

Dynamic Change of Endocannabinoid Signaling in the Medial Prefrontal Cortex Controls the Development of Depression After Neuropathic Pain

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Many patients with chronic pain conditions suffer from depression. The mechanisms underlying pain-induced depression are still unclear. There are critical links of medial prefrontal cortex (mPFC) synaptic function to depression, with signaling through the endocannabinoid (eCB) system as an important contributor. We hypothesized that afferent noxious inputs after injury compromise activity-dependent eCB signaling in the mPFC, resulting in depression. Depression-like behaviors were tested in male and female rats with traumatic neuropathy [spared nerve injury (SNI)], and neuronal activity in the mPFC was monitored using the immediate early gene *c-fos* and *in vivo* electrophysiological recordings. mPFC eCB concentrations were determined using mass spectrometry, and behavioral and electrophysiological experiments were used to evaluate the role of alterations in eCB signaling in depression after pain. SNI-induced pain induced the development of depression phenotypes in both male and female rats. Pyramidal neurons in mPFC showed increased excitability followed by reduced excitability in the onset and prolonged phases of pain, respectively. Concentrations of the eCBs, 2-arachidonoylglycerol (2-AG) in the mPFC, were elevated initially after SNI, and our results indicate that this resulted in a loss of CB1R function on GABAergic interneurons in the mPFC. These data suggest that excessive release of 2-AG as a result of noxious stimuli triggers use-dependent loss of function of eCB signaling leading to excessive GABA release in the mPFC, with the final result being behavioral depression.

Key words: depression; endocannabinoid; medial prefrontal cortex; neuronal activity; neuropathic pain; synaptic transmission

Significance Statement

Pain has both somatosensory and affective components, so the complexity of mechanisms underlying chronic pain is best represented by a biopsychosocial model that includes widespread CNS dysfunction. Many patients with chronic pain conditions develop depression. The mechanism by which pain causes depression is unclear. Although manipulation of the eCB signaling system as an avenue for providing analgesia per se has not shown much promise in previous studies. An important limitation of past research has been inadequate consideration of the dynamic nature of the connection between pain and depression as they develop. Here, we show that activity-dependent synthesis of eCBs during the initial onset of persistent pain is the critical link leading to depression when pain is persistent.

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Introduction

Chronic pain is a major public health challenge that is inadequately addressed. In particular, many patients with chronic pain conditions suffer from depression. Pain research has increasingly demonstrated that functional changes in peripheral sensory neurons are insufficient to fully explain the transition to chronic pain and that substantial alterations in diverse brain centers accompany the development of chronic pain (Saab, 2012). Furthermore, the human experience of pain includes affective features such as unpleasantness, anxiety,

and especially depression. From 30 to 60% of subjects with chronic pain develop depression, with a prevalence of suicidal ideation of 32% (Triñanes et al., 2014). When combined, comorbid pain and depression have a much greater impact on multiple domains of functional status as well as health care usage and increased likelihood of alcohol or substance abuse (Liu et al., 2010) compared with either condition alone. Despite previous preclinical studies exploring this topic (Goffer et al., 2013; Descalzi et al., 2017), the mechanism by which pain induces depression remains unclear. An important limitation of past research has been inadequate consideration of the dynamic nature of comorbid pain and depression as they develop.

One hypothesis is that pain induces depression because it is a stressor (Abdallah and Geha, 2017). However, pain and stress are functionally distinct in their circuitry. Pain targets the limbic system via peripheral A δ and C-fibers that activate the brainstem and thalamus, which relay to multiple cortical and subcortical sites, including limbic areas. In contrast, chronic stress-induced depression is characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis (McEwen et al., 2015), and previous studies have shown that the HPA axis is not altered in neuropathic pain (Ulrich-Lai et al., 2006; Muhtz et al., 2013). Given that pain-induced depression is physiologically distinct from other forms of major depression (Bair et al., 2003), further elucidation of this distinct mechanism of depression is needed to develop novel therapies for this debilitating condition.

The medial prefrontal cortex (mPFC) plays important roles in cognition and mood regulation (Euston et al., 2012). The rat and human mPFC are functionally connected to the thalamus (Klein et al., 2010; Barbas et al., 2011), which in turn receives nociceptive input from the spinal cord dorsal horn, making the mPFC a potential target for amplified nociceptive signaling. Functional magnetic resonance imaging (fMRI) has shown a temporal link between spontaneous pain and mPFC activation in chronic pain patients (Baliki et al., 2010). Deactivation of the mPFC is a consequence of both acute pain (arthritis) through enhanced inhibitory input (Ji and Neugebauer, 2011) and chronic pain, spared nerve injury (SNI), through reduced glutamatergic innervation 2 weeks after injury (Kelly et al., 2016). Together, these observations suggest the mPFC is a key mediator of brain network disruption during pain, but the underlying connectivity and synaptic mechanisms remain unresolved. Elucidation of these mechanisms is necessary to design novel therapeutic interventions.

Most cannabinoid receptors type 1 (CB1Rs) in the mPFC are in γ -aminobutyric acid (GABA)-ergic terminals (Hill et al., 2011). Physiologically, activity-dependent endocannabinoid (eCB) release by the postsynaptic pyramidal neurons suppress GABAergic inhibitory input to mPFC neurons via their presynaptic CB1Rs, thereby keeping the mPFC active. Although manipulations of the eCB signaling system to treat either pain or depression have not shown much promise in preclinical and clinical studies because of low efficacy and significant adverse effects, regulation of the eCB system as a means of preventing or reversing pain-induced depression has been minimally examined. An important limitation of past research has been inadequate consideration of the dynamic nature of the connection between pain and depression as they develop. To address this need, we used a rat nerve injury model to identify the synaptic mechanisms underlying phasic shifts in mPFC excitability that generate depression induced by neuropathic pain.

Materials and Methods

Animals

Male and female Sprague Dawley rats weighing 170–200 g were obtained from Taconic Biosciences (<https://www.taconic.com/rat-model/sprague-dawley>) and were maintained and used according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and in compliance with federal, state, and local laws. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (AUA5615). Animals were housed in a pathogen-free facility, two animals per ventilated cage, in a room maintained at $25 \pm 1^\circ\text{C}$ at 35–45% humidity, with a 12/12 h day/night cycle. Animals had access *ad libitum* to food and water, and bedding was aspen wood chips. At the termination of the study, the animals were killed by decapitation during deep isoflurane anesthesia.

Injury model preparation

Some rats were subjected to spared nerve injury surgery (Fischer et al., 2014). Briefly, during anesthesia by inhalation of isoflurane (1–3%), an incision (~ 2 cm) was made on the lateral mid thigh, the underlying muscles were separated to expose the sciatic nerve, and the tibial and common peroneal were individually ligated with 6.0 sutures and cut distally to the ligation, and 2–3 mm of each nerve was removed distal of the ligation. The sural nerve was preserved, and contact with it was avoided. Muscle and skin were closed using 4.0 monofilament nylon sutures. Sham control rats received sham surgery in the form of skin incision and closure only.

Osmotic pump implantation

In some rats, gabapentin (GBP) was infused subcutaneously with osmotic pumps (ALZET Osmotic Pumps). The pumps were implanted underneath the skin in the back of the rats.

Intra-mPFC injection

Rats were anesthetized with 2–3% isoflurane and placed in a stereotaxic device (David Kopf Instruments). Bilateral guide cannulae (26 gauge; Plastics One) were implanted with a 10° angle from middle line targeting the prelimbic mPFC and also avoiding damage to the dorsal mPFC using the following stereotaxic coordinates (from bregma, anteroposterior, +3.2 mm; mediolateral, ± 1.0 mm; dorsoventral, -3.5 mm; Paxinos and Watson, 2014). Obturators were placed in the guide cannulae, extending 1 mm beyond, and were left there at all times except during microinjections. On the day of injection, the obturator was removed from one of the guide cannulae, and a stainless-steel injector tube (30 gauge; Plastics One) was inserted to a depth 1 mm beyond the end of the guide cannula. The injector tube was connected through polyethylene tubing to a 5 μl Hamilton microsyringe, which was mounted on a single-syringe infusion pump (Masterflex). The injector was kept in the guide cannula for an additional 60 s to ensure adequate diffusion from the injector tip. Similar microinjection was made on the contralateral side.

After completion of all experiments, cannula placements were anatomically verified. The animals were anesthetized with 5% isoflurane and then perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were cut into 40 μm sections and stained with cresyl violet then examined with light microscopy.

Sensory behavioral tests

Sensory testing of the plantar skin included eliciting reflexive behaviors induced at threshold intensity by punctate mechanical stimulation (von Frey test), by noxious mechanical stimulation (pin), dynamic non-noxious mechanical stimulation (brush), and cold stimulation (acetone).

Noxious punctate mechanical stimulation (pin test). Pin test was performed using a standard 22-gauge spinal anesthesia needle that was applied to the lateral third of the hindpaw with enough force to indent the skin but not puncture it. This was repeated for five applications, with at least 10 s intervals between applications, and this set of applications was repeated after 1 min, making a total of 10 touches. Each application induced a behavior that was categorized as either the type typical of uninjured rats, consisting of a very brief (<1 s) withdrawal and

immediate return of the foot to the cage floor, or an alternate behavior that we term a “hyperalgesic response,” consisting of a complex event with sustained elevation of the foot for at least 1 s, variably combined with grooming that included licking and chewing of the paw and shaking of the limb (Hogan et al., 2004). This hyperalgesic behavior is associated with place avoidance (Wu et al., 2010), indicating an aversive experience. Hyperalgesia was quantified by tabulating the number of hyperalgesic responses as a percentage of the total touches. Naïve animals rarely show hyperalgesic behavior to pin stimulation.

Threshold punctate mechanical stimulation (von Frey). The von Frey test was performed using a set of calibrated monofilaments (Patterson Medical). Briefly, beginning with the 2.8 g filament, the filament was applied perpendicularly to the glabrous skin on the lateral third of the plantar aspect of the hindpaw innervated by the sural nerve for 1 s, with just enough force to bend the fiber. If paw withdrawal was observed, then the next weaker filament was applied, and if no response was observed, then the next stiffer filament was applied until a response occurred (termed a “reversal event”). After the reversal event, four more simulations were performed following the same pattern. There was at least a 10 s interval between applications. The forces of the filaments before and after the reversal, and the four filaments applied after the reversal, were used to calculate the 50% withdrawal threshold (Chaplan et al., 1994). Rats not responding to any filament were assigned a score of 25 g. Because the magnitude of mechanical sense is perceived in a logarithmic scale, as described by Weber’s law, von Frey paw withdrawal threshold data were log transformed (Mills et al., 2012). In the absence of a hypersensitivity state, animals often default to the 25 g score.

Dynamic mechanical stimulation (brush). A camel hair brush (4 mm wide) was applied to the lateral plantar skin of the hindpaw by light stroking in the direction from heel to toe for 2 s (Hogan et al., 2004). The response was scored as either positive if the paw was removed or none in the absence of movement. The test was applied three times to each paw, separated by intervals of at least 10 s. Hypersensitivity was quantified by tabulating the number of responses as a percentage of the total applications. Naïve rats and rats with sham injury rarely respond to brush stimulation.

Cold stimulation (acetone). Sensitivity to cold was assessed using the application of acetone, which was expelled through tubing perpendicularly to form a convex meniscus on the end of the tubing that was touched to the lateral plantar skin without contact of the tubing with the skin (Choi et al., 1994). The response was scored as positive if the paw moved, and three repetitions were spaced at least 1 min apart. Hypersensitivity to acetone was quantified by tabulating the number of responses as a percentage of the total applications. Naïve rats and rats with sham injury rarely respond to acetone application.

Depression-like behavioral tests

Open field test (OFT). Behavior of a rat in a new environment (an open field) contains sufficient complexity and sensitivity to a wide range of neurologic diseases. The Behavioral Spectrometer is a newly developed device that is capable of automatically identifying 23 unique behaviors and providing a complete, real-time profile of animal behavior in an open field scenario (Brodtkin et al., 2014). The apparatus (Biobserve) consists of a 40 cm × 40 cm square arena enclosed in a cube box with an edge length of 45 cm. A camera is mounted in the ceiling above the arena to monitor position and posture of the animals. A row of 32 infrared transmitter and receiver pairs is embedded in the walls to monitor the rat behaviors. Rats were placed individually in one corner of the box and allowed to freely explore the arena during a 15 min test session. The distance traveled was calculated. Time in the center is defined as the amount of time that was spent in the central 15 × 15 cm² area of the arena.

Sucrose-preference test (SPT). Rats were individually housed and trained to drink from two bottles that contained 1% sucrose solution and tap water, respectively, for 24 h. After that, rats were deprived of food and water for 8 h, and the consumption of sucrose solution and water over the next 16 h (overnight) was measured. The sucrose preference (%) was calculated as the sucrose solution consumed divided by the total amount of solution consumed.

Novelty-suppressed feeding. The novelty-suppressed feeding (NSF) was conducted similar to a published protocol (Santarelli et al., 2003). Rats were deprived of food for 16 h (overnight) before being placed in a novel environment (a plastic box 45 cm long × 35 cm wide × 20 cm deep) where five food pellets (regular chow) were placed on a piece of white filter paper (11 cm in diameter) in the center of the box. A rat was placed in one corner of the box and the latency to feed was measured. Feeding was defined as biting, not simply sniffing or touching the food. Immediately after the test, the animal was transferred to the home cage, and the latency to feed in the home cage was measured to serve as controls.

Forced swim test (FST). This is a 2 d protocol involving exposure to the water tank 1 d before the test day. On the first day, rats were placed individually into glass cylinders (30 cm diameter, 50 cm tall) filled to a depth of 30 cm with water (23 ± 1°C) for 15 min. On the test day, the rats were tested in the cylinder with water for 5 min. Water was changed between trials. The videos were recorded and analyzed on-line or off-line. Immobility was defined as the cessation of all movements (e.g., climbing, swimming) except those necessary for the rats to keep heads above water (e.g., floating).

EthoVision XT (Noldus Information Technology) was used to analyze those videos in real time or off-line. All behavioral tests were performed blindly; the experimenters performing these tests were blinded from the animal’s treatment.

Immunohistochemistry

Immunohistochemical procedures were based on our previous study with minor modifications (Pan et al., 2011). Rats were deeply anesthetized with 3–4% isoflurane and perfused through the aorta with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. After perfusion, brains were removed and fixed in the same fixative overnight at 4°C. The brains were then cryoprotected in increasing concentrations of sucrose (10, 20, and 30%) in 0.1 M PBS at 4°C, frozen on dry ice, and stored at −80°C until use. Coronal sections were cut at 10 μm thickness with a cryostat and mounted on glass slides. After rinsing three times in PBS, those slices were blocked for 1 h at room temperature with blocking solution (1% bovine serum albumin, 5% normal goat serum, and 1% Triton X-100 in 0.1 M PBS, pH 7.4). Sections were then incubated with 1:500 mouse anti-c-fos antibody, 1:500 rabbit anti-CaMKII antibody, and 1:500 rabbit anti-glutamate decarboxylase-67 (GAD-67) at 4°C for 24 h. After rinsing three times, 5 min each in PBS, sections were incubated in the secondary antibodies: 1:800 goat anti-mouse IgG (Alexa Fluor 594), 1:800 goat anti-rabbit IgG (Alexa Fluor 488) for 1 h at room temperature. All antibodies were purchased from Cell Signaling Technology. Negative control sections were processed with a nonimmune serum in place of the primary antibodies. Sections were analyzed by using a fluorescence microscope (BZ-X800, KEYENCE). For each animal, three sections on each targeted region of interest with 200 μm space were examined, and the mean counts of positive neurons were determined. The experimenters performing slides scanning and analyzing were blinded to the animal’s treatment.

Biochemical detection of endocannabinoids

Control and SNI rats were anesthetized by isoflurane inhalation and decapitated. The brain was immediately removed and rapidly frozen on dry ice, and the prelimbic mPFC was dissected out in a frozen chamber. The eCBs, including 2-arachidonoylglycerol (2-AG) and N-arachidonylethanolamine (AEA), were extracted as previously described (Patel et al., 2003; Wang et al., 2010). Samples were weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 186 pmol [³H]₂-AG. They were homogenized with a round-bottomed rod and sonicated in an ice-cold water bath for 30 min. Samples were incubated overnight at −20°C to precipitate proteins and subsequently centrifuged at 1500 × g for 3 min. The supernatants were transferred to a new glass tube and evaporated to dryness under N₂ gas. The samples were resuspended in 300 μl of methanol to recapture any lipids adhering to the glass tube and dried again under N₂ gas. Dried lipid extracts were suspended in 20 ml of methanol and stored at −80°C until analysis in <1 week. The content of eCBs was determined using isotope dilution

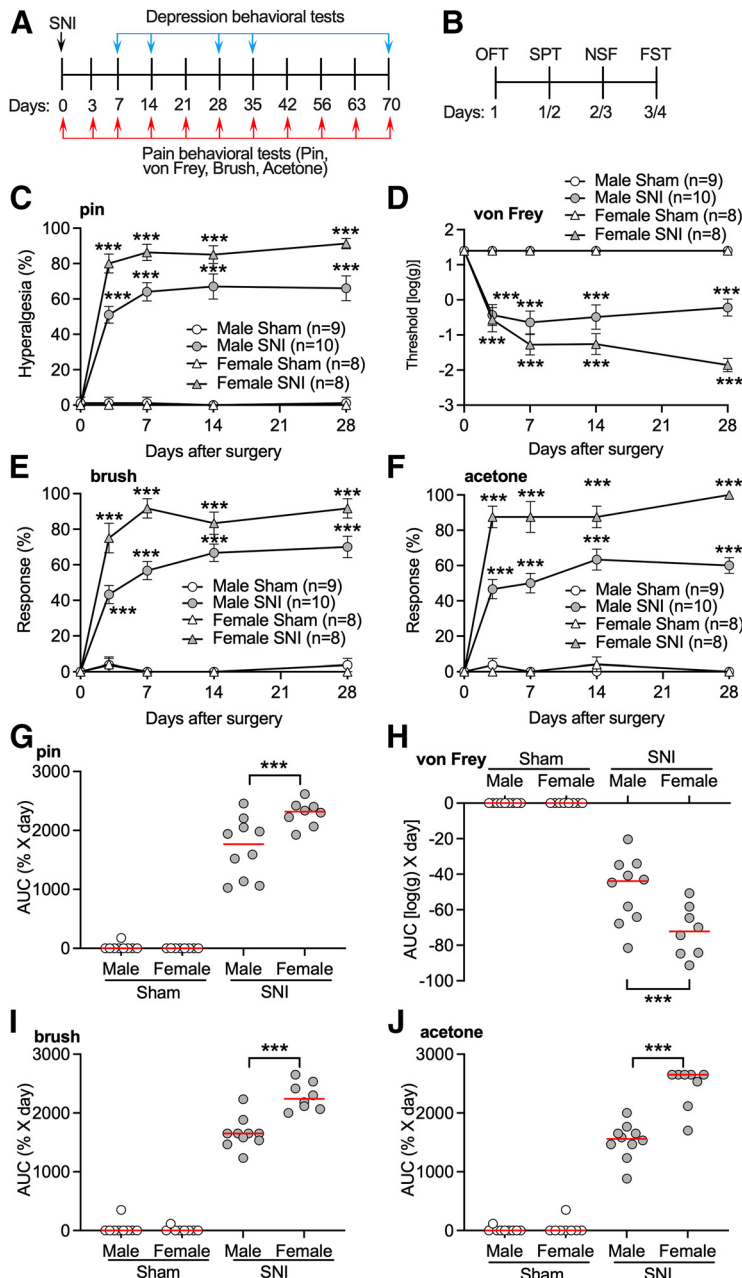


Figure 1. Timeline of procedures and pain behavior in male and female rats after SNI. **A**, Timeline of procedures. Blue arrows indicate the start of depression behavioral tests, and red arrows indicate pain behavioral tests. All depression-like behavioral tests were performed with a different set of rats in different time points to avoid the influence of another test. **B**, Sequence of performing depression-like behavioral tests, with less stressful OFT first and the stressful FST last. **C–F**, Time course of hypersensitivity to (**C**) noxious mechanical stimuli (pin; two-way RM-ANOVA followed by Sidak *post hoc* test, $F_{(12,124)} = 54.1$, $p < 0.0001$, $***p < 0.0001$ compared with time-matched control), (**D**) threshold mechanical stimuli (von Frey; two-way RM-ANOVA followed by Sidak *post hoc* test, $F_{(12,124)} = 13.24$, $p < 0.0001$, $***p < 0.0001$ compared with time-matched control), (**E**) brush (two-way RM-ANOVA followed by Sidak *post hoc* test, $F_{(12,124)} = 22.67$, $p < 0.0001$, $***p < 0.0001$ compared with time-matched control), and (**F**) cold (two-way RM-ANOVA followed by Sidak *post hoc* test, $F_{(12,124)} = 30.07$, $p < 0.0001$, $***p < 0.0001$ compared with time-matched control). **G–J**, The area under the curve (AUC) analysis for comparison between male and female rats show greater sensitivity in females after SNI for pain behavior testing by noxious mechanical stimuli (pin; **G**; two-way ANOVA followed by Sidak *post hoc* test, $F_{(1,31)} = 9.35$, $p = 0.005$, $***p < 0.001$ compared with injury-matched male rats), threshold mechanical stimuli (von Frey; **H**; two-way ANOVA followed by Sidak *post hoc* test, $F_{(1,31)} = 8.18$, $p = 0.008$, $***p < 0.001$ compared with male rats with SNI), brush (**I**; two-way ANOVA followed by Sidak *post hoc* test, $F_{(1,31)} = 25.66$, $p < 0.0001$, $***p < 0.001$ compared with male rats with SNI), and cold (**J**; two-way ANOVA followed by Sidak *post hoc* test, $F_{(1,31)} = 29.61$, $p < 0.0001$, $***p < 0.001$ compared with male rats with SNI). Data are presented as mean $\pm$ SEM.

liquid chromatography-electrospray ionization tandem mass spectrometry (Wang et al., 2010).

Quantitative real-time reverse transcription polymerase chain reaction analysis

Standard quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) procedures were used, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

Tissue extraction and mPFC isolation. Animals were deeply anesthetized under isoflurane and killed via decapitation for the collection of brain tissue at 1, 3, 7, 14, and 35 d after SNI or sham SNI injury. Each brain was immediately submerged in liquid nitrogen after dissection. Using brain matrices (2 mm thickness) for sectioning, brains were sectioned in a Leica CM1950 cryostat to isolate the mPFC, using a rat brain stereotaxic atlas for location reference (Paxinos and Watson, 2014). Brain tissue was stored at -80°C until further use.

RNA Extraction. Tissue was homogenized with stainless steel beads (5 mm) in a TissueLyser LT (Qiagen). Total RNA per sample was extracted via an RNeasy Kit (catalog #74104, Qiagen) per the manufacturer's instructions. Total RNA was quantified on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). The RNA was stored at -80°C until further use.

qRT-PCR. Isolated RNA was synthesized into the first-strand cDNA via SuperScript III (Thermo Fisher Scientific) following the manufacturer's instructions and ran on a C1000 Touch Thermal Cycler (CFX96 Real-Time System, Bio-Rad). Five genes and one housekeeping gene were selected for screening: GAPDH (housekeeping gene), *c-fos*, cannabinoid receptor type 1 (CNR1), diacylglycerol lipase α (DAGL α), N-arachidonylphosphatidylethanolamine-phospholipase D (NAPE-PLD), monoglyceride lipase (MAGL), and fatty acid amide hydrolase (FAAH). PCR primer sequences were used for quantification of genes that encode for GAPDH (Forward: CTCATGACCACAGTCCATGC, Reverse: TTCAGTCTCTGGGATGACCTT), *c-fos* (Forward: GGCAAAGTAGACGACTATCTCT; Reverse: TCAGTCCCTCCTCCGATTTC), CNR1 (Forward: AGGAGCAAGGACCTGAGACA, Reverse: TAACGGTGCTCTTGATGCAG), DAGL α (Forward: GGCAAGACCCTGTAGAGCTG, Reverse: TAAAACAGGTGGCCCTCATC), NAPE-PLD (Forward: GACGAGCTCATCTCTCTCAC, Reverse: AGTC CAGGTGGTCGTAGTGG), MAGL (Forward: TAGCA GCTGCAGAGAGACCA, Reverse: GATGAGTGGG TCGGAGTTGT), and FAAH (Forward: ACITTTGT GGATCCCTGCTTG, Reverse: TCTCATGCTGCAG TTTCCAC). One μl of each cDNA sample was transferred to a 96-well plate with the probes of the chosen genes for screening (20 μl total volume per well). qRT-PCR was performed using ITaq Universal Probes Supermix (2 $\times$; Bio-Rad) containing the gene-specific primers of interest (6 μM). PCR was conducted with the denaturation step of 3 min at 95°C , followed by 40 cycles of 95°C denaturation (20 s duration), and annealing at 60°C (1 min). After each cycle, the plate was read by the C1000 Touch Thermal Cycler (CFX96 Real-Time System, Bio-Rad). PCR-run data were exported from the machine and analyzed via Bio-Rad CFX Maestro Software. Genes of interest were normalized to the housekeeping gene GAPDH in Microsoft Excel and graphed within GraphPad Prism.

In vivo electrophysiological recording

A craniotomy was performed, and the dura was opened to expose the mPFC. Then the brain surface was covered in warm (36°C) mineral oil, and a single-barrel glass micropipette filled with a solution containing 1 M NaCl (with resistance of 15–20 M Ω) was advanced into the mPFC using a microdrive (David Kopf Instruments) at 2 μ m per step, targeting layer IV/V at depths of 3.4–4.2 mm from the brain surface using the stereotaxic coordinates of 3.0–3.4 mm anterior to bregma; 0.7 mm lateral to midline (Paxinos and Watson, 2014). For each rat, spontaneous activity (SA) was sought by the following process. Four vertical pathways with a 100 μ m interval from bregma +3.0 to +3.4 were examined. At each site, SA was searched starting from 3.4 mm from the brain surface, and a 30 μ m span was searched by advancement steps of 1 μ m, after which the electrode was advanced 50 μ m to initiate a similar 30 μ m search. This process was repeated two more times before moving to the next pathway. mPFC pyramidal neurons can be distinguished from interneurons based on their broader action potential waveform and lower baseline discharge rate (Ji and Neugebauer, 2011). Signals were collected with an Axon Axoclamp 900A Microelectrode Amplifier (Molecular Devices), filtered at 1 kHz, and sampled at 10 kHz using a digitizer (Digidata 1440A). Action potentials were isolated by setting the threshold above background noise, and individual units were identified by template matching using Spike2 (Cambridge Electronic Design) or pClamp11 software (Molecular Devices).

Multielectrode array electrophysiology

Rats were anesthetized by isoflurane inhalation and a craniotomy was performed. The dura was opened to expose the mPFC, and a 16-channel platinum/iridium multielectrode array (MEA) microprobe was inserted targeting mPFC. After insertion, the brain surface was covered with a layer of Kwik-Sil silicone adhesive (World Precision Instruments), and then dental cement was used on top of that to secure the MEA. Eight to 10 d after the surgery, neuronal activity was recorded and analyzed with a wireless W2100 data acquisition system (Multichannel Systems), filtered at 2 kHz, and sampled at 10 kHz. On the recording day, rats were given 30 min to 1 h to acclimate to the environment. Spikes were detected by setting the slope and amplitude threshold using Spike Detector (Multichannel Systems).

Slice preparation and electrophysiology

Rats were anesthetized by isoflurane inhalation and decapitated. mPFC Slices (250 μ m) and whole-cell recordings were made as described previously (Hill et al., 2011). Slices were stored in artificial cerebrospinal fluid (aCSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose at room temperature. All solutions were saturated with 95% O₂ and 5% CO₂. All recordings were performed at 32 $\pm$ 1°C by using an automatic temperature controller (Warner Instruments). Patch pipettes, ranging from 2 to 4 M Ω resistance, were formed from borosilicate glass (King Precision Glass) and fire polished. Recordings were made with an Axopatch 700B amplifier (Molecular Devices). Signals were filtered at 2 kHz and sampled at 10 kHz with a Digidata 1440A digitizer and pClamp10 software (Molecular

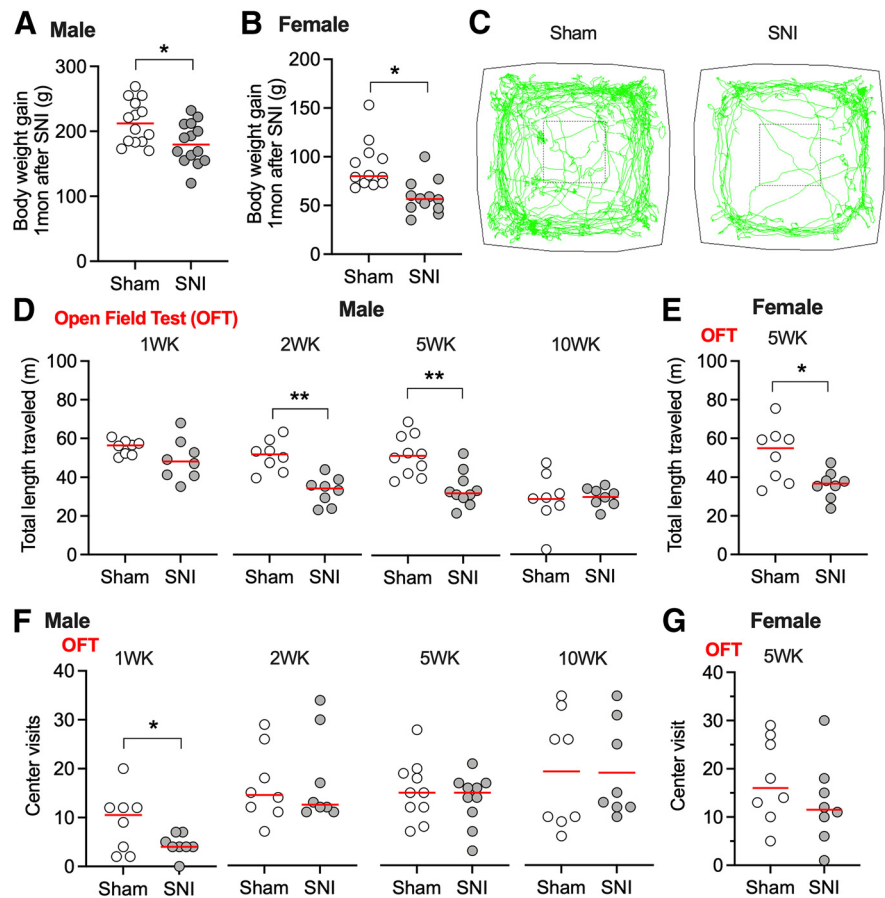


Figure 2. Body weight and open field testing in male and female rats with Sham or painful nerve injury. **A, B**, Body weight gain in 1 month after nerve injury in male (**A**; $t_{(26)} = 2.6$, $*p = 0.015$, two-tailed t test), and female (**B**) rats ($t_{(22)} = 3.68$, $***p = 0.001$, two-tailed t test). **C**, Representative travel tracing in OFT from rats with Sham or SNI surgery. **D, E**, Sustained pain after SNI reduced motor activity in both male (**D**; $t_{(14)} = 4.8$, $***p = 0.003$ in rats 2 weeks after injury, $t_{(18)} = 4.01$, $***p = 0.008$ in rats 5 weeks after SNI, two-tailed t test) and female (**E**; $t_{(14)} = 2.78$, $*p = 0.015$, two-tailed t test) rats in the open field test. **F, G**, SNI also reduced center visits in the early phase of pain for males (**F**; $t_{(14)} = 2.4$, $*p = 0.042$ in rats 1 week after injury), but unchanged center visit in the late phase of pain in both male (**F**) and female (**G**) rats.

Devices). Series resistance (5–10 M Ω) was monitored before and after the recordings, and data were discarded if the resistance changed by 20%.

Excitatory postsynaptic currents (EPSCs) were recorded from mPFC pyramidal neurons, which were identified by spike frequency adaptation during current clamp (Hill et al., 2011), while electrical stimulation was delivered by a bipolar tungsten stimulation electrode (World Precision Instruments) that was placed 100 μ m dorsal part of the recording, using square pulses (duration, 100 μ s; interval, 4–10 s). GABA_A receptor blocker picrotoxin (50 μ M) was present in the aCSF when necessary. Glass pipettes (3–5 M Ω) were filled with an internal solution containing the following (in mM): 135 potassium gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 MgATP, 0.3 Na₂GTP, and 10 Na₂-phosphocreatine (pH 7.2, with KOH). Evoked inhibitory postsynaptic currents (IPSCs) were recorded in the same pattern as for EPSC. Glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX; 20 μ M) and D-2-amino-5-phosphonovaleric acid (D-AP-5; 50 μ M) were present in the aCSF. The internal solution in patch pipettes contained the following (in mM): 80 potassium gluconate, 60 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 MgATP, 0.3 Na₂GTP, and 10 Na₂-phosphocreatine (pH 7.2, with KOH). To examine the effects of WIN 55212–2 on IPSCs, IPSCs were evoked at 10 s intervals. Input-output curves of EPSCs and IPSCs were generated using incremental stimulus intensities of 10–160 mA. For recording of miniature EPSCs and miniature IPSCs, tetrodotoxin was added in the aCSF to block action potentials. In some studies, depolarization-induced suppression of inhibition (DSI) was induced

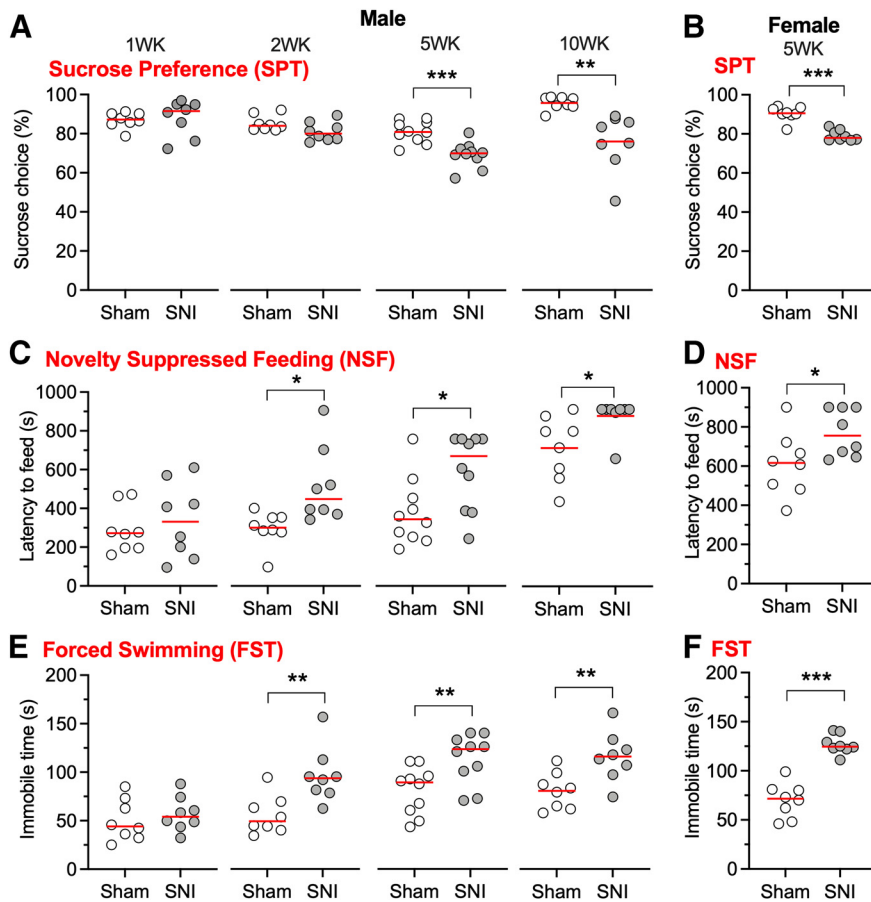


Figure 3. Sustained pain (SNI) induces depression-like behaviors. **A**, Male rats with SNI showed decreased preference to sucrose solution in SPT 5 weeks ($t_{(18)} = 4.39$, $***p = 0.0004$, two-tailed t test) and 10 weeks ($t_{(14)} = 3.74$, $**p = 0.0022$, two-tailed t test) after injury. **B**, Female rats with SNI showed decreased preference to sucrose solution in SPT 5 weeks ($t_{(14)} = 6.87$, $***p < 0.0001$, two-tailed t test) after injury. **C**, Male rats with SNI showed increased latency to food in a novel environment in the NSF test 2 weeks ($t_{(14)} = 2.89$, $*p = 0.012$, two-tailed t test), 5 weeks ($t_{(18)} = 2.62$, $*p = 0.017$, two-tailed t test) and 10 weeks ($t_{(14)} = 2.48$, $*p = 0.027$, two-tailed t test) after injury. **D**, Female rats showed increased latency to food in a novel environment in the NSF test 5 weeks ($t_{(14)} = 2.25$, $*p = 0.041$, two-tailed t test) after injury. **E**, Male rats with SNI showed increased immobility time in FST test 2 weeks ($t_{(14)} = 3.3$, $**p = 0.0045$, two-tailed t test), 5 weeks ($t_{(18)} = 2.93$, $**p = 0.009$, two-tailed t test) and 10 weeks ($t_{(14)} = 3.15$, $**p = 0.007$, two-tailed t test) after injury. **F**, Female rats showed increased immobility time in FST 5 weeks ($t_{(14)} = 7.96$, $**p < 0.0001$, two-tailed t test) after injury.

by depolarization from -60 to 0 mV for 5 s with an internal solution containing the following (in mM): 80 Cs-methanesulfonate, 60 CsCl, 2 QX-314, 10 HEPES, 0.2 EGTA, 2 MgCl_2 , 4 Mg-ATP , 0.3 Na_2GTP , and 10 Na_2 -phosphocreatine, pH 7.2, with CsOH.

fMRI

Blood oxygen level-dependent (BOLD) fMRI responses to noxious hind-limb stimulation were described in our previous report (Pawela et al., 2017). Briefly, each animal underwent an MRI immediately after the anesthetization with 1–2% isoflurane. After the animal was placed in the MR system, isoflurane was discontinued, and dexmedetomidine hydrochloride ($50 \mu\text{g/kg/h}$, DexDomitor, Zoetis) and pancuronium bromide (2 mg/kg/h , Sigma-Aldrich) were administered intravenously to maintain the anesthetic condition. To confirm intact physiological function, visual stimulation with LEDs with a wavelength of 465 nm was tested before limb stimulation to assess the BOLD signal response in each animal. Core body temperature was maintained at 37°C using a small-animal MR compatible heater/air blowing unit (SA Instruments). All experiments were conducted using a 9.4 T small-animal MRI scanner (AVANCE, Bruker). A surface receive coil (catalog #T9208, Bruker) and a linear transmit coil (catalog #T10325, Bruker) were used during the MR imaging protocol. Functional images were acquired using a Gradient Echo-Planar Imaging sequence with the following parameters: repetition time =

2 s, echo time = 18.76 ms, field of view = 35 mm, and 96×96 matrix (zero filled to 128×128 matrix). Acute noxious stimulation using a square-wave electrical generator (S88, Grass Telefactor) consisted of square-wave pulses at 10 Hz frequency, 3 ms pulse width, and 5 mA amplitude, which was administered in a standard block design protocol initiated with 40 s rest followed by three periods of 20 s ON and 40 s OFF (total of 3 min 40 s). Electrical stimulation was triggered by a transistor-transistor logic pulse from the scanner.

Chemicals

All drugs were prepared as concentrated stock solutions and stored at -20 or -80°C before use. All common chemicals were purchased from Sigma Aldrich. CNQX- Na_2 (Sigma-Aldrich) and D-AP-5 (Tocris Bioscience) were dissolved in water. Picrotoxin (Sigma Aldrich) was dissolved in aCSF through sonication. Gabapentin (Sigma Aldrich) was dissolved in normal saline. WIN-55212-2 (WIN), AM4113, and 2-arachidonyl glycerol (2-AG) were obtained from Tocris Bioscience. WIN, AM4113, and 2-AG were dissolved in dimethyl sulfoxide (DMSO). When these drugs were applied to slices, control slices were treated in the same concentration of the respective solvent for a similar exposure time. When these drugs dissolved in DMSO were applied to rats *in vivo*, they were diluted to 20% DMSO/normal saline.

Statistics

Sample sizes were set by the power analysis using our pilot data or previous data. Significance testing and calculation of half-maximal effective concentration (EC_{50}) of WIN on eIPSC was performed with Prism 8 (GraphPad) software. To compare changes from the baseline before stimulation, responses to pin, brush, and cold were evaluated non-parametrically using Friedman repeated-measures ANOVA with *post hoc* Dunn's test. Responses to von Frey were evaluated using repeated-measures ANOVA with *post hoc* comparisons using Dunnett's test. For comparison between groups, t test, paired t test, one-way ANOVA with *post hoc* Tukey's test, repeated-measures ANOVA (RM-ANOVA) with *post hoc* Dunn's test, and two-way ANOVA were used and are specified in each figure. Significance testing of $p < 0.05$ was considered significant. Data are reported as mean $\pm$ SD if they are not specified.

Results

Pain induces depression-like behaviors

SNI was performed on rats to establish a pain-induced depression phenotype, which was confirmed via depression-like and nociceptive behavioral tests. To avoid the effects of repeated testing, each time point used a cohort of animals to examine a depression-like phenotype after SNI (Fig. 1A,B). SNI reliably induced neuropathic pain as shown by the increased hyperalgesic response to noxious stimulus (pin), thermal sensitization to cold (acetone), and mechanical stimuli (brush, von Frey) in both male (Fig. 1C–F) and females (Fig. 1G–J). Consistent with the depression symptom of body mass loss, both male and female

rats with a painful nerve injury showed a slight decline in weight gain compared with Sham control counterparts across a span of 1 month after SNI (Fig. 2*A,B*). The open field test (OFT) was performed 1 week after SNI to assess locomotor activity and anxiety-related behaviors. A reduction in both total distances traveled and crossing time to the center was observed in male and female rats with SNI compared with Sham (Fig. 2*C–G*). A core symptom of depression is anhedonia, which can be assessed via the sucrose-preference test (SPT). Both male and female rats with SNI showed a reduced sucrose intake compared with Sham controls (Fig. 3*A,B*). NSF was used to measure depression and anxiety by forcing the rat to choose between eating or avoiding a novel environment. SNI significantly increased the latency to feed in the novel environment in both male and female rats (Fig. 3*C,D*). Finally, the forced swimming test (FST) detects depression-like behavior via increased immobility, which was observed after SNI in both male and female rats (Fig. 3*E,F*). Thus, our SNI model is followed by the development of multiple behavioral indicators of a depression phenotype.

As stress can induce depression in rats (McEwen et al., 2015), pain, being a potential stressor, must be considered. Stress is characterized by activation of the HPA axis, and animals exposed to chronic stress show adrenal hypertrophy and thymus hypotrophy. We found that adrenal and thymus gland weights were unchanged after SNI in both male and female rats (Fig. 4*A–D*).

As the observed depression behaviors might be a consequence of concurrent pain rather than from depression, the depression tests were performed when pain is controlled by simultaneous administration of the established analgesic GBP (Fig. 5*A*) as well as in the absence of GBP. Another set of experiments with chronic administration of GBP per os (p. o.) was also performed to test whether those depression behaviors can be prevented if the pain is well controlled and to further test the concept that pain causes depression (Fig. 5*B*). To optimize timing of the analgesic effect of GBP (100 mg/kg, i.p.), we measured the timing of its effects on hyperalgesia measured by the pin test and on mechanical hypersensitivity during the von Frey test (Fig. 5*C,D*). These both showed maximum effects at 1 h after injection, so GBP was given 1 h before the animal was tested in SPT, NSF, and FST. As SPT requires 24 h, intradermal perfusion with an osmotic pump was used and showed analgesic effects (Fig. 5*E,F*). Continuous oral administration of GB reversed hyperalgesia and allodynia (Fig. 5*G–J*). Depression-like behaviors (SPT, NSF, FST) were not altered by acute administration of GBP (Fig. 5*K–M*), although GBP treatment increased mobility in the open field test (Fig. 5*N,O*), supporting the interpretation that these behaviors are attributable to depression rather than simply being the direct result of pain. When the pain was well controlled for a prolonged period of time (4–5 weeks) by continuous administration of GBP, depression-like behaviors (SPT, NSF, and FST) did not develop (Fig. 5*K–M*), supporting the idea that pain is the underlying cause of depression.

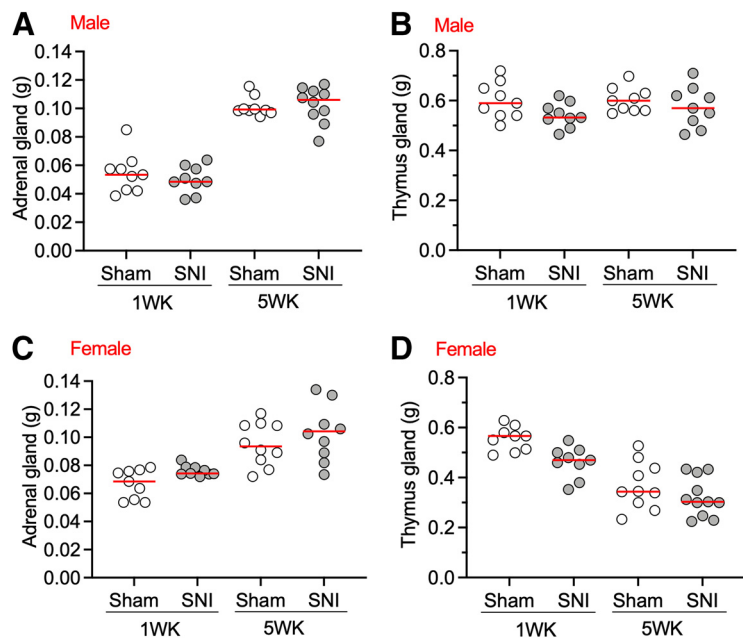


Figure 4. Effects of neuropathic pain on weights of adrenal and thymus glands. ***A–D***, In male rats, SNI did not increase adrenal weight (***A***; two-way ANOVA, $F_{(1,34)} = 0.77$, $p = 0.39$) or thymus weight (***B***; two-way ANOVA, $F_{(1,34)} = 4.6$, $p = 0.06$) compared with time-matched controls, as was also observed in female rats in adrenal weight (***C***; two-way ANOVA, $F_{(1,35)} = 0.84$, $p = 0.36$) or thymus weight (***D***; two-way ANOVA, $F_{(1,34)} = 1.08$, $p = 0.3$).

SNI induces initial hyperactivity of mPFC pyramidal neurons but eventually hypoactivity with persistent pain

The hypothesized trigger for mPFC plasticity during pain is input from the thalamus, driven by ascending nociceptive activity. There is an established temporal link between spontaneous pain and mPFC activation in chronic pain patients (Baliki et al., 2006). To confirm this in a rat model, we used fMRI, which showed strong mPFC activation during noxious stimulation of the paw of naive rats (Fig. 6*A*). SA in ascending nociceptive neurons from a site of injury may provide increased afferent input to the CNS (Chao et al., 2020). *In vivo* teased dorsal root fiber recordings confirmed a high incidence of ongoing SA after SNI, whereas animals with sham surgery had a low incidence of SA, which was evident during the initial onset (day 3) after SNI and continued through the later stage (day 35; Fig. 6*B,C*). Next, expression of the immediate early gene *c-fos* was assessed at these time points. Immunohistochemistry and qRT-PCR data showed increased protein and messenger ribonucleic acid (mRNA) levels of the immediate early gene *c-fos*, early after SNI (Fig. 6*D–F*). Costaining with CaMKII, which is exclusively expressed in glutamatergic neurons in the cortex, and GAD-67, which is expressed in GABAergic neurons, revealed that only glutamatergic neurons were activated 3 d after SNI compared with Sham controls (Fig. 6*G–J*). There was no difference between rats with and without SNI in the numbers of *c-fos* positive (*c-fos*⁺) neurons and the percentage of *c-fos*⁺ neurons in GAD⁺ neurons 35 d after surgery (Fig. 6*K,L*).

To directly examine the effect of neuropathic pain on pyramidal neuron activity, spontaneous firing of pyramidal neurons in the mPFC were recorded in anesthetized rats with sharp electrodes (Fig. 7*A–D*). These studies revealed that SNI rats had elevated firing rates compared with control animals early after SNI but then showed decreased firing compared with controls in the persistent phase (Fig. 7*D*). To further confirm that those with recorded pyramidal neuron activity after painful nerve injury are comparable to those with activity by peripheral

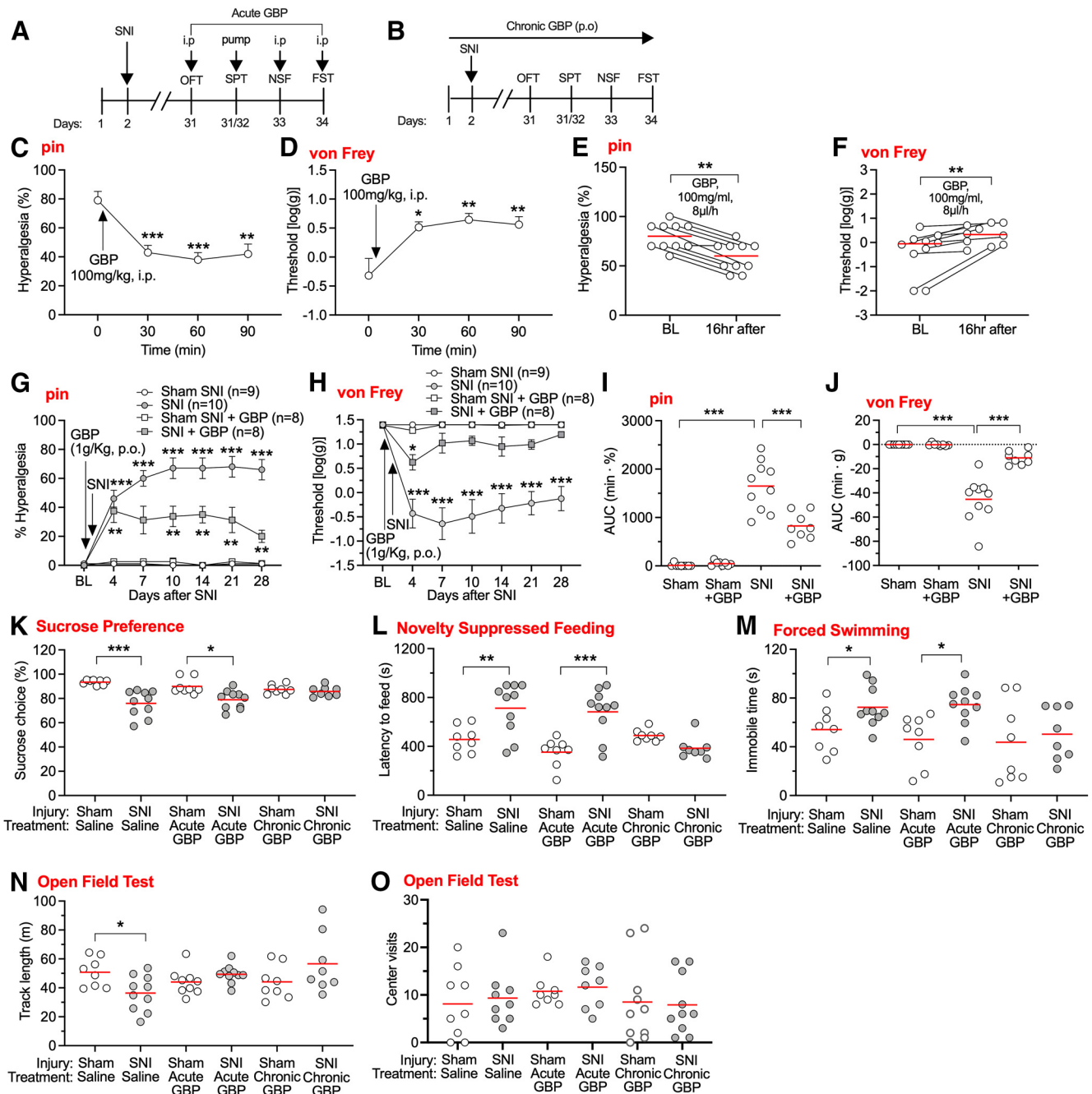


Figure 5. Development of depression-like behaviors is interrupted when pain is controlled. **A**, Timeline of behavioral tests with acute administration of GBP. **B–D**, Timeline of behavioral tests with chronic administration of GBP. The time course for the onset of analgesia by GBP (100 mg/kg, i.p.) in SNI animals is shown for pin (**C**; one-way RM-ANOVA followed by Dunnett's test, $F_{(1,464, 13,17)} = 25.45$, $p < 0.0001$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs baseline at 0 min) and von Frey test (**D**; $n = 10$ rats; one-way RM-ANOVA followed by Dunnett's test, $F_{(1,274, 11,47)} = 14.83$, $p = 0.0016$, $^{*}p < 0.05$, $^{**}p < 0.01$ vs baseline at 0 min). **E**, **F**, Chronic perfusion of GBP with implanted osmotic pumps for 16 h can also reduce hypersensitivity to the pin (**E**; $t_{(9)} = 6.03$, $p = 0.0002$, paired t test) and von Frey test (**F**; $t_{(9)} = 3.25$, $^{**}p = 0.01$ by the paired t test). **G–J**, Chronic administration of GBP in the cage water bottle also reduces hypersensitivity to the pin (**G**) and von Frey test (**H**; two-way RM-ANOVA followed by Dunnett's test, $F_{(18,186)} = 11.93$, $p < 0.0001$ for pin test; $F_{(18,186)} = 6.279$, $p < 0.0001$ for von Frey test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), and AUC analysis for the pin test (**I**) and von Frey test (**J**; two-way ANOVA followed by Dunnett's test, $F_{(1,31)} = 17.00$, $p < 0.0001$ for pin test; $F_{(1,31)} = 25.00$, $p < 0.0001$ for von Frey test, $^{***}p < 0.001$). **K**, Acute administration of GBP has no effects on SPT, and chronic GBP blocks development of reduced sucrose preference by injury (two-way ANOVA followed by the Sidak test, $F_{(1,46)} = 17.16$, $p = 0.0001$ $^{*}p = 0.017$, $^{***}p < 0.001$). **L**, Acute administration of GBP showed no effects on NSF, and chronic GBP reversed development of NSF (two-way ANOVA followed by the Sidak test, $F_{(1,46)} = 15.4$ between with and without SNI, $p = 0.0003$, $^{**}p = 0.002$, $^{***}p < 0.001$). **M**, Acute administration of GBP has no effect on FST, and chronic GBP reversed development of FST after neuropathic pain (two-way ANOVA followed by the Sidak test, $F_{(1,46)} = 9.086$ between with and without SNI, $p = 0.0042$, $^{*}p = 0.029$ between Sham + saline and SNI + saline, $p = 0.019$ between Sham + acute GBP and SNI + acute GBP). **N**, GBP can improve impaired motor activity in OFT (two-way ANOVA followed by the Sidak test, $F_{(2,47)} = 5.63$ between with and without SNI, $p = 0.0064$, $^{*}p = 0.047$ between with and without SNI). **O**, GBP didn't have effects on the time spent in the center. Data are presented as mean $\pm$ SD.

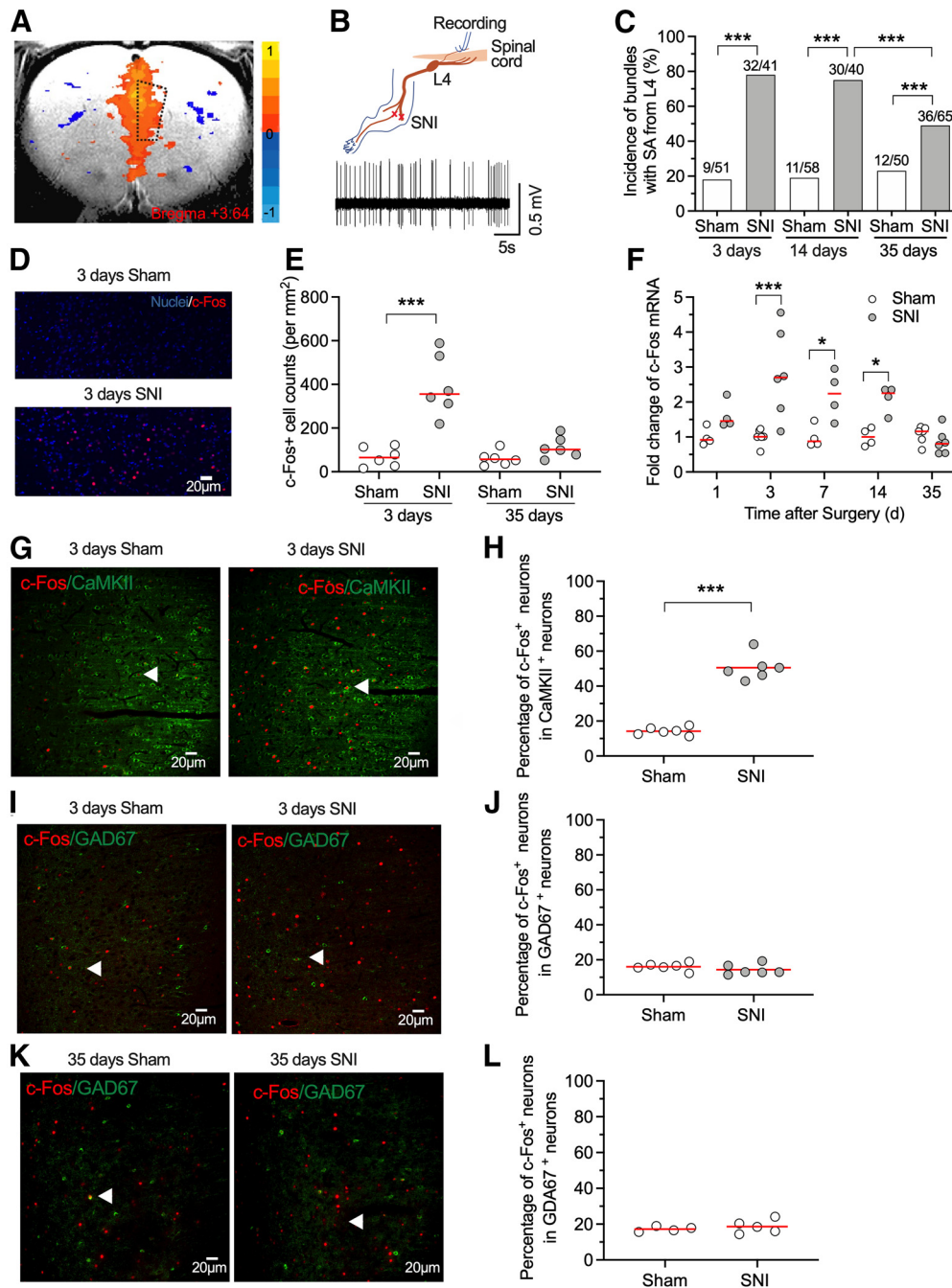


Figure 6. Painful nerve injury can activate mPFC. **A**, Noxious electrical stimulation (10 Hz, 3 ms duration, 5 mA amplitude) to one hindpaw activates the mPFC bilaterally (dotted line, contralateral prelimbic mPFC) with blood oxygen level-dependent fMRI. The colors represent calculated correlation coefficients scaled to -1.0 and 1.0 , representing activity from low to high. Averaged activation map for $n = 18$ rats. **B**, Dorsal root was teased and recorded from rats with and without SNI (top). Representative traces showed spontaneous firings (bottom). **C**, Injury increased the incidence of SA. $***p < 0.001$ by planned comparison with Bonferroni's correction. $n = 3$ rats for each group. **D**, The c-fos protein levels were increased in mPFC 3 d after the SNI surgery. **E**, Summary of c-fos staining (one-way ANOVA followed by Tukey's test, $F_{(3,20)} = 24.18$, $p < 0.0001$, $***p < 0.0001$). **F**, The c-fos mRNA levels were increased in mPFC 3 d after the SNI surgery (two-way ANOVA followed by Sidak *post hoc* test, $F_{(4,28)} = 5.95$, $p = 0.0013$, $*p = 0.024$ vs 7 d control, $*p = 0.045$ vs 14 d control, $***p < 0.0001$ compared with 3 d control). **G**, Costaining of c-fos with CaMKII, a marker of glutamatergic neurons of the cortex, from Sham rats (left) and rats 3 d after nerve injury (right). **H**, Percentage of c-fos⁺ cells in all CaMKII⁺ cells. **I**, Costaining of c-fos with GAD67, a GABAergic neuron marker, from Sham rats (left), and rats 3 d after nerve injury (right). **J**, Percentage of c-fos⁺ cells in all GAD67⁺ cells. $***p < 0.001$, by nonparametric Kolmogorov–Smirnov test. $n = 6$ rats per group. Arrows indicate one sample coexpression of c-fos and CaMKII and GAD67, respectively. **K**, Costaining of c-fos with GABAergic neuron marker, GAD67, from Sham rats, and rats with SNI 35 d after surgery. Arrows indicate one sample coexpression of c-fos and CaMKII and GAD67, respectively. **L**, Percentage of c-fos⁺ cells in all GAD67⁺ cells. Each dot represents one rat.

noxious stimulation, noxious electrical stimulation was delivered to paws of naive rats while recording from mPFC neurons. This stimulation (three trains of 10 Hz, 20 s, with intensity of 5 mA) acutely increased pyramidal neuron activity (Fig. 7E–G), and the firing rates (2.59 ± 0.57 Hz, $n =$

19 neurons from 4 control rats) are comparable to those in neurons recorded in SNI rats (3.33 ± 0.31 Hz, $n = 63$ neurons from 10 rats). By taking advantage of recordings from free-moving rats with implanted MEA, which can provide real-time change of neuronal activity and avoid the impact

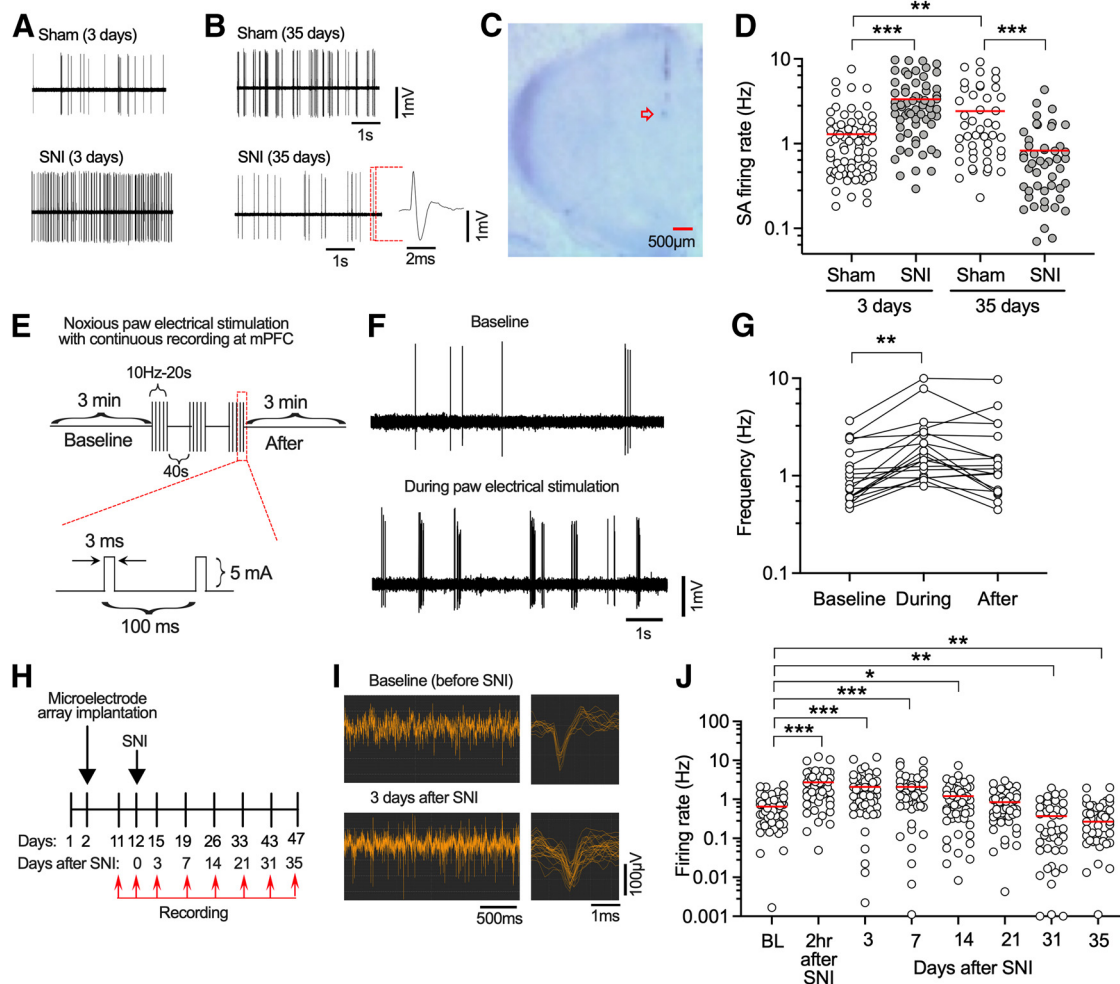


Figure 7. Dynamic change of neuronal activity in mPFC after painful nerve injury. **A, B**, Sample single-unit firing of pyramidal neuron (layer V/VI) from rats with neuropathic pain initial (**A**) and later (**B**) after SNI surgery. Typical individual spike of a pyramidal neuron with spike width $>500 \mu\text{s}$ and firing rate $<10 \text{ Hz}$. **C**, Histologic verification of recording site with trypan blue injected with iontophoresis. **D**, Spike frequency (5 min recording) was increased initially but decreased when pain was persistent (two-way ANOVA followed by Sidak *post hoc* test, $F_{(1,225)} = 53.15$, $p < 0.0001$, $**p = 0.0082$, $***p < 0.0001$). $n = 10$ rats per group. **E**, Noxious electrical stimulation waveform and timeline of recordings. **F**, Sample single-unit firing of pyramidal neuron (layer V/VI) from naive rats before (top) and during (bottom) noxious stimulation to the hindpaw. **G**, Noxious stimulation to hindpaw increased SA firing rates of mPFC neurons (one-way RM-ANOVA followed by Tukey's *post hoc* test, $F_{(2,36)} = 6.776$, $p = 0.0032$, $**p = 0.0023$). **H**, Timeline for the recordings with MEA in free-moving rats. **I**, Sample recordings with MEA (left) and spikes detected in 10 s recordings (right) before and after SNI from the same recording channel. **J**, SNI increased neuronal activities initially and reduced them when pain was persistent [one-way RM-ANOVA followed by Dunnett's *post hoc* test, $F_{(3,348, 162.6)} = 17.05$, $p < 0.00012$, $***p < 0.0001$ baseline (BL) vs 2 h, $***p = 0.0004$ BL vs 3 d, $***p = 0.0002$ BL vs 7 d, $*p = 0.0237$ BL vs 14 d, $**p = 0.003$ BL vs 31 d, $**p = 0.002$ BL vs 35 d]. $n = 4$ rats. Red bars represent means.

of anesthetics used in *in vivo* recordings with sharp electrodes, we found that SNI rats had elevated firing rates early after SNI but then decreased firing in the persistent phase (Fig. 7H–J). From these findings, we conclude that ongoing mPFC hyperactivity occurs initially after injury, followed by delayed hypoactivity during sustained neuropathic pain.

Disrupted synaptic transmission contributes to the abnormal neuronal activity

Neuronal activity plays a critical role in regulating synaptic transmission, and shifts in synaptic strength can also regulate neuronal activity. Excitatory and inhibitory innervation of pyramidal neurons was measured at 3 d and 35 d after SNI, by determining input/output relationships for (1) evoked EPSCs with picrotoxin block of GABA_AR and (2) evoked IPSCs with CNQX block of AMPA receptor, AP-5 block of NMDA receptor in mPFC slice layer V pyramidal neurons, and identified by spike frequency adaptation during current clamp (Hill et al., 2011). Three d after

SNI, the evoked EPSC was elevated (Fig. 8A), but the evoked IPSC was normal (Fig. 8B) versus sham, indicating increased evoked excitatory synaptic transmission that could contribute to the increased resting activity of mPFC neurons (Fig. 7). Thirty-five days after SNI, the evoked EPSC was normal (Fig. 8C), but the evoked IPSC was elevated (Fig. 8D) versus sham, indicating increased evoked inhibitory synaptic transmission that could contribute to the decreased resting activity of mPFC neurons (Fig. 7).

Evoked synaptic transmission can only provide information on transmitter release during induced activity, whereas the resting level of synaptic transmission (spontaneous and miniature IPSC) will provide further information on altered transmitter release that may contribute to regulating resting neuronal activity (Zucker, 2005; Gerkin et al., 2013). As miniature currents represent quantal release of neurotransmitters, change in amplitude is interpreted as a change in postsynaptic function, whereas change in frequency represents a change in presynaptic neurotransmitter release,

according to quantal theory of neurotransmitter release (Del Castillo and Katz, 1954; Stevens, 1993; Choi and Lovinger, 1997). We found that the frequency, not amplitude, of mIPSC, was increased 35 d after nerve injury (Fig. 8E–I), consistent with increased inhibitory presynaptic transmitter release as a contribution to the decreased postsynaptic neuronal activity.

SNI dynamically alters eCB signaling

In our previous report, we found that eCB/CB1R signaling predominantly regulates inhibitory (not excitatory) synaptic transmission in mPFC and that activation of CB1Rs in GABAergic presynaptic terminals reduces IPSCs (Hill et al., 2011). Increased eCB/CB1R signaling at GABAergic synapses in the mPFC might contribute to the increased neuronal activity in the initial stage of neuropathic pain (Fig. 7). At the same time, we expect eCBs will be elevated initially after SNI as mPFC pyramidal neuron activity is driven by thalamic input, and eCBs are synthesized on demand on neuronal activity (Araque et al., 2017). We measured 2-AG and AEA concentrations in mPFC and found elevated 2-AG in the mPFC 3 d after SNI surgery (Fig. 9A). The mRNA levels of receptor CB1R were transiently increased after injury (Fig. 9B). The mRNA levels of the 2-AG synthesizing enzyme DAGL α was transiently increased after injury, consistent with increased 2-AG flux (Fig. 9C). The degradation enzyme MAGL was not affected by SNI or age (Fig. 9D). AEA and its synthetic enzyme NAPE-PLD were not affected by SNI or age (Fig. 9E,F).

If the increased 2-AG in mPFC after SNI contributes to the development of depression, long-term activation of CB1Rs in mPFC should mimic the process of elevated eCB after neuropathic pain. Daily microinjection of 2-AG in the mPFC (0.5 μ g/ μ l/side) for 14 d induced depression behaviors (Fig. 10A–D). On the other hand, interrupting the activated eCB signaling early after painful injury should reduce the development of depression. A low dose of a CB1R neutral antagonist, AM4113, which does not cause depression-like behavior alone (He et al., 2019), was microinjected (1 μ g/ μ l/side) into the mPFC beginning 1 d before nerve injury and continued for 21 d to cover the whole period of time with elevated eCB signaling. This treatment blocked SNI-induced depression behaviors (Fig. 10E–H). Intraperitoneal injection of AM4113 (2 mg/kg body weight) immediately after SNI surgery for 3 weeks reversed the reduced neuronal activity by SNI (Fig. 10I,J). These findings suggest a key role of CB1Rs in the development of pain-induced depression.

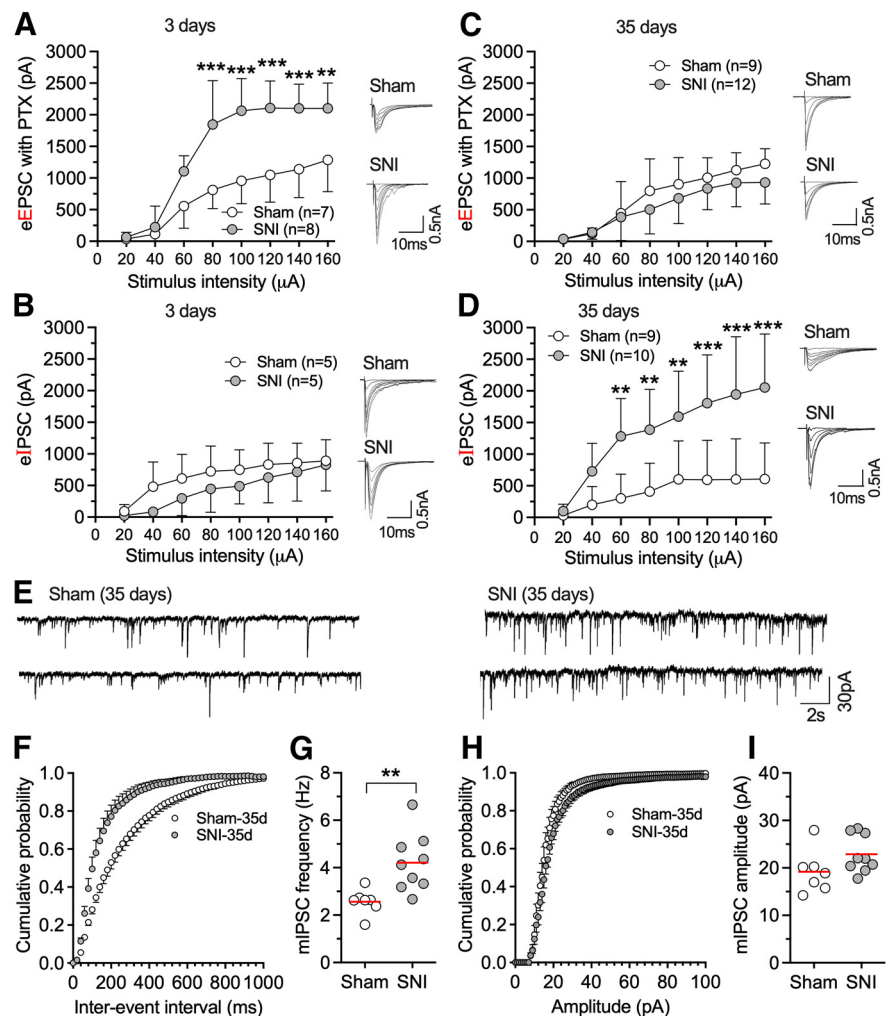


Figure 8. Painful nerve injury altered synaptic transmission to pyramidal neurons in mPFC. **A**, Excitatory synaptic transmission was increased 3 d after SNI (two-way RM-ANOVA followed by Sidak *post hoc* test, $F_{(7,77)} = 6.232$, $p < 0.0001$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with stimulating intensity-matched control; $n = 6$ rats per group). **B**, Inhibitory synaptic transmission was unchanged 3 d after SNI (two-way repeated-measures ANOVA, $F_{(7,56)} = 1.537$, $p = 0.174$; $n = 5$ rats per group). **C**, Excitatory synaptic transmission returned to normal 35 d after SNI (two-way RM-ANOVA, $F_{(7,133)} = 1.7$, $p = 0.1139$; $n = 7$ rats per group). **D**, Inhibitory synaptic transmission was elevated 35 d after SNI (two-way RM-ANOVA followed by Sidak *post hoc* test, $F_{(7,119)} = 12.04$, $p < 0.0001$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with stimulating intensity-matched control; $n = 7$ rats per group). **E**, Representative traces of mIPSC from rats 35 d after SNI and time-matched control rats with sham injury. **F**, **G**, Cumulative probability (**F**) and average (right) frequency (**G**; $t_{(14)} = 3.32$, $^{**}p = 0.0051$ by *t* test) of mIPSC; $n = 7$ rats. **H**, **I**, Cumulative probability (**H**) and average amplitude (**I**) of mIPSC. Data in **A**, **B**, **C**, **D** are presented as mean $\pm$ SD.

Activity-dependent loss of eCB signaling function contributes to pain-induced depression

As with other G-protein-coupled receptors, persistent activation of CB1Rs on agonist binding is known to cause both desensitization and internalization, resulting in loss of function (LOF) of eCB signaling (Sim-Selley et al., 2006; Martini et al., 2007; Wu et al., 2008). Given that most CB1Rs in the mPFC are on GABAergic terminals, we predict that GABA release will be disinhibited, resulting in increased GABAergic innervation and decreased activity of mPFC pyramidal neurons. To examine the number of functional CB1Rs, we tested the effects of the CB1R agonist WIN (1 μ M) on eIPSCs. Although the effect of WIN in suppressing eIPSC from mPFC pyramidal neurons was not altered by nerve injury at 3 d after SNI (Fig. 11A,B), the effect of WIN (1 μ M) was significantly reduced in slices from rats 35 d after SNI (Fig. 11C,D). To further explore the

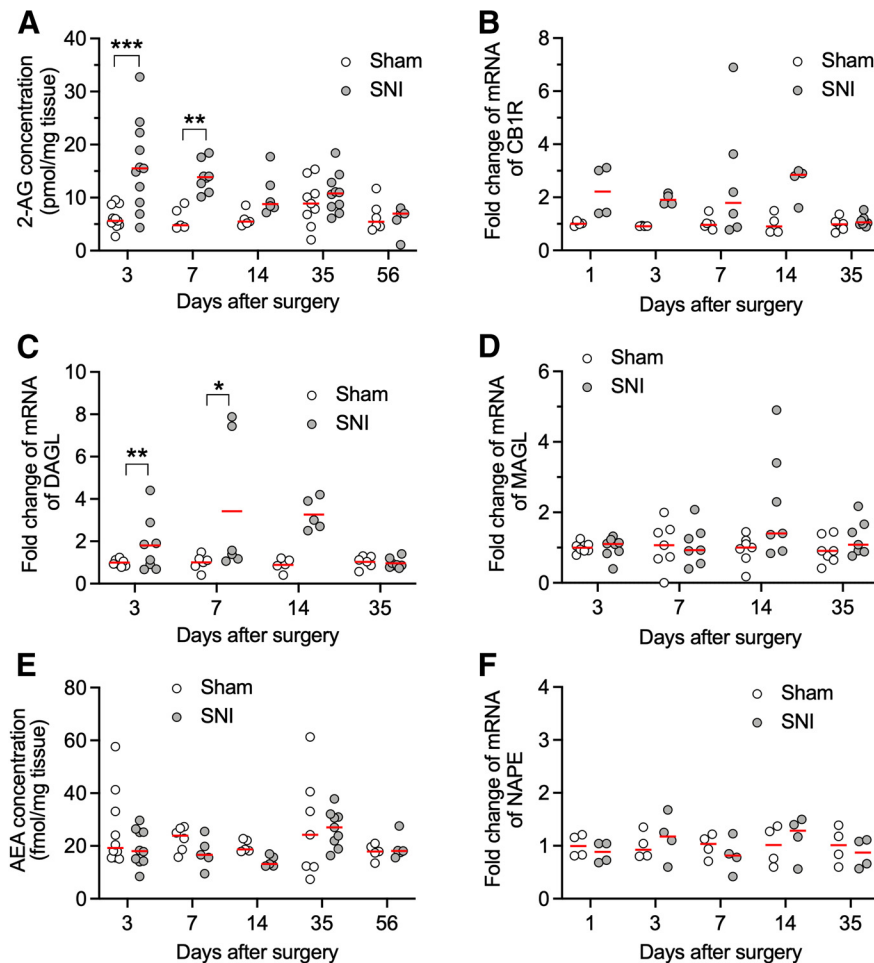


Figure 9. Dynamic change of eCB signaling in mPFC after painful nerve injury. **A**, Initially elevated 2-AG level in mPFC after SNI (two-way ANOVA followed by Sidak *post hoc* test, $F_{(4,66)} = 3.938$, $p = 0.0063$, *** $p = 0.0096$, *** $p < 0.001$ compared with age-matched Sham controls). **B**, Expression of CB1Rs in mPFC after SNI. **C**, Initially elevated DAGL levels in mPFC after SNI (two-way ANOVA followed by Sidak *post hoc* test, $F_{(3,44)} = 2.918$, $p = 0.0415$, * $p = 0.019$, ** $p = 0.007$ compared with age-matched Sham controls). **D**, Temporarily elevated MAGL levels in mPFC after SNI (two-way ANOVA, $F_{(3,50)} = 2.616$, $p = 0.0612$). **E**, **F**, Expression of AEA (**E**) and its synthesis enzyme, NAPE-PLD (**F**) in mPFC after SNI (two-way ANOVA; $n = 4$ –8 rats per time point).

efficacy and potency of WIN on presynaptic vesicle release, effects of WIN with different doses (0.1 μM , 0.5 μM , 3 μM , 10 μM) were tested (Fig. 11E,F). WIN had greater efficacy on presynaptic vesicle release in slices from Sham rats than those from rats with SNI, but potency was similar (EC_{50} values, 0.43 μM in Sham, vs 0.50 μM in SNI; Fig. 11F). CB1Rs are in somatostatin and vasoactive intestinal polypeptide but not parvalbumin-positive interneurons (Bodor et al., 2005; Hill et al., 2007). Parvalbumin-positive interneurons consist of ~40% of GABAergic interneurons in the cortex. It is possible that changed GABA release from parvalbumin-positive interneuron terminals dilute the relative effects of WIN to baseline levels, shown in Figure 11A–E. We compared the input-output curve of WIN-sensitive eIPSC, which was the difference of input-output curves of eIPSC before and 20 min after perfusion of WIN (3 μM), between Sham and SNI. WIN showed less efficacy on GABA release in recordings from rats with SNI (Fig. 11G).

DSI is a form of short-term plasticity mediated by eCB, induced by depolarizing the postsynaptic neuron (Pan et al., 2009) and can be induced at mPFC synapses and is mediated by

2-AG (Hill et al., 2011). At 7 d after SNI, no difference in DSI was found between Sham controls and SNIs (Fig. 11H,I), although DSI was significantly reduced at 35 d after SNI (Fig. 11J,K).

As intra-mPFC injection of 2-AG mimicked the development of depression after SNI (Fig. 10), we used the same approach to ask whether there is LOF of eCB signaling in those animals with chronic elevation of mPFC 2-AG concentrations. The effects of WIN on eIPSC were significantly reduced in rats after 14 d of intra-mPFC injection of 2-AG (Fig. 11L,M).

Finally, we determined whether systemic administration of WIN (1 mg/kg, tail vein intravenous injection) to directly activate CB1R and suppress GABAergic inhibition could return the suppressed mPFC neuronal activity at 35 d after SNI to normal levels. In agreement with our hypothesis, WIN returned SA in rats 35 d after SNI to the same high activity seen in Sham control rats (Fig. 11N,O), compared with Figure 7D.

In summary, our data support the mechanism outlined in Figure 12A–D. At baseline, ongoing mPFC neuronal activity causes the generation of eCBs that suppress GABA inhibitory input onto pyramidal neurons from local interneurons, thus supporting mPFC outflow, which maintains a normal mood (Euthymia; Fig. 12B). At the onset of pain, noxious inputs activate the mPFC and drive eCB production, resulting in reduced GABA release and greater mPFC pyramidal neuron activity (Fig. 12C). However, the strong and consistent activation of CB1R signaling also results in enhanced inhibitory synaptic transmission through LOF of eCB signaling and/or homeostatic compensation by non-CB1R expressing interneurons in the persistent phase of pain to suppress mPFC function and produce depression-like behaviors (Fig. 12D).

Discussion

In this study, we showed that noxious inputs after painful nerve injury activated mPFC pyramidal neurons and initiated activity-dependent eCB synthesis, which chronically resulted in LOF of CB1R-mediated retrograde inhibitory synaptic transmission in the mPFC and thus contributed to the pathophysiology of depression after neuropathic pain.

mPFC neuronal activities in pain is debated. The mPFC is commonly found to be activated in experimental pain studies and shows increased activity in patients with chronic pain (Zhuo, 2008; Seminowicz and Moayed, 2017). In rats subjected to SNI, mPFC neurons show increased excitability regulated by hyperpolarization-activated cyclic nucleotide-gated channels

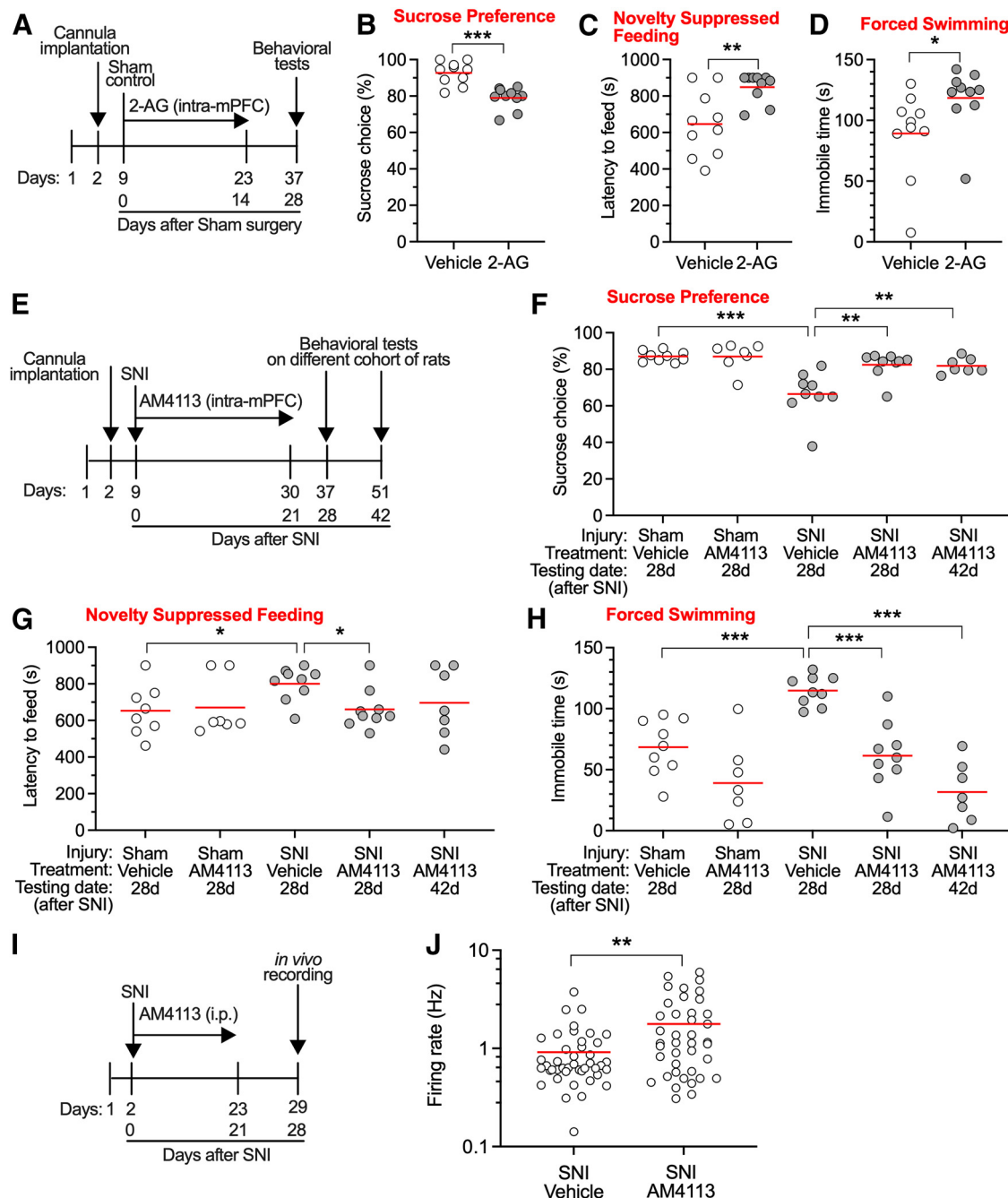


Figure 10. Increased 2-AG in mPFC after painful nerve injury contributes to the development of depression. **A**, Timeline for the intra-mPFC injection of 2-AG and behavior tests. **B–D**, SPT ($t_{(18)} = 5.04$, $p < 0.0001$, t test; **B**), NSF ($t_{(18)} = 3.3$, $p = 0.004$, t test; **C**), and FST ($t_{(18)} = 2.23$, $p = 0.04$, t test; **D**) showed depression phenotype after intra-mPFC injection of 2-AG in naive rats. **E**, Timeline for the intra-mPFC injection of AM4113 and behavior tests in rats with SNI. **F–H**, Intra-mPFC injection of AM4113 reversed depression after nerve injury represented in SPT (**F**; $p = 0.0008$ between Sham+vehicle and SNI+vehicle, $p = 0.004$ between SNI+vehicle and SNI+AM4113 28 d, $p = 0.007$ between SNI+vehicle and SNI+AM4113 42 d, by planned comparison with Bonferroni's correction), NSF (**G**; $p = 0.018$ between Sham+vehicle and SNI+vehicle, $p = 0.025$ between SNI+vehicle and SNI+AM4113 28 d, by planned comparison with Bonferroni's correction) and FST (**H**; $***p < 0.0001$ by planned comparison with Bonferroni's correction) tested 1 week or 3 weeks after the end of AM4113 injection in different cohort rats. Each dot represents one rat. **I**, Timeline for the intraperitoneal injection of AM4113 (2 mg/kg) and *in vivo* single-unit recordings in rats with SNI. **J**, Intraperitoneal injection of AM4113 reversed reduced neuronal activity after neuropathic injury; $t_{(81)} = 3.36$, $***p = 0.001$, by t test; $n = 6$ rats per group. Red bars represent mean.

(Cordeiro Matos et al., 2015). However, there are also reports indicating reduced mPFC neuronal activity in pain. Chronic pain patients given cognitive behavioral therapy have diminished depression and anxiety symptoms and are associated with increased prefrontal activity (Jensen et al., 2012). In the rat SNI model, reduced excitatory glutamatergic currents are found 7 d after injury (Kelly et al., 2016), and optogenetic activation of the PFC 4 weeks after the injury produces strong antinociceptive

effects and reduced affective symptoms of pain (Lee et al., 2015). In an acute arthritis model, mPFC neurons are deactivated immediately after arthritis induction caused by amygdala-driven feedforward inhibition (Ji et al., 2010; Ji and Neugebauer, 2011), which is also found 10 d after SNI in mice (Zhang et al., 2015). These divergent observations may be attributable to different pain models, timing, electrophysiological recording methods (*in vivo* vs *in vitro*) and

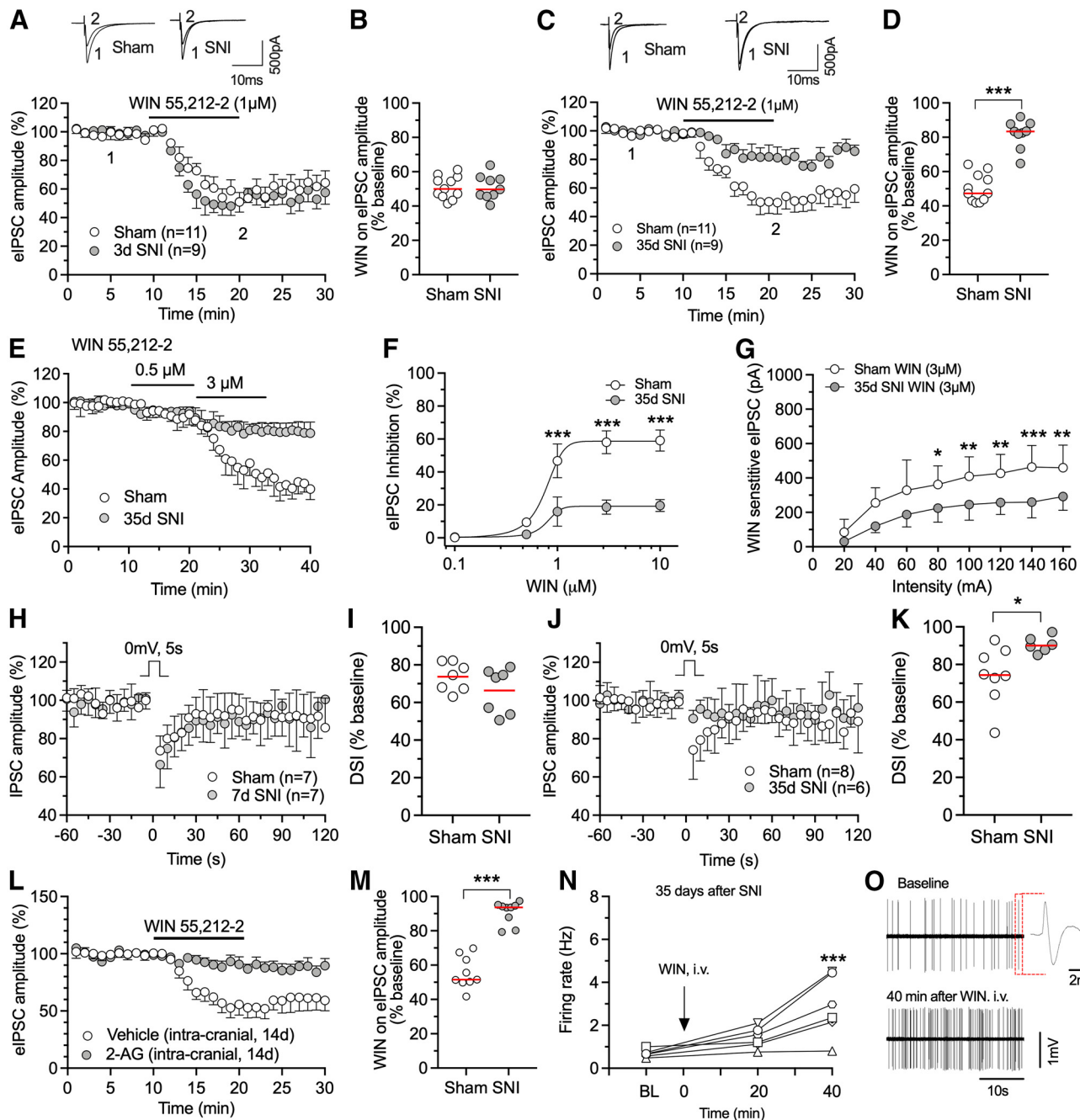


Figure 11. Activity-dependent loss of function of CB1Rs contributes to pain-induced depression. **A**, Suppression of IPSCs by CB1R agonist WIN 55,212-2 (WIN; bath application, 1 μ M) is not affected (vs control) 3 d after SNI. **B**, Summary of effects of WIN on eIPSC in slices 3 d after surgery. **C**, WIN (1 μ M) had less effects 5 weeks post SNI. **D**, Summary of effects of WIN on eIPSC in slices 35 d post surgery. *** $p < 0.0001$ by two-tailed t test, $n = 7$ rats for each group. **E**, WIN at various concentrations also had less effects 5 weeks after SNI compared with Sham. **F**, Dose-response curve of WIN on eIPSC (two-way ANOVA followed by Sidak *post hoc* test, $F_{(4,53)} = 16.58$, $p < 0.0001$, *** $p < 0.001$ compared with age-matched Sham controls, $n = 5$ –9 cells per concentration). **G**, WIN-sensitive currents were reduced in rats with SNI (two-way ANOVA followed by Sidak *post hoc* test, $F_{(1,14)} = 20.01$, $p = 0.0005$ between Sham and SNI, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Sham controls, $n = 8$ cells per group). **H**, **I**, Similar DSIs were induced in mPFC pyramidal neurons from rats SNI and Sham rats 7 d after surgery. **J**, **K**, DSI was diminished in rats 35 d after SNI compared with matched-time control rats ($t_{(12)} = 2.52$, * $p = 0.025$, t test). **L**, Reduction of eIPSC amplitude by bath application of WIN was diminished in slices from intra-mPFC WIN injected rats. **M**, Summary of effects of WIN on eIPSC in slices treated with 2-AG for 14 d ($t_{(16)} = 9.2$, * $p < 0.0001$, t test, $n = 8$ rats per group). **N**, CB1R agonist WIN (1 mg/kg, intravenous injection), returned SA in SNI rats to control levels (one-way RM-ANOVA, $F_{(2,10)} = 14.52$, $p = 0.001$, *** $p = 0.0007$ compared with baseline (BL), $n = 6$ rats). **O**, Sample traces of SA before and after WIN administration. Data are presented as mean $\pm$ SD.

recording from different PFC subregions (anterior cingulate cortex, prelimbic and infralimbic mPFC; Kummer et al., 2020). Our present study is the first to monitor the dynamic shifts of neuronal activity (Fig. 7) and excitatory/inhibitory synaptic transmission (Fig. 8) after painful nerve injury. Thus, in this nonstatic system, observations may

produce divergent findings depending on when recordings are performed.

There are data that HPA axis activation occurs in the context of chronic pain, such as fibromyalgia (Yalcin and Barrot, 2014). In support of this notion, next-generation RNA sequencing has detected increased expression of genes involved in glucocorticoid

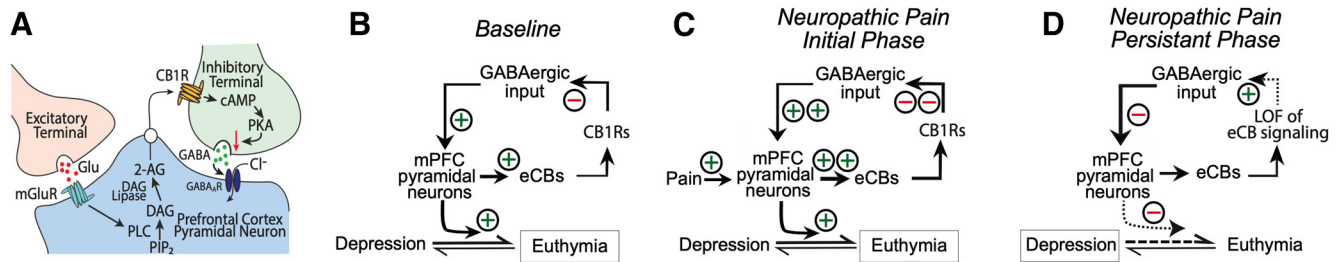


Figure 12. Model of mPFC regulation by eCB after pain. **A**, Synaptic model showing physiological functions of eCB signaling. Glutamate from nearby excitatory axonal terminals acts on metabotropic glutamate receptors (mGluRs) and activates G protein, which in turn activates phospholipase C (PLC). PLC cleaves phosphatidylinositol 1,4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate. DAG is subsequently converted into 2-AG by DAGL. 2-AG travels across the synapse and activates CB1 receptors on the inhibitory axon terminal to decrease GABA release. **B**, At baseline, projections from the mPFC maintain a normal mood (Euthymia). Ongoing mPFC neuron activity causes the generation of eCBs that suppress GABA inhibitory input from local neurons, thus supporting mPFC activity. **C**, Painful injury initially activates the mPFC, causing greater eCB production that further impedes GABAergic inhibition but also leads to LOF of CB1Rs. **D**, During persistent pain, LOF of CB1Rs breaks the feedback loop, leading to unopposed inhibitory input, suppression of mPFC function, and a shift to depression.

signaling in the limbic system of mice with SNI (Descalzi et al., 2017), and other studies provide evidence that stress can contribute to pain sensation and development of depression after pain by activating the HPA axis (Norman et al., 2010; Vachon-Preseu et al., 2013). These studies suggest the hypothesis that pain may induce a stress-like alteration in the HPA axis as an adaptive response (Li and Hu, 2016). On the other hand, multiple studies have found that HPA axis activity is not altered in peripheral neuropathic pain. In particular, neuropathic pain is not accompanied by changes in adrenocorticotrophic hormone or corticosterone concentrations or by activation of neurons in the hypothalamic paraventricular nucleus, which play a central role in initiating HPA axis stress response (Bomholt et al., 2005; Ulrich-Lai et al., 2006; Muhtz et al., 2013; Yalcin and Barrot, 2014), even when anxiety-like phenotypes are observed (Yalcin et al., 2011). Consistent with those reports, we found unchanged adrenal and thymus gland weights after SNI (Fig. 4), suggesting that the HPA axis was not chronically activated in our model. However, we observed decreased center visits in the open field test 1 week after painful nerve injury (Fig. 2), suggesting that the rats with SNI have increased anxiety. There is no agreement on anxiety-like phenotypes after neuropathic pain possibly because of different strains, time points after injury for the test, and nerve injury types (Kremer et al., 2021).

Because preclinical models demonstrate that the eCB signaling system controls mood, emotion, memory, cognition, stress, and pain processes via activation of CB1R (Samat et al., 2008; Hill et al., 2009), therapies targeted to activating the eCB signaling system have been proposed to treat these disorders. CB1Rs are abundant in GABAergic terminals mainly in layers II/III and V/VI of mPFC and other cortex (Bodor et al., 2005; Hill et al., 2011) and in somatostatin and vasoactive intestinal polypeptide, but not parvalbumin-positive interneurons (Bodor et al., 2005; Hill et al., 2007). Parvalbumin-positive interneurons consists of ~40% of GABAergic interneurons in the cortex, which is consistent with our electrophysiological recordings that WIN can maximally reduce the eIPSC amplitude by 60% in control conditions (Fig. 11). Thirty-five days after SNI, WIN-sensitive eIPSC amplitude is reduced by half compared with rats with sham surgery (Fig. 11), suggesting a 50% loss in eCB signaling. GABA release from non-CB1R-expressing interneurons might also be altered after SNI and contributes to the increased overall inhibition, which is an important question as subtypes of interneurons have distinct electrophysiological properties and different roles in cognitive functions (Kim et al., 2016). Further study will be needed to explore the contribution of non-CB1R-expressing interneurons to this process.

WIN's effects on eIPSC represent the process between the activation of CB1Rs and vesicles release (Fig. 12). We expect decreased WIN's effects 3 d after SNI as elevated 2-AG (Fig. 9) might occlude effects of WIN. However, WIN shows similar effects on eIPSC in Shams and SNI rats 3 d after surgery (Fig. 11), which might be because of increased CB1R levels (Fig. 9). DSI represents the whole process of 2-AG synthesis initiated by depolarization-induced Ca^{2+} entry, CB1R activation by 2-AG, and 2-AG hydrolysis by MAGL (Pan et al., 2009). Although we expect elevated DSI 7 d after SNI as there is increased 2-AG concentration and DAGL 7 d after SNI (Fig. 9), similar DSIs are found between Shams and SNIs (Fig. 11). Elevated 2-AG might occlude the effects of newly synthesized 2-AG induced by depolarization.

Our findings contribute to a body of work suggesting that LOF of eCB signaling in the mPFC as a result of excessive CB1R activation represents a final common pathway to the establishment of depression in response to multiple triggers. For example, long-term cannabis users develop depression-like symptoms (Volkow et al., 2014) and synthetic CB1R agonists produce anxiolytic- and antidepressant-like effects in animals (Berrendero and Maldonado, 2002; Patel and Hillard, 2006), both of which would result in overactivation of CB1R signaling and could result in LOF as a result. Furthermore, there is a biphasic influence of stress on eCB signaling, as *c-fos*⁺ neurons were increased after acute stress (Lin et al., 2018), and we have shown that acute stress elevates eCBs in mPFC (Hill et al., 2011), whereas chronic stress associated with depression results in reduced eCBs in the nucleus accumbens (Wang et al., 2010), which also indicates a contribution of LOF in eCB signaling following prolonged activation. From our study, increased neuronal activity and elevated 2-AG were detected early after SNI, whereas decreased neuronal activity and normal eCBs levels were found in the persistent phase of pain (Figs. 7, 9). From these observations, we speculate that eCBs elevated by increased neuronal activity in the early phase, and decreased eCB signaling function in the late phase, might be a shared pathogenic pathway in the development of depression induced by pain and stress. Recent clinical data lend some support to this finding as well (Fitzgerald et al., 2021). In a study of more than 170 individuals with a traumatic injury requiring hospitalization, circulating concentrations of 2-AG at the time of injury were significantly, positively correlated with a diagnosis of depression 6–8 months following the injury. As in the current study, traumatic human injury is accompanied by significant acute pain and we hypothesize that the pain and distress of the injury resulted in excessive release of 2-AG, followed by loss of CB1R signaling, resulting in increased risk for depression.

Reduced influence of eCB signaling on synaptic transmission after painful nerve injury can result from agonist-induced internalization and desensitization of CB1Rs, protein kinase C (PKC) signaling, or cAMP/protein kinase A (PKA) signaling. Agonist-mediated activation of CB1Rs by agonist can initiate internalization and desensitization of CB1Rs (Wu et al., 2008). CB1Rs activity regulates vesicle release by cAMP/PKA signaling pathway (Pan et al., 2008). The phytocannabinoid δ 9-tetrahydrocannabinol can activate PKC isolated from the rat forebrain (Hillard and Auchampach, 1994), and PKC can phosphorylate CB1Rs (Garcia et al., 1998). Further studies will be needed to explore these inter-related mechanisms.

Our findings also have implications for designing treatments targeting the eCB signaling system for depression induced by neuropathic pain so that reducing eCB activity is a viable strategy for treatment in the initial phase of neuropathic pain, indicated by our data with AM4113 (Fig. 10). However, boosting eCB activity will be the proper strategy in the persistent phase of pain (Fig. 11). Excessive activation of CB1Rs may contribute to further LOF of CB1Rs, worsening depression in the long run. The usage of a low dose of FAAH or MAGL blockers (Zhong et al., 2014), or a positive allosteric CB1R modulator to enhance eCB signaling without inducing further LOF of eCB signaling, may be possible treatment strategies to explore in the future.

In summary, our results reveal a novel mechanism by which neuropathic pain induces depression, whereby overactivation of the eCB signaling system by noxious peripheral inputs initially after onset of neuropathic pain causes LOF of eCB signaling in mPFC during prolonged neuropathic pain.

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