Supplementary Material

CRF mediates the anxiogenic and anti-rewarding, but not the anorectic effects of PACAP

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Supplementary Material and Methods

Intracranial Surgery and Microinfusion Procedures

The surgical procedure was performed as previously described (Iemolo *et al*, 2012). Rats underwent unilateral implantation of a 24-gauge stainless steel cannula (Plastics One, Roanoke, VA) under stereotaxic control (David Kopf Instruments, Tujunga, CA, USA) into the left or right lateral ventricle using the following coordinates (from bregma, in mm): AP: -1.0, ML: ±1.5, DV: -2.3 from skull, with incisor bar set at -3.3 mm below the interaural line, according to Paxinos and Watson (Paxinos and Watson, 2007). For the ICSS experiment only the intracerebroventricular coordinates had to be adjusted to allow the cannula and the electrode to fit in the same animal (AP: -1.0, ML: -3.2 with 20° vertical tilt, DV: -2.6 from skull, incisor bar set 5.0 mm above the interaural line). Three stainless steel jeweler's screws were fastened to the rat's skull around the cannula, and dental restorative filled resin and acrylic cement were applied forming a pedestal that firmly anchored the cannula. Rats were then single-housed and allowed to recover for at least 7 days. Cannula placement was functionally verified at the end of the experiments as a positive dipsogenic response (>5mL of water intake within 30 min) to intracerebroventricular (i.c.v.) angiotensin II administration (25 ng/5 µL).

For i.c.v. microinfusions the dummy stylet was removed from the guide cannula and was replaced with a 31-gauge stainless steel injector projecting 2.5 mm beyond the tip of the guide cannula, which was connected via PE 20 tubing to a Hamilton microsyringe driven by a microinfusion pump (KD Scientifics, Holliston, MA). Microinjections were performed in a 6 µL volume delivered over 1 min; injectors were left in place for an additional minute to minimize backflow. Pretreatment time was 30 min in all behavioral experiments, for the Corticosterone and the CRF-like immunoreactivity determination. The pretreatment time was 4 hrs in the quantitative real-time PCR experiment.

Elevated Plus-Maze Test

The elevated plus-maze test was performed as previously described (Cottone *et al*, 2009; Sabino *et al*, 2009). The apparatus was made of black Plexiglas and consisted of four arms (50 cm long × 10 cm wide x 50 cm high), two with 40-cm-high dark walls (closed arms), and two with 0.5-cm-high ledges (open arms). Open arms received 1.5–2.0 lux of illumination. White noise (70 dB) was present during habituation and testing. For the test, rats were individually placed onto the center of the maze for a 5-min period. The primary measures were the percent of total arm time [i.e., 100 × open arm/(open arm + closed arm)], a validated index of anxiety-related behavior (Fernandes and File, 1996), and the number of closed arm entries, a specific index of motor activity (Cruz *et al*, 1994).

Intracranial Self-Stimulation (ICSS) procedure

Surgery for electrode implantation: Surgery for electrode implantation was performed as previously described (Iemolo *et al*, 2012). Rats were unilaterally implanted with a 0.125 mm diameter bipolar stainless steel electrode (Plastics One, Roanoke, VA; length 10.5 mm) into the left or right medial forebrain bundle (MFB) at the level of the lateral hypothalamus, using the following coordinates: AP –0.5 mm from the bregma, ML +1.7 mm, DV -8.7 mm from skull with the incisor bar set 5.0 mm above the interaural line. Rats were anesthetized with isoflurane (2-3% in oxygen) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Two stainless steel jeweler's screws were fastened into the rat's skull around the electrode. Dental restorative filled resin (Henry Schein Inc., Melville, NY) and acrylic cement were applied forming a pedestal that firmly anchored the electrode in place.

Apparatus for intracranial self-stimulation: ICSS training and testing took place in clear polycarbonate/aluminum modular operant chambers encased in individual sound-attenuating and ventilated cubicles (Med Associates, St. Albans, VT) (Blasio *et al*, 2012). Each chamber had grids on the floor and a retractable lever on one of the walls. The electrical stimulation

circuit was provided by bipolar leads and gold contact swivel commutators. Constant current square-wave stimulators were used to deliver electrical brain stimulation through the electrode connection. All programming functions were controlled by a computer with a 10-ms resolution.

Intracranial self-stimulation procedure: The ICSS procedure was performed following a previously published procedure (Blasio *et al*, 2012). Rats were trained to lever press on a fixed ratio 1 (FR1) schedule of reinforcement to obtain an electrical stimulation. Each stimulus consisted of a 500-ms train with a pulse width of 0.2 ms and a delay of 0.2 ms between the positive and negative pulses. All rats first were tested at the 50 Hz frequency, and if the current threshold was below 70 μA or above 120 μA and unstable, then frequencies were adjusted for each animal to reach the desired range of current and were kept constant for the entire experimental procedure (Kenny and Markou, 2005).Once stable FR1 operant self-stimulation responding was established, mean reward thresholds were assessed using a rate-independent discrete-trial current intensity procedure, originally designed by C. Kornetsky (Kornetsky *et al*, 1979). The reward threshold is defined as the minimal current intensity able to produce a response that maintains the self-stimulation behavior. A raise in the reward threshold indicates that stimulus intensities that were previously perceived as reinforcing are no longer perceived as rewarding, reflecting a decrease in reward function. Conversely, lowering of the reward threshold reflects increased reward function (Markou and Koob, 1991). At the beginning of each trial, rats received a non-contingent stimulus (S1), after which they had the opportunity, during a 7.5 s limited period, to lever press, which resulted in the delivery of a contingent stimulus (S2) that was identical to the previous S1. A 7.5–22.5 s (average 15 s) period of time elapsed between S2 delivery and the delivery of the next S1. If no response occurred, this time period began at the end of the 7.5-s period allotted for response. These time periods were randomized so that animal could not “predict” the next S1 delivery. A “trial” consisted of five presentations of S1 at a fixed current intensity (in μA). Three or more responses at that intensity were scored as a plus (+) for that trial, while two or fewer responses were scored a minus (−) for that trial. If the

animal scored a (+) for the first trial, the second trial began at an intensity 5 μA lower than the first. The current intensity continued to decrease by the same fixed intensity until the animal scored a (−) for two consecutive trials. When this occurred, the current intensity at the second trial at which a (−) score was obtained was repeated and current intensities then ascended by 5 μA for each trial until the animal scored a (+) for two consecutive trials. Each set of ascending or descending current intensities was defined as a "column", and a total of six alternating descending/ascending columns were performed for each session. The intensity at the midpoint between (+) and (−) was defined as the column threshold. The threshold for each session was calculated as the mean of the last four column thresholds, and, the first and second column thresholds were, therefore, excluded. To discourage the subject from responding during the ITI, any response during this period postponed the onset of the S1 for an additional 22.5 s (a length of time that exceeded or was equal to the original random duration of the ITI). These "punished" responses were recorded as timeout responses, and represented a measure of impulsivity-like disinhibiting response. Excessive lever responses within 2 s after the initial response had no consequences and were recorded as cluster responses. Response latency was defined as the time between the delivery of the S1 and the animal's lever press. The mean response latency for each test session was defined as the mean response latency of all trials during which a positive response occurred. Rats were tested daily.

Quantitative Real-Time PCR (qPCR)

Tissue CRF and CRFR1 mRNA levels were determined as previously described (Cottone *et al*, 2009; Sabino *et al*, 2011). Rats were anesthetized with isoflurane and sacrificed 4 hrs after drug administration. Brains were quickly removed and coronally sliced in a brain matrix; punches containing the central nucleus of the amygdala (CeA), the basolateral amygdala (BlA), the medial amygdala (MeA), and the paraventricular nucleus of the hypothalamus (PVN) were collected on an ice-cold stage. Total RNA was prepared from each

punch using the RNeasy lipid mini kit (Qiagen, Valencia, CA) as recommended for animal tissue. Total RNA was quantified by Nanodrop 1000 (Thermo Scientific, Wilmington, DE) and then reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA), which includes a DNA removal step. For quantitative real-time PCR, Roche Light Cycler 480 Master-plus SYBR Green mix (Roche Applied Science, Indianapolis, IN) was used. Reactions (10 µl) were carried out in a 96-well plate Realplex2 machine (Eppendorf). The primers (0.5 µM final concentration, Sigma, St. Louis, MO), synthesized with a standard desalting purification, were the following: CRF, TGC TCG GCT GTC CCC CAA CT and CTG CAG CAA CAC GCG GAA AAA (95 °C 10 sec; 59.2 °C 5 sec; 72 °C 10 sec); CRFR1, ACC CTG CCC TGC CTT TTT CTA CGG TG and GCA GAC GGT CGG TGG AGT ACG TGA (95 °C 20 sec; 59.2 °C 30 sec; 72 °C 45 sec); Cyclophilin A, TAT CTG CAC TGC CAA GAC TGA GTG and CTT CTT GCT GGT CTT GCC ATT CC (95 °C 20 sec; 58 °C 15 sec; 72 °C 20 sec). Standard curves were constructed using sequenced PCR products. Results were analysed by second derivative methods and expressed in arbitrary units, normalized to Cyp expression levels. Standards and samples were run in duplicate, and all reactions for a given brain region were performed concurrently. Gene-specific amplification was determined by melting curve analysis as one peak at the expected melting temperature and by agarose gel electrophoresis.

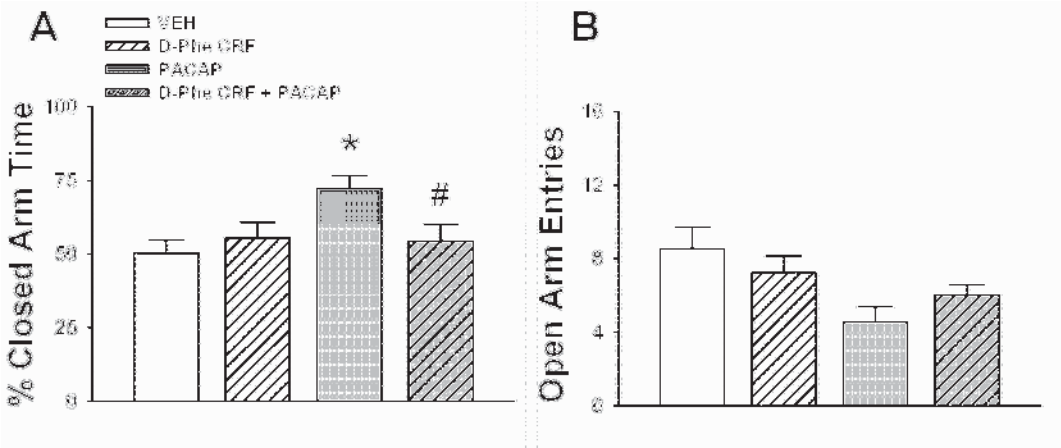
CRF-like immunoreactivity measurement

Tissue CRF levels were determined as previously described (Cottone *et al*, 2009; Zorrilla *et al*, 2001). Punches containing the CeA, BIA, MeA, and PVN were collected as specified in the previous paragraph, and tissue CRF-like immunoreactivity was quantified with a sensitive and specific solid-phase radio-immuno assay adapted from (Zorrilla *et al*, 2001). Following an established procedure for peptide acid extraction, brain regions were ultrasonicated in ice-cold 1 N HCl. Protein content was determined using the Bradford method (Bio-Rad, Hercules, CA). The remaining homogenate was boiled for 10 min and immediately centrifuged (20 min, 7,500

rpm, 4 °C). The supernatant was removed, lyophilized, reconstituted with sodium hydroxide and then diluted to the final volume in gelatin buffer. Immulon-4 96-well plates (Dynatech, Chantilly, VA) were coated overnight with protein A/G (1% *w/v* in bicarbonate buffer). Plates were rinsed with wash buffer to dislodge loose protein A/G and then incubated for 48 h with anti-CRF serum (rC68, 1:200,000 titer; generously provided by Wilye Vale, The Salk Institute, La Jolla, CA). After rinses to dislodge loose antibody, samples or CRF standards (0.03–100 ng/mL) were incubated overnight. The day after, ~10,000 cpm of [^{125}I-Tyr^{0}]r/hCRF (New England Nuclear, Waltham, MA) was added and plates were incubated for an additional 24 h. After plate rinsing, residual radioactivity was counted. Sensitivity of the assay was ~0.3 fmol/well, and inter- and intra-assay coefficients of variation at the ED_{50} dose ranged from 5–10%. Intra- and inter-assay coefficients of variation were <10%. A four-parameter logistic curve fit model was used for interpolation of the standard curves.

Supplementary Figures and Figure Legends

Supplementary Figure 1



Suppl. Fig. 1:

Effects of i.c.v. administration of PACAP (5 μg/rat) and the CRF receptor antagonist D-Phe-CRF(12-41) (10 μg/rat) on the % of the time spent in the closed arms (panel A) and the number of open arm entries (panel B) of an elevated plus maze test. Rats (*n*=39, between-subjects) were tested 30 min after drug administration. Data represent Mean ± SEM. $^{*}p<0.05$, vs. vehicle group; $^{\#}p<0.05$ vs. PACAP group (Newman-Keuls' test).

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