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Circuit-selective properties of glutamatergic inputs to the rat prelimbic cortex and their alterations in neuropathic pain

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Abstract

Functional deactivation of the prefrontal cortex (PFC) is a critical step in the neuropathic pain phenotype. We performed optogenetic circuit dissection to study the properties of ventral hippocampal (vHipp) and thalamic (MDTh) inputs to L5 pyramidal cells in acute mPFC slices and to test whether alterations in these inputs contribute to mPFC deactivation in neuropathic pain. We found that: 1- Both the vHipp and MDTh inputs elicit monosynaptic excitatory and polysynaptic inhibitory currents. 2- The strength of the excitatory MDTh input is uniform, while the vHipp input becomes progressively stronger along the dorsal-ventral axis. 3- Synaptic current kinetics suggests that the MDTh inputs contact distal, while the vHipp inputs contact proximal dendritic sections. 4- The longer delay of inhibitory currents in response to vHipp compared to MDTh inputs suggests that they are activated by feedback and feedforward circuitries, respectively. 5- One week after a peripheral neuropathic injury (SNI), both glutamatergic inputs are modified: MDTh responses are smaller, without evidence of presynaptic changes, while the probability of release at vHipp-mPFC synapses becomes lower, without significant change in current amplitude. Thus, dysregulation of both these inputs likely contributes to the mPFC deactivation in neuropathic pain and may impair PFC-dependent cognitive tasks.

Keywords

pyramidal cell; SNI; channelrhodopsin; hippocampus; thalamus; connectivity

Introduction

Recent studies show that neuropathic pain is associated with wide functional and morphological reorganization of the limbic system (Metz et al. 2009; Ren et al. 2011; Mutso et al. 2012; Chang et al. 2014; Schwartz et al. 2014; Kelly et al. 2016; Ren et al. 2016). Such reorganization is likely to constitute the neurophysiological ground for comorbidity of pain, negative emotional state and some degree of cognitive impairment seen in both chronic pain patients (Baker et al. 2016; Moriarty et al. 2011; Wiech and Tracey 2009) and animal models (Pais-Vieira et al. 2009; Ren et al. 2011). In particular, it has been proposed that global PFC

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Disclosures

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deactivation mediates the cognitive impairments that accompany chronic pain (Ji et al. 2010). Very recently, powerful evidence has appeared linking the deactivation of the PFC not only with the cognitive, but also with the sensory component of pain. Lee et al. (2015) showed that acute optogenetic activation of layer 5 pyramidal neurons of the prelimbic PFC relieves both the aversive cognitive symptoms of neuropathic pain as well as tactile and thermal allodynia. Similarly, Zhang et al. (2015) found that increased activity of parvalbumin-positive interneurons in neuropathic pain decreases the excitability of mPFC layer 5 pyramidal cells and worsens the pain phenotype, while inhibition of these interneurons alleviates it. Thus, multiple lines of evidence concur that deactivation of the prelimbic PFC has a causal role in the pain phenotype. The prelimbic cortex, however, is composed of multiple microcircuits that are defined by their diverse input and output projections. In particular, the mPFC receives three major extracortical glutamatergic inputs from the mediodorsal thalamus, the ventral hippocampus, and the amygdala. Not much is known about the properties of the different excitatory inputs to individual layer 5 neurons. What is the spatial distribution of the inputs? How strong are they? What are the plastic and kinetic properties of these synapses? Even less is known concerning the modulation of these parameters in chronic pain. We hypothesized that the cortical deactivation in neuropathic pain may also depend on alterations in the properties of glutamatergic inputs, as suggested by the decreased glutamate concentration in the mPFC of neuropathic pain rodents (Guida et al. 2015; Kelly et al. 2016). In particular, we focused on the inputs from the medio-dorsal thalamus, which is strongly reciprocally connected with the mPFC (Krettek and Price 1977; Gabbott et al. 2005), and the hippocampal input, as previous work showed that hippocampal function is impaired in chronic pain (Ren et al. 2011; Mutso et al. 2012). We took advantage of selective optogenetic activation to study the properties of the ventral hippocampal (vHipp) and medial dorsal thalamic (MDTh) inputs to layer 5 pyramidal neurons of the prelimbic cortex. We first characterized the basic properties of these two inputs in control conditions, and then we proceeded to investigate how they are affected in neuropathic pain.

Materials and Methods

Animals

All animal procedures were performed with approval of the Northwestern University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River), were housed in conventional rodent housing with unlimited access to food and water, and kept on a 12/12 hour light/dark cycle.

AAV Injections

The channelrhodopsin-AAV viral construct, AAV9.CAG.hChR2(H134R)-mCherry.WPRE.SV40 (Addgene20938M), was obtained from the Penn Vector Core. Viral injections to the ventral hippocampus (vHipp) or medial dorsal thalamic nucleus (MDTh) were performed in 21-24-day old male rats. Animals were anesthetized with 3% isoflurane and maintained at 2.25-3% isoflurane on a nose cone for the duration of the procedure. After immobilizing the rat's head in a stereotaxic frame, the skull was exposed and a 1-2 mm craniotomy performed above the injection site. The viral construct was loaded into a glass microinjection pipette having a 10-20 μ m diameter tip. The tip of the injection pipette was

directed to the desired injection location and virus was expelled using a custom pressure injection system using compressed N₂. For vHipp, 230 nl was injected using the following coordinates (mm from Bregma): -5.3 caudal, 5.0 lateral (right side), and 6.6 ventral to the brain surface. For MDTh, 150 nl was injected at the coordinates (mm from Bregma): -2.8 caudal, 0.5 lateral (right side), and 5.1 ventral to the brain surface. To avoid the excessive bleeding that may result from injecting near the midline, this target was reached by performing a craniotomy -2.8 caudal and 1.9 lateral to Bregma and lowering the injection pipette 5.1 mm ventral to the brain surface at a 15° angle. Recordings were performed ~5 weeks post-injection to allow for abundant Chr2 expression in the mPFC. After obtaining slices for recordings, 300 µm coronal sections of the injection sites were fixed in 4% PFA for at least 12 hours, washed 3 times in PBS and mounted with Mowiol mounting media. mCherry expression was used to confirm injection locations.

Spared Nerve Injury (SNI) pain model

SNI or sham surgeries were performed ~4 weeks after the Chr2 injections, at age p49-50 (200-310g). SNI surgery (Decosterd and Woolf, 2000) is a robust model of persistent neuropathic pain (del Rey et al. 2011). Briefly, isofluorane anesthesia was induced at 3L/min and then kept on a nose cone at 2.5-3L/min for the duration of the surgery. The sciatic nerve was exposed distal to the sural bifurcation, the tibial and peroneal nerves were tightly ligated with 6-0 braided silk sutures (Henry Schein) approximately 2 mm apart, and a 1-2 mm piece of nerve was removed between the ligations. The skin was then sutured with 4-0 nylon sutures (Henry Schein), triple antibiotic ointment was applied to the wound, and the animal was removed from anesthesia. Sham surgeries were the same, except that once the nerve was exposed, it was not further manipulated and the wound was immediately closed.

Behavioral testing to determine the animals' pain threshold was performed immediately prior to surgery and again one week post-surgery, prior to electrophysiological recording. Rats were placed in a chamber with a metal mesh floor and acclimated for at least 20 minutes prior to testing. Testing was performed using von Frey filaments (Stoelting) of different strengths, which were applied to the plantar surface of the hind paws to determine the pain threshold. The 50% withdrawal threshold was calculated using the method described by Chaplan et al., 1994.

Acute brain slices and solutions

7-10 days after sham/SNI surgery, rats were anesthetized with a lethal dose of Ketamine/Xylazine (3:1) and perfused with ice-cold, oxygenated, low-calcium, high-magnesium artificial cerebral spinal fluid (ACSF). The solution contained (in mM) 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₃, 25 glucose, 0.5 CaCl₂ and 7 MgCl₂. Rats were then decapitated and the brain removed into slush, also made from low-calcium, high-magnesium ACSF. 300 µm coronal slices containing the mPFC were cut using a Thermo Scientific Microm Vibratome and stored in low-calcium, high-magnesium ACSF for ~20 min at 32°C, brought to room temperature, and stored up to 8 hours until use. Slices were transferred one at a time into a recording chamber containing standard ACSF (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₃, 25 glucose, 2 CaCl₂ and 1 MgCl₂ (saturated with 95% O₂/5% CO₂).

Electrophysiological recordings and analysis

300 μm slices were obtained 7-11 days after sham/SNI surgery (56-60 day-old rats). The right hemisphere (contralateral to injury) prelimbic region of mPFC was targeted for patch clamp recordings. Pyramidal neurons in layer 5 (L5) – between 550 and 1000 μm from the pial surface – were visualized with an Axioskop 2FS (Zeiss) upright microscope using infrared differential interference contrast video microscopy with a water-immersion 60 $\times$ objective. Cells were selected based on their large pyramidal somata, membrane capacitance (>100 pF) and non-fast-spiking firing patterns. Cell locations were measured using a calibrated microscope stage (Luigs & Neumann). Cells were patched in whole-cell configuration at $\sim 30^\circ\text{C}$ using patch pipettes made from 1.5mm thick-walled borosilicate glass (Sutter #B150-86-10) pulled to a tip size of 5-10 M Ω with horizontal micropipette puller (Sutter). Unless otherwise stated the pipette internal solution contained (in mM): 138 K-gluconate, 2 NaCl, 2 MgCl₂, 2 Na₂-ATP, 0.2 Na-GTP, 0.1 EGTA, 10 HEPES, and 1 mg/mL biocytin, pH 7.3 with KOH. A subset of recordings in which it was necessary vary the holding potentials were performed using an internal solution in which K-gluconate was replaced by iso-osmolar Cs-methanesulfonate to improve the quality of the clamp. The latter solution also contained 5 mM QX-314. Signals were recorded using an Axopatch 200B amplifier (Axon Instruments), filtered at 10 kHz and sampled at 2 kHz. Data were acquired using pClamp9 software running on a PC. Membrane potential values reported were not corrected for liquid junction potentials (-6.5 mV for the Cs-methanesulfonate-based internal and -8.5 mV for the K-gluconate solution, both measured using a 3M chloride agar bridge). Solutions were prepared freshly every day. All drugs were bath applied. Chemicals were from Sigma, except tetrodotoxin (TTX; Alomone).

ChR2 stimulation was achieved delivering 1 ms light pulses (using a 450 nm LED light source; Prizmatix) directed to the slice through a 60 $\times$, 0.9NA water immersion objective. The light intensity was normalized in the different recordings. For each recorded cell it was first established the lowest intensity that would produce a maximal (saturating) response using a 1ms pulse. This light intensity was then used throughout the experiment. 180 μs -long pulses were occasionally used to avoid unclamped spikes when stimulating MDTh inputs. Current amplitudes were measured as the peak response to a single light pulse. Paired-pulse stimulation was performed delivering pairs of 1 ms pulses at either 100 or 200 ms intervals. A 1 minute interval between each stimulus, paired pulse or pulse train allowed for presynaptic recovery and avoid inducing long lasting synaptic plasticity. Synaptic delay was measured as the interval between the time at start of the light stimulus and the first point in the current trace showing a significant deflection from the baseline. The synaptic jitter has the usual definition as the standard deviation of the latency.

Statistics

Data in the text are presented as mean $\pm$ SEM. Box plots in Figures show 75th and 25th quartiles. Whiskers extend to points that fall within the 75th quartile + 1.5*(interquartile range) and 25th quartile – 1.5*(interquartile range). A Wilcoxon rank-sum test (also known as Wilcoxon-Mann-Whitney) test was used to determine statistical differences between groups. Differences were considered significant at $p < 0.05$.

Results

We used an optogenetic approach to determine the properties of the MDTh→L5 mPFC and vHipp→L5 mPFC microcircuits and their potential modulation in a rat model of neuropathic pain (Figure 1). We injected 21-23 day-old rats with an AAV-ChR2-mCherry construct either in the MDTh or vHipp (Figure 1A,C). 4 weeks post-injection, we performed SNI (or sham) surgery on the injected animals. The rats were finally sacrificed for slice recordings 7-10 days later. Tactile allodynia was assessed one week post-SNI, prior to the ex-vivo studies. Figure 1B shows that tactile thresholds were strongly reduced in the injured, left paw in SNI-operated compared to sham-operated rats (SNI: 1.8 ± 0.7 g, 15 rats; sham: 10.7 ± 2.4 g, 17 rats; Student's t-test, two-tailed, $p=0.002$). There was no difference in the tactile threshold of the right (uninjured) paw between the two groups (not shown). All the recordings used for this study were obtained from layer 5 (between 550 and 1000 μm from the pia) pyramidal neurons located in the prelimbic region (PL) of the mPFC (between 1500 and 3400 μm from the apex; Figure 1D).

First, we investigated the basic properties of these two inputs to L5 PL pyramidal cells in control (sham-operated) animals. Neurons were voltage-clamped at -70 mV and stimulated using 1 ms light pulses (Figure 2). 24 of 26 neurons tested in slices from animals injected in the MDTh responded to light stimulation; similarly, 29 of 37 cells responded in slices from hippocampus-injected rats. The mean inward current elicited by activation of the MDTh inputs was larger than the one elicited by stimulation of the vHipp terminals (MDTh: -223 ± 44 pA, 24 cells; vHipp: -102 ± 22 pA, 29 cells; Wilcoxon-Mann-Whitney test, two-tailed, $p=0.013$; Figure 2B). Interestingly, the spatial distribution of the cells that responded to the MDTh and vHipp stimuli differed. While the fraction of responders and non-responders as well as the mean response amplitude to thalamic stimulation were evenly distributed along the dorsal-ventral mPFC axis ($r^2=0.007$, $n=26$ $p=0.67$, Figure 2C), the strength of the vHipp inputs increased along this axis ($r^2=0.21$, $n=37$, $p=0.004$, Figure 2D). This is consistent with reports showing that vHipp terminals in the mPFC are distributed unevenly, and more terminals are present in the ventral region (Dembrow et al. 2015).

We then investigated the first latency and jitter distributions of the responses elicited by activation of these two inputs (Figure 3). The first latency was short for both inputs (MDTh: 2.38 ± 0.56 ms; $n=19$; vHipp: 2.12 ± 0.19 ms; $n=24$; Wilcoxon rank-sum test, two-tailed, $Z=-1.43$, $p=0.15$); the jitter distribution was also similar for the two inputs (MDTh: 2.01 ± 0.42 ms, $n=19$; vHipp, 1.81 ± 0.22 ms, $n=24$; Wilcoxon rank-sum test, two-tailed, $Z=0.43$, $p=0.67$). These data suggest that both inputs mediate responses that are by and large monosynaptic. To confirm the monosynaptic nature of the responses, we tested a subset of cells in the presence of bath applied TTX and 4-Aminopyridine (Petreanu et al. 2009; Tritsch et al. 2012). Under these conditions the responses were still present, thus supporting the idea that these connections are by and large monosynaptic.

Next, we determined the kinetics of the synaptic currents elicited by activation of the two pathways (Figure 4). For this analysis, we excluded responses for which the jitter was larger than the median+interquartile range (to exclude potential polysynaptic inputs). The kinetics of the excitatory current responses evoked by stimulation of MDth and vHipp inputs differed

significantly and the 20-80% rise time was significantly slower in response to MDTh stimulation (3.61 ± 0.48 ms, $n=15$, vs 2.15 ± 0.21 ms for the vHipp, $n=22$; Wilcoxon rank-sum test, two-tailed, $Z=2.89$, $p=0.004$, Figure 4B). Similarly, the decay time constant was longer for MDTh responses (MDTh: 21.97 ± 1.93 ms, $n=15$; vHipp: 12.69 ± 0.83 ms; $n=20$; Wilcoxon rank-sum test, two-tailed, $Z=3.85$, $p=0.0001$; Figure 4C). Thus, both the rise time and decay of the MDTh-elicited currents are slower than vHipp responses, suggesting more distal contacts on the dendritic tree.

To further characterize these microcircuits, we investigated whether these inputs also elicit inhibitory responses in the recorded pyramidal cells. For these experiments the internal solution was Cs based and contained 5 mM QX-314 (to improve clamp conditions, particularly at depolarized holding potentials) and 6 mM Cl^- , so that excitatory and inhibitory responses could be isolated by holding the cells either at -70 mV or at 0 mV respectively (Figure 5A). As may be expected, currents recorded with Cs internal were larger than those in K-gluconate (compare Figure 5 with Figure 2). Importantly, we found that the MDTh inputs were more effective in recruiting both excitatory and inhibitory responses, as 17 of 17 responder cells showed both components, while only 63% (12/19) of the cells receiving vHipp excitatory inputs also displayed an inhibitory current (Fisher's exact test, two-tailed, $p=0.008$).

Interestingly, both the excitatory and inhibitory currents elicited by MDTh inputs were significantly larger than the vHIPP-elicited counterparts (Figure 5B,C). The inhibitory/excitatory current ratio was also larger for MDTh inputs (Figure 5D). Thus, MDTh stimulation recruits inhibitory neurons more efficiently than does the vHipp. The delay of the inhibitory current activation was considerably longer for the vHipp inputs (6.22 ± 0.96 ms; $n=5$ vs. 3.23 ± 0.23 ms for the MDTh; $n=8$; Wilcoxon rank-sum test, two-tailed, $Z=-2.86$, $p=0.004$, Figure 5E). The main properties of the currents elicited by the two inputs are summarized in Table 1.

Having characterized the basic properties of the MDTh and vHipp inputs to L5 pyramidal cells, we examined whether these properties are modified following neuropathic injury (SNI model). We focused our analysis at 7-10 days after surgery, because this is a critical time point for pain-associated brain reorganization, as shown in other brain areas where anatomical and functional changes were found to be causally correlated with the pain behavior (Mutso et al. 2012; Chang et al. 2014; Schwartz et al. 2014; Lee et al. 2015; Ren et al. 2016), and because at this time point changes in glutamatergic activity have been previously described in the mPFC of SNI rats (Kelly et al. 2016). We found that the responses to stimulation of both these inputs were altered in SNI rats. The excitatory currents elicited by blue light stimulation of MDTh terminals were significantly reduced in slices from SNI compared to sham rats (SNI, $n=9$, 387 ± 77 pA; sham, $n=8$, 799 ± 97 pA; Wilcoxon rank-sum test, two-tailed, $Z=-2.36$, $p=0.018$; Figure 6B). Similar to the excitatory responses, the inhibitory responses were also decreased in SNI (SNI, $n=9$, 806 ± 214 pA; sham, $n=8$, 1765 ± 316 pA; Wilcoxon rank-sum test, two-tailed, $Z=-2.36$, $p=0.018$; Figure 6C). We then tested whether the paired pulse ratio (PPR) at this synapses is affected by the SNI surgery. Figure 6E-G shows that no change was detectable in PPR, suggesting that the observed changes in the current amplitude were mainly due to postsynaptic mechanisms. A

similar approach was also used to analyze the potential differences in the vHipp input properties between sham and SNI animals. Although no significant differences were detectable in the size of either the excitatory or the inhibitory currents elicited by this pathway (Figure 7A-D), the vHipp input showed a significant change in PPR, which shifted from neutral to facilitating at both the 100 ms time interval (SNI, $n=23$, 1.33 ± 0.08 ; sham, $n=19$, 1.00 ± 0.08 ; Wilcoxon rank-sum test, two-tailed, $Z=2.80$, $p=0.005$; Figure 7F) and the 200 ms time interval (SNI, $n=21$, 1.39 ± 0.08 ; sham, $n=19$, 1.08 ± 0.09 ; Wilcoxon rank-sum test, two-tailed, $Z=2.65$, $p=0.008$; Figure 7G). Because of the potentially surprising finding of a reduced probability of release without change in current amplitude, we performed a second, independent set of measurements comparing the amplitude of glutamate currents elicited by vHipp synapses. These data were obtained at -70 mV using K-gluconate as internal solution and also failed to detect any significant change (Supplementary Figure 1).

Discussion

Differential properties of vHipp and MDTh inputs to L5 pyramidal cells

We combined in vivo ChR2-AAV transfection of the MDTh and the vHipp with ex-vivo patch clamp recordings from acute slices to study the properties of the afferent inputs from these areas to the PL mPFC. We focused our investigation on layer 5 pyramidal neurons because they provide an integrated output of this brain area and because the output of these cells modulates pain phenotype in the SNI model (Lee et al. 2015; Zhang et al. 2015). We found that the PL L5 pyramidal neurons receive monosynaptic, excitatory inputs from both MDTh and vHipp. The inputs from MDTh elicit responses that are larger and have slower kinetics than vHipp inputs. The larger responses elicited by the MDTh input was a surprisingly robust finding. When using optogenetic stimulation, absolute response magnitudes can vary with slight changes in the injection volume or variation of the injection location. For these experiments, the injection volume was smaller for the MDTh input; therefore it is unlikely that the larger responses are an artifact due to infection of more cells.

While the strength of the thalamic excitatory input is evenly distributed within layer 5, the strength of the ventral hippocampal input increases along the dorsal-ventral axis. Although our recordings were by and large limited to the prelimbic cortex, this distribution matches the strong PL-IL gradient recently shown for BLA inputs to the PFC (Cheriyian et al. 2016). Both the vHIPP and MDTh afferents were also capable of activating inhibitory currents, although the MDTh input was significantly more efficient in this regard, as inhibitory currents were detectable in all cells that exhibited an excitatory response. vHipp stimulation, on the other hand, failed to elicit an inhibitory response in 37% of the cells where an excitatory response was present. Inhibitory responses elicited by thalamic inputs were also larger than those from hippocampal inputs. Additionally, for both inputs the synaptic delay of the inhibitory responses was longer than the corresponding excitatory response, suggesting that the interneurons are activated via a polysynaptic path. The delay of the inhibitory currents elicited by vHipp inputs, however, was significantly longer than that of MDTh inputs. This suggests that MDTh inputs directly activate interneurons in a feed forward circuit, while hippocampal inputs activate interneurons in feedback circuits. Interestingly, because feedback inhibition is by and large provided by basket cells, which

fire with high temporal precision (Jonas et al. 2004), this interpretation would also suggest that the vHipp driven inhibitory responses decay faster. This is indeed the case: Figure 5G shows that the decay of the hippocampal inhibitory responses is approximately two fold faster. Thus our data seem to suggest that perisomatic inhibitory neurons (such as basket cells) mediate the inhibitory response to vHipp inputs, while other interneurons mediate the thalamic evoked inhibition. This picture matches the idea that hippocampal inputs are distributed to deeper layers, while thalamic inputs target superficial layers, where they could also activate feedforward interneurons (summarized in Figure 8). This is in good agreement with previous studies showing that the MDTh inputs contact pyramidal cells as well as different classes of interneurons in layer 3 of the mPFC (Rotaru et al. 2005). Interestingly, the kinetics of the excitatory responses supports the hypothesis that the density of these inputs is uneven across the cortical layers traversed by L5 pyramidal cell dendrites. vHipp inputs display significantly faster rise time and decay times compared to thalamic inputs, as expected if they are located more proximally to the soma. This difference reminds of that described by Little and Carter (2012) for mPFC layer 2 pyramidal neurons. Together, these authors' and our data suggest that the thalamic inputs contact the more distal part of the apical dendrites, while vHipp inputs are closer to the soma.

Reorganization of the inputs to L5 PL cortex in neuropathic pain

Our data show that 1 week after SNI surgery both of the inputs examined in this study are altered in the mPFC contralateral to the injury, although the underlying mechanisms appear to differ. The excitatory current in response to MDTh input is significantly reduced in amplitude, but the PPR is not affected. This suggests a reduced number of contacts or changes in postsynaptic properties such as ion channel density or composition at the individual synapses. Hippocampal inputs, on the other hand, show significant paired-pulse facilitation in SNI animals, suggesting a lower probability of release (Regehr 2012). The hypothesis of a reduction in release probability at these synapses fits with in vivo microdialysis findings showing reduced glutamate concentration in the mPFC of SNI rats at a similar time point (Kelly et al. 2016). Surprisingly, we could not detect a significant change in the size of the postsynaptic currents. Current amplitudes, however, are influenced by multiple pre- and post-synaptic factors; in addition to the release probability, these also include the number of synaptic contacts as well as the density and composition of postsynaptic receptors. Additionally, non-synaptic factors such as slight changes in the injection volume and location and the transfection efficiency might also affect the current amplitude. Thus, the difference in PPR represents a more robust finding than the potential changes in current size. However, it is tempting to speculate on possible mechanisms that may mediate an upregulation of the synaptic current in SNI animals. One such mechanism may simply result from a homeostatic increase in the density of postsynaptic AMPA receptors in response to decreased glutamate release. The hypothesis that the glutamatergic current may be upregulated at the postsynaptic location in response to modified inputs from the vHipp is consistent with the finding by Ryan et al. (Ryan et al. 2013) that the amplitude of miniature glutamatergic synaptic currents is increased in mPFC L5 pyramidal neurons of rats that have a lesion of the ventral hippocampus. The altered presynaptic release, however, would still cause major disruption to the flow of information along the circuit and probably disrupt the plasticity at these synapses.

Inhibitory currents activated by stimulation of the two inputs are also differentially affected in SNI. The inhibitory response to stimulation of ventral hippocampal inputs is not significantly different in neuropathic pain condition, while the inhibitory current in response to thalamic stimulation is reduced proportionally to the excitatory response. Thus, polysynaptic inhibition in response to these specific inputs does not appear to be increased in SNI animals. This result is in agreement with microdialysis data showing no significant change in ambient GABA levels in the mPFC of SNI animals (Guida et al. 2015; Kelly et al. 2016). At the same time, Zhang et al (2016) convincingly showed that inhibitory GABAergic tone is increased in layer 5 of the PL cortex of SNI animals due to activation of parvalbumin-positive interneurons. This may be attributed to increased mGluR1-dependent inhibition mediated by inputs from the basolateral amygdala (Ji et al. 2010; Ji and Neugebauer 2011). Additionally, it is worth noting that although not statistically significant, a trend can be spotted toward a stronger inhibitory component in response to activation of the hippocampal inputs (the inhibitory/excitatory current ratio at this synapse was 0.61 ± 0.26 in sham animals and 1.47 ± 0.46 in slices from SNI rats, $p=0.19$; see Figure 7). The large variability of the responses prevents a rigorous assessment of this ratio with our relatively limited sample size and will need to be more thoroughly explored in future studies. Our results, however, show that the pain-associated reorganization of the mPFC appears microcircuit-specific, which further complicates the idea of using global pharmacological tools to treat this condition, although more global dysfunctions, such as the impaired excitatory cholinergic modulation (Radzicki et al. 2017) may suggest more universal pharmacological targets. Another intriguing result of our study is that the change in excitatory response to MDTh stimulation appears mostly postsynaptic, and yet the inhibitory responses, which on the basis of their short latencies (~ 3 ms) appear part of a feed-forward circuit that receives direct thalamic inputs, are also strongly reduced. A possible explanation of this finding is that both the excitatory and the inhibitory currents are reduced because of a reduction in dendritic length of pyramidal neurons, which has been described in these cells in the same pain model (Kelly et al. 2016). Indeed, if we assume as can be suggested on the basis of their relatively slow kinetics, that the inhibitory currents activated by the thalamic input are from Martinotti-like cells, then the inhibitory terminals would contact pyramidal cells mostly on distal dendrites (Kawaguchi and Kubota 1996; McGarry et al. 2010) and a reduction in dendritic length would similarly affect the excitatory and inhibitory currents.

Functional consequences of PL cortex reorganization

Previous studies have shown that chronic pain is characterized by functional deactivation of the mPFC, a consequence of over-activation of local GABAergic circuitries (Ji et al. 2010; Zhang et al. 2015) and decreased glutamatergic activation of mPFC pyramidal neurons (Kelly et al. 2016); this deactivation leads to decreased output of the prelimbic cortex, which has a causal role in establishing the pain phenotype (Lee et al. 2015; Zhang et al. 2015). Our present findings are in line with the general idea of a functional deactivation of PLC layer 5 pyramidal neurons and suggest that decreased excitatory input from the vHipp and the MDTh contribute to this functional phenotype. Our finding that the ratio of inhibitory/excitatory current elicited by these inputs remains unaltered in SNI rats can be reconciled with the reported increased inhibition of these cells in pain condition (Zhang et al. 2015) as our results are limited to these two specific inputs and the increased inhibition may mostly

be driven by amygdalar inputs (Ji et al. 2010) or inputs from other areas including the contralateral (Dembrow and Johnston 2015) and the infralimbic cortex, which exerts inhibitory modulation on the PL (Ji and Neugebauer 2012). Interestingly, PFC deactivation seems to represent a general mechanism underlying intensified pain perception as it is also observed in exacerbated pain responses such as those following peripheral inflammation (Karshikoff et al. 2016). Apart from the impact on general cortical deactivation, these alterations of the synaptic inputs may also be relevant for the thalamo-cortical dysrhythmia that appears associated with spontaneous pain. According to this hypothesis, reduced thalamic input leads to diminished firing of layer 5 and 6 pyramidal neurons and consequent reduction in inhibitory drive causing abnormal gamma oscillations in adjacent cortex. These abnormal oscillations are suggested to underlie the positive symptoms of central pain, including spontaneous pain and allodynia (Walton and Llinas 2010). Thus, it may be proposed that the ability of mPFC activation to alleviate allodynia as well as cognitive symptoms of neuropathic pain (Lee et al., 2015) may reverse the cortical depression and normalize the oscillatory activity in the thalamo-cortical circuit. Finally, it is interesting that our data show a decreased strength of the glutamatergic input from the MDTh. Because the MDTh-mPFC circuit is critical for the ability to successfully switch behavior following cues (Block et al. 2007), the impairment in this particular network may represent a possible neurobiological basis for the relative cognitive inflexibility observed in chronic pain patients (Karp et al. 2006) and in animal models of chronic pain (Moriarty et al. 2016). Our finding of a reduced amplitude of the PL activation from MDTh inputs in SNI is also in keeping with the conclusions drawn from in vivo recordings in a model of inflammatory pain (Cardoso-Cruz et al. 2013); these authors found a global decrease in connectivity in the mPFC–MD circuit, and in particular in the thalamic input to the PFC and suggested that such this decreased MD-mPFC connectivity underlies the deficits in working memory found in these animals. Thus, working memory deficits in chronic pain appear the result of the potentially synergistic combination of reduced primary thalamic inputs and reduced excitatory cholinergic modulation onto layer 5 PL pyramidal neurons (Radzicki et al. 2017)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Optogenetic dissection of MDTh to mPFC and vHipp to mPFC circuits

A, Experimental design and timeline. B, Allodynic thresholds for the left (injured) paw reveal strong tactile allodynia in SNI compared to sham (SNI, $n=15$, 1.83 ± 0.72 ; sham, $n=17$, 10.73 ± 2.36 ; Student's *t*-test, two-tailed, $p=0.002$). C, Sites of viral microinjections in MDTh (top) and vHipp (bottom). D, Locations of recorded neurons in the prelimbic mPFC (between 1500 and 3400 μm from the apex) in layer 5 (between 550 and 1000 μm from the pia).

Figure 2. Differential effects of MDTh or vHipp input stimulation on layer 5 pyramidal neurons in the prelimbic mPFC

A, Excitatory responses to a 1 ms blue light activation of MDTh (green) and vHipp (purple) afferents. Holding potential: -70 mV; internal solution was potassium based. B, Summary of response amplitudes in sham animals (MDTh, $n=24$; vHipp, $n=29$); vHipp responses are smaller than MDTh responses (MDTh, $n=24$, 223 ± 44 pA, vHipp, $n=29$, 102 ± 22 pA, Wilcoxon rank-sum test, two-tailed, $Z=2.48$, $p=0.013$). C,D, Amplitude of MDTh (C) and vHipp responses (D), vs. cell location on the dorsal-ventral axis. Amplitudes of vHipp responses show a significant positive correlation with distance from the apex ($r^2=0.21$, $p=0.004$).

Figure 3. MDTh and vHipp responses are largely monosynaptic

Analysis of voltage clamp recordings from PL mPFC layer 5 pyramidal neurons in slices from control (sham operated) rats; Holding potential: -70 mV. A, Distribution of the delay to onset in response to stimulation of MDTh ($n=19$, 2.38 ± 0.56 ms) and vHipp inputs ($n=24$, 2.12 ± 0.19 ms). B, Distribution of response jitter for MDTh responses ($n=19$, 2.02 ± 0.42 ms) and vHipp responses ($n=24$, 1.81 ± 0.22 ms). C, Representative traces in slices from sham animals with MDTh or vHipp AAV injections (holding at -70 mV, K-gluconate intrapipette solution). TTX and 4-AP were added to the bath solution to isolate the monosynaptic components.

Figure 4. MDTh and vHipp glutamatergic responses have different kinetics

A, representative excitatory current responses (holding at -70 mV, K-gluconate intra-pipette solution) to stimulation of MDTh (green) and vHipp (purple) projections in PL mPFC slices of control (sham) rats. Exponential fits of the rise and decay are shown in red. B, The 20-80% rise time was significantly longer for the MDTh input (MDTh, n=15, 3.61 ± 0.49 ms; vHipp, n=22, 2.15 ± 0.21 ms; Wilcoxon rank-sum test, two-tailed, $Z=2.89$, $p=0.004$). C, the decay time constant was also significantly longer in response to MDTh compared to vHipp input stimulation (MDTh n=15, 21.97 ± 1.93 ms; vHipp, n=22, 12.69 ± 0.83 ms; Wilcoxon rank-sum test, two-tailed, $Z=3.85$, $p=0.0001$).

Figure 5. MDTh input stimulation elicits larger, slower responses and recruits more local inhibition compared to vHipp

A, Representative excitatory (inward; holding at -70 mV) and inhibitory (outward; holding at 0 mV) current in response to stimulation of MDTh (green) and vHipp (purple) projections. For these recordings, the intrapipette solution contained Cs-methanesulfonate and 5 mM QX-314 was used to improve the voltage clamp. B, The excitatory component of MDTh afferent input is larger than the vHipp inputs (MDTh, $n=8$, 798 ± 97 pA; vHipp, $n=11$, 169 ± 43 pA; Wilcoxon rank-sum test, two-tailed, $Z=3.51$, $p=0.0004$). C, The inhibitory component of the MDTh input is also larger (MDTh, $n=8$, 1765 ± 316 pA, vHipp, $n=9$, 127 ± 196 pA; Wilcoxon rank-sum test, two-tailed, $Z=3.44$, $p=0.0006$). D, The inhibitory/excitatory current ratio is larger for the MDTh input, suggesting that it recruits more

inhibitory circuitry than the vHipp input (MDth, $n=8$, 2.28 ± 0.35 ; vHipp, $n=9$, 0.61 ± 0.26 ; Wilcoxon rank-sum test, two-tailed, $Z=2.86$, $p=0.004$). E-G, The kinetics of the inhibitory component also differs between the two inputs. Responses to MDTh input stimulation have shorter delay (MDTh, $n=8$, 3.23 ± 0.23 ms, vHipp, $n=5$, 6.22 ± 0.96 ms; Wilcoxon rank-sum test, two-tailed, $Z=-2.86$, $p=0.004$) and longer decay time constant (MDTh, $n=8$, 54.42 ± 12.78 ms; vHipp, $n=5$, 17.14 ± 2.72 ms; Wilcoxon rank-sum test, two-tailed, $Z=2.56$, $p=0.010$). No difference is detectable in rise time (MDTh, $n=8$, 3.19 ± 0.51 ms; vHipp, $n=5$, 3.27 ± 0.79 ms).

Figure 6. Neuropathic pain decreases excitatory and inhibitory currents elicited by MDTh inputs

A, Representative excitatory and inhibitory currents (-70 mV and 0 mV holding potentials, respectively; CS-methanesulfonate + QX-314 intrapipette solution), in response to stimulation of MDTh inputs in sham (green) and SNI (gray). B,C, In SNI slices both excitatory (SNI, $n=9$, 387 ± 77 pA; sham, $n=8$, 799 ± 97 pA; Wilcoxon rank-sum test, two-tailed, $Z=-2.36$, $p=0.018$) and inhibitory (SNI, $n=9$, 806 ± 214 pA; sham, $n=8$, 1765 ± 316 pA; Wilcoxon rank-sum test, two-tailed, $Z=-2.36$, $p=0.018$) responses are smaller. D, The inhibitory/excitatory current ratio is unaltered in SNI (2.81 ± 0.82 , $n=9$ vs, 2.28 ± 0.35 , $n=8$ in sham; Wilcoxon rank-sum test, two-tailed, $Z=0.048$, $p=0.96$). E, Representative currents in response to a pair of blue light stimuli, 100 ms apart, for sham (green) and SNI (gray) conditions (-70 mV holding potential, K-gluconate intrapipette solution). F,G, Paired pulse ratio is not significantly altered in SNI for either a 100 ms inter-pulse interval (SNI, $n=15$, 1.12 ± 0.08 ; sham, $n=15$, 0.97 ± 0.04 ; Wilcoxon rank-sum test, two-tailed, $Z=1.45$, $p=0.15$) or a 200 ms inter-pulse interval (SNI, $n=15$, 1.00 ± 0.06 ; sham, $n=14$, 0.88 ± 0.04 ; Wilcoxon rank-sum test, two-tailed, $Z=1.55$, $p=0.12$).

Figure 7. Neuropathic pain alters the release probability at the vHipp inputs

A, Representative excitatory and inhibitory currents (-70 mV and 0 mV holding potentials, respectively; CS-methanesulfonate + QX-314 intrapipette solution), in response to stimulation of vHipp inputs in sham (purple) and SNI (gray). B,C, The SNI condition does not significantly alter the excitatory (SNI, $n=11$, 282 ± 58 pA; sham, $n=11$, 169 ± 43 pA; Wilcoxon rank-sum test, two-tailed, $Z=1.38$, $p=0.17$) or inhibitory (SNI, $n=10$, 429 ± 176 pA; sham, $n=9$, 127 ± 65 pA; Wilcoxon rank-sum test, two-tailed, $Z=1.13$, $p=0.26$) current components. D, The inhibitory/excitatory current ratio is unaltered in SNI (SNI, $n=10$, 1.47 ± 0.46 ; sham, $n=9$, 0.61 ± 0.26 ; Wilcoxon rank-sum test, two-tailed, $Z=1.29$, $p=0.19$). E, Current responses to a pair of blue light stimuli, 100 ms apart, for sham (purple) and SNI (gray) conditions (-70 mV holding potential, K-gluconate intrapipette solution). F,G, Paired pulse ratio becomes significantly facilitating in the SNI condition for both a 100 ms inter-pulse interval (SNI, $n=23$, 1.33 ± 0.08 ; sham, $n=19$, 1.00 ± 0.08 ; Wilcoxon rank-sum test, two-tailed, $Z=2.81$, $p=0.005$) and a 200 ms inter-pulse interval (SNI, $n=21$, 1.39 ± 0.08 ; sham, $n=19$, 1.08 ± 0.09 ; Wilcoxon rank-sum test, two-tailed, $Z=2.65$, $p=0.008$).

Figure 8. Proposed organization of vHIPP and MDTh inhibitory inputs to the prelimbic cortex
 The schematic summarizes the organization of polysynaptic inhibition in PL layer 5 as suggested by the synaptic delays and kinetic properties of inhibitory currents recorded in pyramidal neurons. Activation of the MDTh input leads to an inhibitory response with large amplitude and short delay (t3) whereas activation of the vHIPP input elicits a response that is smaller in amplitude and with longer delay (t4). Green: MDTh network. Red: vHIPP network.

Table 1

Properties of the vHipp and MDTh inputs to mPFC L5 pyramidal cells.

	MDTh	vHipp
Responders (%)	92	78
Response amplitude (excitatory) (pA)	223±44	102±22 *
20-80% RT (ms)	3.61±0.49	2.15±0.21 **
Decay time const. (ms)	21.97±1.93	12.69±0.83 **
Delay to onset (ms)	2.38±0.56	2.12±0.19
Jitter (ms)	2.01±0.42	1.81±0.22
Inhibitory/Excitatory current ratio (Cs-CH ₃ O ₃ S intrapipette solution)	2.28±0.35	0.61±0.26 **
Delay to onset of inhibitory current (ms) (Cs-CH ₃ O ₃ S intrapipette solution)	3.23±0.23	6.22±0.96 **
Decay time const. of inhibitory current (ms) (Cs-CH ₃ O ₃ S intrapipette solution)	54.42±12.78	17.14±2.72 *

Data are reported as mean±SD;

*
p<0.05,

**
p<0.01.

These data were obtained using a K-gluconate-based intrapipette solution unless otherwise noted.