Experimental Neurology 298 (2017) 1–12

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Contribution of amygdala CRF neurons to chronic pain

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ARTICLE INFO

Keywords:
Stress-induced analgesia
Central amygdala
Chronic pain
Neuropathic pain
Corticotropin-releasing factor
Norepinephrine
Descending pain inhibition

ABSTRACT

We investigated the role of amygdala corticotropin-releasing factor (CRF) neurons in the perturbations of descending pain inhibition caused by neuropathic pain. Forced swim increased the tail-flick response latency in uninjured mice, a phenomenon known as stress-induced analgesia (SIA) but did not change the tail-flick response latency in mice with neuropathic pain caused by sciatic nerve constriction. Neuropathic pain also increased the expression of CRF in the central amygdala (CeAmy) and ΔFosB in the dorsal horn of the spinal cord. Next, we injected the CeAmy of CRF-cre mice with cre activated AAV-DREADD (Designer Receptors Exclusively Activated by Designer Drugs) vectors. Activation of CRF neurons by DREADD/Gq did not affect the impaired SIA but inhibition of CRF neurons by DREADD/Gi restored SIA and decreased allodynia in mice with neuropathic pain. The possible downstream circuitry involved in the regulation of SIA was investigated by combined injections of retrograde cre-virus (CAV2-cre) into the locus ceruleus (LC) and cre activated AAV-diphtheria toxin (AAV-FLEX-DTX) virus into the CeAmy. The viral injections were followed by a sciatic nerve constriction ipsilateral or contralateral to the injections. Ablation of amygdala projections to the LC on the side of injury but not on the opposite side, completely restored SIA, decreased allodynia and decreased ΔFosB expression in the spinal cord in mice with neuropathic pain. The possible lateralization of SIA impairment to the side of injury was confirmed by an experiment in which unilateral inhibition of the LC decreased SIA even in uninjured mice.

The current view in the field of pain research attributes the process of pain chronification to abnormal functioning of descending pain inhibition. Our results demonstrate that the continuous activity of CRF neurons brought about by persistent pain leads to impaired SIA, which is a symptom of dysregulation of descending pain inhibition. Therefore, an over-activation of amygdala CRF neurons is very likely an important contributing factor for pain chronification.

1. Introduction

SIA can occur during or after a physical or psychological stressor and it decreases the conscious perception of pain in humans as well as the behavioral response to nociceptive stimuli in animals (Lewis et al., 1980; Long et al., 2016; Benedetti et al., 1999). SIA depends on activation of inhibitory supraspinal projections to the dorsal horn of the spinal cord, which contains the first central nervous system synapses for nociceptive information. The inhibitory projections are collectively referred to as the descending pain inhibitory system or simply descending inhibition (Mayer and Price, 1976; Liebeskind and Mayer, 1971). Descending inhibition affects multiple nociceptive modalities including thermal, inflammatory and neuropathic pain. Descending inhibitory pathways are polysynaptic with significant contributions from the prefrontal and cingulate cortices, amygdala, ventrolateral periaqueductal gray (PAG), LC and rostral ventromedial medulla (RVM) (Millan, 2002). Brainstem projections that contain norepinephrine or serotonin are a major part of the final inhibitory input to the dorsal horn. Norepinephrine and serotonin inhibit nociception via both pre- and postsynaptic mechanisms, which forms the basis for treating chronic pain with serotonin and norepinephrine reuptake inhibitors or receptor agonists (Ossipov et al., 2010). The CeAmy plays an important role in the physiological response to multiple stressors including pain (Oliveira and Prado, 2001; Carrasquillo and Gereau, 2007). The CeAmy receives processed sensory information through the basolateral amygdala (BLA) and a direct nociceptive input via the spino-parabrachial-amygdaloid pathway (Sarhan et al., 2013). The CeAmy is a key element in descending inhibition of pain and is essential for robust SIA (Werka, 1994). The projections of the CeAmy target the bed nucleus of stria terminalis (BNST) and several brainstem nuclei, including the LC (Van Bockstaele et al., 1996; Partridge et al., 2016). There is general agreement that CeAmy CRF neurons do not affect baseline sensory thresholds but their role in pain is not clear. Long lasting inflammatory and neuropathic pain increases CRF expression in the CeAmy (Rouwette

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http://dx.doi.org/10.1016/j.expneurol.2017.08.010
Received 17 April 2017; Received in revised form 3 August 2017; Accepted 18 August 2017
Available online 19 August 2017
0014-4886/

et al., 2012a; Ulrich-Lai et al., 2006; Rouwette et al., 2011). CRF release in the CeAmy causes hypersensitivity via CRF1 receptor and analgesia via CRF2 receptor (Itoga et al., 2016; Ji and Neugebauer, 2008; Ji and Neugebauer, 2007). While low doses of endogenous CRF in CeAmy increase pain sensitivity (Bourbia et al., 2010), high doses of exogenous CRF are analgesic (Cui et al., 2004). One hypothesis that summarizes the role of CRF in pain processing is that the CRF neurons in the amygdala may act as an on/off switch for chronic pain (Rouwette et al., 2012b). The amygdala CRF neurons are well situated for the role of pain switch because they not only receive and respond to nociceptive stimuli but also undergo plasticity in association with chronic nociceptive stimulation and are responsible for the central sensitization and hyperalgesia observed in chronic pain (Ji and Neugebauer, 2007). Furthermore, the CRF projections from the CeAmy to the LC provide a pathway by which information that reaches the amygdala can influence descending inhibition of pain (Van Bockstaele et al., 1996; Tjoumakaris et al., 2003) and it has been well established that the LC and norepinephrine are essential for pain inhibition including SIA (Tamano et al., 2016; Bohn et al., 2000; Hughes et al., 2013). Still, the effects of chronic pain on norepinephrine signaling in the spinal cord are not clear with some reports demonstrating that chronic pain inhibits norepinephrine levels (Matsuoka et al., 2016) and other showing that chronic pain enhances norepinephrine signaling in the spinal cord (Ma and Eisenach, 2003). Recent studies show that augmented descending pain inhibition prevents pain chronification in neuropathic rats and that this prevention is, at least partially, norepinephrine dependent (De Felice et al., 2011). However, the role of CeAmy CRF neurons in regulation of norepinephrine transmission and descending pain inhibition during prolonged nociceptive input remains unexplored.

We tested the hypothesis that CRF neurons in the CeAmy contribute to pain chronification by affecting descending pain inhibition. First, we investigated the effects of long-lasting neuropathic pain on SIA, which depends on the activity of the descending pain inhibitory system. Second, we tested the effects of activation or inhibition of CeAmy CRF neurons on SIA in healthy mice and mice with neuropathic pain. Finally, we examined whether CeAmy projections to the LC are part of the circuitry that inhibits SIA in mice with neuropathic pain.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science (North Chicago, IL) and adhered to the guidelines provided in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6J, B6(Cg)-Crhtm1(cre)Zjh/J and B6.Cg-Tg(Th-cre)1Tmd/J male mice, 9 to 10 weeks old, were purchased from a supplier (The Jackson Laboratory, Bar Harbor, ME) and were housed 3 to 5 mice per cage. All nociceptive tests were done in the morning after an hour of acclimatization to the testing room.

2.2. Neuropathic pain model

Surgeries were performed under isoflurane anesthesia and following aseptic technique. Peripheral nerve constriction surgery was performed by exposing the main branch of the sciatic nerve with forceps and carefully placing a 4 mm long piece of P90 nontoxic, sterile polyethylene tubing that was split lengthwise (inner diameter 0.86 mm and outer diameter 1.27 mm; Becton Dickinson Intramedic, Franklin Lakes, NJ) onto the nerve. After confirmation that the tubing remained loosely on the nerve, the nerve was returned to its position and the incision was closed with wound clips (Dimitrov et al., 2014). Analgesia (flunixin meglumine 2 mg/kg, subcutaneous) was given for the following three days. Control mice were anesthetized with isoflurane and the skin was shaved but the sciatic nerve was not exteriorized in order to prevent behavior alterations and stimulation of CeAmy activity, which is increased in sham animals that undergo surgical incisions of skin and muscle (Morland et al., 2016).

2.3. Application of pharmacogenetics

Stereotaxic surgeries were used to inject DREADD viral constructs into the CeAmy and/or LC. Animals were anesthetized with isoflurane and placed in a Stoelting stereotaxic apparatus. A longitudinal skin incision and removal of pericranial connective tissue exposed the bregma and lambda sutures of the skull. The coordinates for the CeAmy injections were -1.3 mm, $\pm$ 2.5 mm and -4.5 mm in respect to bregma while the LC injections were placed -5.4 mm, $\pm$ 0.8 mm and -4.5 mm to bregma. Small holes were drilled into the skull and the viral solution was injected via a 32 gauge needle connected to an infusion pump (Microsyringe pump, World Precision Instruments, Sarasota, FL) in volume of 0.2 µl over 5 min. All animals were given analgesic and fluids for 3 days after the surgery. B6(Cg)-Crhtm1(cre)Zjh/J mice, with Cre recombinase expression in CRF neurons, were injected bilaterally in the CeAmy with cre activated adeno-associated virus (serotype 5, pAAV-hSyn-DIO-hM3D(Gq)-mCherry and pAAV-hSyn-DIO-hM4D(Gi)-mCherry, titter $\geq 3 \times 10^{12}$ (UNC Vector core, Chapel Hill, NC), referred here as AAV-DREADD-Gq and Gi respectively). C57BL/6J mice were injected unilaterally with retrograde canine adeno-associated cre virus or CAV-2-cre, titter $\geq 2.5 \times 10^{12}$ (Plateforme de Vectorologie de Montpellier, Montpellier, France) into the LC, and with cre activated AAV-diphtheria toxin (serotype 5 AAV-mCherry-FLEX-dtA, titter $\geq 3 \times 10^{12}$ (UNC Vector core, Chapel Hill, NC)) viral construct into the CeAmy. Further behavior and nociceptive tests were performed three to four weeks after the viral injections. During the course of these experiments an AAV variant (rAAV-CAG-eGFP-F2A-Cre, titer $= 1 \times 10^{12}$, NINDS Viral Production Core Facility, Bethesda, MD) with efficient retrograde transport became available (Tervo et al., 2016). Because of the potential benefits of working with AAV we repeated the experiment with retrograde labeling via LC injection and the SIA experiments using the retro-AAV. The results are very similar, providing further support for the initial observation and supporting the suggestion that retro-AAV may be an attractive alternative to CAV2-Cre for this type of experiments.

2.4. Nociceptive and behavior testing

Mice were given at least 1 h to acclimatize to the testing room before nociceptive or behavior testing. Tactile sensitivity was measured using von Frey filaments applied to the plantar surface of the hind paw through a mesh floor. Following the technique for assessment of mechanical allodynia described by Chaplan et al. (1994), six filaments with different stiffness were used for each measurement. The starting filament was always 1.0 g. A quick withdraw, shaking or licking of the paw were considered a positive reaction. The mechanical thresholds were calculated by Dixon's up and down method (Dixon, 1991). Mechanical thresholds were obtained before and in five-day increments following sciatic nerve constriction for up to 15 days.

Thermal pain thresholds were measured by a tail-flick test. Mice were gently restrained in a plastic laboratory tube covered in foil to block environmental stimuli and their tails freely projected out of the restrainer. Next, the mice were placed in the Tail-Flick Analgesia Meter Model 33 (IITC Life Science Instruments, Woodland Hills, CA) in which a high intensity light (beam: 4; sensitivity: 3.5) was directed at their tails. The timer on the meter shut off (automatic shut off at 25 s) immediately once the mice flicked their tails, and these times were recorded as response latencies.

Stress was induced by a forced swim. Mice were placed in a transparent cylinder, 30 cm in diameter, half filled with tap water (21–23 °C) for 5 min. The mice were completely dried with paper towels before undergoing the tail-flick test that followed 1 min after the forced swim.