicle (3 μ l). Behavioural testing was carried out before and after SNI. **(bi)** Scatter plot representing mechanical threshold of individual mice assessed using calibrated von Frey filament before (Bs, Baseline) and after SNI surgery (D1–D30) and **(bii)** Mechanical sensitivity data presented as area under curve (AUC). **(c)** Cold allodynia assessed using acetone drop applied to the hindpaw ipsilateral to the injury (left) before (Bs) and after SNI surgery and **(cii)** Cold allodynia data presented as area under curve (AUC). **(di)** Time course of attending behaviour in response to a one-second von Frey stimulation (.02 g) before (Bs) and after SNI surgery and **(dii)** Attending behaviour data presented as area under curve (AUC). **(e)** Anxiety behaviour measured using elevated plus maze (EPM) represented by frequency of entries to the open arm and time spent in the open arm 30 days after SNI surgery; Student's t-test; SP-BOT, n = 8; Saline, n = 8. Data show means \pm SEM. * P < .05, ** P < .01. For bi, ci and di, * represent results of repeated measure mixed model ANOVA. Abbreviation: ANOVA, Analysis of Variance.

slightly to open the spaces between vertebrae. A 30-gauge needle connected to a 10- μ l Hamilton syringe was used to enter between the vertebrae. After injection, the syringe was rotated and removed, and posture and locomotion were checked. All intrathecally delivered drugs and vehicle solutions were injected in a 3- μ l volume.

Mouse Neuropathic Model: SNI

The SNI surgery was performed as previously described in ref.²⁷ Briefly, under isoflurane anaesthesia, the skin on the lateral surface of the thigh was incised, and a section made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the sural, the common peroneal, and the tibial nerves. The common peroneal and the tibial nerves were tight ligated with 5-0 silk suture and sectioned distal to the ligation. Great care was taken to

avoid any contact with the spared sural nerve. Complete haemostasis was confirmed, and the wound was sutured.

Behavioural Testing

All behavioural experiments were performed by the same experienced female experimenter, who was always blind to the treatment group for all behavioural tests; the test order was randomised with multiple groups being represented in each cage.

von Frey Filament Test (Mechanical Sensitivity)

Mice were placed in Plexiglas chambers, located on an elevated wire grid, and allowed to habituate for at least 1 hour. After this time, the plantar surface of the paw was stimulated with a series of calibrated von Frey's

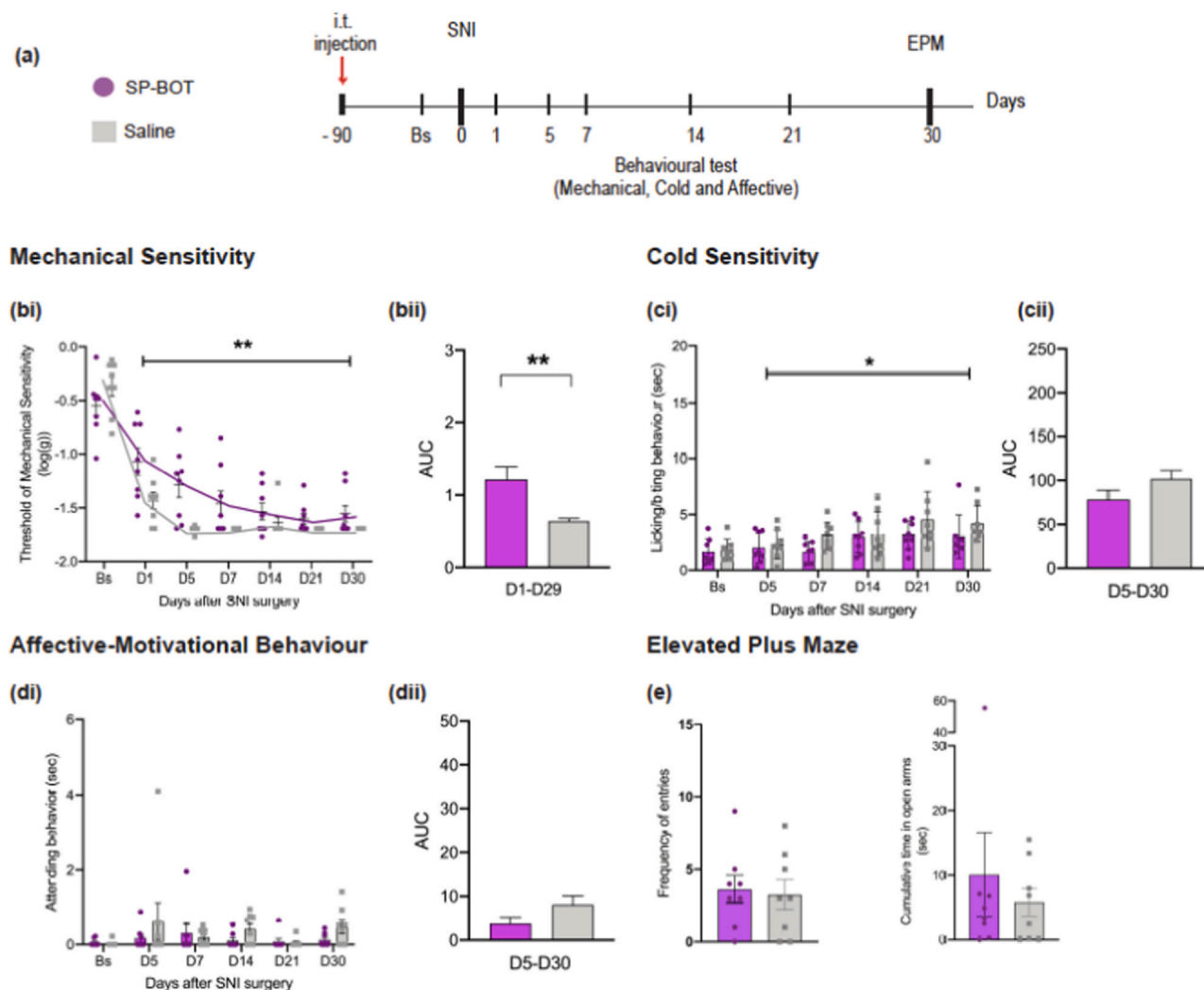


Figure 3. Intrathecal (i.t.) injection of SP-BOT 90 days before spared nerve injury (SNI) had a more limited efficacy in reducing pain behaviours. (a) SNI surgery was performed 90 days after intrathecal injection of SP-BOT (100 ng/3 μ l) or saline vehicle (3 μ l). Behavioural testing was carried out before and after SNI. (b) Scatter plot representing mechanical threshold of individual mice assessed using calibrated von Frey filament before (Bs, Baseline) and after SNI surgery (D1–D29) and (bii) Mechanical sensitivity data presented as area under curve (AUC). (c) Cold allodynia assessed using acetone drop applied to the hindpaw ipsilateral to the injury (left) before (Bs) and after SNI surgery and (cii) Cold allodynia data presented as area under the curve (AUC). (d) Time course of attending behaviour in response to a one-second von Frey stimulation (.02 g) before (Bs) and after SNI surgery and (dii) attending behaviour data presented as the area under curve (AUC). (e) Anxiety behaviour measured using elevated plus maze (EPM) represented by the frequency of entries to the open arm and time spent in the open arm 30 days after SNI surgery; Student’s t-test; SP-BOT, n = 8; Saline, n = 8. Data show means \pm SEM. * $P < .05$, ** $P < .01$. For bi, ci and di, * represent results of repeated measure mixed model ANOVA. Abbreviation: ANOVA, Analysis of Variance.

monofilaments. The threshold was determined by using the up-down method.²⁸ The data are expressed as a log of the mean of the 50% pain threshold \pm SEM.

Acetone Test (Cold Allodynia)

For assessment of cold sensitivity, the acetone test was used as previously described in ref.²⁷ After habituation in Plexiglas chambers, located on an elevated wire grid, a drop (50 μ l) of acetone was applied to the lateral area of the hind paw ipsilateral to the site of injury, avoiding mechanical stimulation of the paw with the syringe. Total time licking/biting of the hind paw was recorded with an arbitrary maximum cut-off time of 20 seconds.

Affective-Motivational Behaviour

The protocol described by Corder et al²⁹ was adapted to evaluate mechanical-induced affective-motivational response. Mice were placed in Plexiglas chambers, located on an elevated wire grid, and allowed to habituate for at least 1 hour. A .02 g von Frey filament was applied for 1 second to the hindpaw, and the duration of attending behaviour (direct licking and biting of the paw, extended lifting or guarding of the paw) was collected for up to 30 seconds after the stimulation.

Anxiety-like Behaviour (Elevated Plus Maze)

EPM was used to assess anxiety behaviour.^{30,31} The maze consisted of two closed arms with high wall but

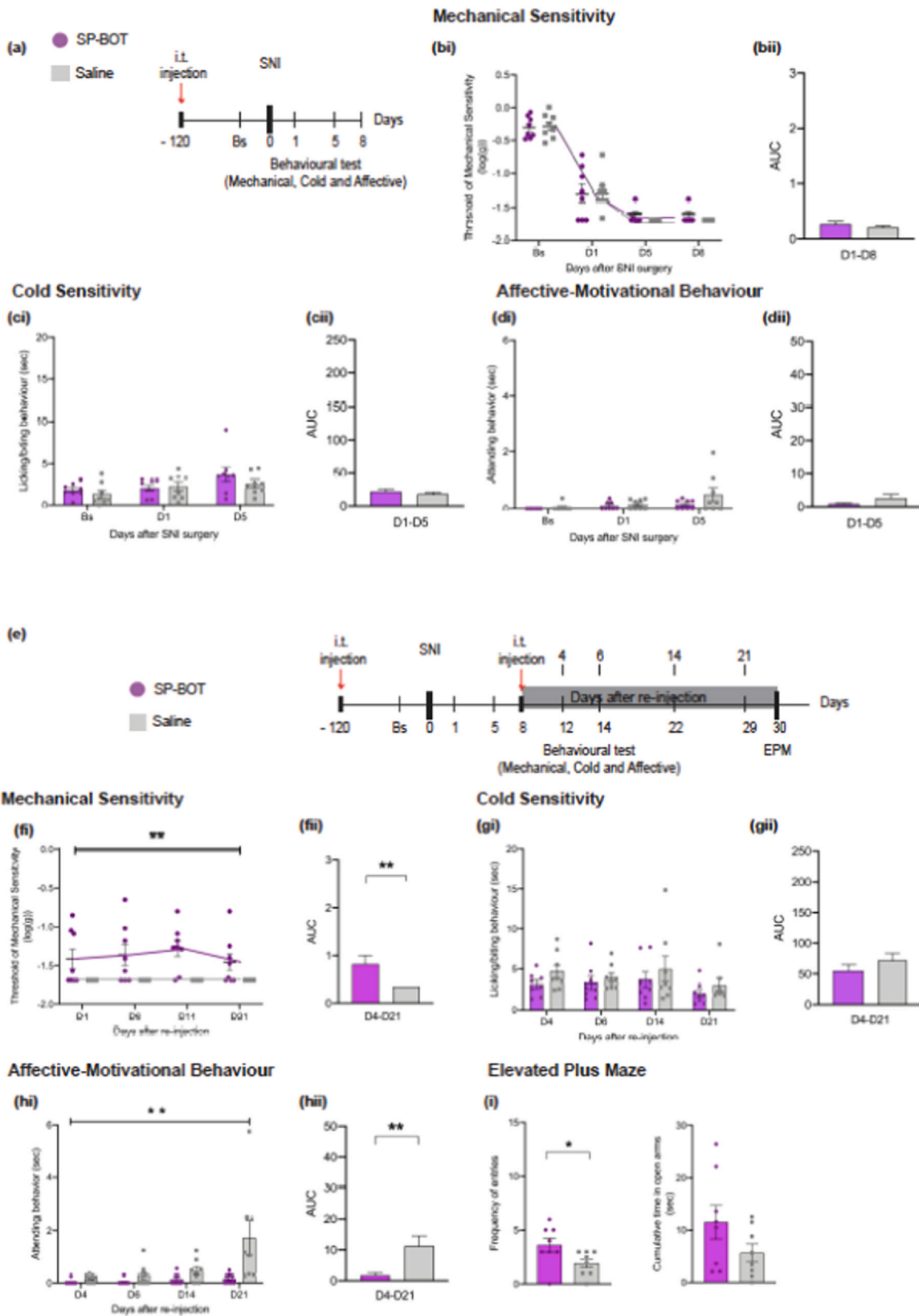


Figure 4. The analgesic impact of the first SP-BOT injection on neuropathic pain behaviour was lost by 120d but restored by a second intrathecal injection of SP-BOT. **(a–d)** SNI surgery was performed 120 days after intrathecal injection of SP-BOT (100 ng/3 μ l) or saline vehicle (3 μ l). Behavioural testing was carried out before and after SNI. **(a)** Timeline of experiments. **(b)** Scatter plot representing the mechanical threshold of individual mice assessed using calibrated von Frey filament before (Bs, Baseline) and after SNI surgery (D1–D8) and **(bii)** Mechanical sensitivity presented as the area under curve (AUC). **(c)** Cold allodynia assessed using acetone drop applied to the hindpaw ipsilateral to the injury (left) before (Bs) and after SNI surgery and **(cii)** Cold allodynia data presented as area under curve (AUC). **(di)** Time course of attending behaviour in response to a one-second von Frey stimulation (.02 g) before (Bs) and after SNI surgery and **(dii)** Attending behaviour data presented as area under curve (AUC). **(e–i)** A second intrathecal injection of SP-BOT (100 ng/3 μ l) or saline vehicle (3 μ l) was performed 8 days after SNI. Behavioural testing was carried out after re-injection. **(e)** Timeline of the experiment **(f)** Scatter plot representing mechanical threshold of individual mice assessed using calibrated von Frey filament after re-injection (D4–D21) and **(fii)** Mechanical sensitivity presented as area under curve (AUC). **(g)** Cold allodynia assessed using acetone drop applied to the hindpaw ipsilateral to the injury (left) after re-injection and **(gii)** Cold allodynia data presented as area under curve (AUC). **(hi)** Time course of attending behaviour in response to a one-second von Frey stimulation (.02 g) after re-injection and **(hii)** Attending behaviour data presented as area under curve (AUC). **(i)** Anxiety behaviour measured using elevated plus maze (EPM) represented by frequency of entries to the open arm and time spent in the open arm 30 days after SNI surgery; Student's t-test. SP-BOT, n = 8; Saline, n = 8. Data show means \pm SEM. * P < .05, ** P < .01. For fi and hi, * represent results of repeated measure mixed model ANOVA. Abbreviation: ANOVA, Analysis of Variance.

not roof, and 2 open arms, (45 * 10 cm) arranged in a cross-like disposition. The 4 arms were connected by a central 10 * 10 cm square and elevated 60 cm off the ground. Before starting the test, mice were habituated to the room to decrease the confound of novel surroundings. Each mouse was then placed in the centre of the maze and allowed to explore for 5 minutes while a camera recorded the mouse's movements. Variables analysed included frequency of entries into open arms and cumulative time spent into open arms. Movements were tracked and analysed with Ethovision (Noldus Ethovision XT 14).

Immunohistochemistry

Mice were anaesthetised with pentobarbital and perfused transcardially with physiological saline containing heparin (5,000 IU/mL), followed by 4% paraformaldehyde (PFA) in a .1 M phosphate buffer (PB; 25 mL per adult mouse). Lumbar spinal cords were dissected out, fixed in 4% PFA for an additional 2 hours, and transferred into a

30% sucrose solution in a PB containing .01% azide at 4 °C for a minimum of 24 hours. Spinal cord sections were cut on a freezing microtome set at 40 μ m. Sections were left to incubate with the primary antibody (anti-NK1R made in rabbit, Sigma-Aldrich S8305) overnight at room temperature at a concentration of 1:40,000. Appropriate biotinylated secondary antibody (biotinylated secondary antibody goat anti-rabbit, Vector Laboratories BA-1000) was used at the concentration of 1:400 in PBS-T solution and left for 90 minutes at room temperature. Then, sections were incubated with avidin-biotin complex in PBS-T (1:250 Vectastain A plus and 1:250 Vectastain B; ABC Kit Peroxidase Standard, Vector Laboratories) for 30 minutes at room temperature, followed by a signal amplification step with biotinylated tyramide solution (1:75 for 5 minutes; Startech). Finally, sections were incubated with Fluorescein Avidin D (1:600, Vector Laboratories) for 2 hours at room temperature. All fluorescent sections were transferred to glass slides and cover slips applied with Gel Mount Aqueous Mounting Medium (Sigma-Aldrich) to prevent fading and stored in dark boxes at 4 °C.

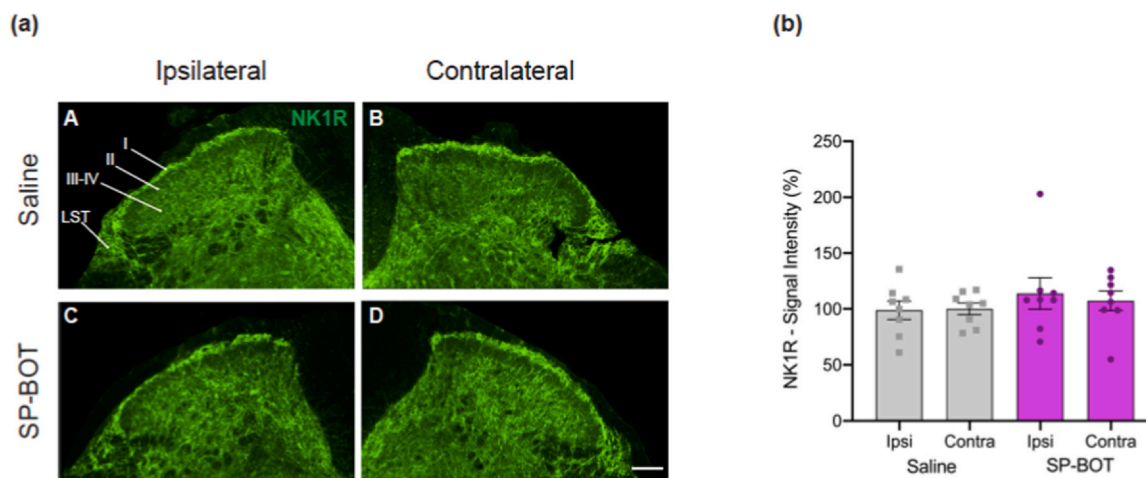


Figure 5. Intrathecal injection of SP-BOT did not reduce NK1-receptor immunoreactivity in the superficial dorsal horn. **(A)** Images of NK1R immunoreactivity in the superficial dorsal horn of mice 30 days after SNI surgery and 150 days after 1st intrathecal injection of SP-BOT or saline (22 days after 2nd injection). Green, NK1R. Scale bar, 100 μ m. I, Lamina I; II, Lamina II; III-IV, Lamina III-IV; LST, Lateral Spinothalamic Tract. **(B)** Quantification of NK1R fluorescence in the ipsilateral and contralateral superficial dorsal horn of mice 30 days after SNI surgery and 150 days after 1st intrathecal injection of SP-BOT or saline (22 days after 2nd injection). All data were normalised to contralateral saline-treated mice (n = 8 per group).

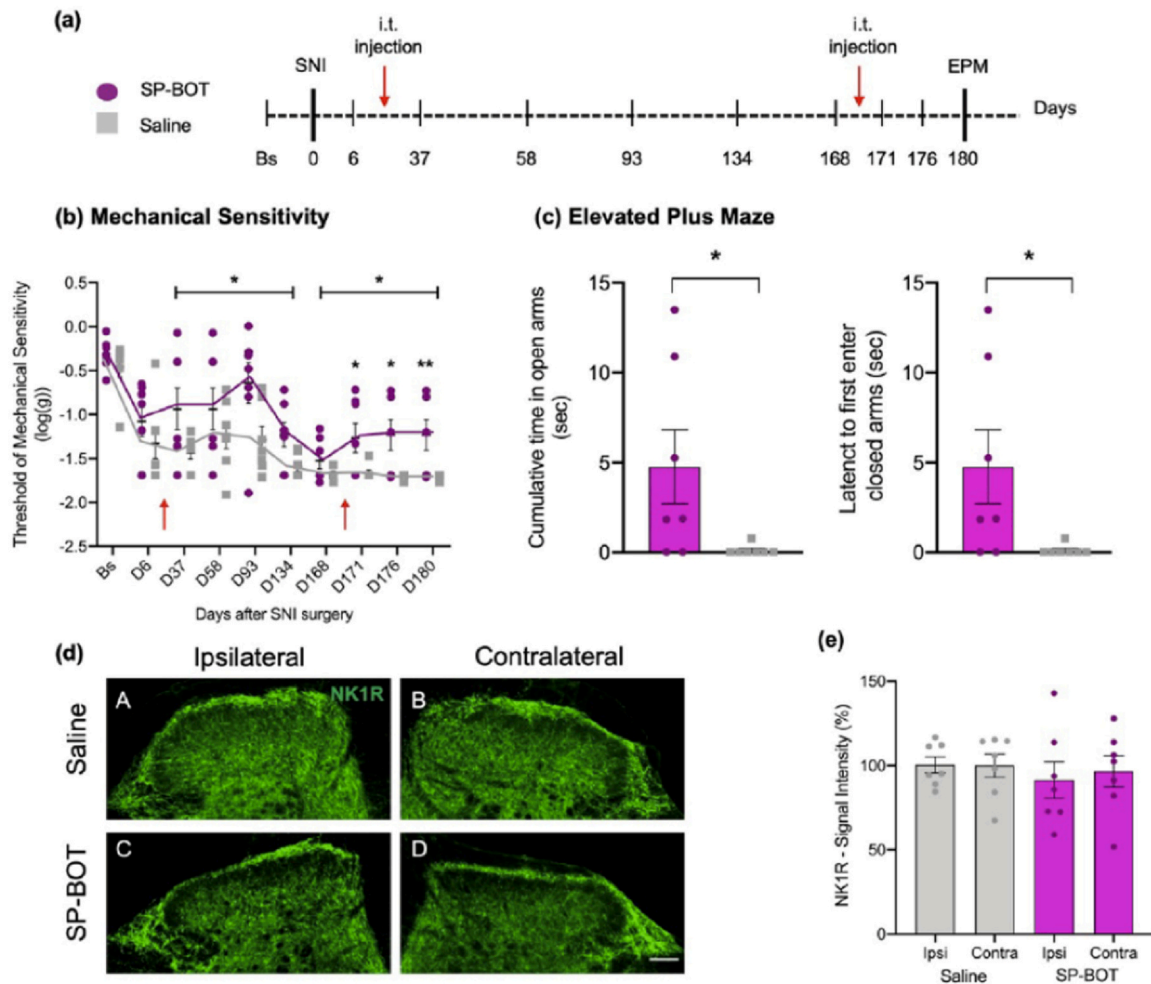


Figure 6. Intrathecal (i.t.) injection of SP-BOT after spared nerve injury (SNI) was effective in reducing pain behaviour. **(A)** Intrathecal injection of SP-BOT (100 ng/3 μl) or saline vehicle (3 μl) was performed after SNI surgery (D6) and a second injection was given when the silencing effect was gone (D168). Behavioural testing was carried out before and after SNI and after re-injection. **(B)** Scatter plot representing the mechanical threshold of individual mice assessed using calibrated von Frey filament before (Bs, Baseline) and after SNI surgery (D6–D180). **(C)** Anxiety behaviour measured using elevated plus maze (EPM) represented by latency to first enter the closed arms and time spent in the open arm 180 days after SNI surgery; Student's *t*-test. SP-BOT, $n = 7$; Saline, $n = 7$. **(D)** Images of NK1R immunoreactivity in the dorsal horn of mice 1,800 days after SNI surgery and 174 days after 1st intrathecal injection of SP-BOT or saline. Green, NK1R. Scale bar, 100 μm . **(E)** Quantification of NK1R fluorescence in the ipsilateral and contralateral superficial dorsal horn of mice 180 days after SNI surgery and 174 days after 1st intrathecal injection of SP-BOT or saline. All data were normalised to contralateral saline-treated mice ($n = 7$ per group). Data show means \pm SEM. $*P < .05$. For b, * represent the results of repeated measure mixed model ANOVA. Abbreviation: ANOVA, Analysis of Variance.

Quantification of Fluorescence

Image J was used to measure optical density values over lamina I and II outer of the superficial dorsal horn. A background optical density reading was taken from the same section but over adjacent unlabelled tissue. Contrast enhancement and fluorescence threshold were kept constant. Four sections were analysed for each animal used in the study.

Component Analysis

Independent component analysis (ICA) was used to find statistically independent components which contribute to the observed pain scores. ICA is a method of blind source separation which models the observed data $X \in \mathbb{R}^{p \times n}$ (where n is the number of observations, and p is the dimensionality of the data, that is, the number of pain metrics) as a linear mixture of k statistically

independent (orthogonal and non-Gaussian) components, $S \in \mathbb{R}^{k \times n}$, i.e.

$$X = WS$$

Here $W \in \mathbb{R}^{p \times k}$ is the mixing-matrix, with elements $\{W\}_{ij}$ representing the weight of component j on pain metric i . The mixing-matrix W and coefficient scores S were estimated using the FastICA algorithm.³² Prior to ICA, data from all time points and all animals was concatenated, z-scored and subsequently pre-whitened using principal component analysis,³³ using the alternating least squares algorithm to estimate missing data points,³⁴ implemented in Matlab's Statistics and Machine Learning Toolbox (version R2021a). The number of components during ICA was set to three, based on the variance explained in the principal component analysis step (3 components explained $\sim 85\%$ of the variance of the z-scored data).

A limitation of ICA is that it is restricted to linear effects. We therefore also calculated Spearman's rank correlation between pain measures to test for non-linear associations.

Data and Statistical Analysis

All experimental data points were included in the scatter plots and data analysis.

All statistical tests were performed using the IBM SPSS Statistic programme (version 25), and $P < .05$ was considered statistically significant. For the behavioural experiments, statistical analysis was performed on the data normalised by log transformation (von Frey data).

Please note that, as in our previous paper,¹⁸ we logged the data of von Frey filaments test to ensure a normal distribution because the von Frey's hairs are distributed on an exponential scale. Mills et al³⁵ recently demonstrated that log transformation makes more "mathematical and biological sense".

Difference in sensitivity was assessed using repeated measure mixed model analysis of Variance (ANOVA). In all cases, "time" was treated as a within-subjects factor and "treatment" was treated as a between-subject factor. Area under the curve (AUC) was calculated for each individual animal, using time course measurements starting from after intrathecal injection (D1) until the end of behavioural testing. OriginLab software was used to integrate each individual curve and determine mathematical area, which were then grouped for statistical analysis. For anxiety-like behaviour and AUC, the statistical significance was determined using Student's t-test.

For immunofluorescence quantification, the results were normalised to the mean of the control value (Saline and contralateral side), and the control value was set as 100%.

Results

We have previously shown that a single injection of botulinum-based constructs can silence NK1R-positive neurons and reduce mechanical hypersensitivity for up to 30 days, when injected following the setting up of inflammatory and neuropathic pain states in rodents, with no effect on baseline mechanical threshold and no signs of motor impairment.¹⁸ However, to establish clinical relevance it was important to know how long this silencing period lasted following a single injection of the botulinum construct. We therefore injected 4 groups of mice with SP-BOT on the same day. At 30, 60, 90, or 120 days after intrathecal injection we performed the spared nerve injury (SNI) model of neuropathic pain in each group and then measured pain behaviours. Using this approach, the silencing of the ascending pain pathways was estimated from the change in neuropathic pain scores. In each group, SP-BOT or saline was injected intrathecally over the lumbar spinal cord of adult C57BL6/J male mice.

Measurements of mechanical hypersensitivity were supplemented by further tests of cold allodynia, affective-motivational behaviour (which measures non-

reflexive responses to mechanical stimulation of the hind paw), and of anxiety-like behaviours using the elevated plus maze (EPM). Changes in these behaviours have previously been shown in rodents to accompany chronic peripheral nerve injury^{29-31,36} and this extended repertoire of behavioural tests can more accurately model the clinical symptoms of neuropathic pain. All mice were tested for up to 30 days following SNI surgery. Motor behaviour assessed with the rotarod was unaffected by SP-BOT injections.³⁷ Toxicity was estimated by measuring levels of NK1 immunoreactivity in the spinal cord in the 120 group of mice. We have previously shown that intrathecal SP-BOT injections do not result in astrogliosis or activation of microglia.¹⁸

Intrathecal SP-BOT Given 30 or 60-days Before SNI Silences NK1R-expressing Neurons and Prevents the Full Development of Pain Related Behaviours

SP-BOT injection 30 days before SNI: Von Frey filaments are widely used to measure sensitivity to mechanical stimulation in both rodents and human patients. A single injection of SP-BOT 30 days prior to SNI (Fig 1A) was able to silence NK1R-expressing neurons and this resulted in a significant improvement of mechanical hypersensitivity for the duration of the testing period (Fig 1Bi, Two-way ANOVA, factor TREATMENT D1 to D30: $F_{1,9} = 9.86$; $P = .012$). In mice that did not receive the botulinum conjugate, mechanical hypersensitivity developed over the 5 days following SNI surgery and was maintained for the remaining 25 days (Fig 1Bi). To summarise the effect of pre-emptive SP-BOT at this timepoint, mechanical threshold scores from all testing days following SNI were used to generate AUC values, which were significantly higher in SP-BOT-treated mice, indicating a lower hypersensitivity throughout the 30 days (Fig 1Bii, t-test $P = .012$).

Cold allodynia, the increased sensitivity to ordinarily non-painful cool temperatures, is a common clinical feature of neuropathic pain.³⁸ Patients can sometimes report severe pain from touching a cold object, and this symptom is not easily or effectively treated with conventional analgesics such as Non-steroidal anti-inflammatory drugs or opioids.⁴ To assess the development of cold allodynia after SNI, we applied the commonly used acetone evaporation test³⁶ and found that licking/biting behaviour in response to acetone increases over time in SNI saline-injected mice, but was dramatically reduced in mice that received SP-BOT 30 days prior to the induction of the pain state (Fig 1Ci, Two-way ANOVA, factor TREATMENT D5 to D30: $F_{1,9} = 16.32$; $P = .003$). AUC values for cold allodynia behaviour post-SNI further confirmed the difference between SP-BOT- and saline-injected mice in this 30-day group (Fig 1Cii, t-test $P = .0012$).

To build a more complete picture of the pain phenotype and thoroughly examine the analgesic properties of SP-BOT, we tested the affective-motivational component of pain behaviour. As adapted from Corder et al,²⁹ mice were placed in Plexiglas chambers located on an elevated wire grid. A .02 g von Frey filament was

applied for 1 second to the hind paw, and the duration of attending behaviours (direct licking and biting of the paw, extended lifting or guarding of the paw) was collected for up to 30 seconds after stimulation. Unlike acute reflex responses to mechanical stimulation, the longer response times of attending behaviours to stimulation requires participation of forebrain circuits.³⁹

No attending behaviours were seen in SP-BOT or saline-treated mice prior to surgery (Fig 1di, Baseline (Bs)). SNI surgery produced a robust affective-motivational response in saline-injected mice which developed incrementally over the 30 days of testing (Fig 1Di). A reduction in attending behaviours was seen in mice who received SP-BOT 30 days prior to SNI (Fig 1, Di, Dii), with the largest difference between conjugate- and saline-treated mice seen at day 30. There was an overall significant effect of SP-BOT treatment in this group (Two-Way ANOVA, factor TREATMENT D5 to D30: $F_{1,14} = 12.64$; $P = .006$) as additionally summarised by AUC values (Fig 1 Dii, t-test $P = .01$).

There is a strong association between anxiety and chronic pain states in both humans and in animals.^{40,41} We used the EPM to monitor anxiety-like behaviour, once per mouse when all other assays had been completed. Both the frequency of entries into the open arms of the maze and the cumulative time spent in the open arms were recorded, with a decrease in these parameters denoting anxiety-like behaviour. Both measures were significantly higher in mice who received intrathecal SP-BOT 30 days prior to SNI compared to control mice, suggesting a reduction of anxiety behaviours in mice treated with the conjugate (Fig 1E).

SP-BOT injection 60 days before SNI: Next, we looked at SP-BOT-generated silencing and consequent analgesia when given 60-days before SNI surgery (Fig 2A). As seen in the 30-day group, SP-BOT prevented the full development of mechanical sensitivity within 5 days following SNI, which persisted for the remaining 25 days (Fig 2Bi; Two-way ANOVA, factor TREATMENT D1 to D30: $F_{1,14} = .002$; $P = .002$) and was confirmed by AUC values (Fig 2Bii). SNI produced a strong response to cold stimulation in the control group, which was significantly reduced by SP-BOT treatment (Fig 2Ci, Cii; Two-way ANOVA, factor TREATMENT day 14 (D14) to D30: $F_{1,14} = 5.23$; $P = .038$), and the affective-motivational response was also reduced by SP-BOT (Fig 2Di, Dii; Two-Way ANOVA, factor TREATMENT day 5 (D5) to D30: $F_{1,14} = 4.70$; $P = .048$). Furthermore, SP-BOT given 60-days prior to SNI improved anxiety-like behaviours after SNI, compared to the control group (Fig 2E).

Effect of Intrathecal SP-BOT Given 90 or 120 days Before SNI

SP-BOT Injection 90 days Before SNI

Preclinical studies and the clinical use of BoNT/A have demonstrated that its endogenous protease activity lasts for approximately 100 days.^{42,43} We therefore assessed how the injection of our botulinum-neuropeptide conjugate 90 days prior to SNI surgery would affect

the development of pain behaviour (Fig 3A). Intrathecal SP-BOT given 90 days prior to SNI caused a significant decrease in the development of mechanical hypersensitivity measures from day 1 after SNI. Overall, there was a significant effect of SP-BOT treatment (Two-way ANOVA, factor TREATMENT Day 1 (D1) to D30: $F_{1,14} = 13.06$; $P = .003$) (Fig 3Bi) as additionally confirmed by AUC scores (Fig 3Bii). These results suggest that SP-BOT was effective in preventing the mechanical hypersensitivity seen after a neuropathic injury for up to 120 days following a single injection of the conjugate.

For cold allodynia, an overall effect of treatment was observed between the 2 groups (Two-way ANOVA, factor TREATMENT Day 5 (D5) to D30: $F_{1,14} = 4.67$; $P = .048$) (Fig 3Ci, 3Cii). However, at this timepoint, no differences were seen between saline and SP-BOT pretreated mice in the affective-motivational or anxiety responses induced by SNI (Fig 3Di, 3Dii, 3E).

SP-BOT injection 120 days before SNI: In mice injected with SP-BOT or saline 120 days prior to surgery, we found no effect of the conjugate on SNI-induced mechanical hypersensitivity, cold allodynia, affective-motivational or anxiety-like behaviours, with comparable scores between SP-BOT and control groups (Fig 4A-D). This confirmed that by 120 days post-injection, the silencing effect of SP-BOT had been lost and pain-induced behaviours had returned to normal.

SP-BOT-induced Analgesia can be Restored by a Second Intrathecal Injection

Injection of SP-BOT 120 days before SNI did not reduce symptoms of the neuropathic pain state. To test whether it was possible to silence the NK1+R neurons again and restore analgesia, a second SP-BOT injection was given to these mice 8 days after SNI surgery and 128 days after the initial injection. We followed the pain behaviours for the remaining 22 days after re-injection (Fig 4E). Re-injection of SP-BOT was able to silence the NK1+R neurons and significantly reduce mechanical hypersensitivity (Fig 4Fi; Two-way ANOVA, factor TREATMENT Day 4 (D4) to D21: $F_{1,14} = 12.07$; $P = .004$) which was reflected in AUC scores following SNI (Fig 4Fii, $P = .007$). Cold sensitivity was not significantly different between treatment groups following re-injection (Fig 4Gi, Gii). The affective-motivational response to SNI was reduced by SP-BOT re-injection (Fig 4Hi, Two-Way ANOVA, factor TREATMENT Day 4 (D4) to D21: $F_{1,14} = 8.16$; $P = .013$), confirmed by a significant difference between treatment group AUC values ($P = .016$) (Fig 4Hii). Anxiety behaviour was also improved by SP-BOT re-injection with increased frequency of entry into EPM open arms observed in mice that received the conjugate (Fig 4I).

These results also suggested that NK1R-positive neurons were not lost during the lengthy period of silencing. In agreement with this, analysis of the superficial dorsal horn of mice 150 days after initial intrathecal injection of botulinum construct and 22 days after 2nd injection showed no difference in NK1R-immunoreactivity between SP-BOT and saline-injected mice (Fig 5 A-B).

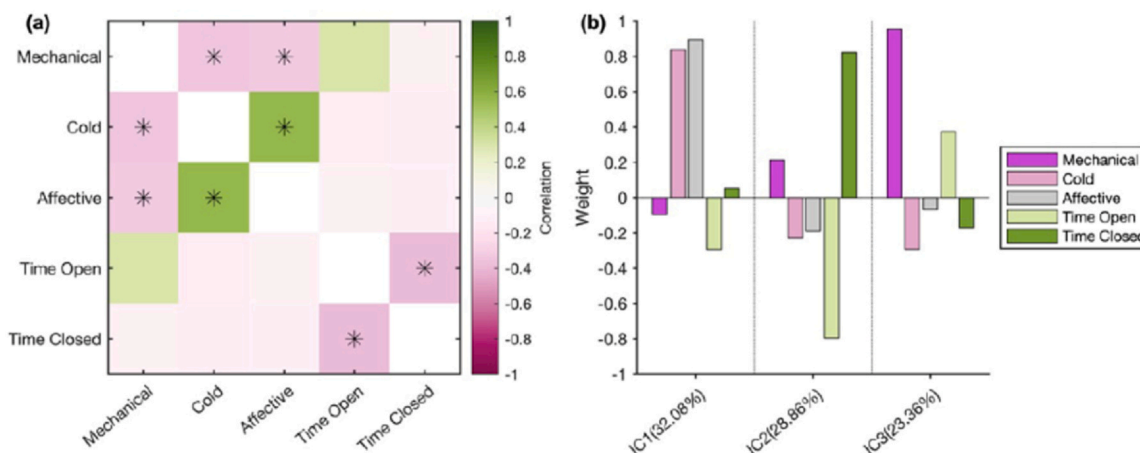


Figure 7. Linear and non-linear associations between pain measures. **(A)** Pairwise Spearman's rank correlation between all pain measures. Asterisks denote false-discovery-rate corrected $P < .05$. **(B)** Weights from ICA. The 3 ICA components are separated by a vertical dashed line. For each component, a given bar shows the influence of the component on the pain scores. Hence, IC1 demonstrates strong weighting towards cold and affective scores; IC2 demonstrates strong positive weighting towards time closed and strong negative weighting towards time open scores (meaning a rodent affected strongly by pain component 2 would have a high time closed score and low time open score); while IC3 demonstrates strong weighting towards the mechanical score.

SP-BOT-induced Silencing is Effective for Several Months When Given After SNI Surgery and it can be Restored by a Second Intrathecal Injection

Neuronal silencing lasted for approximately 120 days after injection of SP-BOT. However, it was unclear if i.t. injection of SP-BOT after establishing SNI would yield the same degree of analgesia. We have previously shown that a single injection of SP-BOT can improve pain-like behaviour for several weeks.¹⁸ Here we have shown that a single injection of SP-BOT given after SNI (Fig 6A) was able to significantly improve mechanical hypersensitivity for several months (Fig 6B, Two-way ANOVA, factor TREATMENT D37 to D168: $F_{1,12} = 4.7$; $P = .050$). In mice that did not receive the botulinum conjugate, mechanical hypersensitivity fully developed following SNI surgery and was maintained for the whole duration of the experiment (Fig 6Bi). The silencing effect of SP-BOT was lost by 168 days; however, a second intrathecal injection was able to silence the ascending pathway and restore analgesia (Fig 6B, Two-way ANOVA, factor TREATMENT D168 to D180: $F_{1,12} = 6.1$; $P = .029$). Furthermore, SP-BOT given after SNI improved anxiety-like behaviours, compared to the control group (Fig 6C).

We have also analysed the immunoreactivity of NK1R-positive neurons in the spinal dorsal horn of mice after this long period of silencing and found that NK1R-positive neurons were not lost 174 days after initial intrathecal injection of botulinum construct as shown by measurements of NK1R-immunoreactivity between SP-BOT and saline-injected mice (Fig 6D-E).

Component Analysis

After extensive behavioural testing, we conducted the component analysis to uncover any relationships that may exist between the diverse pain-related

behaviours we measured. Because of the divergent nature of the data and scores from various behavioural assays we subjected all AUC data points and time spent in open or closed arms of the EPM to an ICA. A preliminary pairwise correlation analysis (Fig 7A) suggests that mechanical hypersensitivity is largely independent of other behavioural metrics, while cold and affective-motivational behaviours were positively associated. Time in the open arms and the closed arms of the EPM were negatively associated as expected (Fig 7A). Three components explained 84.96% of the variance in the data: these components largely corresponded to a cold/affective component, an anxiety component (time open/closed), and a mechanical component (Fig 7B).

Comparison of the components scores between animals (Supplementary Fig 1) suggests that the cold/affective component was lower in those treated with SP-BOT, while the mechanical component was larger. No significant differences were found in the anxiety component.

Discussion

We previously demonstrated that novel substance P-botulinum constructs could alleviate chronic neuropathic and inflammatory pain states in mice following a single intrathecal injection of the conjugate by silencing key NK1R-positive neurons in the spinal dorsal horn. Specificity was demonstrated by showing that these conjugates were only internalised by NK1R-expressing neurons and were ineffective in NK1R knockout mice.¹⁸ Here we investigated for how long NK1R+ neurons were silenced following a single injection of SP-BOT. We used 4 groups of mice all injected intrathecally with SP-BOT on the same day. Silencing of the pain pathway was measured in these groups of mice following the introduction of the pain state (SNI). SNI was performed on group 1 at 30d, group 2 at 60d, group 3 at 90d, or group 4 at 120d after the SP-BOT injection. The degree

of neuronal silencing was then estimated from the reduction in pain scores. Using an extended range of behavioural assays including thermal and affective-motivational measures of pain behaviour we found that pain scores were reduced in 30d, 60d, and 90d mouse groups following SNI but had reduced impact over the next 30d period (up to 120d). We also demonstrated that pain scores could be reduced again by a second injection of SP-BOT, 128 days after the first injection. Finally, levels of NK1R immunoreactivity remained at control levels in all 120d mice indicating no loss of NK1R + neurons. We have also shown that, when given after the induction of the pain state, SP-BOT can silence the ascending pathway for several months resulting in a reduced hypersensitivity to mechanical stimulation and reduced anxiety-like behaviour compared to saline control.

Our study was restricted to male mice which was a limitation given recent reports of sex differences in pain processing. However, to our knowledge, no sex differences were seen when NK1R+ neurons were ablated using SP-SAP,^{21,22} in experimental investigations of NK1R+ projection neurons^{9,23} and in our ongoing experiment with SP-BOT (in preparation). Clinically, in humans, no difference in effect size have been observed between male and female patients undergoing treatment with BoNT/A.²⁴ For these reasons and to limit the numbers of mice we only used male mice in this project.

The time over which neuronal silencing could be observed was comparable to that previously reported in rats using BoNT/A injections into the hind limb.⁴² Once internalised within the motor neuron, the light chain of BoNT/A silenced peripheral motor neurons for several months via the specific proteolytic cleavage of SNAP25 (SNAP25: soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex), a protein essential for synaptic release. SNAP25 is found in neurons but not in glial cells⁴⁴ and is the unique substrate for botulinum protease cleavage. The silencing was slowly reversed as the endopeptidase lost activity and function was usually restored in rats within 100 days. Data on direct injections of BoNT/A into the central nervous system are more limited but recent studies have shown that the functional consequences of injections of BoNT/A directly into the striatum of hemi-Parkinsonian rats were also reversed within 90 to 100 days.⁴³ No toxicity has been reported following BoNT/A injections into peripheral tissues including motor neurons and clinically the process is frequently repeated. We show here that SP-BOT silencing could be reinstated once the effects of the first injection had worn off. This also implied, and we confirmed, that the NK1R positive neurons, which are the substrate for silencing and the alleviation of pain states, are present and have not been lost or remodelled during the lengthy period of silencing.

The original studies by Mantyh and colleagues and recent research demonstrated that NK1R+ spinal projection neurons were essential for the full expression and maintenance of chronic pain behaviours caused by nerve damage or inflammation.^{6,7,9,45} Ablation of NK1R

+ neurons with SP-saporin conjugates was irreversible and alleviated pain states when given either before or after establishing spinal nerve ligation and indeed has been shown to be an effective treatment in alleviating pain in companion dogs with bone cancer.^{7,13} SP-SAP ablation also reduced chronic itch and cold allodynia seen after spinal nerve ligation.^{8,46–48}

However, it is well established that a group of NK1R+ excitatory interneurons are also found within the superficial dorsal horn and could contribute to the pain phenotype.⁴⁹ Here we report that different behavioural responses change with time. Von Frey measurements of mechanical sensitivity were substantially reduced in mice receiving SP-BOT 90 days before SNI while behaviours with a strong affective component such as attending behaviour and the response to cold were no longer sensitive to SP-BOT silencing at this 90d time-point. We presumed this was because the mice were older and had become used to their environment and thus are less anxious. However, ICA also indicated that attending behaviours as well as cold and EPM scores segregated together and separately from mechanical sensitivity. This therefore might suggest that SP-BOT silencing is disrupting 2 independent NK1R+ dorsal horn circuits: one concerned with rapid reflex response to a noxious signal and a second circuit that communicates injury-related information directly to the brain where pain behaviours are elaborated. It is therefore unclear if SP-BOT also silences the population of lightly labelled small diameter NK1R+ interneurons found in the superficial dorsal horn as well as the small group of larger spinal projection neurons that project to the brain and form the major route for nociceptive information to reach the brain. Certainly, the data suggests that SP conjugates may silence several populations of NK1R+ expressing neurons within the superficial dorsal horn but including the NK1R+ subset of lamina 1 projection neurons. These populations may collaborate to organise both reflex and affective behaviours to noxious stimulation. A second, partially nociceptive pathway from lamina 1 to the brain has also been identified⁹ and is predominantly NK1R negative. Silencing this pathway with botulinum constructs together with the NK1 positive pathway may well further reduce pain behaviours seen after SNI.

We also carried out the component analysis of the different pain behaviours investigated in time. Our aim was to identify one or more components which may allow us to identify a metric of the overall pain experience. We have observed one component which suggested that cold sensitivity is positively associated with affective deficit. In addition, this cold/affective component was negatively associated with mechanical sensitivity. This suggests that affective and 'reflex' pain behaviours are under the control of distinct neuronal circuits, both modulated by NK1R+ neurons.

The clinical use of botulinum toxins has largely been restricted to peripheral sites and no serious side effects have been reported. Their use in the human central nervous system has not been reported and there is limited data available on their potential toxicity. We

previously showed that a single intrathecal injection of SP-BOT had no effect on motor performance as measured by the rotarod test and at the cellular level there was no microglial response or astrogliosis in the spinal cord.³⁷ We also showed that the NK1R was key as silencing of neurons did not occur in NK1R knockout mice. Here we also measured NK1R levels using immunohistochemistry in the 120d group and found that levels of NK1R were similar to control mice. This implies that there was no death of NK1R+ neurons months after injection of SP-BOT. If indeed there had been toxicity and the death of NK1R+ neurons then we would have expected reduced pain scores rather than a return to untreated saline group levels with time. We also reinstated silencing by reinjecting mice with SP-BOT and found that analgesia returned. This also suggests that NK1R+ neurons were still present and silenced again by the second injection of SP-BOT and had not been damaged by the previous injection of SP-BOT.

Conclusion

The clinical problem of managing chronic pain whether it be from on-going disease, nerve damage or from previous injury is immense and has proved extremely challenging to deal with. We show here that a single intrathecal injection of a SP-BOT conjugate silences a key pain-signalling pathway to the brain for up to 3 months, that this can occur regardless of whether the conjugate is given before or after nerve damage and that and that the procedure is repeatable and non-toxic to cells of the spinal cord. Therefore, botulinum conjugate treatment may provide substantial alleviation from chronic pain states in both patients and animals.

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Disclosures

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The authors declare that they have no competing interests.

CRediT authorship contribution statement

MM, BD, SPH: Conceptualization. **MM, CL, BD, SPH:** Methodology. **MM, CL, SSH, SFG, LT, FT, SPH:** Investigation. **MM, BD, SPH:** Visualization. **MM, BD, SPH:** Funding acquisition. **MM, BD, SPH:** Project administration. **MM, BD, SPH:** Supervision. **MM, SPH:** Writing.

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Data and Materials Availability

All data associated with this study are present in the paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpain.2024.01.331](https://doi.org/10.1016/j.jpain.2024.01.331).

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