Title

Controlled activation of cortical astrocytes can reverse neuropathic chronic pain

Ikuko Takeda1,2, Kohei Yoshihara3, Dennis L. Cheung1, Masakazu Agetsuma1,4, Makoto Tsuda3, Kei Eto1,5, Andrew J. Moorhouse6, Junichi Nabekura1*

Affiliations

1Division of Homeostatic Development, National Institute for Physiological Sciences, Okazaki, Japan.

2Department of Anatomy and Molecular Cell Biology Graduate School of Medicine, Nagoya University, Nagoya, Japan.

3Department of Life Innovation, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan.

4Division of Molecular Design, Research Center for Systems Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

5Department of Physiology, School of Allied Health Sciences, Kitasato University, Sagamihara, Kanagawa, Japan.

6Department of Physiology, School of Medical Sciences, The University of New South Wales, Sydney, New South Wales, Australia.

*Corresponding author. Email: nabekura@nips.ac.jp
Abstract

Chronic pain is a major public health problem that currently lacks effective treatment options. Here, we report a novel combination therapy that can effectively reverse chronic pain induced by nerve injury in mice. By combing transient nerve block to inhibit noxious afferent input from injured peripheral nerves, with transient concurrent activation of astrocytes in the somatosensory cortex (S1) by either transcranial direct current stimulation (tDCS) or via the chemogenetic DREADD system, we could reverse allodynia previously established by partial sciatic nerve ligation (PSL). Activation of astrocytes initiated spine plasticity to reduce synapses formed shortly after PSL. The cure from allodynia persisted long after ceasing active treatment. Thus, our study represents the first report of a robust, readily translatable approach for treating chronic pain that capitalizes on the causative interplay between noxious afferents, sensitized central neuronal circuits and astrocytic-activation induced plasticity.
Introduction

Chronic pain remains as one of the leading causes of global disability, affecting around 20% of the adult population in the USA\(^1\). Novel approaches to reverse the mechanisms causing the transition from acute to chronic pain are likely to have the greatest therapeutic impact\(^2\). Injury-induced maladaptive neural circuit plasticity at both spinal and cortical levels, amplifies the noxious input and can render other sensory input as painful (allodynia)\(^3,4,5\). Innocuous circuits in the somatosensory cortex (S1) which normally encode the location, intensity, and duration of nociceptive stimuli\(^6\), are transformed during the early post-injury period to become activated by both nociceptive and innocuous stimuli\(^7,8\). Astrocytes are closely associated with neural circuit microstructure, and through both physical contacts and via secreted molecules can regulate a number of aspects of synaptic function, including synaptogenesis, spinogenesis, modulation of synaptic plasticity and the elimination of spines and synapses\(^9,10\). Some of these astrocytic signalling pathways responsible for spinogenesis in development are reactivated following peripheral nerve injury to promote dendritic spine turnover, and thereby contribute to this remodelling of cortical pain circuits\(^11\). We proposed that corrective remodelling of these S1 circuit synapses may effectively reverse chronic pain. We achieved this by blocking the noxious peripheral afferent
inputs while concurrently augmenting the activity of cortical astrocytes using transcranial direct current stimulation (tDCS) or the Designer Receptors Exclusively Activated by Designer Drugs (DREADD) system. This transient therapy induced rewiring of cortical circuit synapses and achieved a remission from allodynia that lasted well beyond the end of active treatment.

Results

tDCS-TTX therapy reverses alldynia in neuropathic model mice
tDCS has been widely applied to humans for a variety of neurological disorders, typically applied at current intensities between 1-4 mA over multiple sessions of 15-30 mins duration\textsuperscript{12}. Although tDCS is devoid of serious adverse effects, evidence for its efficacy in treating chronic pain is poor – at best providing only modest and temporary relief\textsuperscript{12,13,14}. Conventional tDCS is believed to act via modulating spontaneous neuronal activity, but recent rodent studies demonstrated tDCS induced synchronized and widespread astrocytic Ca\textsuperscript{2+} transients in the absence of neuronal activation\textsuperscript{15}. Using a similar protocol, we confirmed that a single tDCS session (0.1 mA for 10 minutes) applied over the cortex of awake mice caused a significant and sustained increase in the frequency and amplitude of Ca\textsuperscript{2+} transients in S1 astrocytes (Fig. 1, a to c).
We asked if modulating astrocyte activity could impact on chronic pain behaviour, and used partial sciatic nerve ligation (PSL)\(^{10}\) to induce a sustained allodynia. tDCS was subsequently applied for a week at 8-hourly intervals, all 2 weeks following the PSL injury. We further reasoned that reducing noxious afferent activity from the injured nerve may also be required to reverse chronic allodynia, so simultaneously delivered tetrodotoxin (TTX) locally to the sciatic nerve via an implanted Elvax drug elution cuff (Fig. 1f). Prior to any treatment, all mice displayed mechanical allodynia following PSL as shown by decreases in paw withdrawal thresholds (Fig 1d). TTX application returned these thresholds back to control levels, indicating relief from pain due to afferent input blockade. In control mice, as TTX elution subsided\(^{11}\), thresholds again decreased to post PSL levels, indicating return of mechanical allodynia. However, in PSL mice with combined tDCS, paw withdrawal thresholds were sustained at pre-injury levels, indicating reversal of allodynia (Fig. 1d). Thermal allodynia was also reversed by combined tDCS and TTX, without any effects on withdrawal thresholds in the uninjured contralateral paw (Supplementary Fig. 4). Conventional therapeutic tDCS, i.e., without simultaneous blockade of noxious inputs with TTX, was ineffective in providing lasting pain relief (Fig. 1e). Together our results show that a brief treatment regime combining tDCS configured to induce S1 astrocytic
activation, combined with TTX-blockade of noxious afferents, i.e., tDCS-TTX therapy, is able to mediate a lasting adaptive response to reverse chronic pain.

Specific chemogenic activation of S1 astrocytes also reverses alldynia

tDCS is known to broadly activate astrocytes across the cortex both ipsilateral and contralateral to the stimulating anode\textsuperscript{15}. To more specifically determine the locus of the therapeutic effect, we used a different and more targeted approach using the DREADD system\textsuperscript{17} (Fig. 2a). We expressed the hM3D receptor in S1 astrocytes by localised AAV injection (Fig. 2b), achieving expression in ~89\% of S1 astrocytes (392 astrocytes from 5 mice). A single intraperitoneal injection of clozapine N-oxide (CNO; 1.0 mg/kg, i.p.), which activates this hM3D receptor, also increased astrocytic Ca\textsuperscript{2+} transients. The increase in the Ca\textsuperscript{2+} transient response was specific to S1 astrocytes; the activity of astrocytes in M1 was not affected by CNO (Supplementary Fig. 5). Repeated CNO administration induced up-regulation of glial fibrillary acidic protein (GFAP) in S1 astrocytes (Supplementary Fig. 6), indicating they adopted an activated phenotype. Consistent with the rapid distribution of CNO\textsuperscript{18}, astrocytic Ca\textsuperscript{2+} activity peaked immediately after administration but then showed a sustained increase from 3 hours onwards (Fig. 2c), perhaps reflecting prolonged signalling or accumulation in brain
tissues. Given that these sustained increases in astrocytic Ca$^{2+}$ were comparable to those seen with tDSC, we used this CNO dose in mice with established PSL-induced allodynia. We administered CNO at 8-hourly intervals for 1 week, whilst again simultaneously suppressing noxious afferent input from the injured sciatic nerve with local TTX elution from the Elvax cuff (Fig. 2a). As before, TTX administration immediately restored paw withdrawal thresholds to pre-injury levels (Fig. 2d), but this was only transient in control mice treated with concurrent saline injections. In contrast, mice given TTX and concurrent CNO injections continued to maintain pre-injury withdrawal thresholds indicating a sustained reversal of allodynia, and this relief could last for at least 2 months in some mice (Figs. 2, d and e). Importantly, CNO treatment by itself (with astrocytic mCherry expression as a control for hM3D) did not reverse allodynia, and similarly there was no effect of CNO in mice expressing hM3D in astrocytes but not given concurrent TTX (Supplementary Fig. 7a). Furthermore, lower doses of CNO that caused significantly smaller astrocytic activation, did not reverse the allodynia following PSL (Supplementary Fig. 8). Thus, a transient post-injury regime of CNO-TTX therapy or tDSC-TTX therapy are both able to prevent long-term allodynia, indicating that the key locus of the therapeutic effect is the selective activation of S1 astrocytes during the period when noxious afferent input is reduced.
The therapy preferentially eliminates dendrite spines associated with noxious circuits

We next examined the mechanisms by which the combination therapy was able to induce this seemingly permanent reversal of the chronic pain response. Previous studies have demonstrated that transient and discrete astrocyte activation in the first week following PSL triggers a period of increased spine formation and elimination in S111,19, which may represent maladaptive plasticity of cortical neural circuits that engenders nociceptive responses to previous innocuous sensory stimuli. We therefore hypothesized that subsequently augmenting astrocyte activity, coupled with an absence of noxious afferent input, could re-awaken spine plasticity and induce an adaptive rewiring of this maladapted circuitry. We therefore imaged S1 layer 5 pyramidal neuron dendritic spines, starting prior to PSL and again during and after the combination therapy, either CNO-TTX (Fig. 3a) or tDCS-TTX (Supplementary Fig. 9a). Spine dynamics were significantly increased by CNO-TTX (Fig. 3, b to c) and tDCS-TTX therapy (Supplementary Fig. 9), with an increase in spine elimination and/or formation rates as compared with control mice (Figs. 3, d and e, Supplementary Fig.10). But spine density kept the same level before, during and after CNO-TTX therapy (Supplementary Fig.
We then stratified eliminated spines into those that were present prior to PSL (pre-PSL spines), and those that appeared just after PSL (early post-PSL spines), reasoning that pre-PSL spines were more likely to represent neuronal circuits for normal somatosensory processing, whereas early post-PSL spines would be more relevant to PSL-triggered maladaptive noxious circuits. Early post-PSL spine elimination was three times higher than pre-PSL spine elimination in CNO-treated mice, but equally labile in control mice (Fig. 3f). Closer examination of the temporal pattern of elimination of these early post-PSL spines (Fig. 3g) showed that while most were eliminated during the long pre-treatment period in all mice, the proportion of persistent spines eliminated during the subsequent treatment period was significantly higher in the CNO mice. These results are consistent with the idea that activated astrocytes provide a temporary window of plasticity where dendritic spines associated with maladaptive noxious circuits (rendered inactive by afferent input blockade) are preferentially eliminated.

Consistently, S1 neuronal activity in PSL mice, and the correlations between neuronal firing within these S1 neurons, was modestly but significantly decreased by CNO-TTX therapy (Fig. 3h, Supplementary Fig. 11), suggesting a reduced functional connectivity in these post-PSL noxious circuits concurrent with the spine elimination. We propose that this preferential loss of early post-PSL spines represents a targeted pruning of the
aberrant neural connections that critically contribute to the chronic tactile and thermal allodynia.

**Preclinical evaluation of combined tDCS and lidocaine therapy**

While TTX effectively blocked the noxious afferent inputs during the therapeutic window, it is too toxic to be integrated into clinical application. Therefore, we evaluated the use of a local anaesthetic, applying lidocaine by osmotic pump (2 mg/kg/hr) from day 0 to 3 (Fig. 4a). While the effects of lidocaine on paw withdrawal thresholds were weaker than TTX, combined transient lidocaine and tDCS therapy still caused a sustained and significant reduction in the extent of allodynia that outlasted the treatment period (Fig. 4b). Combination tDCS-lidocaine therapy did not adversely affect motor coordination in the rotarod assay (Fig. 4c), or locomotion and anxiety-like behaviours in the open-field test (Fig. 4d).

**Discussion**

This study demonstrates that astrocytes can be positively leveraged to treat chronic pain. Specifically, transiently enhancing astrocytic Ca^{2+} activity, either through tDCS or the DREADD system, whilst simultaneously blocking noxious afferent input from the site
of injury, enables increased circuit remodelling which we propose preferentially eliminates maladapted noxious circuits and thereby permanently eliminates mechanical allodynia and returns tactile sensation to normal. This is a conceptual shift in chronic pain treatment, from targeting neuronal excitability and synaptic transmission that reduces the consequences of hyperactive circuits, to targeting astrocytes and cortical circuit plasticity, that may reverse some of the mechanisms by which acute pain transitions to chronic pain.

Our study provides further support for the growing idea that aberrant neural circuit plasticity in S1 significantly underpins intractable nociceptive perceptions in chronic pain\textsuperscript{11,20}. Hyperactivity of L2/3\textsuperscript{20}, loss of effective local circuit inhibition\textsuperscript{21}, and activation of astrocytes to mediate spine plasticity\textsuperscript{11,19} have all been observed during early chronic pain development in rodent models and prophylactically reducing cortical neuronal and astrocyte activity has been shown to prevent robust chronic pain development. Furthermore, enhanced activity of S1 affects other brain areas related to chronic pain, e.g. anterior cingulate cortex\textsuperscript{20}. Our current report shows that targeting S1 circuits weeks after allodynia has developed can still ameliorate pain symptoms and reverse the injury-associated S1 plasticity. We propose that the tDCS-TTX treatment strategy reduces connectivity in S1 noxious circuits. Other spinal and brain regions are
undoubtedly important for chronic pain, and it will be informative to see if and how their activities change in response to therapeutic S1 remodelling.

A key component of this therapeutic strategy was the concurrent application of TTX or lidocaine. We believe the doses and delivery systems used fortuitously and selectively reduced noxious afferent input without large impacts on tactile and motor fibres. A reduction of afferent input can very effectively weaken the synaptic representation of those afferents in cortical areas. We propose reduction in nociceptive afferent activity by TTX weakens the maladapted cortical circuits responding to the painful tactile stimuli, exposing these weakened synapses to be eliminated, by astrocyte or microglial phagocytosis. Because tactile sensation remains, circuits responding to “normal” sensation can be strengthened, returning S1 representations and activity back to control. Concurrent astrocyte activity may either facilitate synapse removal, or strengthen rewiring of “normal” circuits. The current clinical use of nerve block approaches similarly targets excessive nociceptive efferent activity whilst sparing tactile and motor function, and hence may be suitable to complement tDCS to selectively weaken aberrant maladapted cortical noxious circuits.

Our study suggests combined tDCS and afferent nerve blockade warrants further clinical investigation as a therapy for pain. tDCS is being widely trialled for
different neurocognitive applications, particularly depression, stroke recovery and chronic pain, and at typical current intensities is considered safe\textsuperscript{12}. Based on our results in mice, we would envisage twice or thrice daily stimulation over one week with the anode placed over the S1 approximate to the injured region, and a modest stimulation intensity to preferentially activate astrocytes rather than neurons. Regional peripheral nerve block via a catheter implanted under ultrasound-guided surgery is used commonly for postoperative analgesia\textsuperscript{28,29} and we would also envisage concurrent application of a long-lasting local anaesthetic bolus via this route. Alternatively, continuous infusion via a controlled pump for 4-6 days has also been trialled to treat different chronic pain syndromes\textsuperscript{30,31}. However, it should be recognized that our current study only used young male mice, many pain phenotypes differ between sexes with females typically reporting greater pain responses\textsuperscript{32,33}. A further limitation of our study regards translating efficacy from a single pain model in one mouse strain to humans; for example, pregabalin has larger and broader analgesic effects in rodent chronic pain models than observed clinically\textsuperscript{34}.

In conclusion, we report a transient combination therapy that reverses mechanical allodynia in mice. We propose that activating cortical astrocytes while reducing peripheral noxious inputs engineer synaptic plasticity that breaks down
inappropriate neural connections formed during the transition from acute to chronic pain. Given that both nerve block and tDCS are both readily utilized in the clinic, it seems feasible to translate our discovery into clinical practice for the treatment of intractable chronic pain associated with a well defined peripheral injury.
Materials and Methods

Care and use of animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institutes for Natural Sciences, Okazaki, Japan. Every effort was made to minimize the suffering and number of animals used.

All animal experiments were conducted using male, 8-10 week old C57BL/6 mice, M-line mice (C57BL/6 mice expressing enhanced green fluorescent protein (EGFP) in sparse subsets of cortical neurons under the thy-1 promoter) or mGFAP-Cre mice (C57BL/6 mice expressing Cre recombinase in astrocytes under the mouse GFAP promoter (astrocyte specific promoter)). All mice were housed with ad libitum access to standard rodent chow and water under a 12-hour light-dark cycle. Mice were randomly separated to treatment or control groups.

Partial sciatic nerve ligation (PSL) and behavioural testing

The PSL model is a well-established animal model of chronic pain that is characterized by protracted touch-evoked allodynia and hyperalgesia. PSL was performed by ligating the dorsal one-third to half of the right sciatic nerve with 8-0 silk suture under 1.5-1.8% isoflurane anaesthesia.
Mechanical-tactile allodynia was observed for 2 weeks post-PSL before further experiments. Mechanical allodynia and hyperalgesia were assessed manually using the von Frey filament test. All testing was performed during the 12hr daylight cycle. Prior to von Frey testing, mice were habituated individually for 30 minutes in the testing chamber which consisted of a small plastic cage with a mesh floor. The von Frey filaments were applied through the mesh floor so as to perpendicularly touch the hind paw with sufficient force to cause slight filament buckling. If this elicited withdrawal, licking or shaking of the paw by the mice, a positive response (pain) was recorded. Filaments were tested with increasing then decreasing force to establish that which caused a median 50% paw withdrawal threshold\textsuperscript{37}. All von Frey testing was performed in a blinded fashion with the experimenter unaware of the treatments the mice had received. There were no differences in the baseline thresholds for mechanical sensitivity between the three strains of mice used (C57BL/6, M-line and mGFAP-Cre).

Thermal sensitivity was assessed following injury (post-PSL) and after treatment (at days 8 and 14) using the Plantar test\textsuperscript{38} with an infrared heat stimulus and an automated recording of latency to paw withdrawal (Hargreaves Apparatus, ugo baile, Italy). As previously described\textsuperscript{39}, mice were placed in the thermal sensitivity enclosure for 60 mins for each of 2 days prior to testing to habituate to the plantar test.
environment. When mice positioned their hind paw on the infrared emitter/detector, heat stimulation started and continued until the paw was withdrawn. The latency to paw withdrawal was observed over 5 trials, with the averaged value excluded the two trials with the smallest and largest withdrawal times.

The accelerating rotarod test was performed to assess motor coordination as previously described\textsuperscript{40,41}. Briefly, mice were habituated to the rotarod (LE8200, Panlab, Spain) over 30 mins on two consecutive days prior to the trial. For the test trial, the rotation frequency began at 4 rpm and accelerated to 40 rpm over 5 min with the time at which mice fell from the rotarod noted. The rotarod test was repeated twice at 20 min intervals, with the latencies averaged from both trials.

The open field test was performed to assess locomotor activity and anxiety-like behaviour. As previously described\textsuperscript{42}, mice were placed in a 40 × 40 cm chamber and exploratory behaviours recorded for 10 mins and subsequently analyzed using an automated tracking system (Lab Squirrel Pty Ltd, Sydney, Australia). The open field was divided into an inner (20 × 20 cm) and outer zone, to assess anxiety-like behaviour. We measured total locomotion speed, total distance travelled and the total duration spent in the inner zone.
Preparation and implantation of TTX mixed with Elvax, and of the lidocaine osmotic pump

As described previously\textsuperscript{43}, TTX was applied to the right sciatic nerve using an ethylene-vinyl acetate copolymer (Elvax) carrier. Briefly, 100 mg/mL Elvax solution was prepared by dissolving Elvax beads (Dupont) in dichloromethane, before adding 3 mM TTX to give a final TTX concentration of 300 $\mu$M. This TTX-Elvax solution was then stirred for 1 hour to ensure thorough mixing before pouring onto a pre-cooled glass dish, which had been incubated for 2-3 hours at -80°C, and stored at -20°C. This allowed for the complete evaporation of the dichloromethane and for the TTX-Elvax solution to solidify into a sheet that could then be applied around the right sciatic nerve just proximal to the PSL injury (under 1.5-1.8% isoflurane anaesthesia). The estimated duration for drug delivery using Elvax is 5-6 days\textsuperscript{11}.

Lidocaine (2 mg/kg/hr in saline) was applied using an osmotic pump (0.5 $\mu$l/h, 1007D, Alzet, CA) implanted subcutaneously in the back with the tip of the osmotic pump implanted close to the sciatic nerve ligation. After 3 days, the osmotic pump was removed. Implantation and removal surgery occurred under isoflurane anaesthesia with mice.
**Generation of Adeno-Associated Viruses**

pAAV-GFAP-mCherry was prepared by generating a linearized vector from pAAV-GFAP-hM3D(Gq)-mCherry (Addgene, #50478) using restriction enzymes with subsequent vector end blunting and nick sealing mediated by DNA polymerase I Large (Klenow) Fragment and T4 DNA ligase respectively. pAAV-GFAP-GCaMP6f was prepared by incorporating the GCaMP6f sequence from pGP-CMV-GCaMP6f (Addgene, #40755) into a pAAV-GFAP- vector (Addgene, #50478) using the In-Fusion cloning kit (Takara, Mountain View, CA) and restriction enzymes. All plasmids sequences were confirmed by direct-PCR.

Adeno-associated viruses (AAV’s) were generated as described previously. Briefly, HEK293 cells were transfected with pAAV-GFAP-mCherry or pAAV-GFAP-GCaMP6f, pHelper (Cell Biolabs, Inc. San Diego, CA), and pRC5 (Cell Biolabs, Inc. San Diego, CA). The surrounding media from each culture plate was collected 3 days later and purified for AAV particles using a tangential flow filter system (VIVAFLOW50 and Masterflex Economy Drive Peristaltic Pump, Sartorius, Germany). The dialyzed product was concentrated using an Amicon Ultra-15 filter (MWCO, 100kDa, Merck, Germany) before benzonase (Merck, Germany) was added at 100 units per culture plate, then PBS was added and the mixture incubated at 37°C for 30 minutes. The incubated
mixture was then transferred into an ultracentrifuge tube, forming the top solution layer over an iodixanol solution series of increasing concentration (8 ml of 15%, 6 ml of 25%, 5 ml of 40%, and 5 ml of 60%; top to bottom). After 3 hours of ultracentrifugation at 230000g and 18°C, the 40% iodixanol fraction was aspirated using a 16-gauge needle syringe and concentrated using an Amicon Ultra-15 filter to give the finished product. Virus titres were measured by quantitative real-time PCR analysis (Thunderbird SYBR qPCR Mix, TOYOBO CO., Osaka, Japan).

AAV2/5-GFAP-hM3D(Gq)-mCherry generation from pAAV-GFAP-hM3D(Gq)-mCherry (Addgene, #50478) was outsourced for AAV custom vector production (UNC vector core, NC, USA).

**AAV injection**

Mice used for the DREADD-behaviour only experiments were injected with AAV2/5-GFAP-hM3D(Gq)-mCherry as follows, 1-2 weeks prior to the PSL operation. Mice were anesthetized (i.p.) using a ketamine (70 mg/kg) and xylazine (10.5 mg/kg) mixture and mounted into a stereotaxic frame (NARISHIGE, Japan). Approximately 500 nL of AAV was injected into the left S1 at stereotaxic coordinates 0.5 mm posterior and 1.5 mm lateral to Bregma, and at a depth of 300 μm below the brain surface. The injection
pipettes were pulled from filament-containing glass capillaries (GDC-1, NARISHIGE, Japan) and AAV slowly expelled (over 10 mins) using pneumatic pressure (IM 300 Microinjector, Narishige Scientific Instrument Lab., Tokyo, Japan).

**Immunohistochemistry**

Brains were fixed by cardiac perfusion with 4.0% paraformaldehyde (Muto Pure Chemicals Co., Tokyo, Japan) under anaesthesia (i.p.) using ketamine (70 mg/kg) and xylazine (10.5 mg/kg). Following overnight post-fixation in 4.0% paraformaldehyde, the fixed brains were cut using a vibratome (VT1006S, Leica, Germany) into 50 μm thick sections. These were incubated over 2 nights at 4°C with primary antibodies – rabbit anti-S100β (1:500; EP1576Y, ab52642, Abcam, Cambridge, UK), mouse anti-NeuN (1:400; 1B7, ab104224, Abcam, Cambridge, UK), or rabbit anti-GFAP (1:500; ab5804, Merck, Germany). Immunocomplexes were visualized by chemiluminescent detection using HRP-conjugated goat anti-rabbit/anti-mouse secondary antibodies (1:300; Santa Cruz Biotechnology). Images were acquired using an A1R confocal microscope (Nikon, Tokyo, Japan) with NIS-elements software (Nikon, Tokyo, Japan) under a ×20 objective lens (PLanSApo, NA = 0.75) with a z-step size of 0.5 μm. The total number of mCherry-expressing cells co-localized with S100β or NeuN in the S1
was manually counted using the Cell Counter plugin (credit Durt De Vos) within the ImageJ software environment (NIH).

Open-skull chronic cranial window implantation

Mice used for in vivo imaging were injected with the relevant AAV’s and implanted with a chronic cranial window according to the following procedure. The cranial window surgery was undertaken two weeks prior to the PSL operation.

Mice were anaesthetized (i.p.) using a ketamine (70 mg/kg) and xylazine (10.5 mg/kg) mixture. The scalp skin was incised and the entire skull surface was waterproofed with tissue adhesive (3M Vetbond, 3M, MN, USA). A custom-made metal head plate was then directly attached to the skull using resin cement (Estecem II, Tokuyama Dental Corporation, Tokyo, Japan) and dental cement (Fuji Lute BC, GC Corporation, Tokyo, Japan). After the cements had cured, the entire skull surface was covered with dental adhesive (Super-Bond with Catalyst V, Monomer and Polymer, Sun Medical Corporation, Shiga, Japan) which acted as both a reinforcing and waterproofing agent.

Implantation of the cranial window was performed the next day. Mice were anaesthetized with 1.0-1.2 % isoflurane and secured in a stereotaxic frame via the
attached head plate. For S1 imaging, a circular 2-3 mm diameter craniotomy was then drilled over the hind-limb area of the left S1 (craniotomy centre at 0.5 mm posterior and 1.5 mm lateral to Bregma). Following this, approximately 500 nL of AAVs (GFAP-hM3D-mCherry, GFAP-GCaMP6f, GFPA-mCherry) were injected as described above into the centre of the craniotomy site at a depth of 300 µm from the brain surface.

Comparative M1 and S1 imaging used a single rectangular (3 × 2 mm) craniotomy through which approximately 500 nL of AAVs (CamKII-GCaMP6f (UPENN), GFAP-hM3D-mCherry, GFAP-GCaMP6f) were injected into M1 and S1 areas at a depth of 300 µm from the brain surface (Supplementary Fig. 8a). The craniotomy sites were covered with a double glass coverslip (Matsunami Glass, Osaka, Japan) which consisted of a 2 mm diameter coverslip fused to a 4.5 mm diameter coverslip, or a 2 × 3 mm coverslip fused to a 4 × 5 mm coverslip. The coverslip was fixed to the skull using a mixture of dental cement (Fuji Lute BC, GC Corporation, Tokyo, Japan) and dental adhesive (Super-Bond with Catalyst V, Monomer and Polymer, Sun Medical Corporation, Shiga, Japan).

**Ca²⁺ imaging of astrocytes and neurons in S1 or M1 for DREADD and tDCS assessment**
Ca\(^{2+}\) imaging was performed 2 weeks after cranial window implantation using a multiphoton microscope (Nikon A1R MP, Nikon, Tokyo, Japan) fitted with a 25x water immersion objective lens (Nikon Apo LWD 25x/1.10w, Nikon, Tokyo, Japan) and a Ti:Sapphire laser (MaiTai DeepSee, Spectra Physics, CA, USA), tuned to 950 nm for 2-photon excitation of GCaMP6f.

During the imaging session, mice were secured in a custom-built frame via their surgically attached head plate. mGFAP-Cre and C57BL6/J mice anaesthetized with isoflurane (2% for induction, 1% for maintenance) were used to assess CNO-induced Ca\(^{2+}\) activity. Awake C57BL6/J mice were used to assess tDCS-induced Ca\(^{2+}\) activity, after habituation to the head fixation frame for 5 days prior to imaging session by daily, 15-minute fixation with free access to water. To determine neural circuit connectivity, Ca\(^{2+}\) fluorescence activity was recorded from the soma of layer 5 pyramidal neurons in mice freely moving on a moveable running baseplate while head-fixed\(^45\). Mice were habituated to the experimental room, head fixation and baseplate with 10-minute sessions for 2 days prior to imaging.

Astrocyte regions of interest (ROIs) were detected semi-automatically using the GECIquant plugin\(^46\) within the ImageJ software environment (NIH). The ROIs corresponding to neurons were detected using a previously described MATLAB
The change in the Ca\textsuperscript{2+} fluorescence within detected ROIs was subsequently analysed using MATLAB. Baseline mean fluorescence (F\textsubscript{0}) was defined as the mean intensity of the first 50 frames acquired before tDCS or CNO administration. The difference between the observed fluorescence over a given period of time (F) and F\textsubscript{0} was then calculated and normalized against F\textsubscript{0} to give the change in Ca\textsuperscript{2+} fluorescence signal amplitude (\(\Delta F/F_0\), where \(\Delta F = F - F_0\)). Ca\textsuperscript{2+} events were defined as periods where the \(\Delta F/F_0\) values in a single ROI exceeded a \(F_0 + 3*SD\) threshold. Quantitative comparison of \(\Delta F/F_0\) between mice was performed using area under the curve values (AUCs). Neuronal activity and connectivity during locomotion was quantified by the number of Ca\textsuperscript{2+} transient spikes per image frame (20 mins, with spikes determined using a threshold of \(F_0 + 4*SD\)) and connectivity by the pairwise correlations between Ca\textsuperscript{2+} levels across each neuronal pair within an imaging area using Pearson’s correlation coefficient\textsuperscript{48}. The number of Ca\textsuperscript{2+} transient spikes and cell-cell correlation were calculated by integrating the area of each Ca\textsuperscript{2+} event for every ROI in the field of view and summing these together\textsuperscript{49}.

Transcranial direct current stimulation (tDCS)
Transcranial direct current stimulation (tDCS) has traditionally been used to non-invasively modulate neuronal activity. In this study, however, we used a tDCS protocol to selectively activate astrocytes\textsuperscript{15}. In mice used for tDCS-behaviour alone experiments, PSL was performed 1 week after attaching the metal head plate but 2 weeks before preparing the skull for tDCS stimulation. In mice used for tDCS-imaging experiments, cranial window implantation and AAV injection were performed 1 day after attaching a metal head plate and 2 weeks before preparation for tDCS. PSL was not performed in these mice.

To prepare for tDCS, mice were anesthetized (i.p.) using a ketamine (70 mg/kg) and xylazine (10.5 mg/kg) mixture. The scalp skin was incised and the entire skull surface was waterproofed with a tissue adhesive (3M Vetbond, 3M, MN, USA). A custom-made metal head plate was then directly attached to the skull using resin cement (Estecem II, Tokuyama Dental Corporation, Tokyo, Japan) and dental cement (Fuji Lute BC, GC Corporation, Tokyo, Japan). After the cements had cured, the entire skull surface was covered with dental adhesive (Super-Bond with Catalyst V, Monomer and Polymer, Sun Medical Corporation, Shiga, Japan) which acted as both a reinforcing and waterproofing agent.
On the first day of tDCS therapy (day 0), the cathode and anode sites for tDCS were prepared. For the cathode, 0.5 mm diameter silver wire (The Nilaco corporation, Tokyo, Japan) was subcutaneously implanted in the neck. For the anode, the dental adhesive was removed from a 5 mm² diameter circular area of the skull 2 mm posterior to the left S1 (Supplementary Fig. 1). Between tDCS sessions, this anode skull site was covered with silicon to keep the skull clean and moist. During each tDCS session, a silver wire was placed within this anode skull site which was filled with conductive gel (Gelaid, Nihon Kohden, Japan).

tDCS therapy consisted of ten-minute individual sessions performed at 8-hourly intervals over 1 week (21 tDCS sessions in total). During each tDCS session, a constant 0.1 mA of direct current was applied from the anode to the cathode over the 10 minutes using a stimulus isolator (Nihon Kohden, Japan) (Supplementary Fig. 1). During this time, mice were awake but secured in a custom-made frame via their attached head plate. Control mice were subjected to the same procedure without any current stimulation. In experiments where the effect of tDCS on astrocytic or neuronal activity was assessed by simultaneous imaging, the constant current varied between each session from 0.01 mA to 1 mA.
**Long-term in vivo 2-photon imaging of spine dynamics**

In these experiments, M-line mice were injected with the relevant AAV’s and implanted with a chronic cranial window as described above. The first imaging session was performed 14-20 days after cranial window implantation and thus preceded the PSL operation by 3 days. Subsequent imaging sessions were performed at 14, 10 and 7 days prior to the first CNO injection (day 0) and on days 0, 4, 7, 11 and 14 (Fig. 3a).

During each imaging session, mice were anesthetized using 1.0-1.5% isoflurane. The imaging area position was determined as previously described (11) by using intrinsic optical signal imaging and identifying the mCherry expressing area. *In vivo* 2-photon imaging was performed using a multiphoton microscope fitted with a 25x water immersion objective lens and a Ti:Sapphire laser tuned to 800 nm and 950 nm for 2-photon excitation of mCherry and EGFP, respectively. In order to perform reliable long-term imaging of the same specific layer 5 pyramidal neuron dendrite over multiple imaging sessions, the dendritic area of interest was first identified using low-magnification imaging (512 × 512 pixels, 0.99 μm/pixel, 2-μm z step). High-magnification (512 × 512 pixels, 0.12 μm/pixel, 0.5-μm z step) was then used to quantify the specific dendritic region morphology.
For analysis, all images were first imported into the ImageJ software environment (NIH) in order to create 3D image stacks for each dendrite, with motion artefacts corrected using TurboReg. The 3D image stacks were then imported into the AIVIA software environment (DRvision Technologies LLC, DC, USA) which was able to automatically identify and track individual spines on the same section of dendrite across all imaging sessions (with manual cross-checking). All types of dendritic protrusions were included for analysis, except for those which were located in close proximity to the distal tip of a dendritic branch, due to previous reports of their significantly greater instability\textsuperscript{11}. Spine parameters were quantified by other experimenters who did not perform therapy and spine imaging and were blinded to the treatment/control group.

When comparing 2 successive imaging sessions, spines which were present in the latter session but not the former session were referred to as “formed” or “gained” spines whereas spines which were present in the former session but not in a latter session were referred to as “lost” spines. The spine formation rate between 2 successive imaging sessions was calculated as the number of formed spines divided by the total number of spines counted in the former session. Spine elimination rate between 2 successive imaging sessions was calculated as the number of lost spines divided by the
total number of spines counted in the former session. Spine turnover rate between 2 successive imaging sessions was an average of these values - calculated as the sum of the number of formed spines and lost spines, divided by twice the total number of spines counted in the former session. Spine density was calculated as the total number of spines counted, divided by the length of the dendrite. Spine densities were normalized to that counted on day 0.

**Statistical analysis**

All statistical analyses were performed using SPSSver22 (IBM, NY, USA) or MATLAB (The Mathworks, Inc). Comparisons between different sample groups within the same treatment cohort were performed using one-way repeated ANOVA or two-way repeated ANOVA followed by the Bonferroni post-hoc test. Non-parametric multiple comparisons between different treatment cohorts were performed using the Kruskal-Wallis H test followed by the Bonferroni post-hoc test. Comparisons between different treatment cohorts were performed using the Chi-squared test. Single variable comparisons were performed using paired, unpaired $t$-tests and Wilcoxon rank sum test. Values of $p < 0.05$ were considered to be statistically significant. All values were reported as mean +/- 2 standard error.
Data and materials availability

All data associated with this study are present in the paper or Supplementary Materials.
References

1. J. Dahlhamer, J. Lucas, C. Zelaya, R. Nahin, S. Mackey, L. DeBar, R. Kerns, M. Von Korff, L. Porter, C. Helmick, Prevalence of Chronic Pain and High-Impact Chronic Pain Among Adults - United States, 2016. *MMWR Morb. Mortal. Wkly. Rep.* **67**, 1001-1006 (2018).

2. T. J. Price, A. I. Basbaum, J. Bresnahan, J. F. Chambers, Y. De Koninck, R. R. Edwards, R. R. Ji, J. Katz, A. Kavelaars, J. D. Levine, L. Porter, N. Schechter, K. A. Sluka, G. W. Terman, T. D. Wager, T. L. Yaksh, R. H. Dworkin, Transition to chronic pain: opportunities for novel therapeutics. *Nat. Rev. Neurosci.* **19**, 383-384 (2018).

3. C. J. Woolf, Evidence for a central component of post-injury pain hypersensitivity. *Nature* **306**, 686-688 (1983).

4. A. I. Basbaum, D. M. Bautista, G. Scherrer, D. Julius, Cellular and molecular mechanisms of pain. *Cell* **139**, 267-284 (2009).

5. A. Latremoliere, C. J. Woolf, Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J. Pain* **10**, 895-926 (2009).
6. M. C. Bushnell, G. H. Duncan, R. K. Hofbauer, B. Ha, J. I. Chen, B. Carrier,
Pain perception: is there a role for primary somatosensory cortex? *Proc. Natl.
Acad. Sci. U. S. A.* **96**, 7705-7709 (1999).

7. N. Birbaumer, W. Lutzenberger, P. Montoya, W. Larbig, K. Unertl, S. Topfner,
W. Grodd, E. Taub, H. Flor, Effects of regional anesthesia on phantom limb pain
are mirrored in changes in cortical reorganization. *J. Neurosci.* **17**, 5503-5508
(1997).

8. H. Flor, Remapping somatosensory cortex after injury. *Adv. Neurol.* **93**, 195-204
(2003).

9. L. E. Clarke, B. A. Barres, Emerging roles of astrocytes in neural circuit
development. *Nat. Rev. Neurosci.* **14**, 311-321 (2013).

10. W. S. Chung, N. J. Allen, C. Eroglu, Astrocytes Control Synapse Formation,
Function, and Elimination. *Cold Spring Harb. Perspect. Biol.* **7**, a020370
(2015).

11. S. K. Kim, J. Nabekura, Rapid synaptic remodeling in the adult somatosensory
cortex following peripheral nerve injury and its association with neuropathic
pain. *J. Neurosci.* **31**, 5477-5482 (2011).
12. M. Bikson, P. Grossman, C. Thomas, A. L. Zannou, J. Jiang, T. Adnan, A. P. Mourdoukoutas, G. Kronberg, D. Truong, P. Boggio, A. R. Brunoni, L. Charvet, F. Fregni, B. Fritsch, B. Gillick, R. H. Hamilton, B. M. Hampstead, R. Jankord, A. Kirton, H. Knotkova, D. Liebetanz, A. Liu, C. Loo, M. A. Nitsche, J. Reis, J. D. Richardson, A. Rotenberg, P. E. Turkeltaub, A. J. Woods, Safety of Transcranial Direct Current Stimulation: Evidence Based Update 2016. *Brain Stimul.* **9**, 641-661 (2016).

13. M. P. Jensen, M. A. Day, J. Miro, Neuromodulatory treatments for chronic pain: efficacy and mechanisms. *Nat. Rev. Neurol.* **10**, 167-178 (2014).

14. N. E. O'Connell, L. Marston, S. Spencer, L. H. DeSouza, B. M. Wand, Non-invasive brain stimulation techniques for chronic pain. *Cochrane Database Syst. Rev.* **4**, Cd008208 (2018).

15. H. Monai, M. Ohkura, M. Tanaka, Y. Oe, A. Konno, H. Hirai, K. Mikoshiba, S. Itohara, J. Nakai, Y. Iwai, H. Hirase, Calcium imaging reveals glial involvement in transcranial direct current stimulation-induced plasticity in mouse brain. *Nat Commun* **7**, 11100 (2016).
16. Z. Seltzer, R. Dubner, Y. Shir, A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* **43**, 205-218 (1990).

17. B. L. Roth, DREADDs for Neuroscientists. *Neuron* **89**, 683-694 (2016).

18. J. Wess, K. Nakajima, S. Jain, Novel designer receptors to probe GPCR signaling and physiology. *Trends Pharmacol. Sci.* **34**, 385-392 (2013).

19. S. K. Kim, H. Hayashi, T. Ishikawa, K. Shibata, E. Shigetomi, Y. Shinozaki, H. Inada, S. E. Roh, S. J. Kim, G. Lee, H. Bae, A. J. Moorhouse, K. Mikoshiba, Y. Fukazawa, S. Koizumi, J. Nabekura, Cortical astrocytes rewire somatosensory cortical circuits for peripheral neuropathic pain. *J. Clin. Invest.* **126**, 1983-1997 (2016).

20. K. Eto, H. Wake, M. Watanabe, H. Ishibashi, M. Noda, Y. Yanagawa, J. Nabekura, Inter-regional contribution of enhanced activity of the primary somatosensory cortex to the anterior cingulate cortex accelerates chronic pain behavior. *J. Neurosci.* **31**, 7631-7636 (2011).

21. J. Cichon, T. J. J. Blanck, W. B. Gan, G. Yang, Activation of cortical somatostatin interneurons prevents the development of neuropathic pain. *Nat. Neurosci.* **20**, 1122-1132 (2017).
22. C. D. Rittenhouse, H. Z. Shouval, M. A. Paradiso, M. F. Bear, Monocular deprivation induces homosynaptic long-term depression in visual cortex. *Nature* **397**, 347-350 (1999).

23. D. P. Schafer, E. K. Lehrman, A. G. Kautzman, R. Koyama, A. R. Mardinly, R. Yamasaki, R. M. Ransohoff, M. E. Greenberg, B. A. Barres, B. Stevens, Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* **74**, 691-705 (2012).

24. W. S. Chung, L. E. Clarke, G. X. Wang, B. K. Stafford, A. Sher, C. Chakraborty, J. Joung, L. C. Foo, A. Thompson, C. Chen, S. J. Smith, B. A. Barres, Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* **504**, 394-400 (2013).

25. N. J. Allen, C. Eroglu, Cell Biology of Astrocyte-Synapse Interactions. *Neuron* **96**, 697-708 (2017).

26. M. A. Di Castro, J. Chuquet, N. Liaudet, K. Bhaukaurally, M. Santello, D. Bouvier, P. Tiret, A. Volterra, Local Ca2+ detection and modulation of synaptic release by astrocytes. *Nat. Neurosci.* **14**, 1276-1284 (2011).

27. Practice guidelines for chronic pain management: an updated report by the American Society of Anesthesiologists Task Force on Chronic Pain Management
and the American Society of Regional Anesthesia and Pain Medicine.

*Anesthesiology* **112**, 810-833 (2010).

28. B. M. Ilfeld, Continuous Peripheral Nerve Blocks: An Update of the Published Evidence and Comparison With Novel, Alternative Analgesic Modalities. *Anesth. Analg.* **124**, 308-335 (2017).

29. B. M. Ilfeld, Continuous peripheral nerve blocks: a review of the published evidence. *Anesth. Analg.* **113**, 904-925 (2011).

30. B. M. Ilfeld, Liposome bupivacaine in peripheral nerve blocks and epidural injections to manage postoperative pain. *Expert Opin. Pharmacother.* **14**, 2421-2431 (2013).

31. C. Dadure, X. Capdevila, Continuous peripheral nerve blocks in children. *Best Pract. Res. Clin. Anaesthesiol.* **19**, 309-321 (2005).

32. J. S. Mogil, Qualitative sex differences in pain processing: emerging evidence of a biased literature. *Nat. Rev. Neurosci.* **21**, 353-365 (2020).

33. A. Muralidharan, S. G. Sotocinal, J. S. Austin, J. S. Mogil, The influence of aging and duration of nerve injury on the antiallodynic efficacy of analgesics in laboratory mice. *Pain Rep* **5**, e824 (2020).
34. C. A. Federico, J. S. Mogil, T. Ramsay, D. A. Fergusson, J. Kimmelman, A systematic review and meta-analysis of pregabalin preclinical studies. *Pain* **161**, 684-693 (2020).

35. G. Feng, R. H. Mellor, M. Bernstein, C. Keller-Peck, Q. T. Nguyen, M. Wallace, J. M. Nerbonne, J. W. Lichtman, J. R. Sanes, Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28**, 41-51 (2000).

36. A. D. Garcia, N. B. Doan, T. Imura, T. G. Bush, M. V. Sofroniew, GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat. Neurosci.* **7**, 1233-1241 (2004).

37. S. R. Chaplan, F. W. Bach, J. W. Pogrel, J. M. Chung, T. L. Yaksh, Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **53**, 55-63 (1994).

38. K. Hargreaves, R. Dubner, F. Brown, C. Flores, J. Joris, A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**, 77-88 (1988).

39. M. Cheah, J. W. Fawcett, M. R. Andrews, Assessment of Thermal Pain Sensation in Rats and Mice Using the Hargreaves Test. *Bio Protoc* **7**, (2017).
40. W. Kakegawa, Y. Miyoshi, K. Hamase, S. Matsuda, K. Matsuda, K. Kohda, K. Emi, J. Motohashi, R. Konno, K. Zaitsu, M. Yuzaki, D-serine regulates cerebellar LTD and motor coordination through the δ2 glutamate receptor. Nat. Neurosci. 14, 603-611 (2011).

41. P. E. Rothwell, M. V. Fuccillo, S. Maxeiner, S. J. Hayton, O. Gokce, B. K. Lim, S. C. Fowler, R. C. Malenka, T. C. Südhof, Autism-associated neuroligin-3 mutations commonly impair striatal circuits to boost repetitive behaviors. Cell 158, 198-212 (2014).

42. M. L. Seibenhener, M. C. Wooten, Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice. J Vis Exp, e52434 (2015).

43. S. Kakizawa, T. Miyazaki, D. Yanagihara, M. Iino, M. Watanabe, M. Kano, Maintenance of presynaptic function by AMPA receptor-mediated excitatory postsynaptic activity in adult brain. Proc. Natl. Acad. Sci. U. S. A. 102, 19180-19185 (2005).

44. B. Strobel, F. D. Miller, W. Rist, T. Lamla, Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. Hum Gene Ther Methods 26, 147-157 (2015).
45. S. Inagaki, M. Agetsuma, S. Ohara, T. Iijima, H. Yokota, T. Wazawa, Y. Arai, T. Nagai, Imaging local brain activity of multiple freely moving mice sharing the same environment. Sci. Rep. 9, 7460 (2019).

46. R. Srinivasan, B. S. Huang, S. Venugopal, A. D. Johnston, H. Chai, H. Zeng, P. Golshani, B. S. Khakh, Ca(2+) signaling in astrocytes from Ip3r2(-/-) mice in brain slices and during startle responses in vivo. Nat. Neurosci. 18, 708-717 (2015).

47. E. A. Pnevmatikakis, D. Soudry, Y. Gao, T. A. Machado, J. Merel, D. Pfau, T. Reardon, Y. Mu, C. Lacefield, W. Yang, M. Ahrens, R. Bruno, T. M. Jessell, D. S. Peterka, R. Yuste, L. Paninski, Simultaneous Denoising, Deconvolution, and Demixing of Calcium Imaging Data. Neuron 89, 285-299 (2016).

48. M. Agetsuma, J. P. Hamm, K. Tao, S. Fujisawa, R. Yuste, Parvalbumin-Positive Interneurons Regulate Neuronal Ensembles in Visual Cortex. Cereb. Cortex 28, 1831-1845 (2018).

49. L. Reznichenko, Q. Cheng, K. Nizar, S. L. Gratiy, P. A. Saisan, E. M. Rockenstein, T. Gonzalez, C. Patrick, B. Spencer, P. Desplats, A. M. Dale, A. Devor, E. Masliah, In vivo alterations in calcium buffering capacity in transgenic mouse model of synucleinopathy. J. Neurosci. 32, 9992-9998 (2012).
Acknowledgements: We thank M. Kano for the gift of Elvax beads; T. Toda, T. Kobayashi, T Oba for excellent technical assistance and critical discussions on the experiments; H. Hirase, H. Monai for technical advice of tDCS; M. Cooke for offering an analytical software in open field test. Confocal images were acquired at the Spectrography and Bioimaging Facility, NIBB Core Research Facilities. Funding: This work was supported by a Core research for Evolutional Science and Technology (CREST) grant from the Japan Agency for Medical Research and Development (AMED) and from the Japan Science and Technology Agency (JST) (to J. Nabekura); Grant-in-Aids for Scientific Research (A) (17H01530, to J. Nabekura) from the Japan Society for the Promotion of Science (JSPS); a research grant from Takeda Science Foundation (to J. Nabekura); Grant-in-Aid for Young Scientists (17K16133 and 20K16510, to I. Takeda) from JSPS; KAKENHI Grant (18K06536 and 20H05076, to M. Agetsuma) from JSPS; Bilateral Program (JPJSPBP1-20199901, to M. Agetsuma) from JSPS; AMED (20dm0207086, to M. Agetsuma) from JST. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Author contributions: IT, KE and JN conceived and designed the study. IT, KY, MA and JN analysed and interpreted the data and wrote the manuscript. IT, KY,
KE, and DC performed the experiments. All of the authors read and discussed the manuscript. **Competing interest:** The authors declare no competing interests.
Fig. 1. Astrocyte activation by transcranial direct current stimulation reverses mechanical allodynia.
a Representative image of S1 astrocytes expressing GCaMP6f and imaged through a cranial window using 2-photon microscopy in awake mice (upper panel). Ca^{2+} events were observed in the two astrocytes indicated by arrow heads. scale bar = 20 μm. Lower panels show sample Ca^{2+} fluorescence levels for a representative astrocyte illustrating Ca^{2+} transients (black arrow heads) under control conditions (spontaneous, upper trace) and during 0.1 mA for 10 minutes tDCS (lower trace).

b and e A single session of tDCS induces prolonged increases in astrocytic Ca^{2+} activity.

b An example raster plot of Ca^{2+} fluorescence over 10 minutes in 18 individual astrocytes from one mouse before (Spontan.), during (tDCS) and different times after tDCS as indicated. c Graph showing averaged 10 min summed Ca^{2+} responses were significantly increased for up to 10 hours after tDCS (n = 132 astrocytes from 4 mice).

Comparisons between Spontaneous Ca^{2+} activity (Spontan.) and all subsequent observation time points was tested using a one-way repeated measures ANOVA, $F(2.997, 768) = 106.617, p = 0.000$; followed by Bonferroni post-hoc tests, vs tDCS ($p = 0.000$), vs 2 h ($p = 0.000$), vs 4 h ($p = 0.000$), vs 6 h ($p = 0.000$), vs 8 h ($p = 0.016$), vs 10 h ($p = 0.003$). Asterisks indicate the level of significance as follows: *** $p < 0.001$, ** $p < 0.001$, * $p < 0.05$. 

44
**d** Mechanical paw withdrawal thresholds returned to pre-PSL levels following tDCS. Control thresholds (Spontan.) indicate values prior to PSL injury, days 0-21 are relative to the period of tDCS (blue boxes, n=6) or sham-tDCS administration (white boxes, n=3), and as indicated by grey shading (see also panel E). tDCS was accompanied by application of TTX to the injured nerve (see panel E). Statistical significance between tDCS-treated mice and control mice was tested using a two-way repeated measures ANOVA, interaction effect: $F(1.632, 11.423) = 2.168, p = 0.163$, the main effect: $F(1.632, 11.423) = 4.255, p = 0.048$; followed by Bonferroni post-hoc tests, Spontan. ($p = 0.719$), day 0 ($p = 0.234$), day 1 ($p = 0.460$), day 3 ($p = 0.148$), day 7 ($p = 0.117$), day 14 ($p = 0.010$), day 21 ($p = 0.002$). Statistical significance between different treatment days was tested using one-way repeated measures ANOVA, $F(6, 30) = 4.355, p = 0.003$; followed by Bonferroni post hoc tests, Spontan. vs day 0 ($p = 0.000$), Spontan. vs day 1 ($p = 0.201$), Spontan. vs day 3 ($p = 0.282$), Spontan. vs day 7 ($p = 0.399$), Spontan. vs day 14 ($p = 0.957$), Spontan. vs day 21 ($p = 0.354$), day 0 vs day 1 ($p = 0.011$), day 0 vs day 3 ($p = 0.035$), day 0 vs day 7 ($p = 0.062$), day 0 vs day 14 ($p = 0.009$), day 0 vs day 21 ($p = 0.003$).

**e** tDCS or TTX administration by themselves fail to reverse mechanical allodynia.
Fig. 1d in the main text demonstrates combined tDCS-TTX treatment reverses mechanical allodynia induced by prior PSL injury. The data here show that neither tDCS alone (magenta, n = 8, dotted lines show individual data traces) nor TTX alone (white, n = 3) reverses allodynia. Statistical significance in the paw withdrawal thresholds between the 2 treatment cohorts and the various time points was tested using a two-factor repeated measures ANOVA, the interaction effect F(1.656, 14.908) = 2.618, \( p = 0.113 \), the main effect F(1.656, 14.908) = 14.010, \( p = 0.001 \); followed by a Bonferroni post-hoc test, Spontan. (\( p = 0.23 \)), day 0 (\( p = 0.436 \)), day 1 (\( p = 0.141 \)), day 3 (\( p = 0.782 \)), day 7 (\( p = 0.436 \)), day 14 (\( p = 0.533 \)), day 21 (\( p = 0.650 \)). Statistical significance between the paw withdrawal thresholds prior to PSL (Spontan.) and at all subsequent time points in tDCS without TTX treatment mice was tested using a one-way repeated measures ANOVA, F(6, 30) = 4.355, \( p = 0.003 \); followed by Bonferroni post-hoc tests, vs day 0 (\( p = 0.000 \)), vs day 1 (\( p = 0.000 \)), vs day 3 (\( p = 0.000 \)), vs day 14 (\( p = 0.005 \)), vs day 21 (\( p = 0.002 \)). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \). These results confirm that neither the astrocyte activation by tDCS nor the peripheral afferent blockade by TTX are, by themselves, sufficient to cure chronic pain.

* Schematic of the experimental schedule for using tDCS and nerve block to treat PSL-induced mechanical allodynia.
Fig. 2. Specific chemogenic activation of astrocytes reverses mechanical allodynia.

a Schematic of the experimental schedule, using the DREADD system to selectively activate astrocytes following PSL.
b Immunohistochemistry showing selective expression of GFAP-hM3D-mCherry in S1 astrocytes, as identified by S100β expression. Upper right panel, show images across X, Y and Z planes. Bar graphs plot the mean +/- 2SEM. Almost all S100β-positive cells (presumed astrocytes) also showed mCherry-expression (98.8±1.1%, n = 568 cells from 4 mice). In contrast, very few NeuN-positive cells (predominantly neurons) showed mCherry-expression (1.1±1.1%, n = 13 cells from 4 mice). Left figure scale bar = 500 μm (left figure). Right figure scale bar = 10 μm.

c A single injection of CNO (1.0 mg/kg) caused a prolonged increase in Ca²⁺ activity in astrocytes (n = 176 astrocytes from 6 mice). Box plot shows median and interquartile range of Ca²⁺ transient integrals recorded during 10 min intervals before (Spontan.), during (CNO) and at different times after a single injection of CNO (Post). Comparisons between control Ca²⁺ activity (Spontan.) and all subsequent observation time points was tested using one-way repeated measures ANOVA, F (1.012, 177.177) = 42.997, p < 0.001; followed by Bonferroni post hoc tests, vs CNO (p = 0.000), vs Post1h (p =1.00), vs Post2h (p = 0.213), vs Post3h (p = 0.000), vs Post4h (p = 0.010), vs Post5h (p = 0.003), vs Post6h (p = 0.000).

d Mechanical paw withdrawal thresholds returned to pre-PSL levels following activation of astrocytes using CNO. Control thresholds (Spontan.) indicate values prior
to PSL injury, days 0-21 are relative to the period of combined CNO injection (1.0 mg/kg) and TTX application, as indicated with grey shading. Graph shows mean +/- 2SEM for CNO-treated mice (blue, \(n = 12\)) and control mice injected with saline (black, \(n = 5\)). Comparisons between CNO-treated mice and control mice on all days were tested using a two-way repeated measures ANOVA, \(F(3.654, 51.162) = 7.715, p = 0.000\); followed by Bonferroni post hoc tests, Spontan. \((p = 0.974)\), day 0 \((p = 0.494)\), day 1 \((p = 0.850)\), day 3 \((p = 0.001)\), day 7 \((p = 0.000)\), day 14 \((p = 0.000)\), and day 21 \((p = 0.000)\). Statistical significance between different treatment days was tested using one-way repeated measures ANOVA, \(F(3.311, 39.735) = 22.204, p = 0.000\); followed by Bonferroni post hoc tests, Spontan. vs day 0 \((p = 0.000)\), Spontan. vs day 1 \((p = 1.000)\), Spontan. vs day 3 \((p = 1.000)\), Spontan. vs day 7 \((p = 1.000)\), Spontan. vs day 14 \((p = 1.000)\), day 0 vs day 1 \((p = 0.000)\), day 0 vs day 3 \((p = 0.000)\), day 0 vs day 7 \((p = 0.000)\), day 0 vs day 14 \((p = 0.000)\), day 0 vs day 21 \((p = 0.000)\).

Therapeutic effects in reducing mechanical allodynia were prolonged. Graph plots the mean +/- 2SEM of paw-withdrawal thresholds (PWTs) in treated with CNO injection (1.0 mg/kg) and TTX application, as indicated with grey shading (blue, \(n = 5\) mice). Spontaneous reflects baseline PWT, day 0 is after PSL induced injury the day before treatment. Statistical significance between different treatment days was tested
using one-way repeated measures ANOVA, F(3, 12) = 28.632, p = 0.000; followed by Bonferroni post hoc tests, Spontan. vs day 0 (p = 0.017), Spontan. vs day 41 (p = 1.000), Spontan. vs day 54 (p = 1.000), day 0 vs day 41 (p = 0.000), day 0 vs day 54 (p = 0.044), day 41 vs day 54 (p = 1.000).
Fig. 3. Increase in dendritic spine elimination is correlated with the reversal of mechanical allodynia.
a Schematic detailing the experimental schedule in treating PSL-induced mechanical allodynia using the DREADD system (CNO) with spine dynamics observed using \textit{in vivo} 2-photon imaging at times indicated by dark arrows before, during and after CNO treatment, and before PSL injury. The start of CNO-TTX therapy is designated as day 0 and is 2 weeks after PSL, once allodynia is established. The timing for head plate fixation, PSL operation, Elvax-TTX application and CNO therapy was the same as in the previous CNO-TTX experiments.

b Repeated imaging of the same dendritic segment from a layer 5 pyramidal neurons in the left S1 before (day -3 to day 0), during (day 4 to day 7) and after (day11 to day 14) CNO treatment. Arrowheads indicate spine formation (magenta) and spine elimination (blue). Scale bar = 5 µm.

c to e Spine dynamics during and after CNO (with TTX) treatment. The period during CNO-TTX or saline-TTX administration is indicated by grey shading. Asterisks indicate the level of statistical significance when comparing saline and CNO treatment as follows: *$p < 0.05$, **$p < 0.01$. c Normalized spine turnover rates; in saline-treated (white; $n = 16$ dendrites from 7 mice) and CNO-treated mice (green; $n = 19$ dendrites from 5 mice). Spine turnover rate was calculated as the average of spine formation and elimination rates. Comparisons between CNO and saline used a two-way repeated
ANOVA, the interaction effect: $F(4, 132) = 1.461, p = 0.218$, the main effect for group: $F(1, 33) = 6.445, P = 0.016$; followed by Bonferroni post hoc test, day 0 ($p = 1.0$), day 4 ($p = 0.031$), day 7 ($p = 0.385$), day 11 ($p = 0.114$), and day 14 ($p = 0.032$).

Normalized spine elimination rates in saline-treated (white; $n = 16$ dendrites from 7 mice) and CNO-treated mice (blue; $n = 19$ dendrites from 5 mice). Spine elimination rate was calculated as the number of spines that were lost between 2 successive imaging sessions, divided by the total number of spines counted in the first of these imaging sessions, and normalized to the pre-injury elimination rate. Comparisons between CNO and saline was tested using a two-way repeated ANOVA, the interaction effect: $F(4, 132) = 1.852, p = 0.123$, the main effect of group: $F(1, 33) = 18.477, p = 0.000$; followed by Bonferroni post-hoc test, day 0 ($p = 1.000$), day 4 ($p = 0.002$), day 7 ($p = 0.018$), day 11 ($p = 0.285$), and day 14 ($p = 0.008$).

Normalized spine formation rates in saline-treated (white; $n = 16$ dendrites from 7 mice) and CNO-treated mice (pink; $n = 19$ dendrites from 5 mice). Spine formation rate was similarly calculated as the number of spines that were formed between 2 successive imaging sessions, divided by the total number of spines counted in the prior imaging session, normalized to pre injury rate. Comparisons between CNO and saline was tested using a two-way repeated ANOVA,
the interaction effect: $F(4, 132) = 0.741, p = 0.565$, the main effect: $F(1, 33) = 0.465, p = 0.500$.

f CNO-TTX treatment was more likely to be associated with elimination of spines formed during the period just after PSL. Pre-PSL spines were defined as those which were observed both before the PSL operation and before the start of CNO-TTX therapy. Early post-PSL spines were defined as those which only appeared in the week following PSL and which were also still present before the start of CNO-TTX therapy. Pre-PSL spine counts in CNO- and saline-treated mice were 33 spines, 19 dendrites from 5 CNO-treated mice and 17 spines, 16 dendrites from 7 saline-treated mice, respectively. Early post-PSL spine counts in CNO- and saline-treated mice were $n = 25$ spines, 19 dendrites from 5 CNO-treated mice, $n = 7$ spines, 16 dendrites from 7 saline-treated mice. Comparisons between the percentage of spine elimination between spine-types was tested using two-way ANOVA, the interaction effect: $F(1, 66) = 2.165, p = 0.146$, the main effect: $F(1, 66) = 10.969, p = 0.002$; followed by Bonferroni post hoc test, pre-PSL vs early post-PSL in CNO-treated mice ($p = 0.001$), pre-PSL vs early post-PSL in saline-treated mice ($p = 0.216$).

g Early post-PSL spines were further categorized according to their time-course of elimination. Early post-PSL spines appeared only after the PSL operation and thus were
absent at the day-14 imaging time point, but were present at the day-7 time point.

“Before” spines were those which disappeared prior to the start of CNO-TTX therapy, i.e., lost at the day -3 or day 0 time points. “During” spines were those which disappeared during CNO-TTX therapy, i.e., lost at the day 4 or day 7 time points.

“After” spines were those which disappeared in the week after CNO-TTX therapy had finished, i.e., lost at the day 11 or day 14 time point. “Persistent” spines were those which were observed at all subsequent time points following their first appearance.

Below are the proportions of different categories of spines compared between saline-treated (white; \(n = 119\) spines, 16 dendrites from 7 mice) and CNO-treated mice (green; \(n = 171\) spines, 19 dendrites from 5 mice). Spine categories are expressed as proportions of the total number of spines which appeared in the week following PSL. Comparisons between CNO and saline treated mice for each spine category used an unpaired \(t\) test, Before (\(t(33) = 0.395, p = 0.696\)), During (\(t(33) = 2.276, p = 0.029\)) and After (\(t(33) = 0.131, p = 0.896\)), Persistent (\(t(33) = -2.000, p = 0.054\)), \(^*p < 0.05\).

Neuronal activity in S1 during spontaneous locomotion decreased after CNO-TTX therapy. Box plots show the individual neuron frequency (left) and lines show average \(\text{Ca}^{2+}\) transient frequencies from individual mice before and after treatment. Note how the mean frequency of \(\text{Ca}^{2+}\) transients was decreased in most, but not all, mice (with a
significant decrease overall). The right panel graphs show the frequency distribution
histogram and the cumulative probability curve of the cross-correlation between
neuronal activity (right) in CNO-treated mice (post-PSL; \( n = 1151 \) neurons and 16635
pairs in 8 mice, post-therapy; \( n = 1031 \) neurons and 13581 pairs in 8 mice). Before
(blue, post-PSL) and after therapy (magenta). Therapy significantly reduced these
parameters (Wilcoxon rank sum test, mean spikes (\( p = 0.000 \)), positive correlation (\( p =
0.000 \))).
Fig. 4  

**Fig. 4.** tDCS-lidocaine therapy reverses mechanical allodynia without affecting rotarod and open field behaviours.

**a** Schematic of the experimental schedule for using tDSCS and nerve block by lidocaine to treat PSL-induced mechanical allodynia. Lidocaine was applied using a temporary (3
day) subcutaneous osmotic pump delivering lidocaine around the sciatic nerve ligation site.

b Mechanical paw withdrawal thresholds return towards pre-PSL levels following tDCS and lidocaine. The period of tDCS (blue boxes, n=7) or sham-tDCS administration (white boxes, n=3) is indicated by grey shading. tDCS was accompanied by application of lidocaine to the injured nerve. Statistical significance between tDCS-treated mice and control mice was tested using a two-way repeated measures ANOVA, interaction effect: $F(1.632, 11.423) = 2.168, p = 0.163$, the main effect: $F(1.632, 11.423) = 4.255, p = 0.048$; followed by Bonferroni post-hoc tests, Spontan. ($p = 0.719$), day 0 ($p = 0.234$), day 1 ($p = 0.460$), day 3 ($p = 0.148$), day 7 ($p = 0.117$), day 14 ($p = 0.010$), day 21 ($p = 0.002$).

c tDCS-treatment does not effect motor performance on the rotarod test. Graph shows the latency to fall off the escalating rotarod for tDCS/lidocaine, and lidocaine treated mice, before, during and after tDCS treatment. Statistical significance between tDCS-treated mice and control mice was tested using a two-way repeated measures ANOVA, interaction effect: $F(2, 18) = 6.858, p = 0.743$, the main effect: $F(1, 9) = 0.219, p = 0.651$. 


d tDCS does not effect behavioural parameters in the open field assay. Graphs show locomotor activity (speed, left; distance, centre) and anxiety behaviour (time spent in inner zone, right) for tDCS/lidocaine and lidocaine control mice, all tested at the end of tDCS treatment (day 7). Statistical significance between tDCS-treated mice and control mice was tested using unpaired t-test, speed (t(9) = -1.638, p = 0.136), distance (t(9) = -1.523, p = 0.162), duration in inner zone (t(9) = -0.222, p = 0.829).