Published in final edited form as:

ACS Chem Neurosci. 2018 September 19; 9(9): 2252–2261. doi:10.1021/acscchemneuro.8b00022.

Pathway-specific alterations of cortico-amygdala transmission in an arthritis pain model

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Abstract

Medial prefrontal cortex (mPFC) and amygdala are closely interconnected brain areas that play a key role in cognitive-affective aspects of pain through their reciprocal interactions. Clinical and preclinical evidence suggests that dysfunctions in the mPFC-amygdala circuitry underlie pain-related cognitive-affective deficits. However, synaptic mechanisms of pain-related changes in these long-range pathways are largely unknown. Here we used optogenetics and brain slice physiology to analyze synaptic transmission in different types of amygdala neurons driven by inputs from infralimbic (IL) and prelimbic (PL) subdivisions of the mPFC. We found that IL inputs evoked stronger synaptic inhibition of neurons in the latero-capsular division of the central nucleus (CeLC) of the amygdala than PL inputs, and this inhibition was impaired in an arthritis pain model. Furthermore, inhibition-excitation ratio in basolateral amygdala neurons was increased in the pain model in the IL pathway but not in the PL pathway. These results suggest that IL rather than PL controls CeLC activity, and that changes in this acute pain model occur predominantly in the IL-amygdala pathway.

Graphical abstract

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Conflict of Interest: The authors declare no competing financial interest

Author Contributions: V.N. conceived the study and supervised the research. V.N. and T.K. designed the experiments. T.K. performed the experiments. T.K. and V.N. analyzed the data and wrote the manuscript.

Cortico-amygdala connectivity changes in pain

Keywords

Medial prefrontal cortex; amygdala; pain; optogenetics; patch-clamp

INTRODUCTION

The corticolimbic system is closely linked to pain, and functional reorganization of this circuitry has been found repeatedly in pain conditions.^{1,2} Importantly, functional connectivity within the corticolimbic system was identified as a novel predictor for the risk of pain chronification.^{1,2} Within the corticolimbic system, the amygdala and medial prefrontal cortex (mPFC) are especially closely interconnected brain areas,^{3–5} and clinical and preclinical studies have consistently shown pain-related dysfunctions in these structures.^{6,7} Preclinical studies found mPFC neuronal deactivation in models of acute (carrageenan, arthritis)^{8–11} and chronic (neuropathic) pain models,^{12,13} although changes in the opposite direction were observed in some neuronal subpopulations.^{14,15} Emerging evidence implicates the amygdala in pain-related mPFC changes.^{10,11,14} Accordingly, pharmacological strategies centered on metabotropic glutamate receptors have been tested to normalize interactions between mPFC and amygdala.^{9,11,15,16} Impaired interactions between amygdala and mPFC have been implicated in emotional cognitive deficits¹⁷, including those observed in pain conditions.^{6,7} However, identifying the synaptic mechanisms of pain-related changes in these long-range connections had been hampered the lack of suitable tools.

The combination of optogenetics with brain slice physiology has emerged as a powerful approach to examine such long-range functional connectivities¹⁸ and is now widely used to study the mPFC-amygdala circuitry.^{4,5,19–24} In addition to synaptic properties and connectivity, changes in synaptic transmission underlying experience-dependent plasticity such as fear learning and extinction^{25,26} were detected with this method.^{19,21} This approach was also used to determine pathway-specific changes in the mPFC-accumbens circuitry in a chronic pain model²⁷ and to identify differential targeting of amygdala (BLA) projections to mPFC neurons.²⁴ A recent study from our group found that arthritis pain-related changes in the basolateral amygdala (BLA)-mPFC pathway involved BLA-driven increased

feedforward inhibition to reduce mPFC output, leading to pain behaviors and cognitive decision-making deficits.¹⁶

Here we examined the modulation of amygdala neurons by the mPFC, using optogenetics with slice patch-clamp recording to determine mPFC-driven synaptic responses in different types of amygdala neurons and changes in an arthritis pain model. The amygdala consists of several nuclei^{25,28} and plastic changes of synaptic transmission in the latero-capsular division of the central nucleus (CeLC)^{29–31}, BLA¹⁰, and intercalated cell mass (ITC)³² were causally linked to pain behaviors^{6,7}. Pain-related information reaches the amygdala through a rather direct line of nociceptive inputs from the brainstem to the CeLC and through thalamic inputs to the BLA.⁶ Importantly, activation of mPFC terminals can drive synaptic responses in these neurons.¹⁹ Therefore, we focused on the analysis of synaptic transmission in neurons in these regions. We also sought to determine any differences between mPFC subdivisions (infralimbic, IL, and prelimbic, PL) because multiple lines of evidence suggest distinct functions of IL and PL in fear expression and extinction³³ and we previously identified an inverse relationship between IL and PL in pain.³⁴ Here we report that IL drives stronger feedforward inhibition of CeLC neurons than the PL, and this inhibition is impaired in the arthritis pain model.

RESULTS AND DISCUSSION

To investigate the synaptic properties of IL and PL inputs onto amygdala neurons, we stereotactically injected adeno-associated viral vector coding channelrhodopsin 2 (ChR2) and enhanced yellow fluorescent protein (eYFP) under the control of a CaMKII promoter into IL or PL. Histological verification confirmed selective eYFP expression in IL (Figure 1a–c) or PL (Figure 1e–g). Consistent with previous studies,^{19,21} dense eYFP-labeled fibers were observed primarily in the BLA after 4–5 weeks of viral expression (Figure 1d, h). Whole-cell patch-clamp recordings were obtained from amygdala neurons (CeLC, ITC and BLA) in brain slices, and ChR2-expressing mPFC axon terminals were stimulated with blue laser pulses (473 nm).

CeLC neurons

Consistent with previous observations,^{35–37} we encountered three types of neurons in the CeLC based on action potential firing evoked by current injections (regular spiking, low-threshold bursting, late firing, Figure 2c). Although cell-type specific differences in the processing of nociceptive synaptic inputs were suggested in a previous brain slice physiology study,³⁷ perhaps due to the sample size in the present study, we did not detect any significant differences in light-evoked cortico-amygdala responses between the different groups, and so the data were pooled. There was possibly a trend for regular spiking neurons to show predominant synaptic inhibition from IL. Late firing neurons received strong excitatory and inhibitory inputs but represented only 10.0% of neurons in this study (regular spiking, 30.0%; low-threshold bursting, 60.0%). Low-threshold bursting neurons showed only weak excitatory and inhibitory responses. Resting membrane potential (-60.5 ± 1.1 mV) and input resistance (269.2 ± 17.6 M Ω) of CeLC neurons ($n = 22$) matched published data on rat CeLC neurons.^{32,37} Light activation of IL terminals in the amygdala slice evoked

glutamate receptor-driven IPSCs in a large fraction of CeLC neurons (67%; $n = 8/12$) that were blocked by NBQX (10 μ M) or bicuculline (10 μ M) (Figure 3c–f), while EPSCs were small and only detected in a fraction of CeLC neurons (33%). The dominant inhibitory effect of mPFC inputs on CeLC neurons (summarized in Figure 6a–c) is in agreement with a previous study.¹⁹ The amplitude of light-evoked IPSCs was largest when the stimulation area was centered on the ITC area, suggesting that activation of cortical fibers onto ITC neurons rather than onto BLA or other CeLC neurons generated synaptic inhibition. Light activation of PL terminals had little effect on CeLC neurons. PL-evoked IPSCs were only detected in two of 10 CeLC neurons (Figure 3i, j) and were significantly smaller than IL–CeLC IPSCs (summarized in Figure 6a–c), suggesting that cortical control of CeLC neurons through the PL–CeLC pathway is weaker than through the IL–CeLC pathway. These results are consistent with previous studies showing that feedforward inhibition of CeL neurons is closely linked to the activity of the IL.^{38,39} It should be noted that the present study did not examine the detailed connectivity underlying IL-driven feedforward inhibition of CeLC neurons, which could be mediated by ITC cells, BLA interneurons or CeLC interneurons.

BLA neurons

Principal glutamatergic neurons form the majority (80%) of BLA neurons with the remainder (20%) being GABAergic interneurons.⁴⁰ While the regulation of principal neurons by BLA interneurons is crucial for information processing in the BLA,⁴¹ we focused on principal neurons based on our previous studies that showed their important contribution to amygdala pain mechanisms.^{10,30} In agreement with previous reports,^{28,42} principal BLA neurons showed different degrees of spike-frequency adaptation ranging from rapidly adapting (accommodating) to slowly adapting (non-accommodating) firing patterns (Figure 2d). Firing patterns and action potential shapes of principal neurons are different from BLA interneurons (Figure 2e).²⁸ No obvious differences in mPFC-driven synaptic responses were found between principal cell-types and therefore data were pooled. Light activation of IL (Figure 4c, f) and PL (Figure 4i, l) axon terminals in the amygdala slice evoked short latency EPSCs and long latency IPSCs in BLA principal neurons. IPSCs were blocked by NBQX (IL-BLA, Figure 4d; PL-BLA, Figure 4j), suggesting the recruitment of local amygdala interneurons by both pathways. EPSCs were preserved in the presence of TTX (1 μ M) and 4-AP (1 mM) and abolished by subsequent application of AP5 (50 μ M) + CNQX (20 μ M) (IL-BLA, Figure 4e; PL-BLA, Figure 4k), suggesting the existence of monosynaptic glutamatergic inputs^{43,44} from both IL and PL onto BLA principal neurons. Interestingly, mPFC stimulation produced only EPSCs in BLA interneurons (IL-BLA, $n = 2$; PL-BLA, $n = 2$; data not shown), which would be consistent with their role in cortically driven feedforward inhibition of BLA principal neurons.

ITC cells

We focused on ITC cells in the dorsomedial cluster because of their strong projections to the CeL³⁹ and involvement in amygdala pain mechanisms.³² ITC cells recorded here were identified based on their location; they showed characteristics consistent with those reported in the literature,^{32,38,39} including higher input resistance (362.6 ± 33.7 MG) and more negative resting membrane potential (-65.8 ± 1.0 mV) than CeLC neurons as well as distinct morphology such as very small soma (10μ m) and flattened dendritic tree mostly confined

to within the BLA-CeA border³⁸ (Figure 5b, h). Light activation of IL (Figure 5c, d, f) or PL (Figure 5i, j, l) axon terminals in amygdala slices evoked short latency EPSCs and long latency IPSCs in ITC neurons that were blocked by NBQX, which is consistent with glutamate receptor-driven feedforward inhibition in these pathways. The IPSCs could reflect local connections within ITC⁴⁵ or BLA-driven feedforward inhibition evoked by light activation of cortical axons in BLA adjacent to ITC²². EPSCs were preserved in the presence of TTX and 4-AP and abolished by subsequent application of AP5+CNQX (IL-ITC, Figure 5e; PL-ITC, Figure 5k), suggesting the existence of monosynaptic glutamatergic inputs to ITC neurons from IL and to a lesser extent from PL (Figure 6h).

It should be noted that contrasting findings have been reported in other optogenetics and brain slice physiology studies investigating the synaptic connectivity underlying mPFC effects on the amygdala. For example, an earlier study showed that IL neurons monosynaptically connect with ITC cells and all BLA principal neurons,¹⁹ which is consistent with our present results, but a later study concluded that monosynaptic connections are predominantly present in the IL-BLA pathway.²² IL projections to the BLA were also identified with retrograde labeling.^{19,46} A recent study reported that the ventral mPFC (IL and dorsal peduncular cortex) predominantly makes functional monosynaptic connections with basomedial amygdala neurons but not with BLA or ITC, while the dorsal mPFC (PL and cingulate cortex) forms monosynaptic connections with ITC and BLA.²³ Our data suggest that both IL and PL make strong functional connections with BLA neurons, and IL more so than PL also activates ITC cells (Figure 6e and h). Selectively targeting IL and PL using viral vector injection-based strategies has technical limitations, and it is possible that leakage of virus from IL caused minor Chr2-eYFP expression in the PL in the present study.

Pain-related changes: CeLC neurons

Next, we sought to determine mPFC-driven synaptic modulation of amygdala neurons in a pain model. To do so we compared light-evoked IPSCs (Figure 6a, d, g), EPSCs (Figure 6b, e, h) and IPSC/EPSC ratio (Figure 6c, f, i) under control conditions (sham or normal naïve rats) with an arthritis pain condition (5–6 h postinduction; see Methods). As previously reported in the formalin-induced inflammatory pain model,³⁷ the proportion of cell-types was not significantly changed in the arthritis pain model (normal naïve and sham controls: regular spiking, 32.1%; low-threshold bursting, 57.1%; late firing, 10.7%, arthritis: regular spiking, 30.0%; low-threshold bursting, 55.0%; late firing, 15.0%, $P = 0.91$, χ^2 test). We found that IPSCs were decreased in the IL-CeLC, but not PL-CeLC, pathway in brain slices from arthritic rats ($P < 0.01$; $F_{(4, 45)} = 4.843$; one-way ANOVA with Bonferroni posttests compared with sham; Figure 6a). Decreased cortically driven synaptic inhibition of CeLC neurons in this pain model was suggested in our previous study using electrical stimulation of the external capsule which includes mPFC afferents identified by anterograde labeling.³² The selective activation of IL axon terminals in the amygdala in the present study provides direct evidence for our hypothesis that IL-driven control of CeLC activity is impaired in pain, at least at this relatively acute stage.^{6,7}

Pain-related changes: BLA neurons

In the IL-BLA, but not PL-BLA, pathway the IPSC/EPSC ratio was increased in the arthritis pain model ($P < 0.05$; $F_{(2, 21)} = 5.228$; one-way ANOVA with Bonferroni posttests compared with normal; Figure 6f). This net inhibition of BLA principal neurons was due to decreased excitatory transmission from IL and could contribute to decreased feedforward inhibition of CeLC neurons described in the previous section. Interestingly, decreased net inhibition in the PL-BLA, but not IL-BLA, pathway was found in a model of fear learning.²¹ Although we did not analyze pain-related changes of synaptic responses in BLA interneurons, IL and PL inputs could recruit different sets of BLA interneurons, including parvalbumin-expressing (PV) interneurons, as suggested previously.²⁰ Monosynaptic mPFC inputs onto PV interneurons were found to modulate reciprocal interactions between mPFC and BLA.⁴⁷ Cell-type specific responsiveness of BLA interneurons to noxious stimuli was also observed with systems electrophysiology (in vivo recording).⁴⁸ Thus, it is possible that certain BLA interneurons might contribute to increased inhibition of BLA principal neurons under pain conditions, resulting in decreased feedforward inhibition of CeLC neurons. The cell-type specific contribution of BLA interneurons to amygdala pain processing remains to be determined.

Pain-related changes: ITC cells

We did not find significant changes in the IL-ITC and PL-ITC pathways in the pain model (Figure 6g–i). These results are in contrast with our previous study where external capsule-driven EPSCs in ITC neurons were decreased in the pain model,³² probably because of differences in the selectivity and number of fibers stimulated with electrical versus optogenetic activation. One possibility is that ChR2 was expressed on only the subset of IL fibers, leading to relatively small EPSCs (< 50 pA) compared to those evoked by electrical stimulation of external capsule (up to 200 pA), which may not have been sufficient to reveal pain-induced changes. Significant differences were only detected for large EPSC (> 50 pA) in the previous study³², and these external capsule-driven large synaptic responses may have involved inputs from other cortical regions. For example, optogenetic studies did not find ChR2-eYFP labeled fibers from IL or PL²¹ or anterior cingulate cortex⁴⁹ in the external capsule. Functional connectivity at the IL-ITC synapse may also be weak as suggested by anatomical data⁵⁰ and/or relatively stable compared with the IL-BLA synapse as suggested in a previous study on fear extinction¹⁹.

As a note of caution, the activation of ChR2 can cause non-physiological synaptic release.^{18,51} And although ChR2-assisted brain slice electrophysiology is useful to determine the presence or absence of functional synaptic connections across distant brain regions and has been used successfully to detect functional changes in synaptic transmission in models fear learning and extinction^{19,21} and in pain models^{27,37}, it may not always be able to probe endogenous functions and changes. For example, activation of light sensitive channels that are not normally expressed could serve as an “intervention” to stimulate synaptic processes that may not occur physiologically and may in fact be impaired in certain disease models.

Accumulating evidence suggests dramatic functional and structural reorganization of corticolimbic circuits in chronic pain,^{1,52} which is largely distinct from the acute pain

condition that was studied in here. Pain-related plastic changes in the amygdala circuitry have been observed in models of acute and chronic pain^{6,7} It remains to be determined if cortical influences contribute differently to amygdala plasticity in acute versus chronic pain conditions. Other corticolimbic circuits such as those involving the nucleus accumbens and hippocampus have also been linked to pain chronification.^{1,2,53} The relative contribution and interaction of different corticolimbic subsystems to pain and other aversive-affective states^{20,27,44,53–55} remains to be determined.

In conclusion, we show synaptic properties of mPFC inputs to amygdala neurons and their pain-related pathway-specific changes in an arthritis pain model by combining optogenetics with brain slice physiology. The main changes in the pain condition were a decrease in IL-driven inhibition of CeLC neurons and a shift of excitation-inhibition balance in the IL-BLA pathway towards inhibition. The data suggest that decreased cortical inhibitory control may contribute to increased activity of CeLC neurons and amygdala-dependent aspects of pain.

Methods

Animals

Male Sprague Dawley rats were used in this study; they were four weeks old (50–80 g) at the beginning of the experiments that started with viral vector injection, and were 8–9 weeks old (250–350 g) by the time brain slice physiology was done. Animals were housed in a temperature-controlled room under a 12-h light/dark cycle. Water and food were available ad libitum. On the day of the experiment, rats were transferred from the animal facility and allowed to acclimate to the laboratory for at least 1 hr. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at TTUHSC and conform to the guidelines of the International Association for the Study of Pain (IASP) and of the National Institutes of Health (NIH).

Viral vector injection

A viral vector (0.4 μ l) encoding channel rhodopsin 2 (ChR2) and enhanced yellow fluorescent protein (eYFP) under the control of the CaMKII promoter (rAAV5/CaMKIIa-ChR2(H134R)-eYFP; Karl Deisseroth laboratory, packaged by the vector core facility at the University of North Carolina, Chapel Hill) was injected stereotactically into the right PL or IL, using 5 μ l Neuros syringe (33 gauge, Hamilton): 2.3 mm anterior to bregma; 0.3 mm lateral to midline; depth, 3.7 mm (PL) or 5.1 mm (IL).⁵⁶ Animals were allowed to recover 4–5 weeks for viral expression before brain slices were obtained for electrophysiology.

Arthritis pain model

In some rats a mono-arthritis was induced in the left knee joint as described in detail previously.⁵⁷ A kaolin suspension (4%, 100 μ l) was injected slowly into the joint cavity followed by repetitive flexions and extensions of the knee for 15 min. Next, a carrageenan solution (2%, 100 μ l) was injected into the knee joint cavity, and the leg was flexed and extended for another 5 min. This treatment paradigm reliably leads to a localized inflammation confined to one knee joint within 1–3 h, persists for weeks, and is significantly associated with pain behaviors and activity changes in the peripheral and central nervous

system. Electrophysiological experiments were performed 5–6 h after arthritis induction and data were compared to those obtained from normal naïve rats based on our previous works that showed brain activity and behavior of normal rats was not different from those that received intraarticular saline injection⁵⁸ or needle insertion.⁵⁹ Controls included normal naïve rats and sham rats in which a needle was inserted into the knee joint cavity followed by repetitive flexions and extensions of the knee, but without intraarticular injections of any chemicals. A lack of differences between sham rats and normal rats served to validate our use of normal naïve rats as controls.

Electrophysiology

Brain slice preparation—Brain slices containing the amygdala were obtained from normal, sham and arthritic rats as described before.¹⁶ Brains were quickly removed and immersed in oxygenated ice-cold sucrose-based physiological solution containing (in mM): 87 NaCl, 75 sucrose, 25 glucose, 5 KCl, 21 MgCl₂, 0.5 CaCl₂ and 1.25 NaH₂PO₄. Coronal brain slices (400µm) were prepared using a Vibratome (Series 1000 Plus, The Vibratome Co., St. Louis, MO). The amygdala slices were then incubated in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature (21°C) for at least 1h before patch recordings. ACSF contained the following (in mM): 117 NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃ and 11 glucose. A single brain slice was transferred to the recording chamber and submerged in ACSF (31±1°C) superfusing the slice at ~2 ml/min. Only one or two brain slices per animal were used.

Patch-clamp recording—Whole-cell patch-clamp recordings were obtained from visually identified CeLC, BLA and ITC neurons in amygdala slices of the right hemisphere, using infrared DIC videomicroscopy as described previously.¹⁶ Recording electrodes (tip resistance 5–8 MΩ, CeLC, 4–7 MΩ, BLA, 7–9 MΩ, ITC) were made from borosilicate glass and filled with intracellular solution containing (in mM): 122 K-gluconate, 5 NaCl, 0.3 CaCl₂, 2 MgCl₂, 1 EGTA, 10 HEPES, 5 Na₂-ATP, and 0.4 Na₃-GTP; pH was adjusted to 7.2–7.3 with KOH and osmolarity to 280 mOsm/kg with sucrose. On the day of recording, 0.2% biocytin was included in the intracellular solution. Data acquisition and analysis was done using a dual 4-pole Bessel filter (Warner Instr., Hamden, CT), low-noise Digidata 1322 interface (Axon Instr., Molecular Devices, Sunnyvale, CA), Axoclamp-2B amplifier (Axon Instr., Molecular Devices, Sunnyvale, CA), Pentium PC, and pClamp9 software (Axon Instr.). Headstage voltage was monitored continuously on an oscilloscope to ensure precise performance of the amplifier. If series resistance (monitored with pClamp9 software) changed more than 10%, the neuron was discarded. To characterize the electroresponsive properties of recorded neurons, depolarizing and hyperpolarizing current pulses (500 ms, 20 pA step) were applied.

Neurons were voltage-clamped at –70 mV or 0 mV for the study of excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs), respectively. The calculated equilibrium potential for chloride in this system was –68.99 mV (Nernst equation, pClamp9 software). To evoke EPSCs and IPSCs in amygdala neurons, ChR2 expressing afferent fibers from the mPFC were activated optically by laser light pulses (5 ms, 0.05 Hz) generated by a blue laser (473 nm; Thorlabs, Newton, NJ) controlled by an S88 Grass stimulator (Grass Technologies,

Warwick, RI); they were delivered through the 40× (for ITC and BLA) or 4× (for CeLC) objective of the microscope. Illumination area of the 40× objective (0.24 mm²) was centered on the soma of the patched cell (ITC or BLA). For CeLC recordings we used the 4× objective based on our pilot experiments that showed no detectable responses in most of the recorded neurons using the 40× objective (data not shown). Our pilot study showed that the light-evoked response in CeLC neurons was larger when the illumination area of the 4× objective (about 1 mm²) was centered on the ITC rather than on the CeLC (recorded neuron) or the BLA, therefore we used this configuration in all of the CeLC recordings. Light power density was measured using an optical power meter (PM200, Thorlabs) placed under the objective. Maximum laser powers (50 mW at laser source; 2.7 mW/mm² (40×) or 1.2 mW/mm² (4×) under the objectives) were used in all of the experiments. In most cases, we recorded neurons in all three amygdala regions (CeLC, BLA, and ITC) from each animal to ensure that undetectable responses in some CeLC and ITC neurons were not due to insufficient ChR2 expression or other technical issues.

Drugs

The following drugs were used: NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (**AP5**); non-NMDA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione disodium (**CNQX**) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (**NBQX**); GABA_A receptor antagonist **bicuculline**; sodium channel blocker tetrodotoxin citrate (**TTX**); and potassium channel blocker 4-aminopyridine (**4-AP**). All drugs were purchased from Tocris Bioscience (R&D Systems, Minneapolis, MN). Drugs were applied by gravity-driven superfusion of the brain slice in ACSF (~2 ml/min). Solution flow into the recording chamber (1 ml volume) was controlled with a three-way stopcock. Drugs were applied for at least 15 min to establish equilibrium in the tissue.

Histology

ChR2-eYFP expression at the viral vector injection sites (IL and PL) and on the mPFC axons in the amygdala were verified as described before.¹⁶ To confirm the location and to visualize the morphology of recorded neurons, the recorded slices were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 12–24 h at 4°C. After fixation, slices were washed in phosphate buffered saline (PBS) (3 × 10 min), and permeabilized in PBS containing 0.2% Triton X-100 for 60 min. Slices were then incubated in fluorescently-conjugated streptavidin (1:300, Streptavidin, Alexa Fluor 594 conjugate, Life Technologies) for 12–24 h at 4°C. Finally, the slices were washed in PBS (3 × 10 min), mounted on slides with Vectashield mounting medium with DAPI (Vector Laboratories), and imaged under a confocal microscope (Ti-E, A1, Nikon).

Statistical analysis

All averaged values are given as the mean ± SE. Statistical significance was accepted at the level $P < 0.05$. GraphPad Prism 3.0 software (Graph-Pad, San Diego, CA) was used for all statistical analyses performed on the raw data. Student's *t* test was used to compare two sets of data that had Gaussian distribution and similar variances. For multiple comparisons, ANOVA was used with Bonferroni posttests as indicated in the text and figure legends.

Acknowledgments

Work in the authors' laboratory was supported by National Institute of Neurological Disorders and Stroke (NIH/NINDS) grants NS038261, NS081121, and NS106902.

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Figure 1.

Verification of AAV injections in the mPFC and ChR2-eYFP expressing mPFC fibers in the amygdala. (a) Schematic representation of viral injection site in IL. (b) Viral vector-mediated ChR2-eYFP expression in IL (green, ChR2-eYFP; blue, DAPI). Scale bar, 500 μ m. (c) Magnified image of the area indicated by the red rectangle in (b). Scale bar, 100 μ m. (d) ChR2-eYFP expressing IL fibers in the amygdala. Scale bar, 200 μ m. (e–h) Same as (a–d), but for PL injection.

Figure 2.

Firing properties of neurons in the CeLC, BLA and ITC. **(a, b)** Biocytin-labeled CeLC (A), BLA (B) and ITC (C) neurons in a brain slice (area indicated by the red rectangle in the diagram) from an animal with IL (a) and PL (b) viral vector injection to express ChR2 (red, biocytin; green, ChR2-eYFP; blue, DAPI). Scale bars, 1 mm (diagrams), 50 μ m (images). **(c-f)** Current-clamp recordings of membrane potential changes and action potential firing in response to depolarizing (threshold and twice threshold) and hyperpolarizing (-100pA)

current pulses in CeLC (c, regular spiking, low-threshold bursting, late firing), BLA (d, principal neurons, rapidly adapting and slowly adapting; e, interneuron), and ITC (f).

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Figure 3. mPFC-driven synaptic responses of CeLC neurons. **(a)** Experimental setup for optogenetic stimulation of IL axon terminals in amygdala brain slice and patch-clamp recording of CeLC neurons. **(b)** High-magnification image of the CeLC neuron shown in Figure 2a. Scale bar, 20 μ m. **(c)** Example traces of light-evoked EPSCs and IPSCs in the CeLC neuron. Gray traces, individual response; black trace, average of 10 responses. **(d)** Light-evoked responses were blocked by NBQX (10 μ M). **(e)** IPSCs were also blocked by bicuculline (10 μ M). **(f)** Summary of onset latency of light-evoked EPSCs and IPSCs. EPSC, $n = 4$ neurons; IPSC, $n = 8$ neurons. ** $P < 0.01$ compared with EPSC, unpaired t test. **(g–j)** Same as (a–f) but for the PL-CeLC pathway. **(j)** IPSC, $n = 2$ neurons.

Figure 4. mPFC-driven synaptic responses of BLA neurons. **(a)** Experimental setup for optogenetic stimulation of IL axon terminals in amygdala brain slice and patch-clamp recording of BLA neurons. **(b)** High-magnification image of the BLA neuron shown in Figure 2a. Scale bar, 20 μ m. **(c)** Example traces of light-evoked EPSCs and IPSCs in the BLA neuron. Gray traces, individual response; black trace, average of 10 responses. **(d)** Light-evoked responses were blocked by NBQX. **(e)** IL-driven EPSCs in BLA neurons were preserved in the presence of TTX (1 μ M) and 4-AP (1 mM), and were blocked by AP5 (50 μ M) + CNQX (20 μ M). Stronger stimulation parameters (10–20 ms duration) were required to evoke responses in this condition. **(f)** Summary of onset latency of light-evoked EPSC and IPSC. EPSC, $n = 8$ neurons; IPSC, $n = 7$ neurons. *** $P < 0.001$ compared with EPSC, unpaired t test. **(g–l)** Same as (a–f) but for the PL-BLA pathway. **(l)** EPSC, $n = 7$ neurons; IPSC, $n = 6$ neurons. *** $P < 0.001$ compared with EPSC, unpaired t test.

Figure 5. mPFC-driven synaptic responses of ITC neurons. (a–l) Same display as in Figure 4 but for recordings in ITC neurons. (f) EPSC, $n = 8$ neurons; IPSC, $n = 6$ neurons. *** $P < 0.001$ compared with EPSC, unpaired t test. (l) EPSC, $n = 5$ neurons; IPSC, $n = 5$ neurons. ** $P < 0.01$ compared with EPSC, unpaired t test.

Figure 6.

Pain-related changes of IL-but not PL-driven synaptic responses in the arthritis pain model. (a–c) Light-evoked IPSCs (a), EPSCs (b) and IPSC/EPSC ratio (c) in the IL-CeLC and PL-CeLC pathways in brain slices from normal (Nor), sham (Sham) and arthritic rats (Arth). IL-CeLC: normal, n = 12 neurons; sham, n = 8 neurons; arthritis, n = 11 neurons. PL-CeLC: normal, n = 10 neurons; arthritis, n = 9 neurons. *, ** P < 0.05–0.01, one-way ANOVA with Bonferroni posttests compared with sham IL-CeLC. (d–f) Same display as in (a–c) but for recordings in BLA neurons. IL-BLA: normal, n = 9 neurons; sham, n = 8 neurons; arthritis,

n = 7 neurons. PL-BLA: normal, n = 8 neurons; arthritis, n = 8 neurons. * $P < 0.05$, one-way ANOVA with Bonferroni posttests compared with normal IL-BLA. **(g–i)** Same display as in (a–c) but for recordings in ITC neurons. IL-ITC: normal, n = 9 neurons; sham, n = 8 neurons; arthritis, n = 9 neurons. PL-ITC: normal, n = 9 neurons; arthritis, n = 9 neurons.