Long-Term Depression Induced by Optogenetically Driven Nociceptive Inputs to Trigeminal Nucleus Caudalis or Headache Triggers

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The peripheral trigeminovascular pathway mediates orofacial and craniofacial pain and projects centrally to the brainstem trigeminal nucleus caudalis (TNC). Sensitization of this pathway is involved in many pain conditions, but little is known about synaptic plasticity at its first central synapse. We have taken advantage of optogenetics to investigate plasticity selectively evoked at synapses of nociceptive primary afferents onto TNC neurons. Based on immunolabeling in the trigeminal ganglia, TRPV1-lineage neurons comprise primarily peptidergic and nonpeptidergic nociceptors. Optical stimulation of channelrhodopsin-expressing axons in the TRPV1/ChR2 mouse in TNC slices thus allowed us to activate a nociceptor-enriched subset of primary afferents. We recorded from lamina I/II neurons in acutely prepared transverse TNC slices, and alternately stimulated two independent afferent pathways, one with light-activated nociceptive afferents and the other with electrically-activated inputs. Low-frequency optical stimulation induced robust long-term depression (LTD) of optically-evoked EPSCs, but not of electrically-evoked EPSCs in the same neurons. Blocking NMDA receptors or nitric oxide synthase strongly attenuated LTD, whereas a cannabinoid receptor 1 antagonist had no effect. The neuropeptide PACAP-38 or the nitric oxide donors nitroglycerin or sodium nitroprusside are pharmacologic triggers of human headache. Bath application of any of these three compounds also persistently depressed optically-evoked EPSCs. Together, our data show that LTD of nociceptive afferent synapses on trigeminal nucleus neurons is elicited when the afferents are activated at frequencies consistent with the development of central sensitization of the trigeminovascular pathway.

Key words: LTD; optogenetic; PACAP-38; synaptic plasticity; trigeminal ganglion; Trpv1

Significance Statement

Animal models suggest that sensitization of trigeminovascular afferents plays a major role in craniofacial pain syndromes including primary headaches and trigeminal neuralgia, yet little is known about synaptic transmission and plasticity in the brainstem trigeminal nucleus caudalis (TNC). Here we used optogenetics to selectively drive a nociceptor-enriched population of trigeminal afferents while recording from superficial laminae neurons in the TNC. Low-frequency optical stimulation evoked robust long-term depression at TRPV1/ChR2 synapses. Moreover, application of three different headache trigger drugs also depressed TRPV1/ChR2 synapses. Synaptic depression at these primary afferent synapses may represent a newly identified mechanism contributing to central sensitization during headache.

Introduction

Orofacial and craniofacial pain conditions are a prevalent health burden worldwide (Vos et al., 2012) and include primary and sec-

ondary headaches and trigeminal neuralgia, as well as trigeminal inflammatory and neuropathic pain (Hargreaves, 2011; Chichorro et al., 2017). The trigeminovascular pathway is critically involved in mediating craniofacial pain. It consists of peripheral sensory neurons with cell bodies in the trigeminal ganglion (TG) that innervate meninges, cornea, tooth pulp, oral/nasal mucosa and the temporo-

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mandibular joint (Hargreaves, 2011). Centrally, nociceptive afferents synapse onto neurons in superficial and deep laminae of the brainstem trigeminal nucleus caudalis (TNc; Bennett et al., 1983). Prolonged exposure to injurious peripheral stimuli promotes sensitization of the trigeminovascular pathway that contributes to chronic pain. Experimentally, peripheral sensitization can be induced through stimulation, e.g., with capsaicin, low acidic pH, KCl, and inflammatory mediators, all of which increase spontaneous firing of trigeminal ganglion neurons in the range of 1–10 Hz (Strassman et al., 1996; Bove and Moskowitz, 1997; Zhang et al., 2012). Increased firing can also be observed in central TNc neurons upon exposure to algescic compounds including pharmacological headache triggers (Burstein et al., 1998; Jones et al., 2001; Koulchitsky et al., 2009; Akerman and Goadsby, 2015).

Despite data from animal models suggesting that sensitization of this pathway plays a key role in the pathology of headache (Akerman et al., 2011), surprisingly little is known about long-term changes in synapses of primary trigeminal afferents onto second-order TNc neurons (Hamba et al., 2000; Liang et al., 2005). This is due in part to experimental limitations, as it is technically challenging to isolate a healthy brainstem preparation with the trigeminal nerve attached (Hamba and Onimaru, 1998), and until recently this was the only way to enable intracellular recordings during selective activation of trigeminal synapses on TNc neurons. Evidence suggests that synapses from specific excitatory inputs on TNc neurons can be differentially modulated; e.g., cannabinoid receptor 1 (CB1) activation transiently depresses EPSCs at synapses of primary afferent C- and A δ -fibers (Liang et al., 2004); however, locally-evoked EPSCs were unaffected by the same agonist (Jennings et al., 2003). This discrepancy underscores the benefits of using a preparation that allows selective stimulation of defined afferents.

Here for the first time in the TNc we have taken advantage of optogenetics to selectively drive a population of nociceptive afferents by conditionally expressing channelrhodopsin in the TRPV1-cre mouse line. TRPV1 is expressed in many nociceptors (Cavanaugh et al., 2011b), and using TRPV1/ChR2 mice we found that optical stimulation in the trigeminal ganglion activates predominantly nociceptive neurons. In slices from the brainstem TNc, we then used light pulses to activate TRPV1/ChR2 axon terminals specifically, or electrical stimulation in laminae I/II to activate local synapses more globally. Using a stimulation pattern that is in the physiological range of sensitized peripheral sensory neurons, optical low-frequency activation of TRPV1/ChR2 afferents produced robust NO-mediated and NMDAR-dependent long-term depression (LTD) of optical EPSCs without depressing electrically-activated synapses on the same cell. Furthermore, *in vitro* application of drugs known to

Trigeminal ganglion

Figure 1. Trigeminal ganglion TRPV1/TdTom neurons are mainly peptidergic and nonpeptidergic nociceptors. **A–D**, Left, representative pseudocolor images of TRPV1/TdTom (purple); middle, labeling with respective neuronal markers (green); and right, double-labeling of TRPV1/TdTom with respective neuronal markers in the trigeminal ganglion. **A**, Staining for CGRP; 1435 of 3730 TRPV1/TdTom neurons were labeled with CGRP ($38 \pm 2\%$, $n = 8$). **B**, Colabeling with IB4; 816 of 2600 TRPV1/TdTom neurons were double-labeled ($31 \pm 4\%$, $n = 5$). **C**, Of 2583 TRPV1/TdTom neurons, 103 were stained with TH ($4 \pm 0.5\%$, $n = 6$) and (**D**) 181 of 2877 TRPV1/TdTom neurons were positive for NF200 ($6 \pm 0.3\%$, $n = 6$). Scale bars, 20 μ m.

trigger migraine or headache also caused synaptic depression of TRPV1/ChR2 synapses, suggesting intriguing similarities between the central actions of these drugs and activity-dependent plasticity triggered by trigeminal nociceptor activation.

Materials and Methods

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Brown University, Providence. Trpv1-Cre, lox-STOP-lox-ChR2-EYFP, and lox-STOP-lox-TdTomato mice were purchased from The Jackson Laboratory. All mice used in this study were first-generation progeny of homozygous parents. trpv1-Cre^{+/+} mice were mated with either ChR2-EYFP^{+/+} or TdTomato^{+/+} mice to generate trpv1^{+/+}/ChR2-EYFP^{+/+} or trpv1^{+/+}/TdTomato^{+/+} offspring (referred to as TRPV1/ChR2 or TRPV1/TdTomato, respectively). Both male and female mice were used for this study.

Immunohistochemistry. Standard procedures for tissue preparation and staining were used as described recently (Pradier et al., 2018). Briefly, adult (2–4 months old) TRPV1/TdTom animals were transcardially perfused with ice-cold 4% PFA. Brainstems and trigeminal ganglia were removed and postfixed overnight in 4% PFA followed by an incubation in 30% sucrose for 24 h at 4°C. Brain tissues were then snap-frozen, cryosectioned at 30 μ m on a Leica CM3050S cryostat and kept at -80°C

until use. For immunostaining, slices were thawed, washed in PBS, and permeabilized in 0.25% PBS-T for 30 min. After blocking in 5% donkey serum, the primary antibody was applied directly onto the slices and incubation followed overnight in a moist compartment at 4°C. The next day, slices were washed three times for 10 min, and the secondary antibody was applied in 0.5% BSA for 1 h. Next, slices were washed three times before mounting in Fluoromount-G. Sealing with water varnish prevented sections from drying out. Primary antibodies used in this study were raised against calcitonin gene-related peptide (CGRP; Millipore, catalog #PC205L; RRID: AB_2068524, 1:250), tyrosine hydroxylase (TH; Millipore, catalog #AB152; RRID: AB_390204, 1:500), and neurofilament 200 (NF200; Millipore, catalog #MAB5262; RRID: AB_95186; 1:200). We also used AlexaFluor 647-conjugated isolectin B4 (IB4; Invitrogen, catalog #I21411; RRID: AB_2314662) to label nonpeptidergic neurons.

Image analysis. Images were acquired on a Zeiss LSM 800 confocal microscope using a 40× water-immersion objective or for tile scan images a 20× objective at a 1024 × 1024 resolution. Lasers with wavelengths to excite green (488 nm), red (561 nm), and far-red (640 nm) fluorochromes were used. For quantification of trigeminal ganglia cells, we recorded 2–6 non-overlapping images per section and analyzed 4–5 sections per animal from a total of 5–8 mice with a total of 14,911 neurons counted using ImageJ. Immunofluorescently labeled cells were counted for each channel separately in ImageJ, and overlay images were used to identify and count cells that coexpressed TdTom and the respective neuronal marker. For the TNc intensity profile, three to four sections per animal were analyzed from a total of five mice. Intensity of TRPV1/TdTom expression as well as CGRP and IB4 binding were measured in transverse slices mediolaterally from the inner boundary of the trigeminal tract at 30–40 different locations per animal using the line tool in ImageJ.

Preparation of brainstem slices. Electrophysiological recordings were performed as described for spinal cord slices (Chirila et al., 2014) with minor modifications to improve viability of brainstem slices. In brief, under deep anesthesia brainstems of P14–P28 old TRPV1/ChR2-EYFP mice were removed after cardiac perfusion with 34°C prewarmed and oxygenated cutting ACSF (in mM: 125 NaCl, 26 NaHCO₃, 25 glucose, 6 MgCl₂, 2.5 KCl, 1.5 CaCl₂, 1.25 NaH₂PO₄, 1 kynurenic acid). Then, two to three 250-μm-thick coronal brainstem slices were cut on a vibratome (Leica 1200) starting ~0.5–0.6 mm caudal to the obex to yield the caudal trigeminal nucleus. Slices were allowed to rest at 34°C in cutting ACSF for 1 h before they were kept in ACSF (below) at RT until recording.

Whole-cell recording. TNc slices were transferred into a recording chamber, in which slices were continuously perfused with 28–30°C warm ACSF (in mM: 119 NaCl, 26 NaHCO₃, 25 glucose, 4 CaCl₂, 4 MgCl₂, 2.5 KCl, 1.3 Na-ascorbate, 1 NaH₂PO₄) bubbled with 95% O₂/5% CO₂; 100 μM picrotoxin was used to block glycine and GABA_A receptors. This high divalent cation ACSF was used to increase surface charge screening and thereby limit polysynaptic activity upon optical or electrical stimulation (Hille et al., 1975). Patch pipettes (5–7 MΩ) were pulled from borosilicate glass (Sutter Instruments) and filled with a K-glucuronate-based internal recording solution (in mM: 117 K-glucuronate, 2.8 NaCl, 0.2 MgCl₂, 5, CaCl₂, 20 HEPES, 2 Na-ATP, 0.3 Na-GTP, 0.6 EGTA). Membrane potentials were not corrected for the liquid-junction potential. An optic fiber (230 μm diameter; Plexon) was placed at the dorsolateral slice edge to stimulate TRPV1/ChR2 primary afferents with light (Plexon LED; 465 nm, 0.5–1 ms, 1–9 mW) and a concentric bipolar stainless steel electrode was placed within lamina I/II lateral to the recording site to stimulate local synapses (see Fig. 3C). Cells from lamina I/II were identified visually and recorded using whole-cell patch-clamp. Neurons were voltage-clamped at −70 mV; input resistance and series resistance were monitored throughout experiments. Recordings were discarded if these values changed by >20% during the experiment. Optically and electrically evoked EPSCs were stimulated alternately every 30 s (with 15 s interval between light and electrical stimulation). Fifty-two of 84 recordings were found to have polysynaptic optically-evoked EPSCs. In these cases, we only measured the amplitude of the first EPSC. The prevalence of polysynaptic responses made it difficult to measure paired-pulse ratios, and we have therefore not used this measure. DNQX (10 μM), TTX (1 μM), and 4-AP (100 μM) were used to characterize

light-evoked channelrhodopsin currents in TNc neurons. The light-evoked responses were unaffected by strychnine [1 μM; optical EPSCs (oEPSCs): 99 ± 12% of control, *n* = 3] and entirely blocked by DNQX (10 μM) indicating that they are mediated by AMPARs. However, in few recordings we observed a residual current of 5–10 pA that might be mediated through presynaptic release of ATP (mean amplitude in DNQX: 7.7 ± 2.4% of control; *n* = 4).

For optically-induced LTD we stimulated each cell with a light train of 1 Hz for 2 min while voltage-clamped at −40 mV at twofold baseline light pulse duration. Electrical high-frequency stimulation (HFS) was performed with two trains of 100 Hz for 1 s each at an interval of 20 s and 1.5-fold stimulation intensity. For experiments using depolarization alone, recording electrodes were filled with a Cs-glucuronate internal solution (in mM: 117 Cs-glucuronate, 2.8 NaCl, 5 MgCl₂, 0.2 CaCl₂, 20 HEPES, 2 Na-ATP, 0.3 Na-GTP, 0.6 EGTA-Cs) and following a brief baseline recording, cells were voltage-clamped at 0 mV for 90 s. To avoid washout of intracellular factors that might be required for persistent plasticity, electrical HFS, optical low-frequency stimulation (LFS), or depolarization were typically delivered within 5–10 min following establishment of the whole-cell configuration.

Headache trigger drugs were bath-applied for 10 min at the following concentrations: pituitary adenylate cyclase activating peptide 1–38 (PACAP-38) 10 nM, sodium nitroprusside (SNP) 150 μM, and nitroglycerin (NTG) 100 μM. NTG was dissolved in 23 mM ethanol and 18 mM propylene glycol; these solvents were included as vehicle to the ACSF before and after NTG application. During PACAP-38 application, BSA was present at 1 μg/ml throughout the experiment. SNP was readily soluble in water. To characterize the mechanisms of oLFS-induced LTD, TNc slices were incubated for at least 20–30 min in inhibitors (D-APV at 50 μM dissolved in water; L-NAME at 100 or 250 μM dissolved in water; AM251 at 5 μM dissolved in DMSO) before oLFS. In some experiments, the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP; 200 μM, dissolved in DMSO) was bath applied. DMSO was then perfused into the recording chamber at least 10 min before SNAP application. If the EPSCs averaged over 5 min changed by >20% of baseline values at indicated time points, recordings were regarded as potentiated or depressed; otherwise they were considered unchanged.

Statistical analysis. All results are expressed as the mean ± SEM. We evaluated the effect of LFS/HFS and of drug application using paired *t* tests of non-normalized raw data comparing 5 min bins before and at indicated time points after each experimental manipulation, except as noted. Some of the recordings from Figure 4E were interleaved with experimental treatments and are therefore also reported in Figure 5B as “control cells”. *N* values were based on previous experience and represent the number of slices used. Typically one slice was used per animal and experiment, although occasionally 2 and rarely 3 recordings were used per animal. All statistical analyses were performed using GraphPad Prism software. Statistical tests were considered to be significant at a 95% confidence interval, with *p* values reported in the Results section.

Results

Primary trigeminal ganglion afferents expressing TRPV1-cre

We first characterized the neuronal population of TG cells expressing cre-recombinase under the control of the *trpv1* promoter (Cavanaugh et al., 2011b) crossed with animals expressing TdTom in a cre-dependent manner. First we compared immunohistochemical labeling with TdTom expression in the trigeminal ganglion (Fig. 1A–D, purple). Labeling of TG neurons with a marker for peptidergic nociceptors revealed that 38 ± 2% of TdTom⁺ neurons were positive for CGRP (Fig. 1A, green). Additionally, 31 ± 4% of TdTom⁺ neurons bind IB4, a marker for nonpeptidergic nociceptors (Fig. 1B, green), 4 ± 0.5% were co-labeled with TH (Fig. 1C, green), and 6 ± 0.3% colocalized with NF200 (Fig. 1D, green), markers that identify myelinated and unmyelinated low-threshold mechanoreceptors (LTMRs), respectively (Li et al., 2011; Ringkamp et al., 2013; Usoskin et al., 2015). Conversely, 71 ± 3% of CGRP-labeled, 59 ± 2% of IB4-

Figure 2. Peripheral TRPV1/TdTom neurons predominantly terminate in laminae I/II of the trigeminal nucleus caudalis. **A–D**, TRPV1/TdTom expression decreases toward the rostral extent of the TN. TRPV1/TdTom expression (left, purple) decreases from interpolar (**A**, **C**) to oral (**B**, **D**) extent of the TN, consistent with reduced labeling for peptidergic (CGRP; **A**, **B**) and nonpeptidergic (IB4; **C**, **D**) nociceptors (green, overlay, left). **E**, **F**, Representative images of TRPV1/TdTom terminals (left, purple) in the TNc stained for CGRP (**E**, top middle, green), which marks lamina I and outer lamina II or colabeled with IB4, which highlights inner lamina II (**F**, bottom middle, green). Both markers colocalize extensively with TRPV1/TdTom (right). **G**, Quantitative analysis reveals that TRPV1/TdTom fibers terminate in laminae I and II of the TNc ($n = 5$). Scale bars: 200 μm ; insets, 40 μm .

binding, $84 \pm 2\%$ of TH-labeled and $18 \pm 1\%$ of NF200-labeled neurons were positive for TdTom. Consistent with earlier reports (Cavanaugh et al., 2011a; Kim et al., 2012), we also found cell bodies in the TNc that express TRPV1/TdTom. These neurons, however, were very sparse and have previously been shown to be predominantly GABAergic interneurons (Kim et al., 2012). As GABA_A receptors were blocked by picrotoxin in our experiments, light-activated IPSCs from these neurons did not contribute to our recordings.

The somatosensory modality of input encoded by peripheral TG neurons projecting to the trigeminal nucleus differs along its caudal to rostral extent, with TNc receiving predominantly nociceptive and rostral (oral) TN receiving predominantly non-nociceptive sensory input (Villanueva and Nosedá, 2013). Consistent with this, we found the weakest TRPV1/TdTom ex-

pression in the rostral part of the TN, whereas its expression continuously increased toward the caudal extent of the TN (Fig. 2A–D), and was paralleled by increased peptidergic (Fig. 2A, B) and nonpeptidergic (Fig. 2C, D) nociceptor innervations. As we were most interested in nociceptive inputs, we characterized terminals of TRPV1-lineage neurons in the TNc in more detail and found that TRPV1/TdTomato terminals largely overlapped in the superficial layers with markers for peptidergic (CGRP; Fig. 2E) and nonpeptidergic (IB4; Fig. 2F) nociceptors. TRPV1-lineage neurons predominantly terminate 5–60 μm from the inner boundary of the trigeminal tract, and colocalized with peptidergic terminals (Fig. 2G) that terminate 10–50 μm from the trigeminal tract and that identify lamina I and outer lamina II (Todd, 2010). Further, we detected nonpeptidergic fibers that define inner lamina II in the spinal cord, 25–55 μm distant from

Figure 3. Electrical HFS induces plasticity at synapses of TRPV1/ChR2 fibers and non-nociceptive afferents with TNc neurons. **A**, Channelrhodopsin expression in TRPV1-lineage trigeminal ganglion neurons allows faithful optical control of spiking at 1 Hz. Blue ticks indicate optical stimuli. **B**, Light pulses elicited steady-state photocurrents, a characteristic of cells expressing ChR2. **C**, Diagram showing recording setup: an optic fiber was placed at the dorsolateral slice edge to stimulate trigeminal TRPV1/ChR2 primary afferents with blue light (465 nm, 0.5–1 ms, 1–9 mW) and a concentric bipolar stainless steel electrode was placed within lamina I/II lateral to the recording site to stimulate local synapses. Note that there are no dorsal roots in this slice preparation of the TNc, and instead we optically stimulated peripheral afferents at the slice edge. HFS was delivered using the electrical stimulation electrode. **D**, Example traces for monosynaptic (top) and polysynaptic (bottom) optically-evoked EPSCs in different cells from TRPV1/ChR2 synapses. **E**, In neurons of lamina I/II of the TNc, optical stimulation of TRPV1/ChR2 primary afferent terminals elicited EPSCs (black trace) at the first central synapse of the trigeminovascular pathway. Application of TTX ($1 \mu\text{M}$) blocked all synaptic activity (green trace). **F**, After electrical HFS, normalized data show no change at electrically-evoked EPSCs (top) and optically-

the trigeminal tract (Fig. 2G), and these also overlapped extensively with TRPV1-lineage fibers. Together, these data indicate that TRPV1/ChR2 animals provide a valuable tool to study a nociceptor-enriched population of projections from the trigeminal ganglion to the brainstem TNc.

In freely moving TRPV1/ChR2 mice, stimulation of the shaved scalp skin with blue light evoked strong nocifensive responses reminiscent of behaviors seen following light stimulation of the hindpaw in the same mouse line (data not shown; Stemkowski et al., 2016), consistent with TRPV1-lineage neurons comprising nociceptive primary afferents. We next investigated the effect of light-gated currents in TRPV1/ChR2 neurons. Cells in the trigeminal ganglion were stimulated with brief pulses of blue light and responses were recorded in current-clamp. We found that blue light evoked action potentials, and that neurons would readily follow 1 Hz stimulation (Fig. 3A). In voltage-clamp recordings from TG neurons light pulses elicited steady-state photocurrents, a characteristic of cells expressing ChR2 (Fig. 3B).

In the brainstem TNc slice preparation, we stimulated terminals of TRPV1/ChR2 primary afferents by delivering blue light (Plexon LED; 465 nm, 0.5–1 ms, 1–9 mW) through an optic fiber directed at the dorsolateral slice edge, where trigeminal ganglion afferents enter the brainstem (Fig. 3C). Light activation could evoke

evoked EPSCs (bottom). **G**, Time course of normalized, individual experiments following electrical HFS, showing variability of synaptic responses for eEPSCs (top) and oEPSCs (bottom). Two eEPSCs and 4 oEPSCs (shown in gold) potentiated $>20\%$, 3 eEPSCs and 6 oEPSCs (shown in light gray) depressed $>20\%$, 6 eEPSCs and 1 oEPSC (in dark gray) did not change $>20\%$ 15–20 min following eHFS. **H**, Non-normalized data showing eEPSCs (top) and oEPSCs (bottom) at baseline compared with 15–20 min following HFS (eEPSCs: $99 \pm 17\%$; paired t test: $p = 0.83$, $n = 11$; oEPSCs: $99 \pm 16\%$; paired t test: $p = 0.68$, $n = 11$). **I–K**, Depolarization alone of TNc neurons induces plasticity at synapses with TRPV1/ChR2 fibers and non-nociceptive afferents. **I**, Summary of normalized data showing no change at electrically-evoked EPSCs (top) and optically-evoked EPSCs (bottom). **J**, Time course of normalized, individual experiments showing variability of synaptic response following depolarization for eEPSCs (top) and oEPSCs (bottom). Four eEPSCs and 5 oEPSCs (shown in gold) potentiated $>20\%$, 3 eEPSCs and 2 oEPSCs (shown in light gray) depressed $>20\%$, 1 eEPSC, and 3 oEPSCs (in dark gray) did not change $>20\%$ 15–20 min following depolarization. **K**, Non-normalized data showing eEPSCs (top) and oEPSCs (bottom) at baseline compared with 15–20 min following depolarization (eEPSCs: $103 \pm 27\%$; paired t test: $p = 0.87$, $n = 8$; oEPSCs: $136 \pm 27\%$; paired t test: $p = 0.48$, $n = 10$).

Figure 4. Optical LFS depresses EPSCs at TRPV1/Chr2 synapses. **A**, Diagram showing recording setup: an optic fiber was placed at the dorsolateral slice edge to stimulate TRPV1/Chr2 primary afferents with blue light and a concentric bipolar stainless steel electrode was placed within lamina I/II lateral to the recording site to stimulate local synapses. LFS was delivered using the optic fiber. **B**, Representative trace of optically-evoked EPSC that faithfully follow optical LFS at 1 Hz (pulse duration: 1 ms). **C**, Two different frequencies for oLFS (1 and 10 Hz) as well as pairing of 1 Hz oLFS to postsynaptic depolarization (−40 mV vs −70 mV) all induce depression at TRPV1/Chr2 synapses at 15–20 min following oLFS compared with baseline (two-way ANOVA: $p = 0.0007$, $F_{(1,11)} = 21.66$, $n = 4–5$ per group). **D, G**, Representative single experiments showing LTD of optically-evoked EPSCs following oLFS (**D**) and modest potentiation of electrically-evoked EPSCs (**G**) in the same cell. DNQX completely blocked light- and electrically-evoked synaptic transmission. **E, H**, Summary of normalized data showing LTD of oEPSCs (**E**) and modest potentiation of eEPSCs (**H**) following oLFS. **F, I**, Non-normalized data showing oEPSCs (**F**) and eEPSCs (**I**) at baseline compared with 15–20 min following oLFS (oEPSCs: $62 \pm 10\%$; paired t test: $**p = 0.002$, $n = 16$; eEPSCs: $114 \pm 7\%$; paired t test: $*p = 0.02$, $n = 16$). Insets, EPSCs before (black trace, control), 20 min after oLFS (red trace) and following DNQX application (blue trace, $10 \mu\text{M}$). Scale bars: 10 ms, 100 pA. **J**, Monosynaptic light–light paired EPSCs (black–black traces, top) or electrical–light (red–black traces, bottom). EPSCs were evoked at an interstimulus interval of 50 ms. Examples of evoked EPSC pairs from two cells. **K**, Pairs of stimuli like those in **J** were delivered. The amplitudes of the second oEPSC is plotted as evoked either by a light–light pairing (green symbols) or an electrical–light pairing (blue symbols), normalized to the first oEPSC in a light–light pair ($n = 7$). If all of the afferents evoked by light- and electrical-stimulation are shared, the blue symbol should be the same amplitude as the green symbol for a given cell; if none of the afferents is shared, the blue symbol should have the same amplitude as the black symbol (oEPSC1). Although there is some apparent overlap in the two inputs, in most cells the inputs are relatively independent.

EPSCs in cells of lamina I/II that appeared monosynaptic (short latency, low jitter), but in other cells often evoked apparently polysynaptic EPSCs as well (Fig. 3D). Because picrotoxin was used in all of our experiments, the polysynaptic activity was presumably evoked by neurons in the TNc receiving synaptic inputs from TRPV1 lineage primary afferents that then made excitatory synapses on the recorded cell. Application of TTX blocked all synaptic activity indicating that ChR2 currents in TRPV1-lineage axons alone are not sufficient to trigger neurotransmitter release and that presynaptic voltage-gated Na^+ channels are required to elicit postsynaptic EPSCs (Fig. 3E).

Synaptic plasticity following electrical HFS and depolarization alone

In each TNc slice from TRPV1/ChR2 mice, we alternately stimulated two different afferent inputs (Fig. 3C); the optic fiber was used to evoke oEPSCs selectively from TRPV1-lineage afferents, while an electrical stimulating electrode was placed nearby in laminae I/II to activate unidentified mixed afferents and evoke electrical EPSCs (eEPSCs; Fig. 3C). HFS or LFS of afferents can induce long-term potentiation (LTP) or LTD at many synapses, so we first tested the effects of HFS delivered via the electrical stimulating electrode (eHFS; 100 Hz). On average, eEPSCs did not change (Fig. 3F, top; $99 \pm 17\%$; $p = 0.83$), however, responses varied from experiment to experiment. Only 2 of 11 eEPSCs recorded potentiated by at least 20% and 3 of 11 inputs depressed by at least 20% (Fig. 3G,H, top). On average, the amplitude of oEPSCs in the same cells was also unchanged (Fig. 3F, bottom; $99 \pm 16\%$; $p = 0.68$), but the variability of the resulting plasticity was even more marked (Fig. 3G,H, bottom): 4 of 11 oEPSCs potentiated while 6 of 11 inputs depressed.

As the electric field generated by the eHFS could potentially also activate TRPV1/ChR2 axon terminals we measured the correlation between the initial electrical stimulation strength and the percentage potentiation/depression. No significant correlation was observed ($R^2 = 0.01$, $p = 0.74$), indicating that stimulation intensity cannot account for the sign of the plasticity. Notably, we also recorded persistent plasticity in 6 of 11 recordings using stimulation intensities considered subthreshold for C- and A δ -fibers ($<150 \mu\text{A}$; Chen and Sandkühler, 2000). These results suggest that in almost half of our recordings, TRPV1/ChR2 synaptic strength was modified heterosynaptically by activation of non-C- or A δ -fiber activity.

To mimic strong postsynaptic neuronal activity, we depolarized the postsynaptic cell to 0 mV for 90 s without concomitant synaptic activation. Similarly to the eHFS experiment above, neither optically- nor electrically-evoked EPSCs changed on average (Fig. 3I, top; eEPSC: $103 \pm 27\%$, $p = 0.87$; oEPSC, bottom: $136 \pm 27\%$, $p = 0.48$), but again, highly variable LTP or LTD was generated on a cell by cell basis. Four of 8 eEPSCs potentiated and 3 of 8 depressed (Fig. 3J,K, top), while 5 of 10 oEPSCs potentiated and 2 of 10 depressed (Fig. 3J,K, bottom). Thus, postsynaptic depolarization alone persistently potentiates or depresses many TNc synapses sampled using either local electrical stimulation or selective optical stimulation of nociceptor-enriched afferents.

Synaptic plasticity following optical LFS

Next we asked whether optical activation of TRPV1/ChR2 primary afferents at low frequencies could trigger LTP or LTD at synapses on TNc neurons. Again we recorded both o- and eEPSCs (Fig. 4A), before and after delivering a light train at either 1 or 10 Hz, frequencies consistent with the development of pe-

ripheral sensitization of trigeminovascular nociceptors. Light stimulation of presynaptic TRPV1/ChR2 terminals at 1 Hz reliably induced oEPSCs (Fig. 4B). Following a brief baseline period, we delivered optical LFS and observed robust depression (Fig. 4C; $p = 0.0007$, repeated-measures two-way ANOVA, $F_{(1,11)} = 21.66$). Notably, pairing 1 Hz optical LFS with postsynaptic depolarization (a protocol that can trigger LTP at some synapses) also triggered only LTD.

C- and A δ -fiber nociceptors only reliably follow 1–2 Hz stimulation without failures of AP firing (Nakatsuka et al., 2000), and we therefore focused our studies on a single protocol: 1 Hz light pulses paired with modest postsynaptic depolarization to -40 mV (Fig. 4D–I). Optical LFS at 1 Hz produced robust long-term depression at TRPV1/ChR2 terminals measured with optical stimulation (Fig. 4D–F; $62 \pm 10\%$; $p = 0.0009$). Surprisingly, however, eEPSCs evoked in the same neurons rarely had LTD following optical LFS (only 1 of 16 inputs depressed), and in fact 7 of 16 were potentiated (Fig. 4G–I; average eEPSC amplitude post-oLFS: $114 \pm 7\%$; $p = 0.01$). This result suggests that electrical stimulation in lamina I/II drives a population of predominantly non-TRPV1-lineage afferents making synapses on superficial laminae neurons, because oEPSCs and eEPSCs are differentially modulated by oLFS. We further probed the independence of light- and electrically-evoked pathways by comparing responses to pairs of stimuli with a 50 ms interstimulus interval consisting either of the same modality (paired light-light pulses) or each modality (electrical-light pulses). Although in some cells we observed an interaction, in general, the electrical pathway only modestly affected the subsequently activated light pathway; i.e., a light-evoked EPSC was of similar amplitude whether or not it was preceded by an electrically-evoked EPSC (Fig. 4J,K).

LTD requires NMDAR and NO signaling, but not CB1R activation

To investigate the mechanisms required for light-evoked LTD we tested several possible signaling molecules (Fig. 5A). NMDARs play a pivotal role in many forms of LTP and LTD at excitatory CNS synapses (Malenka and Bear, 2004). To test their role in the TNc, we used the NMDAR antagonist D-APV ($50 \mu\text{M}$) to block signaling through these receptors (Fig. 5B). Following optical LFS, LTD of optically evoked EPSCs was blocked (and in fact in 5 of 9 experiments, oEPSCs were potentiated after oLFS; Fig. 5C–E; $111 \pm 19\%$; $p = 0.87$). Interleaved control recordings without D-APV depressed strongly compared with those with D-APV (Fig. 5E; $59 \pm 9\%$; $p = 0.03$, unpaired t test). Thus, activation of NMDARs is required for optical LFS to trigger LTD at optically-activated synapses. Because nitric oxide is involved in sensitization of the TNc and can act as a downstream effector for NMDAR signaling (Brenman and Bredt, 1997; Yonehara et al., 2002), we tested the effects of bath application of the NO donor, SNAP. Application of SNAP alone depressed optically-activated synapses ($33 \pm 7.8\%$, $p = 0.04$, $n = 5$). To probe the involvement of NO signaling in oLFS-induced LTD, we next blocked NO synthase (NOS). In the presence of L-NAME (100 or $250 \mu\text{M}$), optical LFS no longer elicited significant LTD (Fig. 5F–H; L-NAME $100 \mu\text{M}$: $81 \pm 10\%$; $p = 0.13$; $250 \mu\text{M}$: $84 \pm 6\%$; $p = 0.14$). To test whether this form of LTD also requires endocannabinoid signaling we investigated the effect of the CB1R antagonist AM251 (Fig. 5I–K). AM251 ($5 \mu\text{M}$) did not affect oLFS-induced LTD (Fig. 5I–K; $61 \pm 11\%$; $p = 0.03$).

We were concerned whether repetitive light stimulation itself causes desensitization and/or internalization of presynaptic

Figure 6. Headache triggers depress TRPV1/ChR2 synapses. **A, D, G**, Representative single experiments showing depression of optically-evoked EPSCs following 10 min bath application of NTG (100 μ M; **A**), SNP (150 μ M; **D**) and PACAP-38 (10 μ M; **G**). **B, E, H**, Summary of normalized data showing depression induced by NTG (**B**), SNP (**E**), and PACAP-38 (**H**). **C, F, I**, Non-normalized data showing oEPSCs at baseline compared with 15–20 min following bath application of NTG (**C**; $73 \pm 8\%$; paired t test: $***p = 0.0009$, $n = 11$), SNP (**F**; $70 \pm 8\%$; paired t test: $**p = 0.007$, $n = 11$), and PACAP-38 (**I**; $75 \pm 9\%$; paired t test: $*p < 0.03$, $n = 10$). Insets, EPSCs before (black trace, control), and 20 min after drug application (green trace). Scale bars: 5 ms, 25 pA. Insets are averages of six oEPSCs.

ChR2, producing an artifactual depression (Lin, 2011). To control for this, we isolated ChR2-evoked synaptic currents by application of TTX together with 4-aminopyridine (4-AP). TTX blocks voltage-dependent Na^+ channels and action potentials, and 4-AP blocks K^+ channels, often permitting ChR2 expressed in presynaptic terminals to produce sufficient depolarization to allow vesicle release (Cruikshank et al., 2010). As expected, oEPSCs in the presence of TTX and 4-AP had markedly increased onset latency, consistent with slower presynaptic terminal depolarization upon stimulation with light. Importantly, delivery of the same optical LFS protocol used above had no effect on these ChR2 responses (Fig. 5L), showing that changes in presynaptic ChR2 cannot account for the long-term synaptic depression. The lack of LTD was observed in three experiments; in some experiments we also observed an increased onset latency of the oEPSC following oLFS.

Pharmacologic headache triggers induce depression at TRPV1/ChR2 synapses

To further investigate the first central synapse of the trigemino-vascular pathway, we next tested the effects of acute application of drugs known to trigger headache or migraine when given to human patients (Iversen et al., 1989; Schytz et al., 2009; Guo et al., 2013). We first applied two nitric oxide donors (nitroglycerine and sodium nitroprusside), while recording from lamina I/II TNC neurons and stimulating optically. Both drugs depressed optically-evoked EPSCs. Bath-application of NTG (100 μ M) caused a $73 \pm 8\%$ depression of oEPSCs (Fig. 6A–C; $p = 0.0009$, $n = 11$), and bath-application of SNP (150 μ M) similarly depressed oEPSCs (Fig. 6D–F; $70 \pm 8\%$; $p = 0.007$, $n = 11$). The neuropeptide PACAP induces migraine in humans, and in-

creased plasma levels are reported during migraine attack (Edvinsson et al., 2018). Bath application of 10 nM PACAP-38 on the brain slice also caused a $75 \pm 9\%$ depression of oEPSCs in TNC neurons (Fig. 6G–I; $p = 0.03$, $n = 10$). In most recordings, the oEPSCs remained persistently depressed after each drug was washed out. Together, our experiments demonstrate that depression at TRPV1-lineage synapses is a prominent form of plasticity following acute application of headache triggering drugs.

Discussion

We have used a novel approach to selectively test synaptic plasticity in a nociceptor-enriched population of primary afferents on TNC neurons. We found that the majority of TRPV1/ChR2 trigeminal ganglion cells label with nociceptor markers, and that these TG cells heavily innervate the superficial layers of the brainstem TNC. Using light to stimulate axons of these cells in brainstem slices, we found that neurons in laminae I–II receive only excitatory synaptic input. Surprisingly, patterned synaptic activation produced divergent activity-dependent plasticity at inputs evoked either optically or electrically. Optical LFS invariably triggered NMDAR-dependent LTD that was predominantly mediated through NOS, while blockade of CB1Rs had no effect on

LTD. Furthermore, application of several drugs that trigger headache in humans also depressed trigeminal nociceptor-enriched synapses.

TRPV1/ChR2 trigeminal ganglion neurons and their central projections

We found that 70% of TRPV1/TdTom labeled neurons in the TG stained for either CGRP or IB4, consistent with identity as peptidergic or nonpeptidergic nociceptors, while smaller populations also identified myelinated (NF200) and unmyelinated LTMRs (TH). The ratio of peptidergic to nonpeptidergic nociceptors was lower than expected in the TRPV1/TdTom population based on literature using reporter expression or antibodies for TRPV1 (Zwick et al., 2002; Price and Flores, 2007; Cavanaugh et al., 2011b; Ono et al., 2015). Once cre-recombinase expression is turned on in a cell during development, it will remain continuously active, thereby marking a pool of cre-expressing neurons that later in development may not express TRPV1 channels (Cavanaugh et al., 2011b). As nonpeptidergic and peptidergic nociceptors developmentally derive from the same progenitor cells (Chen et al., 2006), it is possible that a subpopulation of IB4 neurons originates from TRPV1/cre-lineage neurons. This would increase the observed ratio of TRPV1/TdTom/IB4 cells while decreasing the ratio of peptidergic TRPV1/TdTom neurons. Despite this caveat, our characterization emphasizes that the TRPV1-cre mouse line is a useful tool to study physiology of synapses from nociceptor-enriched afferents in the TNC.

Optically- and electrically-evoked afferents activate distinct TNc synapses

Surprisingly, we found that the synapses activated electrically or optically behave differently from one another. Although our optically-stimulated pathway is comprised of peripheral primary afferents, the electrical stimulating electrode was not placed in a primary afferent bundle, but instead is likely to activate both local and ascending/descending inputs. HFS delivered via the electrical stimulating electrode rarely produced plasticity of the same magnitude or sign in oEPSCs or eEPSCs from the same cell. Similarly, after optical LFS, we consistently observed depression of oEPSCs but instead, modest potentiation of eEPSCs. As none of the electrically-evoked EPSCs showed LTD but 75% of optically-evoked EPSCs were depressed following oLFS, we conclude that local electrical stimulation in this slice preparation yields a low probability of sampling TRPV1/cre nerve terminals from TG neurons. Moreover, the oEPSCs in paired pulse recordings were minimally affected by previous activation of the electrical pathway, again supporting the idea that the two are largely independent. These observations emphasize the utility of using a defined input to study synapses on TNc cells, and suggest caution in interpreting experiments relying on nonselective afferent stimulation.

Mechanisms of LTD at nociceptor-enriched synapses

Sensitization of peripheral afferents through chemical stimulation, including “inflammatory soup”, IL-1 β , acidic pH, KCl or capsaicin, increases spontaneous discharge frequency of trigeminal ganglion neurons to 1–10 Hz (Strassman et al., 1996; Bove and Moskowitz, 1997; Zhang et al., 2012). Thus our *in vitro* observations using stimulation frequencies of 1 and 10 Hz may model central effects following peripheral sensitization. Optical LFS triggered robust LTD that was blocked by an NMDA receptor antagonist. Importantly, this LTD was not due to desensitization of presynaptic channelrhodopsin. Many studies of craniofacial pain report increased neuronal activation in the TNc following peripheral injection of formalin, capsaicin, NO-donors (NTG, SNP, and SNAP) or complete Freund’s adjuvant (Burstein et al., 1998; Jones et al., 2001; Jung et al., 2009; Koulchitsky et al., 2009; Sixt et al., 2009; Takeda et al., 2012; Romero-Reyes et al., 2013). Craniofacial allodynia or c-fos induction in the TNc is also prevented by the nonselective NOS inhibitor L-NAME or ODQ, which inhibits the downstream NO target, soluble guanylate cyclase (Lassen et al., 1998; Hoskin et al., 1999; Offenhauser et al., 2005; Jung et al., 2009; Ramachandran et al., 2014; Ben Aissa et al., 2018; Pradhan et al., 2018). Our data also support a key role for NO signaling in the LTD of nociceptor synapses, as a NOS inhibitor strongly attenuated the LTD. We also noted that simply depolarizing the postsynaptic neuron, without concomitant synaptic activity, or eHFS often produced either LTD or LTP; it is possible that in some neurons, this protocol causes NO release downstream from Ca²⁺ influx, and that this could contribute to the LTD. Previous work demonstrated LTP following HFS (50–100 Hz) at synapses from isolated trigeminal nerve afferents onto neurons in the TNc slice that is independent of NMDA receptor activation and postsynaptic Ca²⁺, but requires mGluR5 activation (Hamba et al., 2000; Liang et al., 2005). In our experiments, electrical HFS did not consistently evoke LTP, but again the electrically stimulated afferents are largely non-overlapping with nociceptive neurons in our preparation.

This is the first report of optical LFS-induced LTD in the TNc, however LTD has been characterized in the dorsal horn using electrical LFS of dorsal roots (Sandkühler et al., 1997; Chen and

Sandkühler, 2000; Kato et al., 2012; Kim et al., 2015). Using a similar stimulation of C-fibers in the spinal dorsal horn Kato et al. (2012) observed two mechanistically different forms of LTD: one mediated by NMDA receptors and the other dependent on CB1 receptor activation; D-APV alone does not fully block the spinal dorsal horn LTD (Sandkühler et al., 1997; Kato et al., 2012), whereas it was fully blocked in the TNc. However, in contrast to findings in the spinal cord, blockade of CB1R had no effect on LTD in the TNc despite earlier studies demonstrating CB1R activation at C- and A δ -fiber synapses induced short-term depression (Liang et al., 2004). The differences in LTD mechanisms in the TNc versus the spinal dorsal horn might be attributable to sampling of different subsets of afferents or synapses, but may as well reflect region-specific plasticity. Interestingly, with NMDARs blocked (but not with NOS blocked), in half of our experiments oLFS potentiated TNc synapses, suggesting that oLFS may activate two competing signaling pathways: one that elicits LTD, and a second that instead promotes NMDAR-independent LTP, which is unmasked when NMDARs are blocked.

Intriguingly, we also found that in the presence of TTX and 4-AP, conditions that block action potentials and allow only Chr2-induced neurotransmitter release (Cruikshank et al., 2010), oEPSCs were not depressed following oLFS. This experiment confirmed that oLFS does not significantly inactivate Chr2, as we had hoped. However, this result also indicates that TTX and 4-AP block oLFS-induced LTD. Although it is possible that the oLFS-induced LTD is caused by an NMDAR-dependent decrease in postsynaptic AMPAR numbers or function, it is difficult to suggest a scenario by which TTX + 4-AP would prevent this. Instead, we speculate that Chr2-gated Ca²⁺ currents and/or release mechanisms cannot be modified as usual when LTD is induced. Perhaps action potentials and Chr2 promote differential release of dense core vesicles that contain neuropeptides like CGRP or PACAP-38, possibly necessary for LTD induction. Alternatively, 4-AP-sensitive K⁺ or TTX-sensitive Na⁺ channels may be directly required to mediate presynaptic depression, so that when blocked the LTD cannot occur. In either case, our results favor an induction mechanism that requires NMDARs (located either presynaptically or postsynaptically) and NO-release that then may act to weaken synaptic strength by either a presynaptic or postsynaptic mechanism. Further work will be needed to thoroughly define this signaling pathway.

Headache triggers depress TNc nociceptive synapses

We tested the acute effect of bath applying drugs that trigger headaches or migraines in humans, and elicit peripheral and facial allodynia as well as photo- and osmophobia in mouse models for migraine (Iversen et al., 1989; Schytz et al., 2009; Guo et al., 2013; Romero-Reyes and Akerman, 2014; Burgos-Vega et al., 2016). Nitroglycerine, SNP, and PACAP-38 all promoted persistent synaptic depression at optically activated TRPV1/Chr2 synapses on lamina I/II TNc neurons. The similarity to oLFS-induced LTD suggests the possibility that these forms of depression may use overlapping mechanisms.

PACAP-38 is known to modulate excitatory transmission in the hippocampus, increasing NMDAR currents and phosphorylation at concentrations similar to those we used (1–10 nM; Yaka et al., 2003; Macdonald et al., 2005). As increased PACAP-38 plasma levels are reported during migraine attack and it is expressed by CGRP⁺ trigeminal nociceptors (Eftekhari et al., 2015; Edvinsson et al., 2018), it may be released during optical LFS, thereby enhancing NMDAR signaling and promoting NMDAR-dependent LTD. NO production can be triggered through NMDAR-mediated Ca²⁺ signaling,

which subsequently activates neuronal NOS (Brenman and Bredt, 1997) and optical LFS also promotes the release of NO. Interestingly, both mechanisms might also functionally overlap, as mice deficient for PACAP-38 showed no NTG-induced photophobia or neuronal activation in the TNc (Markovics et al., 2012). However, future studies are needed to investigate interactions among PACAP-38-, NO-, and NMDAR-signaling.

Might depression of EPSCs by low-frequency TG nociceptor activation or by headache trigger drugs enhance nociception? As LTD was most easily elicited at excitatory trigeminal nucleus synapses on inhibitory neurons (Kim et al., 2015), one possibility is that oLFS, PACAP-38, and NO-donors depress TRPV1/ChR2 synapses on inhibitory neurons. If the excitatory input to inhibitory neurons is reduced, the inhibitory output will then be decreased, consequently resulting in disinhibition of excitatory transmission. Our data cannot address this; however, future experiments investigating cell-type-specific forms of plasticity will be essential to understand the local circuitry. Our data suggest that the TRPV1/ChR2 brain slice model may prove useful to elucidate central mechanisms of headache and modulation by headache inducing drugs.

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