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Research article

Distinct neuronal populations in the basolateral and central amygdala are activated with acute pain, conditioned fear, and fear-conditioned analgesia

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ABSTRACT

Fear-conditioned analgesia (FCA) is modulated by brain areas involved in the descending inhibitory pain pathway such as the basolateral (BLA) and central amygdala (CEA). The BLA contains Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and parvalbumin (PV) neurons. CEA neurons are primarily inhibitory (GABAergic) that comprise enkephalin (ENK) interneurons and corticotropin-releasing factor (CRF) – neurons that project to the periaqueductal grey. The purpose of our experiment was to determine the pattern of activation of CaMKII/PV and ENK/CRF neurons following the expression of acute pain, conditioned fear, and FCA. A significant reduction was observed in nociceptive behaviors in mice re-exposed to a contextually-aversive environment. Using NeuN and cFos as markers for activated neurons, CaMKII, PV, ENK, or CRF were used to identify neuronal subtypes. We find that mice expressing conditioned fear displayed an increase in c-Fos/CaMKII co-localization in the lateral amygdala and BLA compared to controls. Additionally a significant increase in cFos/CRF co-localization was observed in mice expressing FCA. These results show that amygdala processing of conditioned contextual aversive, nociceptive, and FCA behaviors involve different neuronal phenotypes and neural circuits between, within, and from various amygdala nuclei. This information will be important in developing novel therapies for treating pain and emotive disorders in humans.

1. Introduction

The amygdala is a brain region mediating survival behaviors and it functions as a gateway between sensory input and emotive responses. Two of the most well-defined amygdala responses include conditioned aversive responses and nociception [7,23]. The pairing of conditioned and unconditioned stimuli is processed within the basolateral (BLA) and central amygdala (CEA) and this information is sent to other brain regions [9]. By comparison, the emotional and affective dimensions of nociception are processed within the amygdala [29]. The reduction in nociceptive behavior upon re-exposure to a contextually aversive stimulus, termed fear-conditioned analgesia (FCA), is mediated also by the amygdala [1,31]. However, it is unknown how the amygdala circuitry differs when a subject is in the presence of a conditioned aversive or a nociceptive stimulus, as compared to when both stimuli are present simultaneously resulting in FCA.

The neurons of the BLA and CEA have vastly different electrophysiological and phenotypic properties. The BLA possesses cortical-like features with excitatory output neurons containing Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [19], that are under

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inhibitory control of local parvalbumin (PV) interneurons [18]. By comparison, the CEA has inhibitory local and inhibitory projection neurons that are characteristic of striatal-like regions [27]. These GABAergic inhibitory projection neurons contain corticotropin-releasing factor (CRF) which are under inhibitory control of local enkephalin (ENK) containing neurons [6]. The CEA receives projections from CaMKII-positive neurons of the BLA [28]. The CRF-positive neurons in the CEA then project to downstream effectors such as the periaqueductal grey [6].

The BLA has been identified as integral to conditioned aversive and nociceptive responses. A recent optogenetics and electrophysiology study has implicated the disinhibition of CaMKII principle neurons by PV-positive neurons in the facilitation of cue-conditioned fear [32]. However, it is unclear whether nociceptive signaling or FCA is mediated by PV or CaMKII neurons in the BLA. Nevertheless, GABA receptor antagonism in the BLA can reduce acute nociceptive behavior [14] and it appears to be involved in the recovery of endocannabinoid-mediated FCA [24]. By comparison, in the CEA pharmacological manipulation of CRF [4] and ENK [11] signaling can alter acute nociceptive and conditioned aversive responses in rodents. Despite this fact, it is unclear whether CRF- or ENK-containing neurons in the CEA modulate FCA or whether the activation patterns of these neurons are different during acute pain, conditioned fear, or FCA.

In the present experiments we sought to ascertain the role(s) of these neuronal populations in the expression of conditioned fear, acute pain, and FCA. We hypothesize that mice re-exposed to an arena in which they received foot-shocks will express reduced nociceptive behaviors with the formalin test of persistent pain as this model. Since the amygdala is a locus of the descending inhibitory pathway in which the CEA is the primary output, we hypothesize that expression of FCA will activate neurons in the CEA that permit the descending inhibitory pain pathway to be disinhibited.

2. Materials and methods

2.1. Animals

Twenty-eight, 6–8 week-old C57BL/6J male mice (Jackson Labs, Bar Harbor, ME) were individually-housed 7-days prior to conditioning. Animals were provided with food and water *ad libitum* and housed in a temperature- and humidity-controlled room under a 14:10 h light:dark cycle (lights on 0600–2000 h). C57BL/6J mice were chosen because previous studies used this strain for conditioned fear and formalin-induced nociception [8,13,16]. All *in vivo* experiments were conducted with a protocol approved by the Institutional Animal Care and Use Committee at Duke University Medical School. All efforts were made to minimize discomfort of the animals and every attempt was made to follow the ARRIVE guidelines for reporting *in vivo* experiments [12,25].

2.2. Behavioral procedures

The FCA procedure had been developed previously with C57BL/6J mice [8] and in pilot studies we determined optimal conditions for inducing FCA in these mice (data not shown). The FCA procedure consisted of three phases: habituation, conditioning, and testing (Fig. 1). The experimental design included 4 groups (6–8 mice per group): controls – no fear conditioning + saline hind-paw injection (No FC-Sal); pain – no fear conditioning + formalin hind-paw injection (No FC-Form); conditioned fear – fear conditioning + saline hind-paw injection (FC-Sal); and FCA – fear conditioning + formalin hind-paw injection (FC-Form). Mice were randomly assigned to groups. All procedures were performed between 0900 and 1100 h.

Mice were habituated to handling and paw-pinch each day for 7 consecutive days. On day 8, they were placed into the fear-conditioning chamber (30 × 25 × 22 cm; Med-Associates, St. Albans, VT). All behaviors were videotaped. Twenty-four h later the FC-Sal and FC-Form mice were placed individually into the conditioning chamber and after 2.5 min they received the first of 5 foot-shocks (0.4 mA, 2 s duration spaced 58 s apart). Thirty s after the last foot-shock, mice were returned to their home-cage. The No FC-Sal and No FC-Form mice were placed into the chamber for an equivalent amount of time (8 min) without foot-shocks. In between each mouse, the chamber was cleaned with Labsan 256Q (Sanitation Strategies, Williamston, MI).

Mice were briefly restrained 23.5 h later, the diameter of the dorsal region of the right hind-paw was measured with Vernier calipers, and an intra-plantar injection was delivered into that paw with 15 µl of 0.9% saline or 2.5% formaldehyde in saline. Thirty min later mice were tested for contextual fear where each mouse was placed individually into the conditioning chamber (without foot-shock) for 15 min. Immediately following this test, the diameter of the dorsal region of the right hind-paw was measured and the mice were returned to their home-cages. Note, the pre- and post-trial diameters of the right hind-paw were used to calculate paw swelling.

2.3. Behavioral analysis

Behavior was analyzed using the Observer® 10.0 XT program (Noldus Information Technology, Leesburg, VA) that permitted continuous recording over the fear conditioning and testing. A trained rater, blinded to the experimental conditions, scored the behaviors, which included freezing (cessation of all visible movement except for respiration). Formalin-evoked nociceptive behavior was presented as the total duration of combined licking, biting, or right hind-paw shaking.

2.4. Perfusion and immunofluorescence

Two h after contextual fear testing (2.5 h after intra-plantar injection), mice were administered sodium pentobarbitol (i.p.; Sigma-Aldrich, St. Louis, MO) and transcardially perfused with 0.1 M phosphate buffer (PB; pH 7.4) followed by 4% paraformaldehyde in 0.1 M PB. Brains were removed and post-fixed for 3 h. Serial 50 µm coronal sections were cut on a vibratome into 0.1 M PB with 0.1% sodium azide, and stored < 2 wks at +4 °C until processing for immunofluorescence. Two sets of serial sections were collected from the cingulate cortex through the BLA (bregma: −0.58 to −2.46 mm). Sections were washed 5 x for 5 min each in 0.1 M PB. Following washing and blocking [0.1 M PB with 0.04% Triton X-100 containing 5% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA)], set 1 sections were incubated in 0.1 M PB with chicken anti-NeuN (1:500; Aves Labs, Tigard, OR), goat anti-c-Fos (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-CaMKII (1:100; Abcam, Cambridge, MA), and guinea pig anti-PV (1:500; Synaptic Systems, Goettingen, Germany) antibodies overnight at room temperature (RT). Set 2 sections were washed, blocked, and incubated subsequently in 0.1 M PB with chicken anti-c-NeuN (1:500), goat anti-cFos (1:500), rabbit anti-met ENK (1:125; EMD Millipore, Temecula, CA), and guinea pig anti-CRF (1:5000; Peninsula Laboratories International, Inc., San Carlos, CA) under the same procedure. Sections from sets 1 and 2 were subjected to 3 washes in 0.1 M PB, followed by incubation with donkey anti-chicken 405 (1:100), donkey anti-goat 488 (1:500), donkey anti-rabbit Cy3 (1:500), and donkey anti-guinea pig 647 (1:500) fluorescently-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at RT. Sections were mounted and stored in the dark at RT until viewing under a confocal microscope.

2.5. Confocal microscopy and neuronal counting

All sections were imaged with a Zeiss LSM 710 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) with a Zeiss 20 × 0.8 numerical aperture plan-apochromat objective. The 405, 488, 561, and 633 nm