A basomedial amygdala to intercalated cells microcircuit expressing PACAP and its receptor PAC1 regulates contextual fear

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Title: A basomedial amygdala to intercalated cells microcircuit expressing PACAP and its receptor PAC1 regulates contextual fear.

Abbreviated Title: A peptidergic amygdala microcircuit in contextual fear

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Abstract

Trauma can cause dysfunctional fear regulation leading some people to develop disorders such as posttraumatic stress disorder (PTSD). The amygdala regulates fear, whereas PACAP (pituitary adenylate activating peptide) and PAC1 receptors are linked to PTSD symptom severity at genetic/epigenetic levels, with a strong link in females with PTSD. We discovered a PACAPergic projection from the basomedial amygdala (BMA) to the medial intercalated cells (mICCs) in adult mice. In vivo optogenetic stimulation of this pathway increased c-fos expression in mICCs, decreased fear recall and increased fear extinction. Selective deletion of PAC1 receptors from the mICCs in females reduced fear acquisition, but enhanced fear generalization and reduced fear extinction in males. Optogenetic stimulation of the BMA-mICCs PACAPergic pathway produced excitatory postsynaptic currents (EPSCs) in mICC neurons, which were enhanced by the PAC1 receptor antagonist, PACAP 6-38. Our findings show that mICCs modulate contextual fear in a dynamic and sex-dependent manner via a microcircuit containing the BMA and mICCs, and in a manner that was dependent on behavioral state.

Significance Statement

Traumatic stress can affect different aspects of fear behaviors including fear learning, generalization of learned fear to novel contexts, how the fear of the original context is recalled, and how fear is reduced over time. While the amygdala has been studied for its role in regulation of different aspects of fear, the molecular circuitry of this structure is quite complex. In addition, aspects of fear can be modulated differently in males and females. Our findings show that a specific circuitry containing the neuropeptide PACAP and its receptor, PAC1, regulates various aspects of fear including acquisition, generalization, recall and extinction in a sexually dimorphic manner, characterizing a novel pathway that modulates traumatic fear.

Introduction
Given the very high prevalence of stress-related pathology in society, it is essential to understand the cellular and circuit mechanisms underlying emotion dysregulation (School 2007). One theory of heightened fear in Post-Traumatic Stress Disorder (PTSD) is due to inappropriate inhibitory control over fear, leading even mild reminders of trauma to trigger strong symptoms and reduced propensity to extinguish acquired fear (Fanselow, Lester et al. 1988, Rosen and Schulkin 1998, Grillon and Morgan 1999, Milad, Rauch et al. 2006, Jovanovic, Norholm et al. 2010).

The amygdala and its associated structures play a key role in processing and reacting to emotional stimuli and it is known to be involved in PTSD (Stevens, Kim et al. 2017). The cortex-like regions of the amygdala proper (lateral and basal nuclei of the basolateral amygdala complex-BLA) receive sensory information from neocortex and thalamus (Swanson and Petrovich 1998). Plasticity within these nuclei supports associative learning about sensory information pertinent to positive and negative affect and supports processes such as Pavlovian fear conditioning (Falls, Miserendino et al. 1992, Fanselow and Kim 1994, Blair, Schafe et al. 2001).

On the other hand, generation of most fear-related behaviors is initiated by the nearby medial portion of the striatal-like Central Nucleus (CN) (Swanson and Petrovich 1998). There are several routes of communication between the amygdala and CN, only some of which are direct (Pare, Quirk et al. 2004). Indirect microcircuits include relays in the lateral portion of the CN (Haubensak, Kunwar et al. 2010, Li, Penzo et al. 2013) as well as clusters of GABAergic cells that lie in the capsule separating BLA and CN (Pare, Quirk et al. 2004). There is currently only limited information about which specific aspects of fear learning are selectively served by these separable microcircuits. The capsular, or medial intercalated cell clusters (mICCs), appear to play a role in fear extinction, the loss of fear responses to a stimulus that previously triggered fear due to repeated exposure without any aversive consequences. A majority of the mICCs express mu-opioid receptors on their cell bodies and selective ablation of these neurons partially eliminates recall of fear extinction (Likhtik, Popa et al. 2008). Whether or not the mICCs are involved in other aspects of fear, such as acquisition, recall, and generalization is unknown. Therefore, the present experiments combined a contextual fear conditioning task that allowed us to interrogate each of these behaviors with a novel approach to target the medial ICCs dissecting specific neuronal pathways expressed in this circuitry.
The neurotransmitters and neuromodulators that the basolateral complex utilizes to communicate with the mICCs are partially unknown (Manko, Geracitano et al. 2011, Asede, Bosch et al. 2015). Pituitary adenyl cyclase-activating peptide (PACAP) and its G-protein coupled receptor, PAC1, are expressed in brain areas involved in emotion and arousal including the amygdala and mICCs. Polymorphisms in either the PACAP or PAC1 receptor locus have been linked to PTSD symptom severity, with this genetic link especially strong in females with PTSD (Ressler, Mercer et al. 2011). PACAP enhances contextual fear consolidation and extinction, and enhances excitatory synaptic transmission in BLA to lateral CN circuit (Cho, Zushida et al. 2012, Schmidt, Myskiw et al. 2015, Meloni, Venkataraman et al. 2016, Kirry, Herbst et al. 2018). We investigated PACAP/PAC1 functions within the amygdala microcircuitry regulating aspects of contextual fear in male and female mice. Using mice for genetically targeting PACAP or PAC1 expressing cells, we identified a microcircuit consisting of PACAP expressing neurons in the basal medial nucleus of the amygdala (BMA) that project to PAC1 expressing mICCs. BMA is a crucial part of the BLA complex regulating fear and anxiety, but its functions in relation to other amygdala nuclei is not as clearly defined as the BLA (Amano, Duvarci et al. 2011, Adhikari, Lerner et al. 2015). We discovered a BMA-mICCs microcircuit containing PACAP and PAC1 regulates fear acquisition, generalization, recall and extinction in a distinct and sex-dependent manner.

Materials and Methods

Experimental Models and Subject Details

All experimental procedures were conducted in accordance with the guidelines set by the National Institute of Health and the Institutional Animal Care and Use Committee at the University of California, Los Angeles. All mice were kept on ad libitum access to food and water in a light- and temperature-controlled vivarium. Mice (3–4 months) were housed in clear plastic cages (3-5 mice/cage as littermates) in a vivarium with lights on at 7 AM and off at 7 PM. Experiments were performed between 9 AM and 3 PM.
Three different mouse lines male and female sex were used in all aspects of this study. The first was a Tg (Adcyap1-EGFP) FB22Gsat/Mmucd (RRID: IMSR_MMRRC:012011) reporter mouse line that faithfully expresses enhanced green fluorescent protein in PACAP positive neurons. These mice were generated using a bacterial artificial chromosome (BAC; RP24-358O1) by the Gene Expression Nervous System Atlas (GENSAT) project and obtained from the Mutant Mouse Resource and Research Center. These mice were backcrossed from FVB/NTac to C57BL/6 for at least five generations (Condro, Matynia et al. 2016). The second mouse line was an Adcyap1r1<sup>loxP/loxP</sup> mouse. These mice were generated in a C57BL/6 background with a conditional knockout (KO) allele (PAC1<sup>loxP/loxP</sup> mice) through the NIH-funded knockout mouse project (KOMP). The third mouse line was an Adcyap1-2A-Cre mouse line. These mice (Adcyap1-2A-Cre) target Cre to most populations of PACAP neurons of the brain including the amygdala [10] (Allen Brain Atlas).

Measure of freezing

Freezing is a complete lack of movement except for respiration (Fanselow 1980). Freezing was measured using VideoFreeze (Med-Associates Inc.) that performed real-time video recordings at 18 frames per second. With this program, adjacent frames are compared to provide the grayscale change for each pixel and the sum of pixels changing from one frame to the next constitutes a momentary activity score. To account for video noise and to approximate scoring by a trained human observer a threshold is set at 18 activity units so that an instance of freezing is counted when that the activity score remains below this threshold for 1 sec (Anagnostaras, Gale et al. 2001). Percentage freezing=Freezing Time /Total Time×100 for a period of interest. Data are presented as mean percentages (+/− SEM).

Because Med-Associates software uses the number of pixels changed across the entire video frame to calculate the amount of freezing, we were not able to use it for automated analysis of freezing for our
optogenetic studies. This is because even when the mice were freezing, the movement in the optogenetic cable was calculated by the software as movement. Hence for optogenetic behavioral experiments, we analyzed freezing using ezTrack software (Pennington, Dong et al. 2019), which enables removal of this cable artifact. In brief, videos were cropped to reduce the influence of optogenetic cables in the upper portion of the field of view. Subsequently, the number of pixels whose grayscale value changed from one frame to the next was calculated. Freezing was then scored when this number dropped below an experimenter-defined threshold for at least 30 frames (1 second). The freezing threshold was determined by visual inspection of the video and by comparing a subset of the results obtained to the results of manual scoring. All cropping and thresholding parameters were identical across sessions. Data are presented as mean percentages (+/− SEM).

**Determining BMA to mICCs PACAPergic Innervation**

Immunofluorescence for visualizing expression of PACAP-EGFP neurons and VGLUT2

For immunofluorescence labeling, 40-micron coronal brain sections were cut from (Adcyap1-EGFP) mice (N=4; M=2, F=2). Sections were blocked and permeabilized in a solution containing PBS + 10% normal goat serum (NGS) + 1% bovine serum albumin (BSA) + 0.5% TritonX-100 for 1 hour. An anti-GFP primary antibody (A11122, Life Technologies), anti-VGLUT2 antibody (ab178538, Abcam), or anti-NeuN antibody (MAB377, Millipore) were diluted 1:500 in PBS + 5% NGS + 1% BSA and sections were incubated overnight at 4°C, then washed in PBS, and subsequently incubated with an anti-rabbit AlexaFluor 488 (Life Technologies Cat#A11008, RRID:AB_10563748) or anti-mouse Cy3 (Abcam Cat#Ab97035, RRID:AB_10680176) secondary antibodies diluted 1:400 for 2-4 hours at room temperature. Sections were washed in PBS and mounted on slides, and coverslipped with Prolong Gold Antifade Reagent (Life Technologies). Fluorescence images were acquired with a Keyence widefield microscope (BZ-X710).

Intersectional viral method for labeling PACAPergic neurons from BMA to mICCs
Intersectional viral technique uses conditional single-AAV system to express opsins or fluorescence depending upon multiple cell-type features using Boolean logical operations allowing labeling cells that are genetically wired (Fenno, Mattis et al. 2014). Therefore, to further validate the existence of PACAPergic projections from BMA to mICCs, we utilized an intersectional viral labeling method. For this, we used a hEF1α-LS1L-mCherry-IRES-flpo virus (Harvard Vector Core), with either AAV5-EF1a-fDIO-ChR2-eYFP-WPRE or AAV5-EF1a-fDIO-eYFP-WPRE (UNC Vector Core) viruses. Adcyap1-2A-Cre mice (N=3) went through stereotaxic surgeries with BMA co-ordinates: L/M: +/- 3.25; A/P: -1.7; D/V: -4.75) and mICCs co-ordinates: -L/M: +/- 2.7; A/P: -1.06; D/V: -4.2. We first microinfused hEF1α-LS1L-mCherry-IRES-flpo into the mICCs and the AAV5-EF1a-DIO-hChR2(H134R)-mCherry virus into the BMA. The hEF1α-LS1L-mCherry-IRES-flpo virus is a (Cre) recombinase dependent and retrogradely transporting virus, so when injected into the mICCs of Adcyap1-2A-Cre mice, it only expresses in PACAP-containing neurons as they express Cre in the mICCs terminal where it is injected. This virus in turn contains flippase (FLP) recombinase in its sequence. So, we then microinjected a FLP-dependent AAV5-EF1a-fDIO-ChR2-eYFP-WPRE or AAV5-EF1a-fDIO-eYFP-WPRE virus into the BMA. This allows the ChR2 or eYFP (control) to be expressed specifically in PACAPergic neurons that project from the BMA to mICCs. The volume of viral injections in mICCs was 0.1-μl over 2 minutes and in the BMA was 0.3 μl over 6 minutes with 10 minutes of diffusion time for each infusion. Following the surgical procedure, in all experiments, mice were allowed to recover for 21 days to allow viral transduction.

Visualizing expression of PACAPergic neurons

After recovery from surgery, mice were euthanized, brains extracted, and 40-micron brain sections were cut. Sections were mounted on slides and coverslipped with Prolong Gold Antifade Reagent (Life Technologies). Fluorescence images were acquired with a Keyence imager. We also conducted immunohistochemistry on some slices using an antibody against FoxP2 protein, which is highly expressed in the mICCs and used as a marker of labeling in this region.

In vivo Optogenetic Stimulation of BMA-mICCs PACAPergic Neurons and Fear Behaviors
We wanted to determine if altering the activity of PACAPergic neurons that innervate the mICCs changes fear behavior. A previous study showed that the ex vivo optogenetic method can be used to analyze and study the role of intercalated cells in regulating fear behaviors, so we utilized optogenetics to answer our question (Bosch, Asede et al. 2016). For these experiments, we used the Adcyap1-2A-Cre mice and carried out the same intersectional virus labeling strategy described previously. Briefly, we injected the Cre-dependent hEF1α-LS1L-mCherry-ires-flpo in the mICCs and AAV5-EF1a-fDIO-ChR2-eYFP-WPRE or AAV5-EF1a-fDIO-eYFP-WPRE in the BMA for specifically expressing ChR2 in PACAPergic neurons that project from BMA to ICCs. Two 200 µm diameter optic fibers were also implanted bilaterally above mICCs or BMA (available from Prizmatix) and cut at the length of 4.6 mm or 5 mm, respectively. The core diameter of the fibers was 250um and an outer diameter of 275 µm. The numerical aperture (NA) was 0.66. For the stimulation, the optical fiber was connected to an LED emitting blue light (473 nm, 20 Hz train of 25 ms on/off pulses, 10 min duration). Optogenetic stimulation was carried out bilaterally in each mouse throughout the duration of the experiment.

Behavioral Procedure

Conditioning Apparatus: Mice were run individually in sound and light attenuated conditioning boxes (Med Associates Inc., Georgia, VT) (Fig. 5). The boxes were equipped with Near Infra-Red Video Fear Conditioning System and could be configured to represent different contexts by changing the internal structure, floor texture, illumination, and odor. Context A (28 × 21 × 21 cm) had a clear Plexiglas back wall, ceiling, and front door with aluminum sidewalls. It also had a grid floor with evenly spaced stainless-steel rods cleaned and scented with 50% Windex. The floor in context A was connected to a scrambled foot shock generator. Context B had a clear Plexiglas back wall, ceiling, and door with aluminum sidewalls. The chamber was altered by adding a white curved sidewall that extended across the back wall. The floor of context B consisted of an acrylic white board.
Behavioral design

We designed our behavioral tests to capture effects on all aspects of contextual fear regulation including acquisition, generalization, recall and extinction. This design was chosen mainly because mICCs are known to modulate fear extinction, but PAC1 and PACAP modulation could have effects on other aspects of fear. As the circuit mechanisms we are studying have not been examined previously, instead of simply designing a behavioral assay to measure acquisition and extinction, we also added the components to measure generalization and retention in the same animals. For acquisition, we used 5 days of training based on prior work in the lab that has shown that 5 days of training is sufficient to produce an asymptotic level of freezing (e.g., (Young and Fanselow 1992)).

For each behavior day, mice were lightly restrained, the optic cable was connected to the indwelling fiber optics in their head and placed in the testing chamber. We tested acquisition, generalization, and extinction of fear as measured by freezing. For optogenetic stimulation studies, we chose to activate throughout sessions to keep the gain of function and loss of function experiments with PAC1 deletion consistent with each other.

Acquisition: For acquisition we placed the animals in Context A for four minutes and thirty seconds every day around the same time for 5 days. At the fourth minute each day, the mice received a 1 second .65 mA shock. After 29 seconds they were removed from the chamber and put back in their home cages, where they were housed with littermates. Mice were transported to the laboratory together in their home cages. There was one rest day between acquisition and generalization testing.

Generalization Test: The animals were placed in a completely different Context (B) for four minutes and thirty seconds. No shocks were delivered.
Recall Test: One day after generalization testing the animals went through fear recall tests in Context A. Mice were placed in the context for 4 minute 30 seconds without any shocks.

Extinction Test: For the next 5 days animals went through fear extinction, again in context A. Extinction sessions were 30 minutes long. We measured freezing for the first 4 minutes, but the animals remained in the chamber for the entire 30 minutes.

**Deletion of PAC1 Receptors From the mICCs and Measurement of Fear-related Behavior**

For experiments involving deletion of PAC1 receptors, we microinfused AAV2-hsyn-GFP-Cre or AAV2-hsyn-GFP into mICCs using the stereotaxic co-ordinates L/M: +/- 2.7; A/P: -1.06; D/V: -4.2. Although it is challenging to precisely target small structures like the medial ICCs, we have shown feasibility of confining virus infusion to such a small structure by using a specialized digital stereotax (Model 1900, David Kopf Instruments) and pulled glass pipettes that are commonly used for electrophysiological recordings with single cell resolution. We verified through various methods that we were able to precisely target the mICCs, including DAPI infusion (Fig. 1C), infusion of AAV expressing mcherry (Fig. 1C), infusion of AAV2-hsyn-GFP (Fig. not shown), and then co-labeling our intersectional viral technique with an antibody against FoxP2, protein that is highly expressed in the mICCs (Fig. 1E). Using these methods, we found that viral infusions were constrained by the surrounding capsule if the placement was accurate. The behavioral procedure and design were identical to the optogenetic experiments. The only difference was for measuring freezing, automated Medassociates Videofreeze software was used as described previously.

Validation of PAC1 receptor deletion using dual *in situ* hybridization with RNAscope

After the behavioral trials were complete the mice were sacrificed, and their brains extracted and immediately stored in at -80 degrees celsius. The brains were sliced at 15 microns in a cryostat and slices containing the
amygdala were collected on microscope slides. The deletion of PAC1 receptors was verified using RNAscope® for analyzing expression of RNA tissue sections (ACD Biotechne). Briefly, we performed in situ hybridization steps following RNAscope® 2.5 HD Duplex Assay protocol for fresh frozen sections. After completion of the labeling, sections were cover-slipped using Prolong Gold (Thermo Fisher Scientific) with 4',6-diamidino-2-phenylindole (DAPI) and the edges were sealed with clear nail polish. PAC1 mRNA puncta counts were carried out in 4 serial sections containing the mICCs that started at the same rostral plane that were captured with a 40X objective. mICCs frame was 25 microns x 25 microns size. Analysis of dots was conducted using FIJI software. For each image DAPI+PAC1 mRNA and DAPI+GFP were analyzed separately. For counts, DAPI positive cells and PAC1 RNA dots were counted separately and expressed as mean grain count.

**Measurement of cfos Expression After in vivo Optogenetic Stimulation of BMA-PACAP Neurons that Innervate the mICCs**

Viral surgeries and in vivo optogenetic stimulation

For these experiments, we utilized the same intersectional approach described above for labeling PACAPergic neurons projecting from BMA to mICCs by infusing hEF1α-LS1L-mCherry-IRES-flpo into the mICCs (N=4, 2 males and 2 females) and AAV5-EF1a-fDIO-ChR2-eYFP-WPRE or AAV5-EF1a-fDIO-eYFP-WPRE into the BMA. A 200 μm diameter optic fiber was also implanted bilaterally above BMA (available from Prizmatix) and cut at the length of 5 mm. The core diameter of the fibers was 250um and the outer diameter was 275 μm. The numerical aperture (NA) was 0.66. After 21 days, mice were anesthetized with isoflurane and the optical fiber was connected to a LED emitting blue light (473 nm, 20 Hz train of 10ms on/off pulses, 10 min duration). Optogenetic stimulation was carried out unilaterally and the hemisphere of stimulation was counter-balanced between mice. Ninety minutes following the simulation, mice were sacrificed, and brains extracted, cryoprotected and frozen. During the ninety minutes, the mice were in their home cages without anesthesia.

Immunohistochemistry for measuring cfos expression
The brains were processed for cfos immunohistochemistry. First, 40-micrometer coronal sections containing the amygdala were collected serially. On day 1, tissue sections were washed in 1×TBS three times for five minutes, then blocked in 1mL of 1×TBS with 5% Normal Donkey Serum, 0.1% BSA and 0.3% Triton-X for 1 hour. Then the tissue sections were incubated overnight at 4 degrees Celsius with the primary goat polyclonal to cfos (1:500, 24 h, abcam; RRID: SCR_012931) primary antibody. According to the manufacturer, this antibody is a ‘synthetic peptide conjugated to Blue Carrier Protein by a Cysteine residue linker corresponding to the internal sequence amino acids 283–295 of Human c-Fos (NP_005243.1). On the second day, the sections were washed in 1×TBS three times five minutes each and then incubated in the Alexa 488 donkey anti-goat secondary antibody (1:200, Life Technologies) for 2 hours at room temperature. After washing with 1×TBS for 3 times for 5 minutes each, tissue sections were mounted on glass slides and cover-slipped using Prolong Gold (Thermo Fisher Scientific) with 4',6-diamidino-2-phenylindole (DAPI) and the edges were sealed with clear nail polish. Positive cfos immunolabeling was analyzed and quantified in brain sections containing the mICCs.

Cell counts for cfos experiments

Sections in the rostrocaudal extents of the BMA (bregma −1.06 to −2.3) were collected and processed for immunohistochemistry or RNAscope+immunohistochemistry, as described. The numbers of cfos+ and VGLUT2+ cells were manually counted in the region of the mICCs by two trained researchers that were blind to the treatment conditions. For VGLUT2, all PACAP cells, VGLUT2 cells and PACAP+VGLUT2 cells were manually counted first for the tissue sections from PACAP-EGFP mice with VGLUT2 and GFP immunohistochemistry. For tissue sections from wild type mice, with immunohistochemistry for VGLUT2 and RNAscope for PACAP mRNA, VGLUT2-positive cells, and colocalization with PACAP were counted manually using Fiji (NIH) software. For RNAscope in situ hybridization and immunohistochemistry experiments we counted PACAP labeled cells in BMA and assessed colocalization with either VGLUT2 using Fiji software. For RNAscope, at least 5 PACAP mRNA puncta were counted that were in close proximity to a DAPI-labeled
For both, immunohistochemistry and RNAscope experiments, three to four sections from each animal were counted.

**Electrophysiological recordings of mICCs neuronal activity and *ex vivo* optogenetic stimulation of PACAPergic neurons that innervate mICCs**

For *ex vivo* electrophysiology experiments, we infused in the BMA of the Adcyap1-2A-Cre mice a Cre-dependent AAV5-EF1a-DIO-hChR2(H134R)-mCherry virus (UNC Vector Core) (N=6; M=3, F=3). After a 21-day recovery from surgery, animals were deeply anesthetized with isoflurane and decapitated amygdala slices were prepared. The brains were placed in ice-cold modified artificial cerebrospinal fluid (aCSF, containing, in mM: 194 sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, and 10 D-glucose) and cut into 300 µm-thick coronal slices containing the intercalated cell (ICC) layer of the amygdala. The slices were then allowed to equilibrate for 30 min at 32–34°C in normal aCSF (containing in mM; 124 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 1.2 NaH₂PO₄, and 10 D-glucose) continuously bubbled with a mixture of 95% O₂/5% CO₂, stored at room temperature in the same buffer, and used for experiments within 6 hr of slice preparation.

Electrophysiological methods were described previously (Tong, Ao et al. 2014, Octeau, Chai et al. 2018). Cells were visualized with infrared optics on an upright microscope (BX61WI, Olympus). pCLAMP10 software and a MultiClamp 700B amplifier was used for electrophysiology (Molecular Devices). For these recordings the intracellular solution in the patch pipette contained the following, in mM: 135 potassium gluconate, 3 KCl, 0.1 CaCl₂, 10 HEPES, 1 EGTA, 8 Na₂-phosphocreatine, 4 Mg-ATP, 0.3 Na₂-GTP, pH 7.3 adjusted with KOH and filtered with a 0.2 µm syringe filter. For biocytin labeling, 2 mg/ml biocytin (Tocris) was dissolved in the intracellular solution and cells were dialyzed for 20 minutes. For all recordings, the patch-pipette tip resistance was ~5 MΩ. The initial access resistance was less than 25 MΩ for all cells and if this increased by more than 5 MΩ the cell was discarded.
We identified mICCs by their somatic morphology and location in the dorsal ICC nucleus, elevated membrane resistance and the presence of a slowly accommodating inward current upon hyperpolarization, in agreement with past work (Asede, Bosch et al. 2015). The concentration of drugs applied onto brain slices via bath perfusion were: 10 μM CNQX (Cayman chemical), 250 nM PACAP 6-38 (Tocris), 20 μM Bicuculline (Cayman chemical), and 0.5 μM Tetrodotoxin (Cayman chemical). All compounds were stored at -20°C as stock solutions and diluted in ACSF just prior to use. ChR2-mediated responses were evoked by 470 nm light flashes from an LED source (Sutter Instrument) at power of 0.025 mW/mm² and for a flash duration of 25 ms each.

Viruses

For the intersectional method, we used hEF1α-LS1L-mCherry-IRES-flp (MIT and Harvard Vector Core) and AAV5-EF1a-fDIO-ChR2-eYFP-WPRE or AAV5-EF1a-fDIO-eYFP-WPRE (UNC Vector Core) constructed by Dr. Rachel Neve and Dr. Karl Deisseroth, respectively. For ex vivo electrophysiology experiments, we used Cre-dependent AAV5-EF1a-DIO- hChR2(H134R)-mCherry virus (UNC Vector Core). For deletion of PAC1 receptors we used the AAV2-hsyn-GFP-Cre or AAV2-hsyn-GFP (UNC vector core).

Microscopy for All Experiments

The tissue sections were analyzed using a Keyence BZ-X700 -All-in-One Fluorescence Microscope. Images were analyzed with Fiji image processing software (NIH, Bethesda, MD; RRID: SCR_002285). Images were converted to binary mode (black and white image). For ex vivo studies, a confocal microscope was used for analyzing the expression of biocytin-filled cells in the mICCs.

Experimental design and the statistical analyses

To be consistent, for every phase of testing (days 1-14), we measured freezing for the first 4 minutes of the session. This corresponds to the preshock period on the acquisition days, providing a measure of
contextual fear that is not confounded by the unconditional behavioral effects of the shock. For the behavioral experiments in which acquisition and extinction were measured, a three-way analysis of variance (ANOVA) was used to measure differences in means with two between (sex and group) and one within (day) factors. For the other behavioral experiments, a two-way ANOVA was carried out to measure differences in the means with group and sex as between group factors. Significant effects indicated by the ANOVA were further analyzed with a post-hoc Bonferroni post-hoc analysis. The level of significance used for all analyses was p < 0.05. For behavior experiments, analysis was carried out using the SPSS statistics software.

For electrophysiology, statistical tests were run in GraphPad Instat 3. Summary data are presented as mean ± s.e.m. Note that in some of the graphs, the bars representing the s.e.m. are smaller than the symbols used to represent the mean. In all cases the individual n numbers are reported on a scatter plot and defined for each experiment. For each set of data to be compared, we determined within GraphPad Instat whether the data were normally distributed or not. If they were normally distributed, we used parametric tests. If the data were not normally distributed, we used non-parametric tests. Paired and unpaired Student's t tests (as appropriate) and two-tailed Mann–Whitney or Wilcoxon tests were used for most statistical analyses with significance declared at P < 0.05, but stated in each case with a precise P value. When the P value was less than 0.0001, it is stated as such to save space on the figure panels and text. N is defined as the numbers of cells or mice throughout on a case-by-case basis depending on the particular experiment; the unit of analysis is stated in each figure or figure legend. Where appropriate, key statistics are also reported in the text. No data points were excluded from any experiment.

Results

1. PACAP-expressing neurons in the BMA innervate the medial ICCs

We examined EGFP expression in the Adcyap1-EGFP mice, which restricts EGFP expression to PACAP expressing neurons (Condro, Matynia et al. 2016). Although distributed broadly in the amygdala, EGFP+ cells were enriched in the lateral and basomedial nucleus (BMA) subregion with fibers in the mICCs (Fig. 1A and 1B). We evaluated the local PACAPergic efferents in the mICCs and found a high innervation of dorsal and ventral mICCs by PACAPergic neurons (Fig. 1A and 1B). By comparison, we found little to no innervation of the CN by EGFP+ neurons (Fig 1A and 1B). The pattern of expression corresponds with PACAP mRNA expression, which is high in the BMA and some expression in the mICCs (Allen Brain Atlas Mouse Brain in Situ hybridization). To specifically target the mICCs, we first injected DAPI (4',6-diamidino-2-phenylindole) using glass pipettes. While mICCs are difficult to target because of their small size, we were able to localize our DAPI injections to these cells into the capsule of mICCs (Fig. 1C). We also confirmed that our injections would be restricted to the mICCs by injecting AAV5-EF1a-DIO-hChR2(H134R)-mCherry into the mICCs and were able to restrict the injection in the mICCs by this method (Fig. 1C). We then wanted to determine if monosynaptic PACAPergic projections in the mICCs arise from the BMA. Using intersectional approach as
described in Fenno et al. 2014 (Fenno, Mattis et al. 2014), we injected a retrogradely trafficked Cre-dependent HSV virus (hEF1α-LS1L-mCherry-IRES-flpo) into the mICCs (dorsal portion) and a Flp-dependent AAV5-EF1a-fDIO-ChR2-eYFP-WPRE into the BMA of Adcyap1-2A-Cre mice. Adcyap1-2A-Cre mice express Cre specifically in PACAP-containing neurons. With the intersectional approach, the HSV virus, which is Cre-dependent and expresses FLP, retrogradely transports allowing expression specifically in PACAPergic neurons in the BMA. The AAV virus in turn is Flp-dependent, so therefore expresses only in neurons that have FLP. This allows labeling of specific projections from the BMA to mICCs (Fig. 1D). Our analysis showed that mcherry expressing soma were present in the BMA and fibers in the mICCs (Fig. 1D, 1F and 1G). To confirm, mcherry fiber localization in the mICCs, we also co-labeled the sections with antibody against FoxP2, a marker for mICCs and found that the viral labeling overlapped with FoxP2 (Fig. 1E). Thus, using two different approaches (in Adcyap1-EGFP and Adcyap1-2A-Cre mouse lines), we confirmed that mICCs receive PACAPergic innervations and some of those innervations arise in the BMA.

2. *In vivo* optogenetic activation of PACAP-expressing neurons in the BMA enhances expression of cfos in the mICCs

Next, we wanted to validate whether the mICCs projecting BMA PACAPergic neurons are glutamatergic. We first characterized co-localization of PACAPergic neurons with glutamatergic neurons with immunohistochemistry using an antibody against vesicular-glutamate transporter 2 (VGLUT2), a marker for glutamatergic neurons, and green fluorescent protein (GFP) in tissue sections containing the BMA from Adcyap1-EGFP mice (N=6, 3M and 3F) (Fig. 2A). We analyzed the percentage of overlap between VGLUT2-expressing neurons and PACAP. For this, we first counted the number of VGLUT2+ and PACAP+ cells separately in the same area of BMA. We then counted the VGLUT2+ that were either PACAP+ or PACAP-. Then we expressed these counts as fraction of overall VGLUT2 cells and expressed as percentage (Paired T-Test PACAP+/VGLUT2=23% +/- 0.006; PACAP-/VGLUT2=77% +/- 0.015; P=0.0001, t(5)=26.15). Our analysis showed that around 23% of VGLUT2 cells in BMA express PACAP-GFP. We further conducted *in situ* hybridization for PACAP using the RNAscope technology and immunohistochemistry using an antibody against VGLUT2 in tissue sections containing the BMA in a separate group of wild type mice (N=8 (4M and 4F), Fig.
2B). We analyzed the percentage of overlap between VGLUT2 immunoreactive cells with PACAP mRNA puncta. We found that PACAP mRNA puncta were present throughout the BMA. For our analysis, we first counted VGLUT2+ cells. Then we counted the PACAP mRNA puncta surrounding VGLUT2+ cells by setting the criterion of counting more than 5 puncta surrounding the VGLUT2 and DAPI expressing cell. This analysis revealed that about (49% +/- 3%) VGLUT2 cells expressed PACAP mRNA (Fig. 2B). Our analysis shows that there is a significant difference in the expression of PACAP mRNA and PACAP protein expression within the BMA, with higher expression of the PACAP mRNA overlapping with VGLUT2 containing neurons.

3. **In vivo** optogenetic activation of PACAP-expressing neurons in the BMA enhances expression of cfos in the mICCs

Next, we wanted to determine the functional effect of stimulating PACAPergic projections that innervate the mICCs. We chose to measure changes in expression of cfos immunoreactivity after optogenetic stimulation of the BMA-mICC PACAPergic pathway. Using the same intersectional approach as described above in the Adcyap1-2A-Cre mice, we first injected the Cre-dependent HSV virus (hEF1α-LS1L-mCherry-IRES-flpo) into the mICCs (dorsal portion) and a Flp-dependent AAV5-EF1a-fDIO-ChR2-eYFP-WPRE into the BMA of Adcyap1-2A-Cre mice in both hemispheres. We bilaterally implanted optic fibers targeting the BMA (Fig. 3A).

After recovery and viral expression, we performed *in vivo* optogenetic stimulation of the BMA in anesthetized mice in one hemisphere and analyzed changes in expression of cfos immunoreactivity in the mICCs of that hemisphere comparing it to the opposite side. For this, we carried out immunohistochemistry for labeling cfos in tissue sections containing the BMA and mICCs using an antibody against cfos. For analysis, we counted the absolute number of cfos+ cells in the mICCs in both hemispheres. Only, distinct soma-like puncta were counted for the analysis. Optogenetic stimulation of ChR2-containing cells in the BMA significantly enhanced expression of cfos in the mICCs in the hemisphere with optogenetic stimulation compared to mICCs of the hemisphere without optogenetic stimulation (N=4 (2M:2F), Paired t-test; t(3) = 7.816, p=0.0024) (Fig. 3A, 3B, 3C and 3D). We used the Paxinos and Watson Brain Atlas to determine the anatomical boundaries between CN and mICCs for analysis of cfos and restricted it to the mICCs in the dorsal region. These results show that
altering activity of PACAPergic neurons in the BMA modulates activity of neurons in the mICCs, showing a functional effect on neuronal activity in the mICCs.

4.  

Ex vivo optogenetic stimulation of the BMA-ICC PACAPergic pathway enhances EPSCs in the mICCs that is further enhanced by application of a PAC1 receptor antagonist

Next, we sought to characterize the electrophysiological properties of mICCs neurons when PACAPergic projections to this region were optogenetically stimulated. We specifically tested if stimulation of BMA PACAPergic terminals changes synaptic activity of mICC neurons. For this, we performed ex vivo electrophysiological recordings in combination with optogenetic stimulation of BMA PACAPergic fibers expressing ChR2 (Fig. 4A). We injected AAV5-EF1a-DIO-hChR2(H134R)-mCherry virus into the BMA of Adcyap1-2A-Cre mice, which expresses Cre in PACAP-containing neurons (both males and females) (Fig. 4A and 4B). This allowed expression of ChR2 in the efferent pathways of the BMA containing PACAP. We then performed whole-cell patch clamp recordings from the dorsal mICCs region (Fig. 4B). The mICCs neurons showed a sagging current upon hyperpolarization and action potentials in response to stepwise changes in current (Fig. 4B). For all electrophysiological experiments, we confirmed that the recording sites were within the mICCs by filling the recorded neurons with biocytin and confirming the appropriate location of the neurons post-hoc (Fig. 4B, 4J). The electrophysiological and morphological properties of mICC neurons in our studies matched previously described properties of mICCs neurons (Huang, Chen et al. 2014, Aseed, Bosch et al. 2015, Bosch, Aseed et al. 2016).

Optogenetic stimulation of PACAPergic neurons from BMA was carried out with a 473nm light pulsed with a 20 Hz train of 25 ms single pulse or multiple pulses using an LED (Fig. 4B). The stimulation parameter was based on a previous study published in the lab (Hersman, Cushman et al. 2017). A single flash produced excitatory postsynaptic currents (EPSCs) in a majority of mICC neurons (Fig. 4C). In some cases, EPSCs were followed by inhibitory post-synaptic potentials (IPSCs) (Fig. 4C). Out of 32 recorded neurons, 43.8% showed EPSCs, 9.4% showed IPSCs, 25% showed EPSCs followed by IPSCs and 21.9% of cells showed no response (N=8, 22 cells). Mann-Whitney, unpaired test showed that average amplitude of EPSCs was enhanced compared to
IPSCs (U=187; P=0.0011; Fig. 4C). For the neurons that showed EPSCs followed by IPSCs, Unpaired T-Test showed that the IPSC event latencies were much longer compared to the EPSCs (t(30)=10.215; P= 0.0001; Fig.4C). Wilcoxon matched pairs, 2 tailed test showed that application of TTX completely abolished EPSCs and IPSCs (EPSCs; W(17) =-153; P= 0.0001; IPSCs; W(10)=55; P= 0.002; Fig 4D). Wilcoxon matched pairs, 2 tailed test also showed optogenetic light evoked EPSCs and IPSCs were blocked by the application of CNQX in both cases indicating that the BMA neurons release glutamate (N=6; 11 cells (EPSCs); 6 cells (IPSCs)); EPSCs; W(11) =-66; P=0.001; IPSCs; W(6,21; P= 0.0313; Fig. 4E). Paired T-test showed application of the PAC1 receptor antagonist peptide, PACAP 6-38 in the bath significantly enhanced EPSCs (N=6; 14 cells; t(13)=2.441; P=0.0297; Fig. 4F). However, Wilcoxon matched pairs, 2 tailed test showed that application of PACAP 6-38 did not alter paired-pulse ratio (W(14)=51; P= 0.1189; Fig. 4F).

With bursts of optogenetic stimulation, EPSCs amplitude decreased with an increase in stimulus number (Fig. 4G; N=6; 8 cells). Wilcoxon matched pairs, 2 tailed test showed that the IPSCs that were observed in some neurons after EPSCs, were blocked by application of bicuculline (N=6; 11 cells; W(11)=62; P= 0.0029; Fig. 4H). In a small sub-set of neurons, paired T-test showed that the application of TTX suppressed EPSC responses and TTX + 4-AP application increased EPSC amplitude (t(3)=2.584; P=0.0408; Fig. 4I). These results indicate that PACAP neurons produce a predominantly excitatory glutamatergic influence on mICCs, which in turn triggers local GABAergic inhibition between mICCs. Overall, these results show that the PACAPergic neurons from BMA to mICCs are synaptically coupled. These results also suggest that PACAP could be co-released with glutamate from BMA neurons mitigating the post-synaptic influence of glutamate. In our studies, we did not find any significant differences in the electrophysiological properties between males and females and therefore the data for males and females were combined but represented as blue and pink colors in Figure 4.

5. In vivo optogenetic stimulation of BMA PACAPergic input to the mICCs decreases fear recall and increases fear extinction
Given that BMA-PACAPergic neurons modulate electrophysiological properties of dorsal mICCs neurons, next, we wanted to test if, and how, this BMA (PACAP) to mICCs (PAC1) pathway contributes to the learning and expression of fear behaviors. Our goal was to also determine if gain of function in this pathway modulates any specific or all aspects of fear behaviors, so we tested the effects of optogenetic stimulation of BMA-mICCs pathway on fear acquisition, generalization, recall and extinction. We chose to look at all these aspects of fear in the same animals because mICCs have previously been shown to modulate fear extinction, but we wanted to interrogate whether this pathway has effects on other aspects of fear besides extinction in the same experimental preparation. Using previously described intersectional approach in the Adcyap1-2A-Cre mice, we conducted an optogenetic gain of function experiment. We carried out bilateral in vivo optogenetic stimulation of PACAPergic fibers in the mICCs that emanate from the BMA and tested different aspects of contextual fear behavior including acquisition, generalization, recall and extinction. The behavioral procedure is shown in Fig. 5A and 5B. These experiments were carried out in both males (N=8 (ChR2); N=9 (EYFP)) and females (N=9 (ChR2); N=7 (EYFP)). Acquisition measures the ability of animals to learn the association between the context and shock and an asymptotic level of conditional fear is graded in a manner that is proportional to shock intensity. Analysis of Variance (ANOVA) revealed a main effect of the Day (ANOVA; F (4, 145) =66.503; P=0.001, but no Sex X Group interaction (ANOVA; F (1, 145) =0.833; P=0.363) or Day X Sex X group interaction ANOVA; F (4, 145) =30.979; P=0.980) in fear acquisition indicating that the animals acquired/learned fear in a similar manner (Fig. 5C). Learned responses generalize to other contexts and overgeneralization occurs in anxiety disorders reflecting fear in an inappropriate context. Moderate levels of freezing in this alternate context indicate that the mice exhibit over-generalized fear. However, we found that the Sex X Group interaction in the generalization test was not significant, indicating that the animals did not differentially generalize fear to a novel context (Fig 5D) (ANOVA; F (1, 29) =0.594; P=0.447).

The recall test was designed to measure the ability of animals to maintain a long-term fear memory of the context in which they acquired fear. ANOVA revealed a significant main effect of Group (ANOVA; F (1, 29) =13.08; P=0.001) in the recall test, such that the groups that received optogenetic stimulation of the
PACAPergic neurons from BMA to mICCs showed a significantly reduced level of freezing compared to the EGFP controls (Fig. 5E). However, there was no significant Group X Sex interaction (ANOVA; \( F_{(1, 29)} = 0.008; \) \( P = 0.993 \)).

Extinction is the loss of expression of learned behavior with repeated exposure to the conditional stimulus (e.g. context) without the unconditional stimulus (e.g. shock). We found a main effect of Group (ANOVA; \( F_{(1, 145)} = 20.128; \) \( p = 0.0001 \)) and day (ANOVA; \( F_{(4, 145)} = 26.907; \) \( p = 0.0001 \)) during the extinction test, but no Group X Sex interaction (ANOVA; \( F_{(1, 145)} = 0.1; \) \( P = 0.753 \)) or Group X Sex interaction (ANOVA; \( F_{(4, 145)} = 0.247; \) \( P = 0.911 \)) (Fig 5F). These results show that stimulation of PACAPergic pathway from BMA to mICCs alters specific aspects of fear behaviors and similarly between males and females. Specifically, our results indicate the activation of PACAPergic pathway from BMA to mICCs decreases recall of fear potentially indicating that these animals had a weaker memory of the context where they acquired fear behaviors. Even though the recall was decreased, these animals also showed a propensity for enhanced extinction of fear in both males and females, indicating that activation of PACAPergic pathway from BMA to mICCs reduces fear of the context in which the context and shock associations were learned. While the freezing levels on extinction day 1 were higher than on the recall test, it is hard to know whether recall influenced extinction of fear.

6. Deletion of PAC1 receptors from the mICCs enhances fear generalization and decreases fear extinction in males but not in females

Next, we wanted to determine the effects of loss of function of PAC1 containing cells in the mICCs on the same fear behaviors that were measured with optogenetic gain of function of the BMA-mICCs PACAPergic pathway. Previous studies have shown that forebrain-specific deletion of PAC1 receptors leads to an impairment of contextual fear conditioning, but in these studies other aspects of contextual fear learning such as generalization and extinction were not examined (Otto, Kovalchuk et al. 2001). Also, mICCs have been shown to modulate fear extinction, but similar to the optogenetic stimulation experiments, we chose to specifically look at all phases of fear including acquisition, generalization, recall and extinction. This also allowed us to compare
the loss of function experiment with PAC1 deletion in mICCs with the gain of function experiment with optogenetic stimulation of PACAPergic pathway to mICCs. *In situ* hybridization shows that PAC1 receptor mRNA expression is high in the mICCs compared to BLA/BMA or CN (Allen brain Atlas). Thus our loss of function experiment of PAC1 deletion in mICCs was designed to determine how a corresponding loss of mICCs PAC1 receptor gene expression affects the same set of fear behaviors as we tested with the optogenetic gain of function experiments (Fig. 6A and 6B). For this, we bilaterally injected the AAV2-hsyn-GFP-Cre or AAV2-hsyn-GFP as control into the dorsal mICCs of the mice with floxed PAC1 receptor gene. AAV2-hsyn-GFP-Cre allows Cre-mediated site-specific deletion of PAC1 receptors from the mICCs. Only the animals that had bilateral and specific viral expression localized in the mICCs were used in the behavioral analysis. We carried out testing in both males (N=14 (Cre); N=16(GFP) and females (N=15 (Cre); N=18 (GFP). ANOVA revealed a main effect of Day (ANOVA; F (4, 285) =201.186, P=0.0002), Sex (ANOVA; F (1, 285) =10.586, p=0.001) and a Sex X Group interaction (ANOVA; F (1, 285)=4.757, p=0.046) in acquisition (Fig. 6C and 6G). ANOVA revealed there was a main effect of Group (ANOVA; F (1, 59) =3.858, P=0.05) and a Sex x Group interaction (ANOVA; F (1, 59) =9.352, P=0.003) on the generalization test (Fig. 6D and 6H). Post-hoc Bonferroni analysis showed that males with PAC1 deletion were significantly different from control (P<0.05). ANOVA revealed no effect of Sex or Group or Group x Sex interaction on the recall test (Fig.6E and 6I) (ANOVA; F (1, 59) =2.463; p=0.122). ANOVA revealed a main effect of Day (ANOVA; F (1, 295) =49.711, P=0.0004), Sex (ANOVA; F (1, 295) =12.513, p=0.0002) and Day x Sex x Group interaction (ANOVA; F (4, 295) =2.545, P=0.04) in extinction. Post-hoc analysis revealed that extinction rate in males with PAC1 deletion was significantly higher than controls on days 2, 3 and 4 of extinction (P<0.05) (Fig. 6F and 6J). Females showed decreased fear acquisition, but males showed enhanced fear generalization and decreased fear extinction (Fig. 6C-Fig.6F). We verified that viral expression was localized in the mICCs with post-mortem analysis using the RNAscope technique; PAC1 mRNA in the mICCs was significantly reduced after injection of AAV-Cre compared to AAV-GFP control (Fig. 6K and 6L and Fig. 7; Paired T-Test; t(4)=P<0.05).

**Discussion**
We described a previously uncharacterized amygdala microcircuit containing the neuropeptide PACAP and PAC1 receptors in contextual fear regulation, whereby BMA PACAPergic neurons innervate the mICCs, that in turn express PAC1 receptors. BMA PACAP neurons regulate heterogenous aspects of fear behaviors and mICCs electrophysiological properties. PAC1 containing neurons in the mICCs, in turn, alter fear in a sexually dimorphic manner. Our results were supported by gain of function optogenetic, loss of function viral-mediated receptor deletion and electrophysiology using genetically modified mice (Working model: Fig. 8). While mICCs have previously been shown to be important for fear extinction (Likhtik, Popa et al. 2008), we uncovered that mICCs also play a role in influencing other components of fear memory including acquisition, generalization and recall.

We used a novel contextual fear conditioning procedure to assess fear acquisition, generalization, recall and extinction together. Optogenetic stimulation of BMA-mICCs PACAP reduced fear recall and extinction. Deletion of PAC1 receptors from mICCs led to increased fear generalization and decreased extinction in males and decreased acquisition in females. Using two complementary approaches we found that the BMA sends dense PACAPergic projections to mICCs. Previous anatomical studies using the phytohemagglutinin lectin anterograde labeling method suggested that BMA projections to mICCs are sparse. While this method of labeling provides useful information about regional association, it is limited in providing information about transneuronal connections and genetically defined cell populations or defining functional synapses (Petrovich, Risold et al. 1996, Strick and Card 2011).

Our electrophysiology results corroborated physiologically that mICCs are similar to medium spiny neurons, and functionally that these neurons show high excitability and input resistance (Millhouse 1986, Royer, Martina et al. 2000). Ex vivo optogenetic stimulation of the PACAPergic BMA-mICCs projections enhanced EPSCs with a short latency (10 ms) in mICCs neurons. CNQX abolished the EPSCs, indicating that these responses were mediated by glutamatergic AMPA receptors. In a subset of neurons, TTX application suppressed EPSCs and TTX + 4-AP application increased EPSC amplitude, suggesting BMA-mICCs pathway is monosynaptic. Application of PAC1 receptor antagonist (PACAP 6-38) concurrently with BMA-mICCs optogenetic stimulation,
surprisingly, further enhanced EPSCs. This suggests PACAP is co-released with glutamate, validated by our immunohistochemistry results showing VGLUT2 colocalized with PACAP. Blockade of PAC1R enhanced EPSC amplitude without changing paired pulse ratio, arguing against the influence of PACAP 6-38 on presynaptic inputs. The molecular mechanism of how PAC1 blockade enhances EPSC amplitude remains to be precisely determined in future work. PAC1R are known to couple to K\(^+\) channels and their activation can enhance K\(^+\) conductance in multiple cell types (Ichinose, Asai et al. 1998, Baron, Monnier et al. 2001). One hypothesis is that the shunting inhibition promoted by basal tone of PACAP is relieved by application of PACAP 6-38, predicting EPSC enhancement from multiple sources outside of BMA. There were no sex differences in these measures. Our result suggests BMA monosynaptic PACAPergic neurons drive behavioral effects by innervating a subset of mICCs neurons.

In vivo optogenetic stimulation of the PACAP BMA-mICCs pathway decreased contextual fear during the recall test in both males and females, indicating BMA-mICCs monosynaptic pathway may not be sexually dimorphic in and of itself. Following the recall test, we found extinction to be decreased over time. Given that recall itself was altered, we cannot conclude whether extinction changes are attributable to optogenetic manipulation during extinction or due to changes in recall. The differences in freezing during extinction emerged over the course of days even though the groups were at equivalent levels in the beginning, suggesting that PACAP BMA-mICCs pathway modulates extinction, consistent with the known role of ICCs (Likhtik, Popa et al. 2008). Higher freezing on day 1 of extinction than recall, suggests that extinction alterations may be independent of recall. While acquisition and generalization were not altered by our manipulation, it is not inconceivable that effects on fear encoding during acquisition influenced later recall and extinction. It is important to highlight that our main goal was to determine if the BMA-PACAP circuit regulates any or all aspects of fear. Our findings indicate that BMA-mICCs PACAP circuit diminishes specific aspects of learned fear expression rather than a general fear reduction, per se. Overall, our results lay a foundation for future experiments to precisely parse how this pathway affects specific aspects of fear (acquisition, generalization, etc.). For instance, does recall of generalization produce long term plasticity affecting extinction? Other important future directions include determining mechanisms of optogenetic
inhibition of BMA-mICCs (PACAP) pathway, interrogating if fear is changed by altered gating of BMA neurons or via direct influence on CN output neurons to promote behavior output. One limitation of our stimulation parameter where stimulation occurred throughout sessions instead of restricting to a phase of a session is the impact on future fear learning or unwanted effects. This was done to match the PAC1 deletion experiment, where the deletion was present throughout entire sessions.

PAC1 receptor deletion from mICCs slightly decreased fear acquisition, without altering generalization or extinction in females, somewhat modulating the asymptote of the learning curve. In males, PAC1 deletion from mICCs enhanced fear generalization and reduced extinction, indicating that these receptors regulate both fear generalization and extinction. Thus, in males, PAC1 receptors in BMA-mICCs anatomical juncture may be necessary for regulating fear in an inappropriate context, whilst ablating them enhances fear. Reduced extinction indicates a failure to inhibit acquired fear when the PAC1 receptors are lacking. Overall, the results show males and females show different fear expression when PAC1 receptors are absent in mICCs.

PAC1-containing mICCs neurons project to various regions besides the CN to modulate distinct aspects of fear via these downstream projections. Our finding that sex differences in aspects of fear emerged only with the mICC receptor manipulation indicates that PAC1 containing mICCs neurons receive innervation from other areas besides the BMA that drive behavioral output. While additional research is required, we hypothesize that PAC1 expressing mICCs neurons and their downstream projections may be sexually dimorphic.

Our optogenetic stimulation and PAC1 deletion in the mICCs affected different aspects of fear. The optogenetic stimulation experiments were designed to modulate the upstream pathways of mICCs from the BMA, while the PAC1 deletion experiments modulate the downstream pathways. The mICCs are known have a complicated morphology due to direct or indirect projections to the CN and could have different functional roles in fear regulation. Since more is known about the downstream projections of mICCs than the upstream projections to this region, our hypothesis is that the upstream pathways to mICCs are less heterogenous, but the heterogenous downstream projections affect multiple aspects of fear. Our conjecture fits with studies.
showing mICCs modulate distinct aspects of fear via specific neuropeptide/neurotransmitter microcircuits via projections to different structures (Amir, Amano et al. 2011, Busti, Geracitano et al. 2011, Duvarci and Pare 2014). Also not in the scope of our paper, but sub-divisions of the mICCs could also modulate different aspects of fear also via PACAP/PAC1.

We decided not to monitor cycle when comparing males and females because it is not possible to equate the stress from taking samples for cytology in females with males’ experience. Therefore, we could not determine if hormonal cycle played a role in the observed behavioral differences. Statistical analysis of heterogeneity of variance between males and females revealed similar variance for the male and female groups suggesting that cycle may not play a major role in our observations. In future work we will examine cycle effects directly by comparing only females at different stages of the cycle as that allows equivalent handling of all animals.

Amygdala microcircuitry is heterogeneous and complex in its morphology and function and mICCs are major cell groups in this microcircuitry for processing specific aspects of fear-related information (Royer, Martina et al. 1999, Likhtik, Popa et al. 2008, Ehrlich, Humeau et al. 2009, Palomares-Castillo, Hernandez-Perez et al. 2012, Pare and Duvarci 2012). Our finding that PACAP and PAC1 influence specific fear properties suggests that PACAP/PAC1 is a critical neuropeptide system in the BMA-mICCs amygdala node for differentially regulating sensory and associative information. Despite a complex cytoarchitecture, holistically, mICCs are plastic in their response to sensory information and modulate fear behaviors in a manner dependent upon the incoming information (Amano, Duvarci et al. 2011, Huang, Chen et al. 2014, Asede, Bosch et al. 2015).

While fear is a natural response that keeps organisms safe when faced with danger, fear dysregulation as in PTSD, cripples an individual’s ability to function. Traumatic stress results in enhanced acquisition of conditional fear that over-generalizes to safe contexts or enhances recall and is less susceptible to extinction. We looked at all these aspects of fear in our study. PACAP and PAC1 have previously been shown to be important for fear regulation and high blood levels of PACAP, especially in females, and PAC1 methylation in a sex-independent manner are associated with PTSD (Ressler, Mercer et al. 2011). Disentangling a microcircuit like...
BMA-miCCs in fear regulation via specific neuropeptide systems like PACAP/PAC1 provides better anatomical knowledge regarding substrates for targeted therapies for ameliorating symptoms in disorders like PTSD (Fendt and Fanselow 1999).

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Figure Legends

Fig.1. PACAP-expressing neurons in the BMA innervate medial intercalated cells

A. Representative photomicrographs of high resolution imaging showing immunohistochemistry for green fluorescent protein (GFP) in Adcyap1-EGFP mice. PACAPergic neurons are highly expressed in the lateral amygdala and basomedial amygdala (BMA). PACAPergic terminals are observed in the mICCs primarily but not in the central amygdala (CN) (Left and right panel). PACAP-EGFP is shown in green. Scale bar: 500 μm.
B. Example of section containing the basolateral (BLA) complex and PACAPergic innervation in the mICCs as shown by arrow labeled with PACAP-EGFP (green). Scale bar: 100 μm (top) and 50 μm (bottom).

C. Top panels: Representative photomicrographs showing DAPI (blue) injection into the mICCs using specially constructed glass pipettes as shown by arrows. Scale bar: 200 μm. Bottom panel: Representative image showing injection of AAV5-ChR2-mcherry virus into the mICCs. Scale bar: 100 μm.

D. Left panel: Cartoon depicting the injection strategy for the intersectional approach in Adcyap1-2A-Cre mice. Top cartoon: Representative diagram of the viral constructs that were injected for the intersectional viral injections in Adcyap1-2A-Cre mice. The Cre-dependent LTHSV-lox-STOP-lox-mCherry-IRES-Flp virus that was injected in the mICCs is shown on the top and the AAV5-IDIO-ChR2-eYFP was injected into the BMA is shown in the bottom. Right panel: Photomicrograph shows the intersectional virus strategy labeled PACAPergic neuronal in the BMA and terminals in the mICCs (red).

E. Representative photomicrographs confirming that the mICCs terminals labeled with intersectional virus were in the mICCs by co-labeling with the marker FoxP2. FoxP2 was only used here to confirm terminal labeling in the mICCs with the intersectional virus. Arrow indicates the FoxP2 expression in mICCs.

F and G. Photomicrographs showing the intersectional viral strategy of injecting LTHSV-lox-STOP-lox-mCherry-IRES-Flp virus into the dorsal mICCs and AAV5-IDIO-ChR2-eYFP into the BMA led to EYFP (green) and mcherry (red) expression from the LTHSV-lox-STOP-lox-mCherry-IRES-Flp and AAV5-IDIO-ChR2-eYFP viruses in the mICCs and BMA respectively (F: mICCs, G: BMA).

**Fig. 2. Immunohistochemistry and In situ hybridization analysis and quantification of overlapping PACAP and VGLUT2 expressing cells in the BMA**

A. Representative photomicrographs from tissue sections containing the BMA from PACAP-EGFP mice showing expression of VGLUT2 (red) and PACAP-EGFP (green) processed with immunohistochemistry for GFP and VGLUT2. White arrows represent overlapping VGLUT2 and PACAP signals. Green arrow shows an example of PACAP cell without VGLUT2 expression. Scale bar: 25 μm. Right graph shows quantification of overlap. (N=6, 3 M and 3F df=5).
B. Representative photomicrographs from tissue sections containing the BMA from WT mice showing overlapping VGLUT2 (green) and PACAP mRNA (red) fluorescence in the BMA. Tissues were processed with RNAscope technique for PACAP mRNA and then immunohistochemistry for VGLUT2. Right Venn diagram shows quantification of overlap between VGLUT2 and PACAP mRNA. (N=5, 2 males and 3 females; df=4).

Fig. 3. *In vivo* optogenetic activation of PACAP-expressing neurons in the BMA enhances expression of cfos in the mICCs

A. Cartoon depicting optogenetic stimulation strategy in the BMA in anesthetized Adcyap1-2A-Cre mice. Cre-dependent LTHSV-lox-STOP-lox-mCherry-IRES-Fip virus that was injected in the mICCs and the AAV5-fDIO-ChR2-eYFP was injected into the BMA. Optogenetic stimulation was carried out in unilateral hemispheres and control stimulation was in the opposite hemisphere of the same mice. Stimulation parameters are shown above the cartoon on the top.

B. Representative photomicrographs of hemispheres containing mICCs and other regions showing cfos expression after optogenetic stimulation in the BMA of anesthetized mice. Arrows indicate cfos expression. Scale bars: 100 μm.

C. Graphical representation showing number of cfos positive cells in mICCs. Right panels: Representative images showing cfos immunoreactivity expression in the mICCs in the hemisphere that was optogenetically stimulated and the control. (N=4, 2 males and 2 females; df=3, p<0.001).

D. Representative photomicrographs of mICCs showing cfos expression in the hemisphere where optogenetic stimulation occurred (left panel) and the hemisphere where the stimulation was absent (right panel). Scale bars: 100 μm.

Fig. 4. *Ex vivo* optogenetic stimulation of BMA-ICCs PACAPergic pathway enhances EPSCs in the mICCs

A. Cartoon depicting the location of the ChR2-expressing cells within the BMA that innervate the mICCs.

B. Left panel: Cartoon depicting the whole-cell patch clamp of a neuron in the mICCs. Whole-cell electrophysiological properties of mICCs with the current injection parameters depicted below. Middle:
Example of a biocytin filled mICC neuron (left) and reconstruction (right). Right panel: Example trace depicting the electrophysiological responses of mICCs neurons relative to the LED flash. In this example, the neuronal response comprises of an EPSC followed immediately by an IPSC response. Scale bar: 25 μm.

C. Graphs depicting the EPSC and IPSC amplitudes (left), response latency of either EPSC or IPSC events (middle) and pie chart depicting the classification of response types from mICCs. Majority of mICCs neurons showed EPSCs (N=8, Mann Whitney U test p<0.01). Pink dots=females, Blue dots=males.

D. Representative current trace (left) from mICC neuron in response to single flashes with or without TTX applied. Average EPSC (middle) or IPSC (right) amplitude in response to single flashes with or without TTX are also shown (N=8, Mann Whitney U test p<0.002). Pink dots=females, Blue dots=males.

E. Left panel: Representative current trace from mICC neuron in response to single flash with or without PACAP 6-38 application. Average EPSC amplitude (middle) or paired-pulse ratio (right) in response to single flashes with or without PACAP 6-38 is also shown (N=6, P<0.05). Pink dots=females, Blue dots=males.

F. Left panel: Representative current trace from mICC neuron in response to single LED flash with or without CNQX application. Average EPSC (middle panel) or IPSC (right panel) amplitudes in response to single LED flash with or without CNQX (N=6, p<0.05). Pink dots=females, Blue dots=males.

G. Representative current traces (left) from mICCs in response to trains of blue light flashes with or without CNQX present and average EPSC amplitude (right) at each flash for a train of 40 flashes. Inset shows zoomed in trace with both EPSC and IPSC present.

H. Representative current trace (left) from mICCs in response to single flashes with or without bicuculline application. Average IPSC amplitude (right) in response to single flashes with or without bicuculline (N=6, P<0.05). Pink dots=females, Blue dots=males.

I. Representative current traces in mICCs in response to light stimulation of ChR2 in PACAPergic neurons from BMA-mICCs in response to single flashes in the presence of 1 μM TTX and 100 μM TTX+4-AP. Average evoked EPSC amplitudes (middle) and normalized evoked EPSC amplitudes (Right) in response to single flashes with TTX and TTX+4-AP (N=3, p<0.05). Pink dots=females, Blue dots=males.
J. Representative image of one example neuron filled with biocytin in the mICCs after electrophysiological recordings. Inset image shows confocal image of a biocytin-filled mICCs neuron that has morphological properties and processes that looks like a medium spiny neuron. Scale bar 30 mm.

Fig. 5. In vivo optogenetic stimulation of BMA-mICCs PACAPergic inputs decreases fear recall and increases fear extinction

A. Cartoon depicting the intersectional viral injection strategy in the BMA and mICCs and the optogenetic stimulation strategy and parameters for stimulation. LTHSV-lox-STOP-lox-mCherry-IRES-Flp virus was injected into the dorsal mICCs and AAV5-fDIO-ChR2-eYFP into the BMA. Males (N=8 (ChR2); N=9 (EYFP)) and females (N=9 (ChR2); N=7 (EYFP)) were used in these experiments.

B. Cartoon diagram depicting viral injection surgery timeline and behavior protocol. Mice were tested on fear acquisition in context A, generalization test in context B, recall test in context A and extinction in context A. Optogenetic stimulation with parameters shown in the top of A occurred during these test sessions.

C. Graphs showing changes in freezing during acquisition in females and males in response to optogenetic stimulation of PACAPergic pathway from BMA-mICCs. There was no significant Sex X Group interaction in fear acquisition ((males (N=8 (ChR2); N=9 (EYFP)) and females (N=9 (ChR2); N=7 (EYFP)); F=0.833).

D. Graphs showing freezing during fear generalization test in females and males in response to optogenetic stimulation of PACAPergic pathway from BMA-mICCs. The Sex X Group interaction in the generalization test was not significant, indicating that the animals did not differentially generalize fear to a novel context ((males (N=8 (ChR2); N=9 (EYFP)) and females (N=9 (ChR2); N=7 (EYFP)); F=0.594).

E. Graph showing freezing during fear recall test in females and males in response to optogenetic stimulation of PACAPergic pathway from BMA-mICCs. There was a significant main effect of Group N=8 (ChR2); N=9 (EYFP)) and females (N=9 (ChR2); N=7 (EYFP)); F=13.08; p=0.001). Post-hoc comparison indicated that the group that received optogenetic stimulation of the PACAPergic neurons from BMA to mICCs showed a significantly reduced level of freezing compared to the controls. However, there was no significant Group X Sex interaction (F=0.008).
Graph showing freezing during extinction in females and males after optogenetic stimulation of PACAPergic pathway from BMA-mICCs. There was a main effect of Group during the extinction test N=8 (ChR2); N=9 (EYFP)) and females (N=9 (ChR2); N=7 (EYFP)); F=20.128; p=0.0001), but no Group X Sex interaction (F=0.1; p>0.05).

Fig. 6. Deletion of PAC1 receptors from the mICCs of PAC1<sup>loxp/loxp</sup> mice enhances fear generalization and decreases fear extinction in males but not in females

A. Cartoon depicting the AAV2-Cre-GFP or control virus injections in the mICCs. Males (N=14 (Cre); N=16(GFP) and females (N=15 (Cre); N=18 (GFP) were used in these experiments.

B. Example panels showing PAC1 expression levels in the mICCs from mice with AAV-GFP (left panel) and AAV-Cre-GFP (middle panel) using the RNAscope in situ hybridization technique. Control sections with positive and negative controls are shown on the right. Arrows show PAC1 mRNA puncta. Scale bars: 25 μm.

C and G. Graphs showing freezing during acquisition in males and females. ANOVA revealed a main effect of Day (F=201.186, P=0.0002), Sex (F=10.586, p=0.001) and a Sex X Group interaction (F=4.757, p=0.046) in acquisition.

D and H. Graphs showing freezing during fear generalization test in males and females. There was a main effect of Group (F=3.858, P=0.05) and a Sex x Group interaction (F=9.352, P=0.003) on generalization test. Post-hoc analysis showed that males with PAC1 deletion were significantly different from control (P<0.05).

E and I. Graphs depicting fear recall test after deletion of PAC1 receptors from the mICCs in males and females. There was no effect of Sex or Group or Group x Sex interaction on the recall test (F=2.463; p=0.122).

F and J. Graph showing freezing during extinction. There was a main effect of Day (F=49.711, P=0.0004), Sex (F=12.513, p=0.0002) and Day x Sex x Group interaction (F=2.545, p=0.04) in extinction. Post-hoc analysis revealed that extinction rate in males with PAC1 deletion was significantly higher than controls on days 2, 3 and 4 of extinction (P<0.05).

K. Representative photomicrographs showing AAV-Cre-GFP injection into the mICCs. Arrow shows GFP expression in mICCs.
L. Left: Representative photomicrographs showing PAC1 mRNA puncta differences in mice that received AAV-Cre-GFP (top) or Control AAV-GFP (bottom) in the mICCs. Right: Graphs showing mean grain count of PAC1 mRNA in the mICCs with AAV-Cre-GFP (bottom) and control AAV-GFP (top). Mean grain count was significantly reduced after injection of AAV-Cre compared to AAV-GFP control (P<0.05). Arrows show PAC1 mRNA puncta. Scale bars: 25 μm.

Fig. 7. Composite example photomicrographs depicting AAV-Cre-GFP mediated deletion of PAC1 receptors in mICCs or control AAV-GFP without deletion across all groups.

A. Image panels and 4 example images showing viral targeting of mICCs in females with AAV-Cre-GFP mediated PAC1 deletion. Arrows show mRNA puncta absence. Scale bars: 25 μm.

B. Image panels and 4 example images showing viral targeting of mICCs in females with AAV-GFP and no PAC1 deletion. PAC1 mRNA are shown in red puncta. Arrows show mRNA puncta. Scale bars: 25 μm.

C. Image panels and 4 example images showing viral targeting of mICCs in males with AAV-Cre-GFP mediated PAC1 deletion. Arrows show mRNA puncta absence. Scale bars: 25 μm.

D. Image panels and 4 example images showing viral targeting of mICCs in males with AAV-GFP and no PAC1 deletion. PAC1 mRNA are shown in red puncta. Arrows show mRNA puncta. Scale bars: 25 μm.

Fig. 8. Working model of PACAP in the BMA and PAC1 in the mICCs in fear regulation through amygdala microcircuitry

A representative model of the amygdala microcircuitry containing PACAP neurons and PAC1 receptors in the BMA and mICCs respectively. i) BMA PACAPergic neurons are co-localized with glutamatergic neurons. ii) Optogenetic activation of PACAPergic pathway from BMA to mICCs enhances excitatory postsynaptic potentials, which are further enhanced by application of PAC1 receptor antagonist. iii) Stimulating PACAPergic terminals in the mICCs decreases fear recall and fear extinction. iv) Deletion of PAC1 receptors from mICCs in females decreased fear acquisition, but deletion of these receptors from mICCs in males enhanced fear generalization and decreased fear extinction. This could occur through downstream projections of PAC1 expressing neurons in the mICCs.
