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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Signaling in the Prefrontal Cortex Modulates Cued Fear Learning, but not Spatial Working Memory, in Female Rats

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Abstract

A [genetic polymorphism](#) within the gene encoding the [pituitary adenylate cyclase- activating polypeptide](#) (PACAP) receptor type I (PAC1R) has recently been associated with hyper-reactivity to threat-related cues in women, but not men, with [post-traumatic stress disorder](#) (PTSD). PACAP is a highly conserved peptide, whose role in mediating adaptive physiological stress responses is well established. Far less is understood about the contribution of PACAP signaling in emotional learning and memory, particularly the encoding of fear to discrete cues. Moreover, a neurobiological substrate that may account for the observed link between PAC1R and [PTSD](#) in women, but not men, has yet to be identified. Sex differences in PACAP signaling during emotional learning could provide novel targets for the treatment of PTSD. Here we investigated the contribution of PAC1R signaling within the [prefrontal cortex](#) to the acquisition of cued fear in female and male rats. We used a variant of [fear conditioning](#) called trace fear conditioning, which requires sustained attention to fear cues and depends on [working-memory](#) like [neuronal activity](#) within the prefrontal cortex. We found that cued fear learning, but not [spatial working memory](#), was impaired by administration of a PAC1R [antagonist](#) directly into the prelimbic area of the prefrontal cortex. This effect was specific to females. We also found that levels of mRNA for the PAC1R receptor in the prelimbic cortex were greater in females compared with males, and were highest during and immediately following the proestrus stage of the estrous cycle. Together, these results demonstrate a sex-specific role of PAC1R signaling in learning about threat-related cues.

Keywords

Memory formation, Fear conditioning, Sex difference, Working memory, PTSD, Neuropeptide

1. Introduction

Women are more than twice as likely as men to develop [post-traumatic stress disorder](#) (PTSD), yet the neurobiological basis of this sex difference is unknown ([Kessler et al., 1995](#), [Kilpatrick et al., 2013](#)). Recently, dysregulation of [pituitary adenylate cyclase-activating polypeptide](#) (PACAP) signaling has been implicated in [PTSD](#) selectively in women ([Ressler et al., 2011](#)). Elevated PACAP blood levels and a [single nucleotide polymorphism](#) (SNP) in the PACAP receptor type-1 (PAC1R) gene are associated with hyperarousal symptoms in women but not men with PTSD ([Ressler et al., 2011](#)). The [SNP](#) is located within an [estrogen](#) response element of the gene and interferes with DNA binding of an estradiol-ER α complex, which suggests a link between [ovarian hormones](#) and altered PAC1R expression in these patients ([Mercer et al., 2016](#)). PACAP is a highly-conserved peptide important for mediating adaptive

physiological stress responses, and alterations in PACAP signaling may contribute to the development or maintenance of PTSD in women ([Hammack and May 2015](#)). PACAP may also contribute to PTSD by modulating the formation of emotional memories. PACAP and its receptors are widely distributed throughout the [central nervous system](#), including areas important for memory such as the [hippocampus](#), [amygdala](#), cingulate and [frontal cortices](#), and [thalamus](#) (for a review see [Vaudry et al., 2009](#)). This raises the possibility that PACAP signaling may normally contribute to fear memory encoding in these areas. Indeed, the PAC1R [genetic polymorphism](#) is associated with increased reactivity of fear circuitry to threat-related cues and impaired discrimination of threat and safety cues in women with PTSD ([Ressler et al., 2011](#), [Stevens et al., 2014](#)). Currently, very little is known about the neurobiology of PACAP signaling in emotional learning, especially cued fear learning. [Preclinical studies](#) suggest that PACAP signaling influences contextual fear memory. Mice lacking the PAC1R either globally or in the hippocampus and [neocortex](#) showed impaired contextual [fear conditioning](#) ([Otto et al., 2001](#)). PACAP delivered intracerebroventricularly in rats enhanced the consolidation of a passive avoidance memory at low doses ([Sacchetti et al., 2001](#)) and temporarily impaired contextual fear memory at high doses ([Meloni et al., 2016](#)). PACAP delivered directly into the hippocampus enhanced the consolidation of a contextual fear memory ([Schmidt et al., 2015](#)). Endogenous PACAP may also contribute to the consolidation of contextual fear memory given that blocking the activation of the PAC1R with the [antagonist](#) PACAP6-38 in the hippocampus or the amygdala attenuated the consolidation of contextual fear memory ([Schmidt et al., 2015](#)). With the exception of the Schmidt et al. study, the contribution of PAC1R signaling in individual brain regions to learning has not been explored. The [prefrontal cortex](#) in particular warrants closer investigation. Dysfunction within prefrontal cortical regions in humans is associated with aberrant cognitive and emotional regulation in PTSD, including heightened reactivity to threat-related stimuli, possibly through interaction with the amygdala ([Fani et al., 2012](#), [Shin et al., 2006](#), [Taylor and Whalen, 2015](#)). Moreover, abnormal activity in dorsal prefrontal cortical areas may even be a predisposing factor in the development of PTSD ([Admon et al., 2013](#)).

Here we tested the contribution of PAC1R signaling in the prefrontal cortex to memory formation using trace fear conditioning in female and male rats. Unlike standard cued fear conditioning, the trace variant of fear conditioning critically depends on the prefrontal cortex, which makes this variant useful for probing prefrontal contributions to emotional learning that may be altered in pathological fear and anxiety ([Gilmartin et al., 2013b](#), [Gilmartin et al., 2014](#), [Gilmartin and Helmstetter, 2010](#), [Guimaraes et al., 2011](#)). In trace fear conditioning, subjects learn to anticipate a shock that is delivered several seconds after a cue presentation. Successful acquisition is associated with sustained attention and prefrontal firing to the cue during the stimulus-free trace interval before the shock is delivered – a [working-memory](#) like function that depends on the dorsomedial prefrontal cortex in rats ([Baeg et al., 2001](#), [Gilmartin and McEchron, 2005](#), [Gilmartin et al., 2013b](#), [Han et al., 2003](#)). We tested the importance of PAC1R in trace conditioning and [spatial working memory](#) using direct injections of PACAP₆₋₃₈ into the prelimbic (PL) cortex. PAC1R is the primary target of this peptide in the PL given the apparent lack of vasoactive intestinal peptide receptor type-2 (VPAC2R) in rodent frontal cortices ([Lein et al., 2007](#), [Sheward et al., 1995](#), [Ussdin et al., 1994](#)). Importantly, we conducted this study using both female and male rats. Preclinical studies of PACAP's role in memory have been limited to male subjects. However, the sex-specific link between the genetic

polymorphism in the PAC1R gene in humans and PTSD demands the inclusion of female subjects in preclinical studies. We found that the administration of the PAC1R antagonist PACAP₆₋₃₈ into the PL prior to conditioning attenuated the formation of cued fear memory in females, but not males. In contrast, the performance of a spatial working memory task was unaffected by PACAP₆₋₃₈. Interestingly, females had significantly higher levels of mRNA for the PAC1R within the PL compared with males. These results show that PAC1R signaling within the prelimbic cortex has sex specific effects on the formation of cued fear memory and suggest that differences in PAC1R transcripts may contribute to this sex difference.

2. Materials & methods

2.1. Subjects and surgery

Adult female (Experiments 1, 2, and 4) and male (Experiments 3 and 4) Long-Evans rats (225 g females; 325 g males; Envigo, Indianapolis, IN.) were used in this study. Rats were housed individually and received food and water *ad libitum*. All procedures were in accordance with the National Institutes of Health guidelines and approved by the Marquette University Institutional Animal Care and Use Committee. After three days of handling, rats in Experiments 1-3 underwent cannula-implantation surgery under [isoflurane](#) anesthesia (1%-2% isoflurane in 100% O₂). Peri-operative pain was managed with daily administration of [carprofen](#) in an edible supplement (5 mg/kg in MediGel[®] CPF; ClearH₂O, Westbrook, ME) the day before, the day of, and the day after surgery. During surgery, the rat's head was shaved and then secured in a stereotax (David Kopf, Tujunga, CA). A midline incision was made in the scalp and fascia retracted to expose the skull. Small [craniotomies](#) were drilled above the target sites and [stainless steel](#) guide cannulae (26 ga; Plastics One, Roanoke, VA) were stereotaxically lowered to the prelimbic (PL) region of the [medial prefrontal cortex](#) at a 14° angle (AP +3.2, ML ± 1.6, DV -3.2 mm from the skull). The cannulae were secured to the skull with dental cement and stainless steel skull screws. Dummy cannulae (33 ga) were inserted into the guide cannulae to prevent clogging. Rats recovered in their home cages for a minimum of seven days before the experiment began.

2.2. Drug injections

As described previously, following recovery from surgery, rats were acclimated to transport from their home cage to the procedure room over three consecutive days ([Gilmartin et al., 2012](#)). During this time, the rats were acclimated to gentle restraint in a towel and to the sound of the [infusion pump](#) that would be used for intracranial injections. On the third day of acclimation, rats received mock injections in that the injection cannulae were lowered into the guide cannulae, but no drug was delivered. PACAP₆₋₃₈ was obtained from AnaSpec (Fremont, CA) and dissolved in 0.9% sterile saline on the day of injection. On the day of conditioning, rats received bilateral injections of the PAC1R [antagonist](#) PACAP₆₋₃₈ (0.5 µL/side; females received 1 mM, 2 mM, or 3 mM, and males received 2 mM or 3 mM) or sterile saline vehicle (0.5 µL/side) into the PL 1 h prior to training. These doses of PACAP₆₋₃₈ were chosen to fall within the rather wide range (0.1 mM–8.8 mM) used in the literature to attenuate endogenous or exogenous PACAP directly in the [BNST](#), [amygdala](#), or [hypothalamus](#) ([Farnham et al., 2012](#), [Missig et al., 2014](#), [Resch et al., 2011](#), [Roman et al., 2014](#)). The drug was infused at a rate of 0.5 µL per

minute ([Gilmartin and Helmstetter, 2010](#), [Gilmartin et al., 2012](#)). Injectors were left in place for 90 s after injection to allow the drug to completely diffuse away from the cannula. Rats were then returned to their home cages between injection and training.

2.3. Treatment conditions and group sizes

For Experiment 1, 48 female rats were run in two cohorts (cohort 1: Saline, 1 mM and 2 mM PACAP₆₋₃₈; cohort 2: Saline, 2 mM and 3 mM PACAP₆₋₃₈; n = 8-9/group/cohort). One saline-injected rat was excluded from all analyses due to very high baseline freezing (>70%) in the novel testing chamber. This level was greater than 2 standard deviations of the mean. One rat was removed from the 1 mM PACAP₆₋₃₈ group after histological analysis (see Results). There was no effect of replication on the data from the groups common to both runs (i.e., Saline and 2 mM PACAP₆₋₃₈; ps > 0.37), therefore all data was combined for analysis using the following final group sizes: Saline, n = 15; 1 mM PACAP₆₋₃₈, n = 7; 2 mM PACAP₆₋₃₈, n = 16; 3 mM PACAP₆₋₃₈, n = 9).

For Experiment 2, a subset of female rats (n = 11) from cohort 2 underwent delayed alternation training and testing (drug history: n = 4, Saline; n = 7, PACAP₆₋₃₈ 2 mM). All rats received additional injections of saline, 2 mM PACAP₆₋₃₈, and [muscimol](#) during the course of testing (see section [2.5](#)).

For Experiment 3, 40 male rats were run in two cohorts (cohort 1: Saline, 2 mM PACAP₆₋₃₈; cohort 2: Saline, 3 mM PACAP₆₋₃₈; n = 10 per group/cohort). Two saline-injected rats were excluded from all analyses due to very high baseline freezing (>70%; >2 sd of the mean) in the novel testing chamber. One of these two rats was also excluded based on tissue damage at the cannula site (see Results). There was no effect of replication on the data from the control group common to both runs (i.e., Saline; ps > 0.45), therefore all data was combined for analysis using the following final group sizes: Saline, n = 18; 2 mM PACAP₆₋₃₈, n = 10; 3 mM PACAP₆₋₃₈, n = 10).

2.4. Fear conditioning

Training and testing were conducted as previously described ([Gilmartin et al., 2012](#), [Gilmartin et al., 2013b](#)). During training, rats were placed in Med Associates (St. Albans, VT) conditioning chambers (internal dimensions: 30.5 × 24.1 × 29.2 cm), housed in a sound attenuating outer chamber and illuminated with a 7.5-W white incandescent lamp attached to the outer chamber. Ventilation fans in the outer chamber provided 55-60 dB background noise and the auditory cue was delivered through a speaker centered in one side wall of the conditioning chamber. Stainless steel bars (4.8 mm diameter, spaced 16 mm apart) on the floor of the chamber served to deliver a scrambled footshock. The chamber was cleaned and scented with 5% [ammonium hydroxide](#) before training each rat. The training session ([Fig. 1a](#)) consisted of a 6-min baseline period, followed by 6 pairings of a 10-s white noise conditional stimulus (CS; 72 dB) and a 1-s footshock [unconditional stimulus](#) (UCS; 0.8 mA). The CS offset and UCS onset were separated by an empty 20-s trace interval and the intertrial interval (ITI) was 240 ± 20 s.

During training, rats learn to associate both the auditory CS and the training context with the shock UCS. Rats were tested for memory of each association separately the day after training. Conditional freezing to the CS was tested in a novel chamber (internal dimensions: 30.5 × 24.1 × 29.2 cm) housed in a sound attenuating outer chamber with 55–60 dB background noise. Testing chambers were in a separate room and differed from the training chambers in illumination (infrared house lamp), texture (solid, textured floor), and odor (5% [acetic acid](#) solution). In addition, transport between the colony room and the testing room was altered from that used for training: rats were covered during transport and an alternate route was used. In trace conditioning, subjects exhibit conditional responses to CS-offset in addition to the CS itself ([Buhusi and Meck, 2000](#)). Thus, the CS retention test was designed to measure rats' fear to both the CS and CS-offset ([Gilmartin et al., 2012](#)). This test consisted of a 2-min pre-CS baseline period followed by a single 10-s CS (same duration as was used in the conditioning procedure). This CS was followed by a 2-min stimulus-free period (SFP) to assess freezing in response to CS-offset. After this 2-min period, the CS was presented for 5 min to assess freezing in response to the CS. Contextual fear memory was assessed by measuring conditional freezing during a 10-min re-exposure to the original training chamber.

2.5. Delayed alternation

The delayed alternation paradigm is a [spatial working memory](#) task, which depends on intact prefrontal cortical function ([Wang and Cai, 2006](#)). This task typically uses delay periods in the range of 5–60 s ([Wang and Cai, 2006](#), [Zahrt et al., 1997](#)), which overlaps with the trace interval duration used in Experiment 1. Therefore, in Experiment 2 we used delayed alternation with a 20-s delay to test the contribution of PAC1R to spatial working memory. The training protocol used here is adapted from previous reports ([Berridge et al., 2006](#), [Zahrt et al., 1997](#)). A subset of females (n = 11) from cohort 2 in Experiment 1 were food-restricted to maintain 88–93% of their free-feeding body weight; rats were fed 9–10 g of standard lab chow (Teklad rodent diet #8604; Envigo, Indianapolis, IN) once daily. Once testing began, food was given 1 h after testing. Rats were trained in a [T-maze](#) with opaque black floors and walls (overall dimensions: 81 cm wide x 89 cm long; runway: 19 cm wide x 30 cm tall). Prior to training, rats were introduced to food reward (Chocolate Cheerios® General Mills, Minneapolis, MN) and habituated to the T-maze for 2 days. During these sessions, rats were allowed to freely explore the maze for 10 min with the Cheerios® dispersed throughout the maze. Following [habituation](#), rats were trained in an alternation task with a minimal intertrial delay (<10 s), 8–12 trials per day. On the first trial, both arms were baited at the end of the arm. On all subsequent trials, rats were rewarded with ½ pieces of Cheerios® for choosing the arm not chosen on the previous trial. After approximately one week of training, the intertrial delay was increased to 20 s and rats were trained with 12 trials per day. During the delay, rats were placed in a separate holding cage and returned to the start box 5 seconds before the end of the delay. Training continued until rats reached criterion performance, which was defined as >60–70% correct choices for two consecutive days with <10% difference between days ([Berridge et al., 2006](#)). Once all rats reached criterion (<7 days), bilateral injections of 2 mM PACAP₆₋₃₈ (0.5 µL/side) were administered to the PL 1 h prior to testing. An injection of saline vehicle (0.5 µL/side; 30 min pre-test) was given four days later, followed by a second injection of 2 mM PACAP₆₋₃₈ the next day. Lastly, after 1–2 drug-free testing days, rats received one final drug session, in which the GABA_A [agonist](#) muscimol (1 µg/µL; 0.5 µL/side; MP Biomedicals, Santa

Ana, CA) was administered 30 min prior to testing. This served as a positive control for the prefrontal-dependence of our protocol.

2.6. Quantitative RT-PCR

Twenty female rats and 20 male rats were used in Experiment 4 to quantify the relative expression of PAC1R (*ADCYAP1R1*) and PACAP (*ADCYAP1*) mRNA in the PL cortex. All rats were handled for 14 days, followed by 3 consecutive days of transport to the training rooms. Half of the rats (n = 10/sex) received a single session of trace [fear conditioning](#) as described above. Ninety minutes after the end of training, the rats were rapidly decapitated, and 1 mm bilateral punches of the PL cortex were collected and flash frozen in liquid nitrogen. The remaining rats (n = 10/sex) served as untrained home cage controls and were sacrificed for tissue collection time-matched to the fear-conditioned rats. Total RNA was extracted from the tissue samples using the MasterPure Complete DNA & RNA Purification Kit (Epicentre; Madison, WI). Assessment of RNA purity and quantity was performed on a NanoDrop One [spectrophotometer](#) (Thermo Scientific, Waltham, MA). Single stranded cDNA synthesis was conducted with 1 µg of total RNA using the Promega [Reverse Transcription](#) System (Madison, WI). Real-time [quantitative PCR](#) was performed using the QuantStudio 3 real-time PCR system (Applied Biosystems; Carlsbad, CA) with PerfeCTa [SYBR Green](#) FastMix containing ROX (Quanta Biosciences; Gathersberg, MD). Relative quantification of target gene expression was normalized to the reference gene [glyceraldehyde-3-phosphate dehydrogenase](#) (GAPDH) using the $\Delta\Delta C_t$ method ([Schmittgen and Livak, 2008](#)). The primers were designed to be intron-spanning and lack secondary structures using the online [primer design](#) tools Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and Oligo Calc (<http://biotools.nubic.northwestern.edu/OligoCalc.html>). The primers were as follows: PAC1R forward- 5' TGC CTG TGG CTA TTG CTA TG 3'; PAC1R reverse- 5' TTT AGT CCC ATC AGG TCG TTG 3'; PACAP forward-5' AAC CCG CTG CAA GAC TTC TA 3'; PACAP reverse- 5' CTT TGC GGT AGG CTT CGT TA 3'; [GAPDH](#) forward- 5' CTC CCA TTC TTC CAC CTT TGA 3'; GAPDH reverse- 5' ATG TAG GCC ATG AGG TCC AC 3'. A single, unique product from amplification was confirmed by melt curve analysis, which along with in silico BLAST analysis confirmed target specificity. Amplification efficiency of all genes was approximately 95%.

2.7. Estrous cycle tracking

The stage of the estrous cycle was determined through daily vaginal lavages of the 20 females in Experiment 4. The males in this experiment were handled for approximately the same amount of time each day. At approximately the same time each day (10:00 a.m. \pm 1 h), the females were lightly restrained and 120 µL of deionized water was used to flush the vaginal canal and obtain loose vaginal cells. Samples were expelled onto slides, allowed to dry, and stained using a Papanicolaou stain ([Hubscher et al., 2005](#)). Each stage of the estrous cycle was determined with a light microscope using a standard protocol ([Hubscher et al., 2005](#)). Briefly, the following characteristics were used: proestrus is marked by nucleated basophilic cells, estrus is marked by enucleated cornified [squamous cells](#), metestrus is marked by clumped, enucleated and nucleated cells and [leukocytes](#), and diestrus is marked by mucus and waning leukocytes. Transitions between stages were identified using detailed analysis of cellular morphology and composition ([Montes and Luque, 1988](#), [Paccola et al., 2013](#)). We

used these transitions to more accurately predict circulating [estradiol](#) (E2) levels and the dynamic interaction of E2 and PAC1R in cycling females ([Butcher et al., 1974](#)). Females were ordered in terms of their cycle stage at tissue collection and classified as High-E2 or Low-E2. High-E2 included females in the transition from diestrus to proestrus through the transition from proestrus to early estrus ([Montes and Luque, 1988](#), [Paccola et al., 2013](#)). Low-E2 included females in late estrus through diestrus. Cycle stage was assessed for at least five days prior to training to ensure rats were cycling regularly.

2.8. Analyses

Freezing was defined as the cessation of all movement except that needed for respiration and was used as the measure of conditional fear during all training and testing sessions ([Fanselow and Bolles, 1979](#)). Freezing was scored automatically using FreezeScan 2.0 (CleverSys; Reston, VA). Scoring parameters were chosen to produce scores that match hand-scored results, conducted by a trained observer ($r = 0.99$). All statistical analyses were performed with Statistica version 13 (Statsoft, Tulsa, OK). Group differences in freezing were analyzed using one-way Analysis of Variance (ANOVA)s for context retention and mixed model ANOVAs with repeated measures for acquisition and CS retention. Mixed model ANOVAs varied a between-subjects factor of Group (PACAP₆₋₃₈ vs Saline) across a within-subjects factor of Period (for acquisition: *Baseline, Trials 1 through 6*, for CS retention: *Baseline and CS*). We typically analyze only the first 2 min of the CS when conditional responding is strongest and to temporally match baseline ([Gilmartin et al., 2012](#), [Gilmartin et al., 2013b](#)) (Experiments 1 and 3), but in this study we also analyzed freezing across all 5 min to examine potential sex differences in the expression of learned fear (see Results and [Fig. 4](#)). For delayed alternation, the number of correct choices on injection days was converted to a change score based on the 3-day baseline period before any drugs were administered ([Berridge et al., 2006](#)). Change scores from both PACAP₆₋₃₈ injection days were averaged together. These scores were analyzed using repeated measures ANOVA. Fisher's least significant difference tests were used to make pairwise comparisons when ANOVAs revealed an appropriate significant main effect or interaction. Effect sizes were calculated using Cohen's d . An α level of 0.05 was required for significance in all analyses.

PAC1R and PACAP mRNA levels were referenced to GAPDH and expressed as a fold-change relative to a control condition prior to analysis. One male sample from the home cage control group was an outlier (PAC1R mRNA > 2 standard deviations from the mean of all males tested) and was eliminated from the analyses. In addition, samples which failed to produce reliable plate readings were excluded from analysis (PAC1R: 2 females, 0 males; PACAP: 2 females, 2 males).

2.9. Histology

At the end of Experiments 1-3, rats were deeply anesthetized with concentrated isoflurane in a narcosis chamber, transcardially perfused with 0.1M [phosphate-buffered saline](#) followed by 10% buffered [formalin](#), and the brains were placed in fixative overnight. The following day, brains were transferred to a 30% sucrose solution (in 0.1M phosphate buffer) for

cryoprotection. Brains were then frozen, sectioned coronally, mounted on glass slides, and stained with [cresyl violet](#). Cannula placement was assessed with light microscopy.

3. Results

3.1. Experiment 1: prefrontal PAC1R and fear learning in females

All forty-eight female rats implanted with bilateral cannulae had successful placements in the PL. Placements for the Saline and PACAP₆₋₃₈ 2 mM dose groups are shown in [Fig. 1](#)b. One rat was excluded from all analyses due to having some tissue damage at the injection site (PACAP₆₋₃₈ 1 mM group).

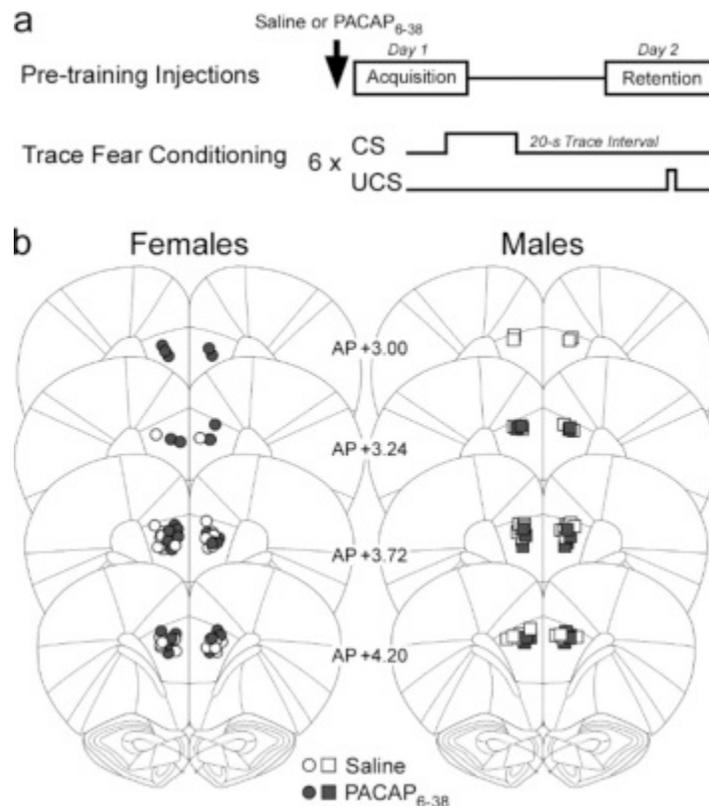


Fig. 1. Experimental timeline and prelimbic cannula placements. (a) PL injections of Saline or PACAP₆₋₃₈ were given 1 h prior to training, and memory retention was assessed the following day in the absence of drug. Trace [fear conditioning](#) consisted of six CS-UCS pairings, in which the offset of the CS and onset of the UCS were separated by 20 s. (b) Cannula placements for females and males treated with Saline (open symbols) or 2 mM PACAP₆₋₃₈ (closed symbols). Placements for 1 mM (females) and 3 mM (females and males) PACAP₆₋₃₈ rats were within this range. Coronal diagrams were adapted from [Paxinos and Watson \(2007\)](#) with permission from Elsevier.

Pre-training injection of the PAC1R [antagonist](#) PACAP₆₋₃₈ impaired the formation of cued, but not contextual fear memory ([Fig. 2](#)). The administration of PACAP₆₋₃₈ did not affect the within-session acquisition or expression of freezing during the training session ([Fig. 2a](#)). Each comparison revealed a significant main effect of Trial ($F_s > 45$; $p_s < 0.00001$), but no main effect of Drug ($p_s > 0.08$) or Drug x Trial interaction ($p_s > 0.18$). During the CS presentation in a

novel chamber the day after training, rats in the 2 mM PACAP₆₋₃₈ group exhibited less freezing compared with Saline-injected controls (Fig. 2b). This observation was supported by a significant Drug x Period interaction ($F_{(1, 29)} = 5.632$, $p = .024$). Planned comparisons confirmed that PACAP₆₋₃₈ rats exhibited significantly less freezing during the CS than the control rats ($p = .007$; Cohen's d : 0.84). Rats which had been injected with 3 mM PACAP₆₋₃₈ showed a trend for a Drug main effect during the CS test ($F_{(1, 22)} = 3.421$, $p = .078$; Cohen's d : 0.82). In contrast, rats injected with 1 mM PACAP₆₋₃₈ did not show an impairment in conditional fear memory compared with vehicle control rats: there was neither a main effect of Drug nor a Drug x Period interaction (p s > 0.28). Contextual fear memory was unaffected by pre-training administration of PACAP₆₋₃₈ (Fig. 2c; all p s > 0.13). Freezing to CS-offset measured in the SFP of the CS test (data not shown) was largely unaffected as well. While rats injected with the low dose of PACAP₆₋₃₈ did exhibit greater freezing at CS-offset compared with controls ($91.6\% \pm 3.86\%$ s.e.m. (1 mM PACAP₆₋₃₈) vs. $65.06\% \pm 7.92\%$ s.e.m. (Saline); main effect of Drug ($F_{(1, 20)} = 8.014$, $p = .01$), neither of the two higher doses affected CS-offset freezing. Together, these results suggest that pre-training PACAP₆₋₃₈ in the [prefrontal cortex](#) selectively impairs the formation of memory for the CS-UCS association while leaving the acquisition of contextual fear intact.

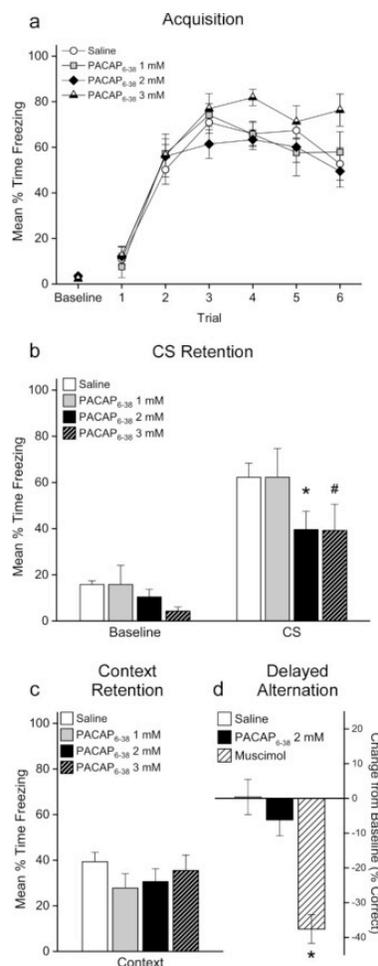


Fig. 2. PACAP₆₋₃₈ dose-dependently impaired the formation of cued, but not contextual, fear memory in females. (a) All subjects exhibited similar levels of freezing across trials during the acquisition session.

Each point in (a) represents the average (\pm s.e.m.) freezing during the 10-s CS + 20-s trace interval on each trial. (b) When tested for retention of fear to the CS the following day, rats which had received either of the two higher doses of PACAP₆₋₃₈ before training exhibited reduced freezing to the CS. Bars represent the average (\pm s.e.m.) freezing for each group. * $p < .05$, # $p = .078$ compared with Saline. (c) All rats showed intact memory for the context-shock association, which was tested separately from the CS memory one day after training. Bars represent the average (\pm s.e.m.) freezing for each group. (d) [Spatial working memory](#) was unaffected by PACAP₆₋₃₈ injection, but was impaired by [muscimol](#) injection. Bars show the average (\pm s.e.m.) change in performance from baseline for each injection. * $p < .05$ compared with Saline or PACAP₆₋₃₈.

3.2. Experiment 2: prefrontal PAC1R and spatial working memory in females

Activity in PL is necessary to bridge the stimulus gap in trace conditioning ([Gilmartin and Helmstetter, 2010](#), [Gilmartin et al., 2013b](#)), a role that may engage [working-memory](#) like mechanisms. To determine if PACAP₆₋₃₈ contributes to prefrontal-dependent working memory in addition to associative fear learning, PACAP₆₋₃₈ was administered to rats during a [spatial-working memory](#) paradigm: delayed alternation. [Fig. 2d](#) shows the change in performance (percentage of correct trials) on injection days compared with the 3-day baseline period before any drugs were administered. [Muscimol](#), but not PACAP₆₋₃₈, produced a significant impairment in performance compared with Saline as confirmed by significant post-hoc tests on the main effect of Injection ($F_{(2, 20)} = 28.513$; $p = .000001$; Cohen's d : 2.48, 0.41, respectively). These results suggest that PACAP signaling in the prefrontal cortex does not contribute to working memory per se, but does contribute to the acquisition of fear to threat-predictive cues.

3.3. Experiment 3: prefrontal PAC1R and fear learning in males

The results of Experiment 1 demonstrate that PAC1R signaling in the PL of female rats contributes to the strength of a cued fear memory. Experiment 3 tested whether the same effect is observed in male rats. All 40 male rats implanted with bilateral cannulae had successful placement in the PL ([Fig. 1b](#)). One rat was excluded from all analyses due to tissue damage around the cannula site (Saline group).

Drug administration in males had no effect on freezing during the acquisition session or on the formation of the contextual fear memory. All groups acquired fear similarly during acquisition, which was confirmed by a lack of a Drug x Trial interaction ($ps > 0.18$) or Drug main effect ($ps > 0.37$; [Fig. 3a](#)). Additionally, PACAP₆₋₃₈ treatment did not impair the formation of the contextual fear memory, as there were no differences between groups during the contextual fear retention test the following day ([Fig. 3c](#); $ps > 0.30$). In contrast to the results in female rats, pre-training PACAP₆₋₃₈ had no reliable effect on the formation of the cued fear memory ([Fig. 3b](#)). This was supported by the lack of a Drug x Period interaction ($ps > 0.10$) or Drug main effect (2 mM: $p = .12$; Cohen's d : 0.54; 3 mM: $p = .44$; Cohen's d : 0.45). These results suggest that prefrontal PAC1R signaling plays a role in fear learning in females, but not males.

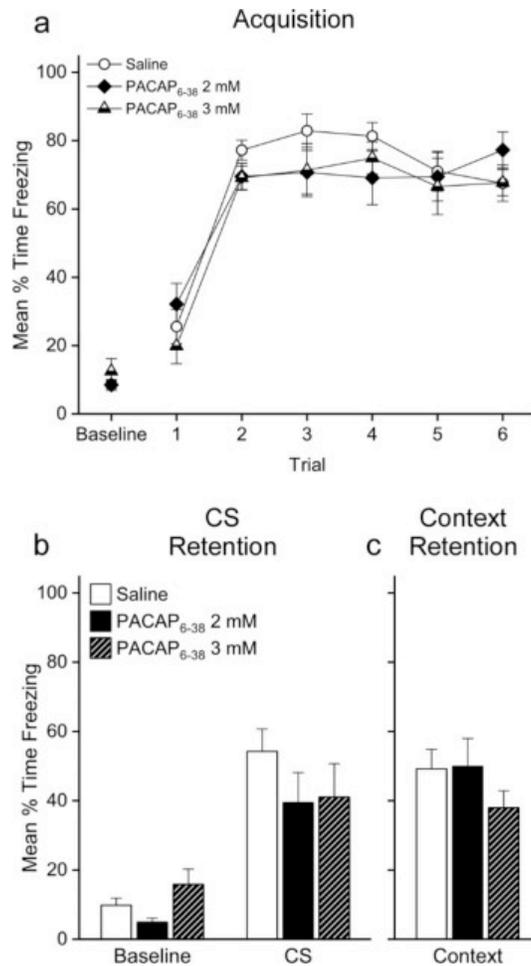


Fig. 3. PACAP₆₋₃₈ does not affect learning in males. (a) Subjects in both groups exhibited similar within-session acquisition of freezing across trials. Each point in (a) represents the average (\pm s.e.m.) freezing during the 10-s CS + 20-s trace interval on each trial. (b) When tested for retention of fear to the CS (left panel) and context (right panel) the following day, no differences in freezing were observed. Bars represent the average (\pm s.e.m.) freezing for each group.

3.4. Sex differences in cued fear memory

The results of Experiments 1 and 2 raised the question of how females and males differed in trace conditioning and in their response to PACAP₆₋₃₈. We extended our analysis of cue-elicited freezing in males and females across the entire 5-min CS presentation (Fig. 4) and directly compared them in the same analysis. For each sex, rats which had received the 2 or 3 mM dose of PACAP₆₋₃₈ were combined into one drug group and compared with Saline control rats (females: Saline n = 15; Drug n = 25; males: Saline n = 18; Drug n = 20). Female control rats showed sustained freezing that gradually reduced across the 5 min of the CS, with drug-treated rats showing consistently less freezing during this time. Male control rats showed a more rapid reduction in freezing within the first 3 min, with drug-treated rats showing similar levels of fear as controls. This observation was supported by a mixed model ANOVA with both Sex and Drug as between factors and Minute (Baseline and 5 CS minutes) as the within factor, which revealed a Sex x Drug x Minute interaction ($F_{(5, 370)} = 2.575$, $p < .026$). Post-hoc tests showed that control females maintained freezing levels significantly above baseline for all

5 min ($p < 0.001$) while control males showed higher-than-baseline levels for the first 2 min only ($p < 0.001$). Female rats injected with PACAP₆₋₃₈ showed a significant reduction in freezing compared with control females during minutes 2, 3, and 5 of the CS ($p < 0.024$). In contrast, male rats injected with PACAP₆₋₃₈ did not differ from control males at any time during the CS ($p > .09$ min 1, $p > 0.12$ min 2-5). Indeed, the larger drug effect exhibited by the females was confirmed by comparing the relative effect sizes of conditional freezing to the CS collapsed across all 5 min (Cohen's d : 0.83 for the females vs. 0.05 for the males).

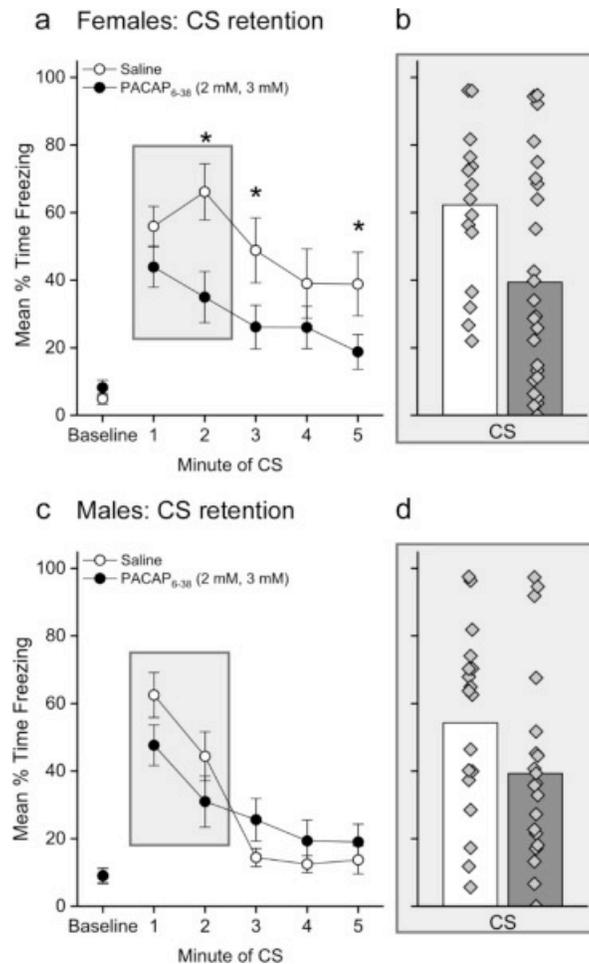


Fig. 4. PACAP₆₋₃₈ may reduce the persistence of learned fear in females. (a) Females maintained fear expression across all 5 min of the CS test. Each point in (a) represents the average (\pm s.e.m.) freezing during each minute of the 5-min CS presentation. (b) Individual freezing scores (grey diamonds) during the first 2 min of the CS are shown for female rats in the Saline and PACAP₆₋₃₈ groups (2 and 3 mM doses combined). Most of the PACAP₆₋₃₈-treated rats showed a profound reduction in freezing, but a subset showed intact fear – an observation that may suggest the presence of individual differences in sensitivity to PAC1R disruption within the population of cycling females. (c) Males exhibited a reduction in fear within the first 3 min of the CS. (d) Individual freezing scores during the first 2 min of the CS show that drug-treated male rats also exhibit variability in CS responding, but similar to that observed in control males. * $p < .05$ compared with Saline.

We also observed individual differences in the rats' responses to PACAP₆₋₃₈. Fig. 4 shows the distribution of individual freezing scores (panels b and d), averaged across the first 2 min of

peak fear expression during the CS presentation, for each group of females and males shown in [Fig. 4a](#) and c. Variability in freezing during the CS was observed in both sexes, but the clear bimodal distribution in drug-treated females suggests that an additional factor(s) may mediate sensitivity or susceptibility to the fear-reducing effects of blocking endogenous PACAP signaling in the prefrontal cortex. As these were cycling females, one possible factor may be the relative levels of [ovarian hormones](#) prior to training (discussed below). Clearly, this variability warrants further scrutiny in future studies. All together our findings suggest that PACAP has a sex-specific role in cued fear acquisition.

3.5. Sex differences in PAC1R mRNA

The results of Experiment 3 suggest that females are more sensitive to the learning-impairing effects of PACAP₆₋₃₈ in the PL compared with males. Here we tested whether female and male rats differ in the expression of PAC1R before or after [fear conditioning](#). [RT-qPCR](#) was used to quantify the relative expression of PAC1R and PACAP mRNA following training and in untrained controls. The estrous cycle of the females was tracked and female rats were categorized as High-E2 (n = 9) or Low-E2 (n = 9) based on the predicted [estradiol](#) (E2) levels of their cycle phase (see Methods). We found no training effect on the expression of PAC1R or PACAP mRNA within either sex ($p > 0.55$). We therefore combined training and home cage groups to look for sex differences in PL mRNA levels with males as the reference group. Overall, PAC1R transcripts were significantly greater in females than in males ($F_{(1, 33)} = 20.412$, $p < .0001$; Cohen's d: 1.58). High-E2 females exhibited the greatest increase in PAC1R expression compared with males ([Fig. 5](#)). A one-way ANOVA revealed a significant effect of Sex ($F_{(2, 32)} = 19.830$, $p < .00001$; Cohen's d: 2.23). Post-hoc tests showed that both Low-E2 and High-E2 females had greater PAC1R expression compared with males ($p < .041$; Cohen's d: 1.16 and $p < .00001$, Cohen's d: 2.61, respectively) and PAC1R levels in High-E2 females were significantly greater than Low-E2 females ($p < .002$, Cohen's d: 1.36). Since there was no difference within either sex between trained and untrained rats, this suggests that PAC1R expression in the prelimbic cortex is not dynamically regulated by the fear conditioning protocol, but may be dynamically regulated by estradiol.

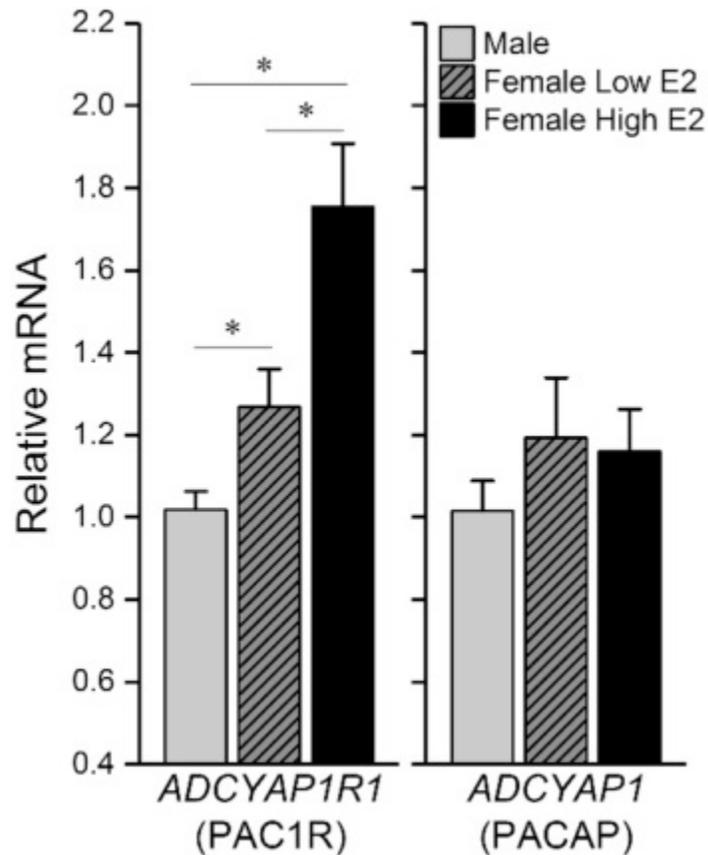


Fig. 5. Females express greater levels of mRNA for PAC1R compared with males. Each bar shows the average (\pm s.e.m.) mRNA levels expressed as a fold-change relative to male samples for PAC1R (left panel) or PACAP (right panel). The [prefrontal cortex](#) was extracted from rats 90 min after trace [fear conditioning](#) or from time-matched home cage controls. Because no training-related differences in expression were observed, the data were collapsed within each sex. Females were classified into high and low [estradiol](#) groups based on their estrous cycle stage at the time of tissue collection. * $p < .05$.

There was no effect of training on PACAP mRNA within each sex ($p_s > 0.56$), and as we did for our PAC1R analysis, we combined training and home cage groups to examine sex differences in PL PACAP mRNA levels using the males as the reference group. There were no differences between the sexes in PACAP transcripts overall ($F_{(1, 32)} = 3.053$, $p = .09$, Cohen's d : 0.60) and no differences between High-E2 and Low-E2 groups ($F_{(2, 31)} = 1.510$, $p = .237$, Cohen's d : 0.62). However, when considering the entire estrous cycle, the distribution of the data strongly suggested that PACAP mRNA levels were elevated during the estrus stage only (mean (\pm s.e.m.) relative expression: Males $n = 19$, 1.015 (± 0.043); Female Estrus $n = 7$, 1.385 (± 0.157); Female Non-Estrus $n = 9$, 1.016 (± 0.051)). A one-way ANOVA revealed a significant effect of cycle stage when comparing males, females in estrus, and females in any other stage ($F_{(2, 31)} = 6.621$, $p = .004$, Cohen's d : 1.31). Post-hoc tests showed that the females in the estrus stage had higher levels of PACAP mRNA compared with males and with females in the other stages of the cycle ($p_s < 0.005$, Cohen's d s: > 1.25). Together, this experiment provides support for estradiol-mediated PAC1R expression to regulate sensitivity to PACAP signaling in an area important for maintaining attention to fear cues.

4. Discussion

Administration of the PAC1R [antagonist](#) PACAP₆₋₃₈ directly into the PL dose-dependently impaired the formation of associative fear memory. This effect was specific to females and to cued fear learning. Females had greater expression of mRNA for the PAC1R in the PL compared with males, regardless of training, and expression levels varied with the estrous cycle. These results suggest that PACAP signaling in the [prefrontal cortex](#) is a means by which fear to threat-predictive cues is strengthened in females. This finding provides a potential neurobiological substrate for the link between [estrogen](#), a [genetic polymorphism](#) in the PAC1R gene, and symptom severity in women with [PTSD](#) ([Mercer et al., 2016](#), [Ressler et al., 2011](#)).

Pre-training infusion of the PAC1R antagonist into the PL impaired cued fear memory formation in females, but not males. Although males did show a slight reduction in freezing early in the CS test, the magnitude of the effect was much greater, more statistically reliable, and more persistent in females. Females also showed greater expression of mRNA for the PAC1 receptor. These results suggest that the prefrontal cortex is a sexually dimorphic site in trace [fear conditioning](#): females are more sensitive to the effects of PACAP on emotional memory formation in this region. The implication of these observations is that the prefrontal cortex may be one site of action for endogenous PACAP that underlies the sex-specific link in humans between the PAC1R genetic polymorphism and PTSD. The [single-nucleotide polymorphism](#) (SNP) *rs2267735* in the PAC1R gene in females, but not males, with PTSD strongly correlated with the severity of hyperarousal symptoms ([Ressler et al., 2011](#)). The [SNP](#) is found within an [estrogen-response element](#) in the promoter region for the PAC1R, and Ressler and colleagues recently showed in the [BNST](#) and in HEK293T cells that the expression of PAC1R mRNA is increased by [estradiol](#) ([Mercer et al., 2016](#)). In women with PTSD, blood levels of PAC1R mRNA positively correlate with estrogen levels ([Mercer et al., 2016](#)). Our [quantitative PCR](#) results are consistent with these observations. Females showed greater expression of PAC1R mRNA in the PL compared with males, and within the females, PAC1R levels were the highest following the peak in estradiol concentrations. This pattern was independent of training and suggests that [ovarian hormones](#) may regulate the expression of PAC1R in the prefrontal cortex. Although the estrous cycle of females was not tracked in the [behavioral pharmacology](#) experiment, it is plausible that changes in PAC1R expression across the estrous cycle contributed to the bimodal response to the PAC1R antagonist in females: the majority of the rats showed a strong reduction in cued fear memory, but a subset was unaffected by the treatment. By regulating PAC1R expression, ovarian hormones may dynamically regulate the sensitivity to PACAP signaling in the PL, which may in turn modulate arousal or attention to cues and the strength of fear memory encoding across the estrous cycle. A disruption in this dynamic regulation would be expected to lead to aberrant fear learning. Indeed, the PAC1R SNP in women with PTSD is associated with impaired cued fear discrimination ([Ressler et al., 2011](#)). Given that in women with PTSD, PAC1R levels are lower in subjects with the SNP than in those with the control alleles and that blood levels of PAC1R mRNA are inversely correlated with PTSD symptom severity ([Mercer et al., 2016](#)), a reduction in PAC1R signaling throughout the brain may contribute to enhanced hyperarousal through enhanced stress reactivity and impaired fear learning. Our results suggest that the prefrontal cortex is important for PACAP's role in the latter.

The specificity of our finding to cued fear conditioning compared with contextual fear conditioning is consistent with PACAP acting on prefrontal mechanisms of cue encoding or plasticity. We have previously shown that a population of cells within the PL exhibits sustained firing in response to cues that predict shock, but not to cues that do not predict shock ([Gilmartin and McEchron, 2005](#)). Specifically, the PL is required for the cue-shock association only when the two events are separated in time ([Gilmartin and Helmstetter, 2010](#), [Guimaraes et al., 2011](#)). Using [optogenetic](#) silencing of PL cells selectively during this cue-shock trace interval, we have shown that prefrontal activity is crucial for cued fear learning ([Gilmartin et al., 2013b](#)). This trace-interval activity may reflect a [working-memory](#) or attentional process to support associative learning to fear cues ([Connor and Gould, 2016](#), [Gilmartin et al., 2014](#)). Signaling at PAC1R may contribute to this maintenance signal in females, facilitating the association of the cue and subsequent shock. However, the antagonist PACAP₆₋₃₈ did not affect working memory performance in the spatial delayed-alternation task, which suggests that PAC1R signaling is not critical for cue maintenance per se. Instead, PAC1R signaling may promote the recruitment of cue-related attentional or working memory prefrontal networks following an aversive event. Alternatively, PAC1R signaling may facilitate the consolidation of the cue-shock memory in the prefrontal cortex, similar to PACAP-enhanced consolidation of contextual fear memories in the [hippocampus](#) ([Schmidt et al., 2015](#)).

The cellular mechanism by which PAC1R activation promotes associative fear learning in the prefrontal cortex is unknown, but one possibility is through facilitation of [glutamatergic](#) signaling at the GluN2B-containing [NMDA receptor](#). GluN2B-containing NMDA receptors are enriched in prefrontal [synapses](#) compared with other cortical regions ([Wang et al., 2008](#)) and because the GluN2B subunit confers a longer deactivation window to the NMDA receptor ([Cull-Candy and Leszkiewicz, 2004](#), [Monyer et al., 1994](#)), it is able to promote persistent firing within prefrontal networks ([Wang et al., 2013](#)). Recent work has shown that PACAP enhances the [phosphorylation](#) of, and thus activity of, GluN2B in hippocampal slices ([Yaka et al., 2003](#)) and in the [ventromedial nucleus](#) of the [hypothalamus](#) (VMN) *in vivo* ([Resch et al., 2014](#)). Activation of PKA/adenylate cyclase by the PAC1R is thought to mediate this enhancement. Moreover, [behavioral effects](#) of exogenously administered PACAP can be attenuated by pre-treatment with the [NMDA receptor antagonist](#) APV in the hippocampus ([Schmidt et al., 2015](#)) or VMN ([Resch et al., 2014](#)). Given our previous finding that GluN2B-containing NMDA receptors in the PL are important for trace cued, but not contextual fear learning ([Gilmartin et al., 2013a](#)), it is possible that the selective attenuation of cued fear following the pre-training injection of a PAC1R antagonist in this study is a result of reduced activation of GluN2B during acquisition. It is important to note that the function of GluN2B in trace conditioning is not known. While work in primates suggests that it is necessary for persistent firing in working memory ([Wang et al., 2013](#)), a recent study by Floresco and colleagues suggests that blocking GluN2B activity in the prefrontal cortex does not affect working memory in male rodents ([Auger and Floresco, 2017](#)). We have also found that injection of the GluN2B antagonist Ro25-6981 (2 µg/µL; 0.5 µL) into the PL does not affect delayed alternation in female rats (unpublished observations; n = 8; F > 1, p > .38). In contrast, Ro25-6981 delivered to the PL does impair cued trace, but not contextual, fear conditioning ([Gilmartin et al., 2013a](#)). GluN2B in the PL may be necessary for PL sustained firing in trace conditioning, but it is also possible that GluN2B supports trace conditioning through another mechanism, such as activation of [intracellular signaling](#) needed for network plasticity or [memory consolidation](#) specific to the

cue-shock association ([Gao et al., 2010](#)). It is also possible that the cellular mechanism of PAC1R signaling does not involve phosphorylation of GluN2B, but rather involves the regulation of synaptic glutamate levels. Recent data suggests that PAC1R is expressed on both cortical neurons and [astrocytes](#) and can regulate the expression of glial glutamate transport systems ([Figiel and Engele, 2000](#), [Kong et al., 2016](#)). PAC1R activation may thus affect the [post-synaptic](#) effects of cue-elicited firing through modulation of extracellular glutamate levels. We predict that the behavioral effects of PACAP₆₋₃₈ in this study are mediated by its action at the PAC1R, which we have shown to be present in the PL in both sexes and more abundant in females. We cannot completely rule out a role for the vasoactive intestinal peptide receptor type-2 (VPAC2R), which is also inhibited by PACAP₆₋₃₈ ([Dickinson et al., 1997](#), [Robberecht et al., 1992](#)); however, the adult rodent appears to have little to no prefrontal cortical expression of VPAC2R mRNA ([Lein et al., 2007](#) and Experiment 1104 from the Allen Brain Atlas: <http://mouse.brain-map.org/experiment/show?id=1104>; [Sheward et al., 1995](#), [Usdin et al., 1994](#)). Future studies will determine the source of PACAP released in the PL during fear conditioning. We did not observe increases in PL PACAP mRNA as a result of training, which leads us to hypothesize that PACAP is primarily released by afferents originating in distal regions mediating stress and arousal.

A role for PAC1R signaling in fear conditioning is consistent with the importance of PACAP in adaptive stress responses. PACAP released in the PL during emotional arousal or stress may serve to enhance the salience of or attention to cues during associative learning. Disruption of PAC1R signaling within frontal cortical regions may thus explain, at least in part, the correlation between hyper-reactivity to threat cues in PTSD and the PAC1R genetic polymorphism in women. Hyperactivity in the dorsal [anterior cingulate](#) of human [frontal cortex](#), which shares homology with the rodent PL, is associated with PTSD symptom severity and may even be a risk factor for developing PTSD in some patients ([Admon et al., 2013](#), [Shin et al., 2011](#), [Shin et al., 2009](#), [Shin et al., 2001](#)). PL has several potentially interrelated functions in emotional learning that are relevant to PTSD. Not only is the PL necessary for maintaining network activity to fear cues during trace conditioning, but it mediates the expression of fear to previously learned cues ([Burgos-Robles et al., 2009](#), [Corcoran and Quirk, 2007](#), [Gilmartin and McEchron, 2005](#), [Gilmartin et al., 2013b](#), [Sierra-Mercado et al., 2010](#), [Vidal-Gonzalez et al., 2006](#)). Furthermore, the PL is necessary for down-regulating attention to irrelevant or less predictive cues as revealed by fear blocking studies ([Furlong et al., 2010](#), [Yau and McNally, 2015](#)). Moreover, the prefrontal cortex is a sexually dimorphic area in the effects of acute stress on learning ([Maeng and Shors, 2013](#), [Maeng et al., 2010](#), [Wood and Shors, 1998](#)). Together, these findings from [preclinical and clinical studies](#) suggest that disrupted PACAP signaling in the prefrontal cortex has the potential to contribute to exaggerated fear and hyper-reactivity to threat-predictive cues as well as increased generalization of fear in non-threatening situations.

In conclusion, PAC1R signaling in the prefrontal cortex is an exciting new target of study in the neurobiological basis of PTSD and the sex differences that contribute to the development and maintenance of the disorder. Future work on the interplay of ovarian hormones and PAC1R signaling, as well as the identification of downstream effectors of PAC1R activation will be instrumental in understanding the conditions in which PACAP exerts its sex-specific effects on memory.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgments

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