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PACAP Controls Endocrine and Behavioral Stress Responses via Separate Brain Circuits

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ABSTRACT

BACKGROUND: The neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) is a master regulator of central and peripheral stress responses, yet it is not clear how PACAP projections throughout the brain execute endocrine and behavioral stress responses.

METHODS: We used AAV (adeno-associated virus) neuronal tracing, an acute restraint stress (ARS) paradigm, and intersectional genetics, in C57BL/6 mice, to identify PACAP-containing circuits controlling stress-induced behavior and endocrine activation.

RESULTS: PACAP deletion from forebrain excitatory neurons, including a projection directly from medial prefrontal cortex to hypothalamus, impairs c-fos activation and corticotropin-releasing hormone (CRH) messenger RNA elevation in the paraventricular nucleus after 2 hours of restraint, without affecting ARS-induced hypophagia, or c-fos elevation in nonhypothalamic brain. Elimination of PACAP within projections from lateral parabrachial nucleus to extended amygdala, on the other hand, attenuates ARS-induced hypophagia, along with extended amygdala fos induction, without affecting ARS-induced CRH messenger RNA elevation in the paraventricular nucleus. PACAP projections to extended amygdala terminate at protein kinase C delta type (PKCδ) neurons in both the central amygdala and the oval bed nucleus of the stria terminalis. Silencing of PKCδ neurons in the central amygdala, but not in the oval bed nucleus of the stria terminalis, attenuates ARS-induced hypophagia. Experiments were carried out in mice of both sexes with $n \geq 3$ per group.

CONCLUSIONS: A frontocortical descending PACAP projection controls paraventricular nucleus CRH messenger RNA production to maintain hypothalamic-pituitary-adrenal axis activation and regulate the endocrine response to stress. An ascending PACAPergic projection from the external lateral parabrachial nucleus to PKCδ neurons in the central amygdala regulates behavioral responses to stress. Defining two separate limbs of the acute stress response provides broader insight into the specific brain circuitry engaged by the psychogenic stress response.

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The flight-or-fight response mediated by catecholamine release from the adrenal medulla (1,2), the cortisol response to stress, and the concept of the stress response itself (3), were elucidated in the early 20th century. Investigations of how interoceptive and exteroceptive stress cues trigger brain responses, and how brain regulation of behavior is subsequently affected, proceeded from these seminal observations (4). McEwen (5) and others established that cortisol/corticosterone (CORT) acts on the brain to mediate long-term consequences of stress. The catecholaminergic alerting system of the locus coeruleus mediates interoceptive stress effects in the central nervous system (6), in parallel with catecholamine release in the periphery (7). The activation of the hypothalamic-pituitary-adrenal (HPA) axis is initiated at corticotropin-releasing hormone (CRH) neurons of the hypothalamic paraventricular nucleus (PVN) (8,9) and constitutes the brain's endocrine response to stress. Stress signaling initiated by both internal and external cues is processed for salience in corticolimbic brain structures, which project to hypothalamus to modulate HPA axis activation and to other brain regions controlling behavioral responses. The HPA axis has long been considered a final common pathway for both endocrine stress responses, that is, CORT elevation, and behavioral responses (via CORT effects on behavioral brain circuits) (4). The involvement of CRH as both hypothalamic hormone and amygdalar neurotransmitter in registering and generating stress-driven affective states contributed to the hypercortisolemic hypothesis of melancholic depression and identification of the CRH receptor as a neurotherapeutic target in treatment of affective disorders, including depression (10,11). However, these studies also contributed to an emerging realization that HPA axis–independent pathways, engaged for behavioral modulation of the stress response, must also exist.

Neuropeptide neurotransmitters besides CRH have recently been invoked in the neurobiology of the stress response. The neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) is a cotransmitter with acetylcholine in the sympathoadrenal system required for elevation of plasma epinephrine and norepinephrine levels during both systemic and psychogenic stress [(12,13) and references therein]. PACAP was

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subsequently shown, through phenotypic analysis of PACAP-deficient mice, to mediate HPA responses to psychogenic stressors, such as social defeat and restraint, but not systemic stress responses, including cold, inflammation, hypoglycemia, and circadian rhythmicity (12,14–17). These studies culminated in the demonstration that PACAP and its receptor are linked to genetic risk for posttraumatic stress disorder in humans (18).

PACAP mediates both endocrine and behavioral responses to psychogenic stress. Restraint stress (RS), both acute and chronic, triggers CRH messenger RNA (mRNA) elevation, c-fos activation, and increased secretion from CRH-expressing neurons of the PVN, the first step in HPA axis activation leading to CORT elevation. RS also causes decreased eating, resulting in loss of body weight. Both HPA axis activation and hypophagia are attenuated or abolished in PACAP-deficient mice (19,20). Chronic social defeat over a 2-week period results in persistent CORT elevation; depressive-like behavior, including decreased social interaction and increased immobility in the forced swim test; and FosB elevation in the PVN and multiple stress-related central and limbic nuclei in mice. All these effects of chronic social defeat are abolished in PACAP-deficient mice (16), as are stress responses induced by open-field exposure (14).

The delayed effects of PACAP deficiency on CORT elevation secondary to its effects on CRH gene transcription, compared to the immediate effects of PACAP deficiency on hypophagia following acute restraint stress (ARS), highlight the dissociation between PACAP's actions within the endocrine and the behavioral domains of the stress response (20). These findings have set the stage to use intersectional genetics to probe endocrine and behavioral aspects of stress regulation by PACAP to probe for separate endocrine and behavioral stress circuits for PACAP in the brain, similar to the concerted but separate actions of CRH in hypothalamic and limbic stress circuits (21). Importantly, the differential temporal PACAP dependence of stress-induced HPA axis activation and decreased eating indicate that hypophagia after acute restraint is not secondary to CORT elevation during ARS (20) and that the hypophagic (behavioral) effect of ARS must therefore require PACAP actions within circuits other than those controlling the HPA axis.

Here, we used regionally precise abrogation of PACAP expression in mice to test the hypothesis that 2 separate PACAPergic circuits mediate the coordinated, but distinct, behavioral and endocrine responses to acute stress. These data have implications for redefining the stress response as proceeding from activation of multiple parallel input circuits and, potentially, to identify multiple drug targets for treatment of the symptoms of stress-related disorders.

METHODS AND MATERIALS

Detailed methods and materials are provided in the Supplement.

Animals and Drugs

Mice (wild-type, transgenic, or knockout) on C57BL/6 background were housed 2 to 5 per cage and acclimatized to 12-hour light/dark cycle with food and water ad libitum. Animal care was approved by the National Institute of Mental Health Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines. PACAP$^{-/-}$ deficient mice and the floxed PACAP mouse strain PACAP$^{fl/fl}$ were generated as described previously (12,22). Camk2α-cre mice (Cre expression restricted to Camk2α-expressing excitatory neurons in the forebrain) and Sim1-cre mice (Cre expression restricted mainly to the hypothalamus) were obtained from The Jackson Laboratory (stock Nos. 005359 and 006395) and bred with PACAP$^{fl/fl}$ mice to obtain Camk2α-cre::PACAP$^{fl/fl}$ and Sim1-cre::PACAP$^{fl/fl}$ conditional knockout mice. Adcyap1-2A-cre mice (Cre expression restricted to PACAP-expressing neurons of adult) were from The Jackson Laboratory (stock No. 030155). Description of PKCδ-cre (Prkcd-glc-1/CFP,-cre)EH124Gsat) mice can be found at (https://www.mmrrc.org/catalog/sds.php?mmrrc_id=11559) (23). Clozapine *N*-oxide (BMLNS105-0005; Enzo Life Science-BIOMOL) was freshly dissolved in saline (0.9% NaCl) to a concentration of 0.4 mg/mL and intraperitoneally injected 30 minutes before 2-hour restraint at 2.5 mg/kg for hM4Di silencing.

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry was conducted as previously described (24). In situ hybridization used the RNAscope Multiplex Fluorescent V.2 Kit (Cat. No. 323100) and RNAscope 2.5 HD Reagent-RED Kit (Cat. No. 322350; ACD Bio), as directed in the manual. Information about all antibodies and probes used can be found in the Supplement.

Mouse Surgery and Viral Injection

Surgeries were conducted and viral injection was administered according to National Institutes of Health Animal Research Advisory Committee guidelines for survival rodent surgery, as previously described (24,25).

Restraint Stress

Acute and chronic RS, food and weight measurement, tail blood sampling, and plasma corticosterone assays were carried out as previously described (19,20).

Experimental Design and Statistical Analysis

Mice of both sexes were used in all studies. The sample size (*n*) per group and statistical analyses are described in detail in the figure legends and Supplemental Methods and Materials. Data are displayed using histograms and scatter plots to represent mean ± SEM and individual animals in each group.

RESULTS

PACAP Elimination From Forebrain Excitatory Neurons Impairs Endocrine but Not Behavioral Effects of ARS

PACAP mRNA is eliminated from forebrain excitatory neurons in Camk2α-cre::PACAP$^{fl/fl}$ mice as confirmed by RNAscope hybridization for cortical areas, including the medial prefrontal cortex (mPFC), the piriform cortex, and the entorhinal cortex (Figure 1A). PACAP mRNA was also reduced in the hippocampus, amygdala, habenula, ventromedial, and mammillary nuclei of the hypothalamus (Figure S1A). Deletion of PACAP

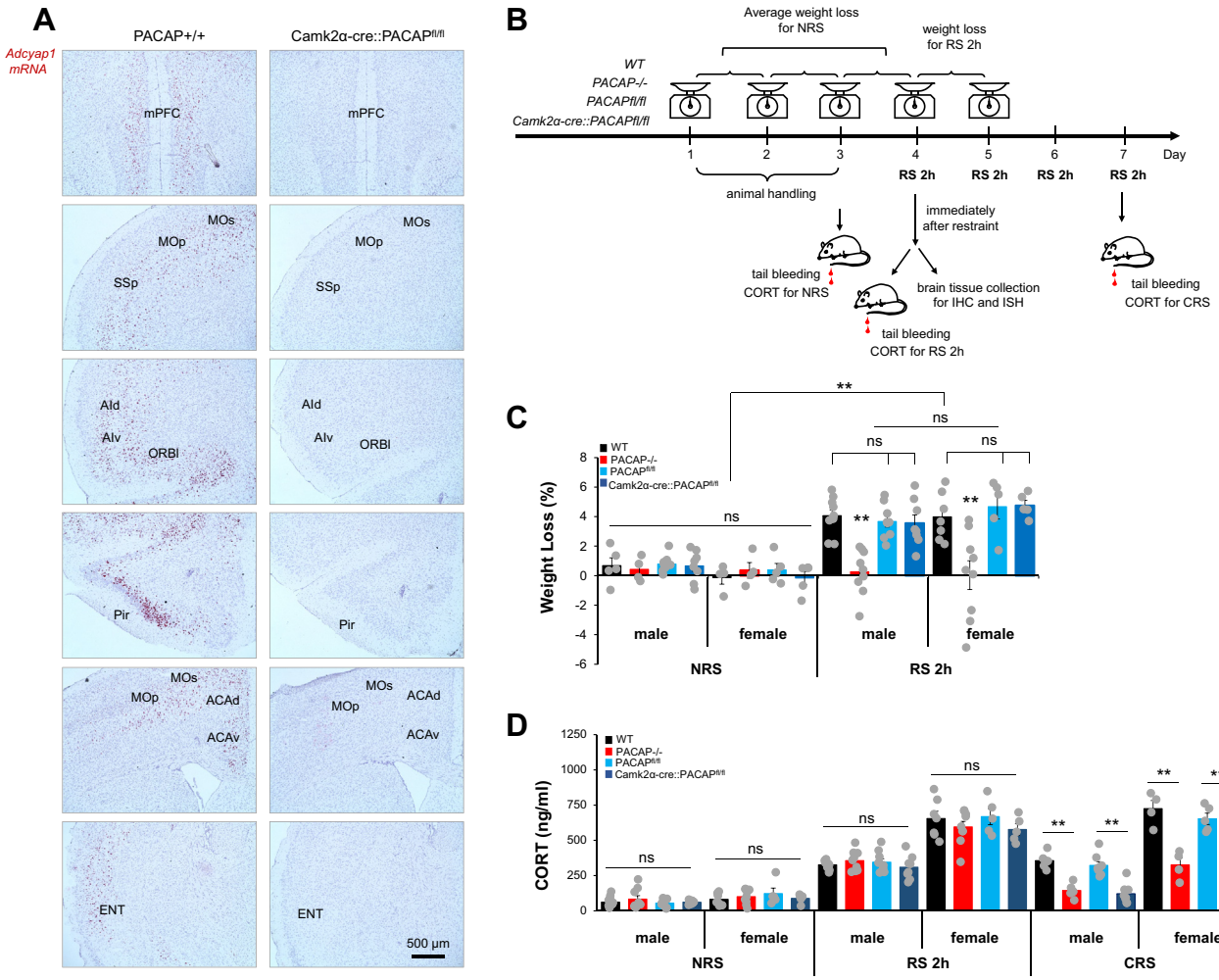


Figure 1. PACAP expression eliminated from forebrain excitatory neurons showed endocrine but not hypophagic effect induced by 2-hour acute restraint. **(A)** PACAP expression eliminated from forebrain (especially limbic cortex areas including the mPFC, cingulate cortex, piriform cortex, entorhinal cortex) excitatory neurons. Camk2α-cre::PACAP$^{fl/fl}$ was generated by crossing Camk2α-cre with PACAP$^{fl/fl}$ mice. PACAP expression throughout the whole adult brain was examined using RNAscope ISH. Adcyap1 mRNA for PACAP is indicated by red signals. **(B)** Scheme of experimental design for acute and chronic RS studies. Mice were acclimatized for 3 days to achieve habituation to handling and blood sampling. Mice were then restrained for 2 hours (RS 2 hours) or chronically for 4 days, 2 hours daily (CRS). Weight loss over a 24-hour period for animals without restraint (NRS) or after 2-hour restraint was collected. Tail blood sampling for CORT assay was performed for animals without RS (for NRS), immediately after 2-hour restraint (for RS 2 hours), or immediately after the fourth restraint (for CRS). Brain samples were collected for IHC or ISH immediately after 2-hour restraint. **(C)** Elimination of PACAP from forebrain excitatory neurons did not affect restraint-induced hypophagia, as seen in PACAP constitutive knockout (PACAP$^{-/-}$) mice. Male and female WT, PACAP$^{-/-}$, PACAP$^{fl/fl}$, or Camk2α-cre::PACAP$^{fl/fl}$ mice were subjected to the RS paradigm shown in **(B)**. Three-way analysis of variance showed no significant sex effect ($F_{1,90} = 0.00651$, $p = .936$) but significant stress effect ($F_{1,90} = 84.356$, $p < .001$), genotype effect ($F_{3,90} = 10.524$, $p < .001$), and stress × genotype interaction ($F_{3,90} = 10.631$, $p < .001$). Post hoc all pairwise multiple comparison with Bonferroni *t* test showed significant stress effect of 2-hour restraint on WT, PACAP$^{fl/fl}$, or Camk2α-cre::PACAP$^{fl/fl}$ (RS 2 hours vs. NRS, $^{**}p < .001$), but not PACAP$^{-/-}$ mice ($p = .694$). And Camk2α-cre::PACAP$^{fl/fl}$ showed a level of weight loss in 24 hours similar to that of WT or PACAP$^{fl/fl}$ after 2-hour restraint ($p = 1$), unlike PACAP$^{-/-}$ mice, which are impaired in hypophagia reflected by weight loss (PACAP$^{-/-}$ vs. WT, or PACAP$^{fl/fl}$, or Camk2α-cre::PACAP$^{fl/fl}$, $^{**}p < .001$). $n = 4$–10 mice for each group. **(D)** Elimination of PACAP from forebrain excitatory neurons attenuated CORT elevation induced by CRS, similar to that observed in PACAP$^{-/-}$ mice. Three-way analysis of variance showed significant sex ($F_{1,144} = 247.822$, $p < .001$), stress ($F_{2,144} = 406.297$, $p < .001$), and genotype ($F_{3,144} = 26.524$, $p < .001$) effects. CORT release immediately after 2-hour restraint is not PACAP dependent: CORT levels in the tail blood samples collected immediately after 2-hour restraint do not differ between WT and PACAP$^{-/-}$ mice (post hoc Bonferroni *t* test, WT vs. PACAP$^{-/-}$, $p = 1$ within RS 2 hours). However, attenuated CORT elevation was observed in PACAP-deficient mice after repetition of RS over a 4-day period (CRS) (WT vs. PACAP$^{-/-}$, $p = .001$ within CRS), indicating that sustained corticotropin-releasing hormone effects on CORT elevation during chronic restraint are PACAP dependent. Attenuated CORT elevation induced by CRS was also observed in mice with PACAP elimination from forebrain excitatory neurons (Camk2α-cre::PACAP$^{fl/fl}$ vs. WT or PACAP$^{fl/fl}$, after CRS, in either males or females, $^{**}p < .001$; Camk2α-cre::PACAP$^{fl/fl}$ vs. PACAP$^{-/-}$ mice, $p = 1$), demonstrating impaired endocrine effect after 2-hour acute RS. $n = 4$–10 animals for each group. ACAd, anterior cingulate area dorsal; ACAv, anterior cingulate area ventral; AId, agranular insular area dorsal; AIv, agranular insular area ventral; CORT, cortisol/corticosterone; CRS, chronic restraint stress; ENT, entorhinal area; IHC, immunohistochemistry; ISH, in situ hybridization; MOp, primary motor area; MOs, secondary motor area; mPFC, medial prefrontal cortex; mRNA, messenger RNA; NRS, no restraint stress; ns, not significant; ORBl, orbital area, lateral part; PACAP, pituitary adenylate cyclase-activating polypeptide; Pir, piriform area; RS, restraint stress; SSp, primary somatosensory area; WT, wild-type.

from these brain areas did not affect neurotransmitter phenotypes in general, based on expression of VGluT1, VGluT2, or VGAT mRNAs, as shown for the mPFC (Figure S1B, C). Camk2α-cre::PACAP$^{fl/fl}$ and corresponding control (PACAP$^{fl/fl}$) mice were subjected to an RS paradigm (Figure 1B; described in detail in the Supplement). Body weight loss and/or food intake was measured during the 24-hour period following 2-hour restraint (RS 2 hours). Tail blood was collected for measurement of plasma CORT levels 1 day before restraint (NRS) or immediately after acute 2-hour restraint (RS 2 hours) or after chronic restraint for 2 hours daily for 4 days (CRS).

Anxiety-associated behavior reliably emerges after chronic, but not acute, RS (16,26–28). However, hypophagia, measured either as decreased food intake or as body weight loss, is a stable behavioral acute stress response in rodents (19,20) (Figure S2). Weight loss caused by ARS is eliminated in constitutive PACAP knockout mice, but preserved after deletion of PACAP from forebrain excitatory neurons (Camk2α-cre::PACAP$^{fl/fl}$) (Figure 1C). In contrast, Camk2α-cre::PACAP$^{fl/fl}$ mice exhibited an impaired endocrine response to stress, as reflected in attenuated CORT elevation after CRS in both male and female mice (Figure 1D).

We compared the stress phenotype of Camk2α-cre::PACAP$^{fl/fl}$ with Sim1-cre::PACAP$^{fl/fl}$ mice, in which PACAP expression is deleted in hypothalamic neurons, to assess the role(s) of hypothalamic and extrahypothalamic PACAP on activation of PVN^{CRH} neurons, the first step in the HPA stress response. PACAP expression arising from intrinsic hypothalamic sources including the preoptic area, PVN and peri-PVN, anterior hypothalamic nucleus, and ventromedial, mammillary, and other hypothalamic nuclei putatively involved in metabolic homeostasis is eliminated in Sim1-cre::PACAP$^{fl/fl}$ mice (Figure 2A), while PACAP expression in cortical areas, notably the mPFC, is largely unaffected (Figure 2B). Induction of c-fos and CRH mRNA was compared in the PVN of hypothalamic (Sim1-cre::PACAP$^{fl/fl}$), constitutive (PACAP$^{-/-}$), and forebrain (Camk2α-cre::PACAP$^{fl/fl}$) PACAP knockout mice. Both c-fos induction and CRH mRNA upregulation in the PVN during ARS were eliminated in PACAP$^{-/-}$ and Camk2α-cre::PACAP$^{fl/fl}$ mice but unaffected in Sim1-cre::PACAP$^{fl/fl}$ mice (Figure 2C). Thus, PACAP dependence for endocrine effects of RS via PVN^{CRH} neurons arises from ablation of PACAP from extrahypothalamic, rather than intrinsic hypothalamic, neurons.

To establish whether hypothalamic PACAP participates in any aspect of HPA axis activation following stress, we measured CORT elevation after ARS and repeated RS in Sim1-Cre::PACAP$^{fl/fl}$ mice. Despite unimpaired c-FOS and CRH mRNA activation in Sim1-Cre::PACAP$^{fl/fl}$ mice, CORT elevation after RS was reduced (Figure S3A). Therefore, we examined the effect of PACAP knockout on expression of arginine vasopressin (AVP) mRNA in the PVN magnocellular neurons (Figure S3B), which also play a role in adrenocorticotropic hormone secretion during stress (29). AVP mRNA was significantly decreased in PVN in both PACAP$^{-/-}$ and Sim1-Cre::PACAP$^{fl/fl}$ but not in Camk2α-cre::PACAP$^{fl/fl}$ mice (Figure S3C). The small population of non-CRH neurons in the PVN that are fos-positive after restraint may represent AVP neurons (Figure S3D). Our data confirm that activation of the HPA axis after ARS requires sustained release of both AVP and CRH and that maintenance of mRNA levels encoding both AVP and CRH sufficient to support this sustained release depends on PACAP release from hypothalamic and forebrain PACAPergic neurons, respectively.

PACAPergic Projections From the mPFC to the PVN Control CRH-Dependent Endocrine Responses to RS

To identify the source of extrahypothalamic PACAP projections to the PVN, we performed retrograde tracing studies after injection of AAV2-hSyn-DIO-EGFP (retrograde) into Adcyap1-2A-cre mice (Figure 3A). Most PACAP-positive neurons that project to the PVN arise within the mPFC (Figure 3A; Figure S4A). This was confirmed by anterograde tracing of PACAP projections from the mPFC to the hypothalamus after injecting AAV1-hSyn-Flex-tdTomato-T2A-synaptophysin-EGFP into the mPFC of Adcyap1-2A-cre mice (Figure 3B; Figure S4B): injection site is indicated by td-Tomato and PACAP terminals are visualized by synaptophysin-EGFP. The tracing result showed that PACAPergic projections from the mPFC to the PVN terminate within a peri-PVN zone (Figure 3B; Figure S4B).

We next asked whether elimination of PACAP expression in mPFC neurons could influence the activation of PVN CRH neurons induced by acute restraint. To eliminate PACAP specifically from neurons with cell bodies in the mPFC, AAV-cre virus was injected bilaterally into the mPFC of PACAP$^{fl/fl}$ or wild-type (WT) control mice (Figure 3C). Four to six weeks later, mice were subjected to 2-hour RS, and c-fos and CRH mRNA levels in the PVN were assessed. RNAscope with c-fos and crh probes indicated that c-fos and CRH upregulation induced by restraint were largely attenuated in the PVN when PACAP expression was knocked out in the mPFC (Figure 3C). Thus, PACAPergic projections from the mPFC to the PVN regulate HPA axis activation (fos and crh mRNA induction in the PVN) after RS.

PACAP Nerve Terminals in the Extended Amygdala Project Mainly or Exclusively From Lateral Parabrachial Nuclei

The lateral parabrachial nucleus (LPBn), especially the external subregion, in mouse brain is densely populated with PACAP-positive neurons coexpressing slc17a6 (VGluT2) and largely overlapping with Calca (CGRP-expressing) neurons (30,31) (Figure S5A–D). These neurons project to several diencephalic structures, including the central amygdala (CeA) and oval bed nucleus of stria terminalis (BNSTov), consistent with previous data for PACAPergic projections to the extended amygdala (EA) from the PBn (Allen Brain Atlas; http://connectivity.brain-map.org/projection/experiment/301016900 and http://connectivity.brain-map.org/projection/experiment/552284594) (31) and with PACAPergic projections from PBn to BNSTov, and coexpressing CGRP, in the rat (32,33). We note here that PACAP expression is unaltered in LPBn in either Camk2α-cre::PACAP$^{fl/fl}$ or Sim1-cre::PACAP$^{fl/fl}$, emphasizing that PACAPergic projections from the cortex to hypothalamus, and within the hypothalamus itself, are genetically insulated from PACAPergic projections from the PBn to the EA in these mice (Figure S5E). We further characterized this projection with respect to its termination onto specific cell populations of the EA. AAV (adeno-associated virus) containing a

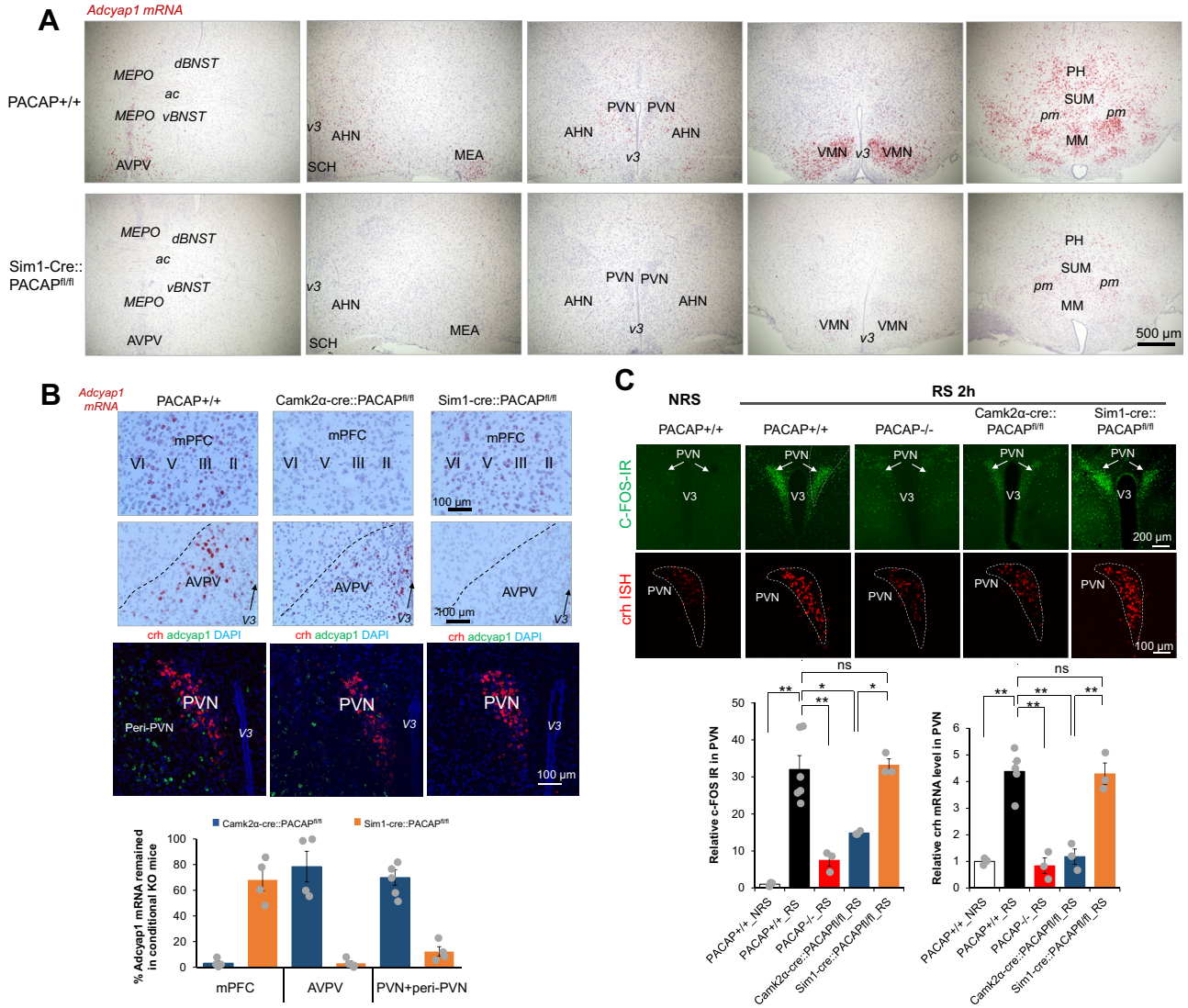


Figure 2. Conditional ablation of PACAP expression in extrahypothalamic areas (especially the limbic cortex) contributes to the endocrine effect. **(A)** PACAP expression eliminated throughout the hypothalamus in Sim1-cre::PACAP$^{fl/fl}$ mice. Adcyap1 mRNA for PACAP is indicated by red signals. **(B)** PACAP expression in the mPFC (layers II, III, V, VI) is largely unaffected in Sim1-cre::PACAP$^{fl/fl}$ mice. However, Camk2α-cre::PACAP$^{fl/fl}$ mice show elimination of PACAP in the mPFC but preservation in the preoptic area and PVN of the hypothalamus. PACAP expression in mouse brain was examined by RNAscope. Adcyap1 (PACAP) mRNA is indicated by red signals in the upper 2 panels. RNAscope shown in the lower panels was conducted with Multiplex Fluorescent V.2 Kit. Adcyap1 mRNA is indicated with green signals and crh mRNA in the PVN is indicated with red signals. Quantification of PACAP mRNA signal in Camk2α-cre::PACAP$^{fl/fl}$ mice with Fuji ImageJ demonstrated that Adcyap1 mRNA remains in the anteroventral periventricular nucleus (78.39% ± 12.03%) and peri-PVN and PVN areas (69.88% ± 5.95%) but is largely eliminated in the mPFC (3.21% ± 1.57%). In Sim1-cre::PACAP$^{fl/fl}$ mice, PACAP expression is largely retained (68.09% ± 8.49%) in the mPFC but eliminated from the anteroventral periventricular nucleus (3.11% ± 1.71%) and in peri-PVN and PVN (12.27% ± 3.73%). n = 4–5 mice per group. **(C)** Attenuated c-fos and CRH biosynthesis elevation after RS in the PVN of Camk2α-cre::PACAP$^{fl/fl}$ mice but not in the PVN of Sim1-cre::PACAP$^{fl/fl}$ mice. Hypothalamic-pituitary-adrenal axis activation was examined by c-FOS-IR (immunoreaction) with c-FOS antibody and crh ISH with RNAscope. c-FOS-IR and CRH mRNA signals in the PVN were quantified by measuring the mean gray values of signal intensity in the PVN with Fuji ImageJ. c-FOS IR or CRH mRNA levels from different groups was normalized to the average value from PACAP$^{+/+}$ mice without RS to obtain relative c-FOS-IR or relative CRH mRNA levels. One-way analysis of variance followed by all pairwise multiple comparison Bonferroni t test showed significant increase of c-FOS-IR and CRH mRNA levels in the PVN of PACAP$^{+/+}$ mice after restraint (NRS vs. RS 2 hours, $^{**}p < .001$). PACAP deficiency eliminated c-fos and CRH mRNA elevation induced by 2-hour restraint in PACAP$^{-/-}$ mice (PACAP$^{-/-}$ vs. PACAP$^{+/+}$ within RS 2 hours, $^{**}p < .001$; RS 2 hours vs. NRS within PACAP$^{-/-}$, $p = 1$). Sim1-cre::PACAP$^{fl/fl}$ showed no attenuation of either c-FOS induction or CRH upregulation in the PVN induced by 2-hour restraint (Bonferroni t test following one-way analysis of variance, Sim1-cre::PACAP$^{fl/fl}$ vs. PACAP$^{+/+}$, $p = 1$). However, impaired c-fos and CRH mRNA elevation after RS were observed in the PVN of Camk2α-cre::PACAP$^{fl/fl}$ mice (Camk2α-cre::PACAP$^{fl/fl}$ vs. PACAP$^{+/+}$ or Sim1-cre::PACAP$^{fl/fl}$, $^{**}p < .001$ for CRH mRNA; $^{*}p < .05$ for c-FOS-IR). n = 3–6 mice per group. ac, anterior commissure; AHN, anterior hypothalamic nucleus; AVPV, anteroventral periventricular nucleus; CRH, corticotropin-releasing hormone; dBNST, bed nuclei of the stria terminalis dorsal; ir, immunoreaction; ISH, in situ hybridization; KO, knockout; MEA, medial amygdala nucleus; MEPO, median preoptic nucleus; MM, medial mammillary nucleus; mPFC, medial prefrontal cortex; mRNA, messenger RNA; NRS, no restraint stress; ns, not significant; PACAP, pituitary adenylate cyclase-activating polypeptide; PH, posterior hypothalamic nucleus; pm, principle mammillary tract; PVN, paraventricular hypothalamic nucleus; RS, restraint stress; SCH, suprachiasmatic nucleus; SUM, supramammillary nucleus; V3, third ventricle; vBNST, bed nuclei of the stria terminalis ventral; VMN, ventromedial nucleus.

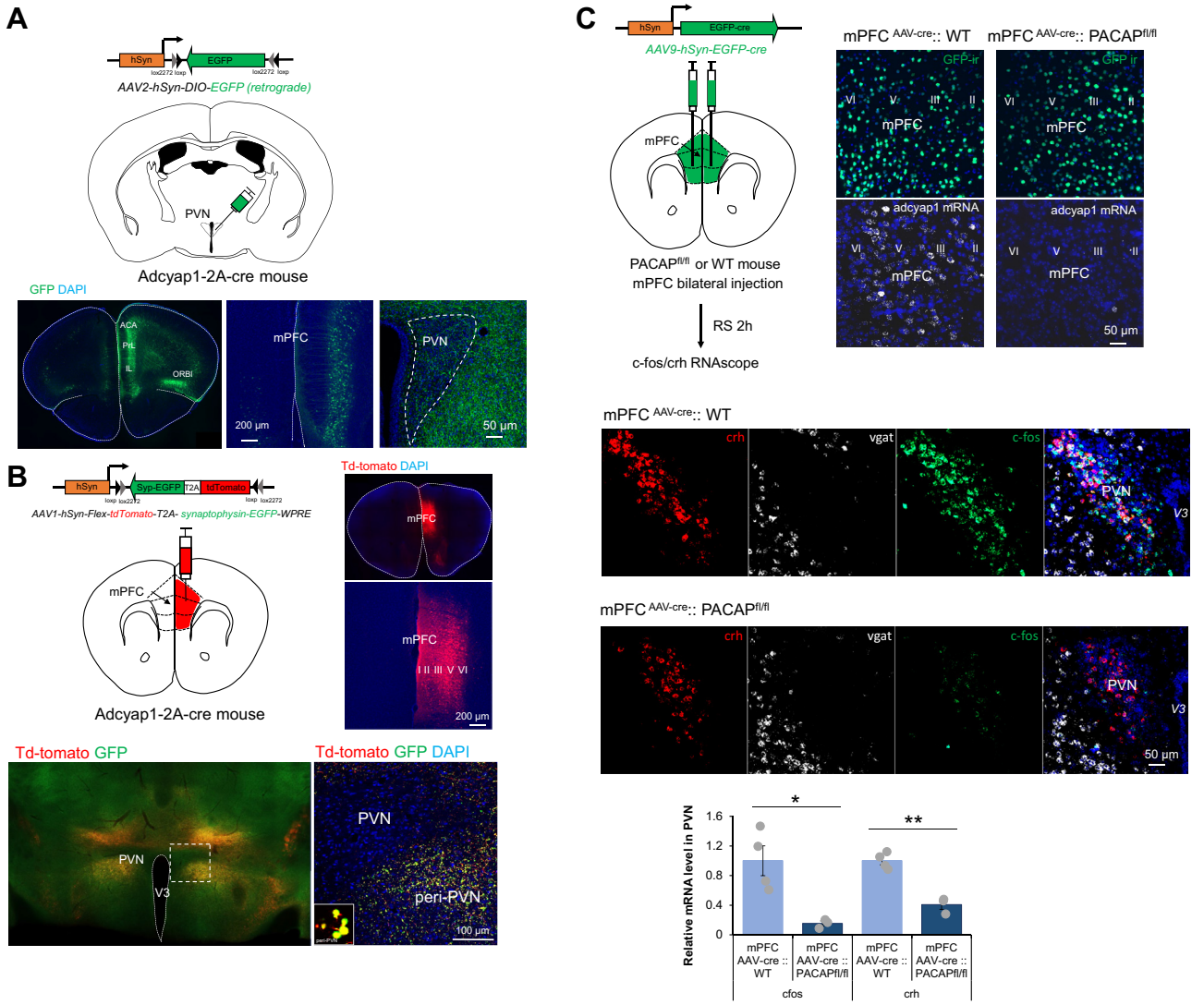


Figure 3. PACAPergic projections from the mPFC to the PVN. **(A)** Retrograde tracing of PACAPergic projections to the PVN: mPFC showed the most PACAP-positive neurons that project to the PVN. AAV2-hSyn-DIO-eGFP (retrograde) was injected into the PVN of Adcyap1-2A-cre mice unilaterally. Four to six weeks later, coronal sections from the viral injected mice were stained with GFP antibody. **(B)** Anterograde tracing of PACAPergic projections from the mPFC to the PVN. AAV1-hSyn-Flex-tdTomato-T2A-synaptophysin-EGFP-WPRE was injected into the mPFC of Adcyap1-2A-cre mice unilaterally. Targeted PACAP neurons in the mPFC were indicated by tdTomato and axonal nerve terminals were visualized with EGFP. **(C)** Elimination of PACAP expression specifically in the mPFC caused abrogation of c-fos and CRH mRNA induction in the PVN after 2-hour RS. AAV9-hSyn-eGFP-cre was injected bilaterally into mPFC of $PACAP^{fl/fl}$ ($mPFC^{AAV\text{-}cre}$::$PACAP^{fl/fl}$) or WT control mice ($mPFC^{AAV\text{-}cre}$::WT). Four to six weeks later, mice were subjected to 2-hour RS. GFP-IR (in green) was assessed to examine the location of injected AAV9-hSyn-eGFP-cre. RNAscope using *adcyap1* probe (in white) indicated that PACAP mRNA in the mPFC was eliminated in $mPFC^{AAV\text{-}cre}$::$PACAP^{fl/fl}$, compared with $mPFC^{AAV\text{-}cre}$::WT mice (upper panels on the right). RNAscope with cfos (in green) and crh (in red) probes (lower panels) indicated that c-fos and crh mRNA upregulation in the PVN induced by restraint were largely eliminated (Student's *t* test, $^{*}p < .05$ for c-fos mRNA and $^{**}p < .001$ for crh mRNA, $mPFC^{AAV\text{-}cre}$::$PACAP^{fl/fl}$ vs. $mPFC^{AAV\text{-}cre}$::WT). n = 3–4 mice per group. ACA, anterior cingulate area; CRH, corticotropin-releasing hormone; EGFP, enhanced GFP; GFP, green fluorescent protein; IL, infralimbic cortex; mPFC, medial prefrontal cortex; mRNA, messenger RNA; ORBl, orbital area, lateral part; PACAP, pituitary adenylate cyclase-activating polypeptide; PrL, prelimbic cortex; PVN, paraventricular hypothalamic nucleus; RS, restraint stress; V3, third ventricle; WT, wild-type.

synapsin promoter driving a Cre-dependent channelrhodopsin ChrimsonR and td-Tomato gene (AAV9-Syn-Flex-ChrimsonR-tdTomato) was injected into the LPBn of Adcyap1-2A-cre mice, mainly targeted to external LPBn. Four to six weeks later, PACAPergic (td-Tomato-positive) nerve terminals were observed in the BNSTov and capsular and lateral parts of the central nucleus of the amygdala (CeC/L) (Figure 4A).

There are 2 non-overlapping neuronal groups in the mouse CeC/L, expressing either protein kinase C delta type (PKCδ) or somatostatin, which together constitute the majority of local GABAergic (gamma-aminobutyric acidergic) neurons in

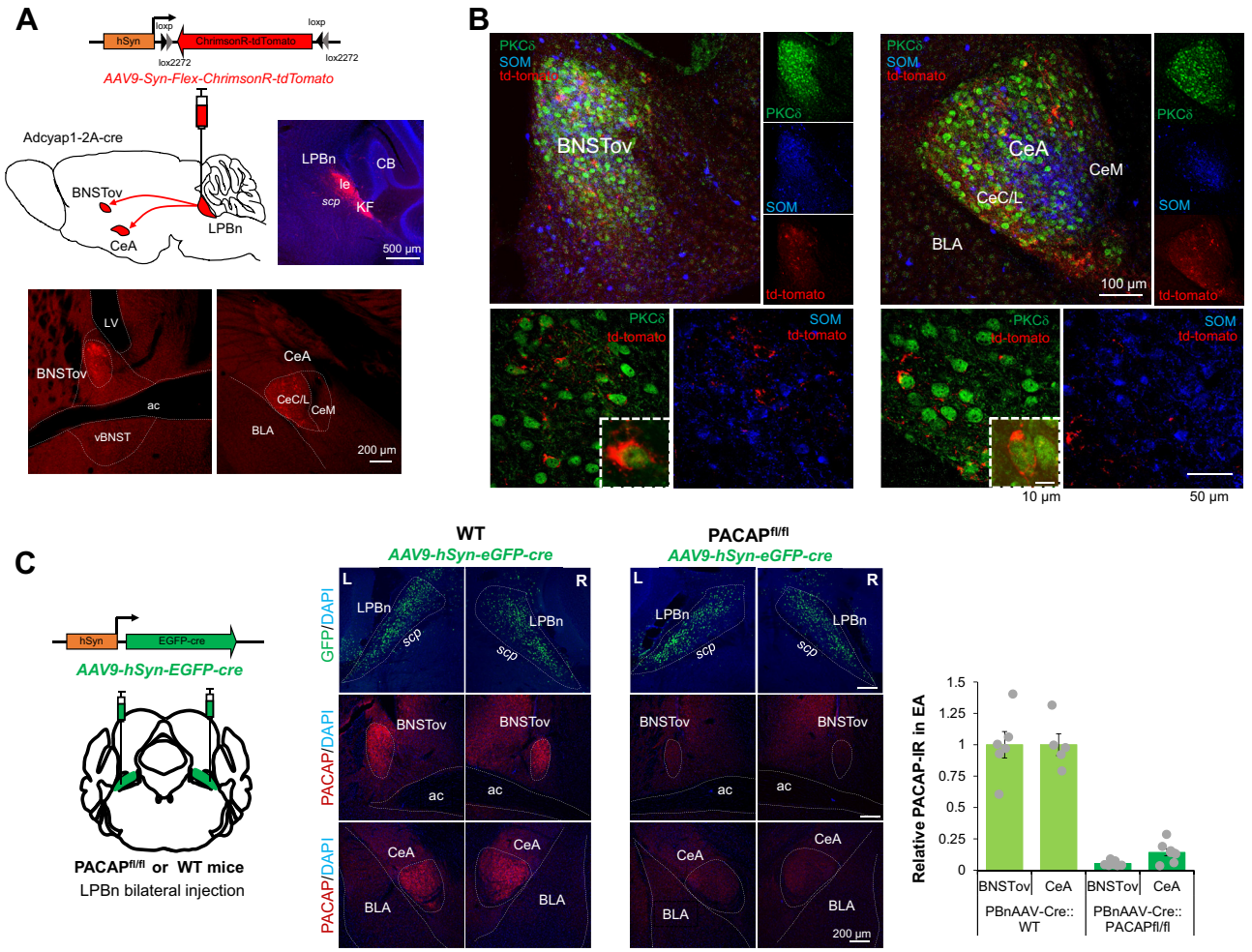


Figure 4. PACAPergic projections in the EA are mainly derived from LPBn. **(A)** PACAP neurons in the LPBn projecting to the EA. AAV9-Syn-Flex-ChrimsonR-tdTomato was injected into the LPBn of Adcyap1-2A-cre mouse. Channelrhodopsin ChrimsonR tagged with tdTomato was presynaptically expressed in PACAP neurons of the LPBn. The ChrimsonR-tdTomato positive axons (PACAPergic) from the LPBn external subregion and KF mainly innervate to BNSTov and CeA (especially CeC/L) identified by td-Tomato immunostaining (in red). **(B)** PACAPergic projection from the LPBn to PKCδ neurons in the EA. PACAPergic terminals in the EA, including BNSTov and CeA, indicated by tdTomato (in red), were further characterized by immunodetection with PKCδ and somatostatin antibodies. PACAPergic projections from the LPBn mainly innervate PKCδ neurons (in green), not somatostatin neurons (in blue), in the EA. Images with higher magnification (white boxes in the lower panels) showed axosomatic PACAPergic terminals indicated by tdTomato on PKCδ neurons in the BNSTov and CeA from the LPBn (red, PACAPergic terminal; green, PKCδ neuron). **(C)** PACAP was almost completely ablated in nerve terminals of BNSTov and CeA when PACAP expression was eliminated in PACAP neurons in the LPBn. AAV9-hSyn-eGFP-cre was bilaterally injected into the LPBn of PACAP$^{fl/fl}$ (PBn$^{AAV\text{-}cre}$::PACAP$^{fl/fl}$) or corresponding control WT mice (PBn$^{AAV\text{-}cre}$::WT). Neurons with cre expression tagged with enhanced GFP were indicated by green epifluorescence in the LPBn. PACAPergic nerve terminals in the BNSTov and CeA were detected with PACAP antibody immunostaining (in red). PACAP IR signals in the EA were quantified with Fuji ImageJ by measuring the mean gray value of signal intensity, then normalizing to the average value in the BNSTov or CeA from PBn$^{AAV\text{-}cre}$::WT mice. ac, anterior commissure; BLA, basolateral amygdala; BNSTov, oval nuclei of bed nucleus of stria terminalis; CB, cerebellum; CeA, central amygdala; CeC/L, capsular and lateral parts of the central nucleus of the amygdala; CeM, medial central amygdala; EA, extended amygdala; GFP, green fluorescent protein; IR, immunoreaction; KF, Kolliker-Fuse nucleus; L, left; le, external lateral subnucleus of LPBn; LPBn, lateral parabrachial nucleus; LV, lateral ventricle; PACAP, pituitary adenylate cyclase-activating polypeptide; PKCδ, protein kinase C delta type; R, right; scp, superior cerebellar peduncle; SOM, somatostatin; vBNST, ventral BNST; WT, wild-type.

this region (23,34–36). Further study of PACAPergic innervation (indicated by td-Tomato-positive nerve terminals) of the EA from PBn revealed that these projections are mainly to the soma of PKCδ neurons, but not to somatostatin neurons (Figure 4B), consistent with the previous observation of specialized projections of the external subregion of LPBn neurons to this population in the EA (34). When AAV9.hSynap.HI.eGFP-Cre.WPRE.SV40 was injected bilaterally into the LPBn of PACAP$^{fl/fl}$ (PBn$^{AAV\text{-}cre}$::PACAP$^{fl/fl}$), PACAP-positive terminals were completely ablated from the BNSTov and CeA approximately 4 to 6 weeks later (Figure 4C). Thus, the external subregion of LPBn is the major source of PACAPergic nerve terminals in the EA, consistent with previous reports of substantial loss of PACAPergic nerve terminals in the BNSTov of the rat following NMDA-induced lesioning of neurons of the PBn (33).

Activation of EA PKCδ Neurons Induced by RS Is PACAP$^{LPBn \rightarrow EA}$ Dependent

Acute 2-hour RS induces c-Fos activation in various discrete brain regions, including the PVN (Figure 2C) and EA (BNSTov and CeA) (Figure 5A). Stress-induced Fos activation in BNSTov and CeA is PACAP dependent since it is not detected in PACAP$^{-/-}$ mice following 2-hour RS (Figure 5A). Furthermore, c-fos is induced by ARS in the LPBn, the putative source of PACAPergic fibers in the EA, and this activation persists in PACAP$^{-/-}$ mice, suggesting that stress-activated inputs to the PBn mediating stress-induced hypophagia are not themselves PACAPergic (Figure S6A).

Because PACAPergic nerve terminals in the EA arise mainly from the LPBn (Figure 4), we examined whether c-Fos activation

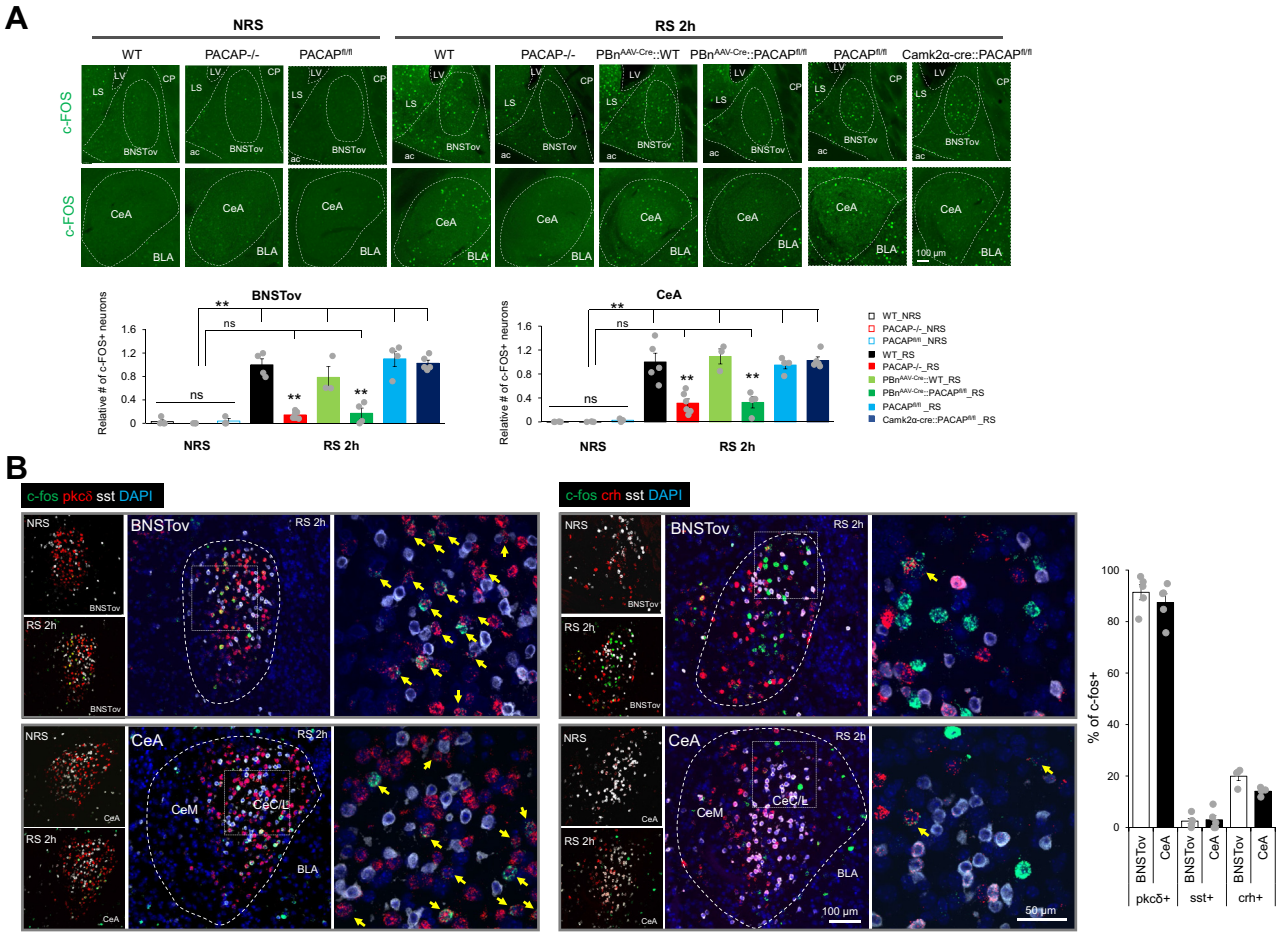


Figure 5. Activation of extended amygdala PKCδ neurons induced by RS is PACAP$^{LPBn \rightarrow extended\ amygdala}$ dependent. **(A)** c-FOS activation in CeA and BNSTov after 2-hour restraint is abolished in mice with elimination of PACAP expression in LPBn, similar to PACAP$^{-/-}$ mice. c-FOS immunostaining in BNSTov and CeA was quantified by counting FOS+ neurons with Fuji ImageJ. The number of FOS+ neurons from different groups was normalized to the average number of FOS+ neurons from WT mice with 2-hour RS to obtain the relative number of c-FOS+ cells. Two-hour RS induces c-FOS activation in the extended amygdala in PACAP$^{+/+}$ mice (one-way analysis of variance followed by all pairwise multiple comparison Bonferroni *t* test, for both WT and PACAP$^{fl/fl}$: RS 2 hours vs. NRS, $^{**}p < .001$ for both BNSTov and CeA). However, stress-induced c-FOS activation in the BNSTov and CeA is ablated in PACAP$^{-/-}$ mice (RS 2 hours vs. NRS, $p = 1$ for BNSTov, $p = .769$ for CeA). Similarly, PBn$^{AAV\text{-}cre}$::PACAP$^{fl/fl}$ mice showed significant attenuation of c-FOS induction in the extended amygdala after acute restraint (PBn$^{AAV\text{-}cre}$::PACAP$^{fl/fl}$ vs. the corresponding control PBn$^{AAV\text{-}cre}$::WT or WT or PACAP$^{fl/fl}$, $^{**}p < .001$; PBn$^{AAV\text{-}cre}$::PACAP$^{fl/fl}$ vs. PACAP$^{-/-}$, $p = 1$). Camk2α-cre::PACAP$^{fl/fl}$ mice showed no effect on c-FOS induction in the extended amygdala in response to restraint (poststress, Camk2α-cre::PACAP$^{fl/fl}$ vs. PACAP$^{fl/fl}$ or WT, $p = 1$). n = 3–6 mice per group. **(B)** c-fos messenger RNA elevation predominantly occurs in PKCδ neurons in the extended amygdala in response to 2-hour RS. Immediately after 2-hour restraint, sections from WT mouse brains were collected for RNAscope in situ hybridization with cfos, pkcδ, sst, and crh probes. Right panels show the images outlined in white within the corresponding left panels at higher magnification. Neurons double positive for cfos/pkcδ or cfos/crh are indicated by yellow arrows. Neuron numbers in the BNSTov or CeA with c-fos mRNA signals were counted in the pkcδ, sst, or crh mRNA-positive neuronal populations. Among c-fos+ neurons induced by 2-hour restraint in the BNSTov, 91.37% ± 2.94% were pkcδ+, 2.52% ± 1.05% were sst+, and 19.94% ± 1.69% were crh+; among cfos+ neurons in the CeA, 87.44% ± 3.35% were pkcδ+, 3.01% ± 1.69% were sst+, and 14.11% ± 0.79% were crh+. ac, anterior commissure; BLA, basolateral amygdala; BNSTov, oval nuclei of bed nucleus of stria terminalis; CeA, central amygdala; CeC/L, capsular and lateral parts of the central nucleus of the amygdala; CeM, medial central amygdala; CP, caudate putamen; LV, lateral ventricle; LS, lateral septum; mRNA, messenger RNA; NRS, no restraint stress; ns, not significant; PACAP, pituitary adenylate cyclase-activating polypeptide; PKCδ, protein kinase C delta; RS, restraint stress; WT, wild-type.

in the EA can be ablated by elimination of PACAP expression in the LPBn. AAV9.hSynap.HI.eGFP-Cre.WPRE.SV40 was injected bilaterally into LPBn of $PACAP^{fl/fl}$ or WT control mice. Brain tissues with EA from $PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$ and their controls $PBn^{AAV\text{-}cre}$::WT were collected immediately after 2-hour restraint for c-FOS immunohistochemistry. Similarly to $PACAP^{-/-}$, $PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$ mice showed significant attenuation of c-FOS induction in the EA after acute restraint (Figure 5A). Elimination of PACAP expression in the forebrain of Camk2α-cre::$PACAP^{fl/fl}$ mice had no effect on c-FOS induction in the EA after restraint (Figure 5A), despite a significant deficit in PVN c-FOS induction (Figure 2C). Thus, c-FOS expression in the EA induced by RS is $PACAP^{LPBn}$ dependent.

Further studies with RNAscope using pkcδ, sst, crh, and c-fos probes indicated that Fos activation occurs mainly in PKCδ neurons (Figure 5B) after RS. This is consistent with our observations that PACAPergic projections from the LPBn establish anatomical and functional connections mainly with PKCδ neurons in the EA.

These results support the notion that activation of EA PKCδ neurons induced by RS is $PACAP^{LPBn}$ dependent, that these neurons also express CGRP (Figure S5C, D), and that PKCδ neurons in the EA are activated by PACAPergic projections from the LPBn and mediate the behavioral effects of RS. Altered expression of PACAP, PAC1, and CRH mRNAs in the EA have been reported in the rat subjected to chronic stress; however, no significant changes in CRH or PACAP expression were observed in the EA after 2-hour RS (Figure S6B, C), suggesting that PACAPergic neurons within the EA itself are not involved in this response.

$PACAP^{LPBn \rightarrow CeA}$ Contributes to RS-Induced Hypophagia

To investigate whether $PACAP^{PBn \rightarrow EA}$ neurons contribute to behavioral (2-hour restraint-induced hypophagia) effects of acute stress, $PACAP^{fl/fl}$ or WT mice received bilateral LPBn injection of AAV-cre virus ($PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$ or $PBn^{AAV\text{-}cre}$::WT) and were subjected to a 2-hour acute restraint paradigm (Figure 6A). Mice with PACAP ablation in the LPBn ($PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$) showed attenuation of body weight loss, compared with corresponding control mice ($PBn^{AAV\text{-}cre}$::WT), in response to 2-hour restraint (Figure 6B). However, $PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$ and $PBn^{AAV\text{-}cre}$::WT mice showed similar levels of CORT elevation after acute or chronic restraint (Figure 6C). These observations eliminate the possibility that weight loss after RS is secondary to stress-induced CORT elevation. Consistent with the functional independence of these 2 pathways, c-Fos and CRH upregulation in the PVN induced by 2-hour RS were not influenced by PACAP elimination in the LPBn (Figure 6D).

The studies described above demonstrate that PACAP neurons in the LPBn project to both the CeA and BNSTov, and c-Fos activation in PKCδ neurons is induced by acute restraint in both the CeA and BNSTov. We next wished to determine whether activation of PKCδ neurons in both the CeA and BNSTov, or only one of these, is required to elicit RS-induced hypophagia. For these inquiries, we turned to a chemogenetic method. AAV9-hSyn-DIO-hM4D(Gi)-mCherry was injected into the CeA or BNSTov of PKCδ-cre mice to allow expression of the inhibitory DREADD (designer receptor exclusively activated by designer drugs) hM4Di in PKCδ neurons in the CeA or BNSTov. Clozapine *N*-oxide was subsequently administered 30 minutes prior to 2-hour restraint, to activate the Gi-coupled DREADD, enabling the electrical silencing of PKCδ neurons (Figure 6E). Silencing of PKCδ neurons in the CeA attenuated weight loss after restraint, as did the deletion of PACAP from the LPBn projection to the EA. Silencing of PKCδ neurons in the BNSTov did not affect restraint-induced hypophagia (Figure 6F), and silencing of PKCδ neurons in either the CeA or BNSTov was without effect on stress-induced CORT elevation (Figure 6G).

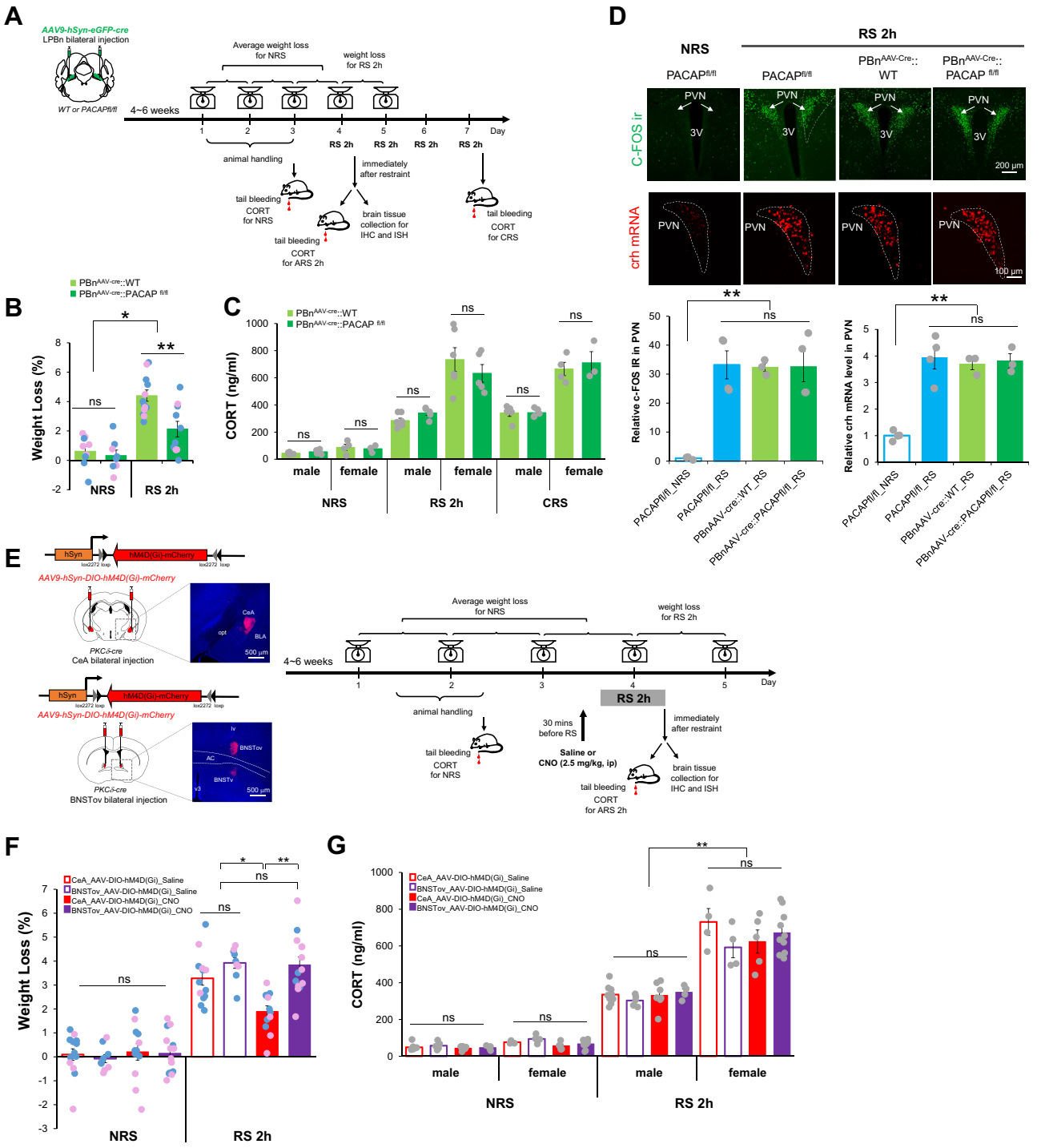
DISCUSSION

We previously observed that RS-dependent CORT elevation and hypophagia are differentially dependent on stress duration (20). Here, regionally specific genetic ablation of PACAP expression identifies 2 neuronal populations, arising from the frontal cortex and brain stem, that independently regulate the endocrine and behavioral effects, respectively, of ARS. These findings contrast with an earlier view that behavioral consequences of stress are mediated largely through the final common pathway of HPA axis activation and CORT effects on the brain (37). They invite consideration that modulation of multiple stress pathways could mitigate separate components of the stress response.

A newly described PACAPergic projection from the mPFC to the hypothalamus mediates activation of PVN CRH neurons and maintenance of CRH biosynthesis, but is dispensable for depression of feeding behavior, after acute stress. The $mPFC^{PACAP}$ projection to the PVN terminates in the PVN, and in a pronounced peri-PVN pattern. PACAP release may cause PVN^{CRH} neuronal activation via release onto extensively arborized CRH neuronal dendrites, and/or via paracrine effects in the peri-PVN region, as described by Chen *et al.* (38) for noradrenergic activation of CRH neurons in the PVN. Learning how frontocortical PACAP projection neurons are activated will help unravel the complete input-to-output brain circuit arcs that constitute the psychogenic stress response. These also include a frontocortical projection to the EA (anterior BNST) activating inhibitory projections to the PVN to attenuate HPA axis activation during stress (39), a PACAP pathway from the amygdala to the ventromedial hypothalamic nucleus that modulates fear extinction by immobilization/RS (40), and PACAPergic neurons within the infralimbic cortex itself apparently dissociated from PACAP-dependent HPA axis activation (41). Antagonistic or differential effects of these multiple pathways during acute versus chronic stress may be key to understanding the interactions between brain state and sensory input that determine whether maladaptive or resilient responses manifest clinically in human subjects after trauma exposure (18,42,43).

A second and separate PACAPergic pathway from the LPBn to the CeA is required for hypophagia induced by ARS and is dispensable for PACAP-dependent activation of PVN^{CRH} neurons. This reveals an anatomical location for the functional role of PACAP in mediating the behavioral effects of acute stress. The PBn was first described as a sensory pathway conveying pain sensation from primary sensory afferents to the CeA (44) and is now known as a relay station for alarm responses, including pain, proximal danger/threat cues, bitter tastants, and other stimuli signaling salience for aversion and promotion of avoidance behavior (45–50). The LPBn is

divided into sections that function in secondary sensory relay from brainstem nuclei to either the hypothalamus or the EA (51,52). The PACAPergic projection described here is congruent with the so-called N19 PBn cluster defined by transcriptomic and multiplex in situ hybridization histochemistry in the mouse (31). Multiple neuropeptides are used as first messengers/neurotransmitters within this pathway, including CGRP, PACAP, substance P, and neurotensin (31). CGRP expression in the PBn is required for pain transmission (45,46,53,54), and a role for PBn PACAP in



chronic pain and anxiety is implied by pharmacological effects of PACAP infused into the lateral part of the central nucleus of the amygdala (CeL) on pain sensitivity and anxiety (32,50).

Multiple studies of PBn→EA circuitry have employed neuropeptide-specific Cre driver mice to gain access to neurons of the LPBn to silence or excite them to inhibit or mimic behaviors exhibited by threatening or aversive stimuli (45–50). Despite this, how these neuropeptides function, together with glutamate release, to gate behaviors based on sensory experience and its salience, and in the filtering of sensory input based on behavioral state (satiety, anxiety, etc.), and, indeed, when their expression is required for patent information transfer in this pathway leading to specific behaviors, has yet to be carefully examined. Here, we used a convergent genetic approach to ablate PACAP from the PBn projections to the amygdala and to silence the PACAP-targeted cell populations there. These experiments allowed us to parse the necessity of a specific neuropeptide first messenger for signaling in a distinct circuit mediating brain-dependent stress response. Stress-induced neuronal activation (c-Fos induction) is PACAP dependent in the EA as in the PVN. Furthermore, this activation is specific to a major GABAergic subgroup of the capsular and lateral parts of the central nucleus of the amygdala (CeC/L) and BNSTov neurons expressing PKCδ, which are differentially innervated by PACAPergic projections from the LPBn and are also implicated in mediating other anorexigenic signals in addition to stress (55). PKCδ neurons in the CeC/L, but not in the BNSTov, are required for mediation of hypophagic behavior following stress. Apparently, BNSTov and CeL PKCδ neurons are not functionally redundant, although stimulated by the same PACAPergic projections from the LPBn. PBn PACAP projections to the BNSTov may modulate other aspects of the threat/stress response (56). PACAP signaling in this pathway is necessary, but may not be sufficient, for stress-induced behaviors such as hypophagia. The approach used here for defining PACAP's role may be useful for defining how other first messengers, including glutamate and CGRP, and perhaps other neuropeptides, signal combinatorially via the PBn→EA to convey pain, gustatory, imminent threat cue, and RS information relevant to affective state and motivation for purposeful action (eating, escape, or avoidance). Such investigations may reveal how this projection integrates state-dependent and immediate sensory information during presentation to limbic structures to convey salience and determine behavioral responses.

PACAP is indeed a master regulator of the stress response (57), conveying separate consequences of stressful stimuli through distinct neuronal circuits to endocrine and behavioral outputs in the mammalian stress response. Stress responses culminating in hypophagia occur in parallel to endocrine (HPA) activation, rather than exclusively downstream of CORT elevation, as in previous models for the psychogenic stress response (37). CORT acting conditionally on these circuits (58,59) may explain the inability of steroid treatment alone to

Figure 6. $PACAP^{PBn\rightarrow CeA}$ contributes to RS-induced hypophagia. **(A)** Scheme of experimental design for elimination of PACAP in the LPBn and the following RS studies. AAV9-hSyn-eGFP-cre was injected into the LPBn of $PACAP^{fl/fl}$ ($PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$) or the corresponding control WT mice ($PBn^{AAV\text{-}cre}$::WT) bilaterally. Animals were subjected to acute 2-hour restraint or (chronic) 2 hours daily restraint for 4 days. Tail blood was collected to examine the CORT level for NRS, RS 2 hours, or CRS; body weight was measured for hypophagic effect; and brain tissue was collected for IHC and ISH. **(B)** PACAP expression eliminated from the LPBn led to an attenuation of restraint-induced hypophagia. Because female and male mice have no difference in weight loss in response to 2-hour restraint (as showed in Figure 1C), data for females and males are displayed together: blue dots indicate male mice and pink dots indicate female mice. Two-way analysis of variance, stress effect $F_{1,37} = 43.647$, $p < .001$; genotype effect $F_{1,37} = 8.728$, $p = .005$; stress × genotype $F_{1,37} = 5.006$, $p = .031$. Post hoc Bonferroni *t* test, RS vs. NRS, $p < .001$, for $PBn^{AAV\text{-}cre}$::WT; $p = .006$ for $PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$. $PBn^{AAV\text{-}cre}$::$PACAP^{fl/fl}$ vs. $PBn^{AAV\text{-}cre}$::WT for RS 2 hours, $p < .001$. $n = 8$–13 mice per group. $^{*}p < .05$; $^{**}p < .001$. **(C)** PACAP expression eliminated from LPBn did not affect CORT elevation induced by 2 hours acute RS or CRS for 4 days, 2 hours daily. Three-way analysis of variance, stress effect $F_{2,45} = 116.097$, $p < .001$; sex effect $F_{1,45} = 87.693$, $p < .001$; genotype effect $F_{1,45} = 0.000312$, $p = .986$. $n = 5$–7 mice per group. **(D)** PACAP expression eliminated from the LPBn did not affect c-FOS induction or crh mRNA elevation in the PVN induced by 2-hour restraint. Immediately after 2-hour restraint, mice were perfused and brain sections were collected for immunostaining with c-FOS antibody, or freshly frozen brains were cryostat sectioned for RNAscope with crh probe. $n = 3$–4 mice per group. $^{**}p < .001$. **(E)** Scheme of experimental design for inactivation of PKCδ neurons in the extended amygdala during 2-hour RS. AAV9-hSyn-DIO-hM4D(Gi)-mCherry was injected into CeA or BNSTov of PKCδ-cre mice bilaterally. Expression of hM4D(Gi) tagged with mCherry in the PKCδ neurons of CeA or BNSTov was confirmed 4 to 6 weeks later. Mice were subjected to 2-hour RS paradigm and CNO (2.5 mg/kg) or saline was ip injected 30 minutes before RS. **(F)** Inactivation of PKCδ neurons in the CeA, not in the BNSTov, led to attenuation of 2-hour restraint-induced hypophagia. Three-way analysis of variance, stress effect $F_{1,88} = 250.618$, $p < .001$; CNO vs. saline, $F_{1,88} = 2.011$, $p = .16$; injection site CeA vs. BNSTov, $F_{1,88} = 8.888$, $p = .004$. PKCδ-cre mice bilaterally injected with AAV9-hSyn-DIO-hM4D(Gi)-mCherry into the CeA or BNSTov showed no difference in weight loss in the 24 hours prior to ARS (post hoc Bonferroni *t* test, CeA vs. BNSTov, $p = .169$). After RS, mice injected with virus in the BNSTov or CeA treated with saline similarly showed significant body weight loss in 24-hour poststress period [post hoc Bonferroni *t* test following 3-way analysis of variance, RS 2 hours vs. NRS, $p < .001$; BNSTov_AAV-DIO-hM4D(Gi) vs. CeA_AAV-DIO-hM4D(Gi), $p = .116$]. Inactivation of PKCδ neurons in the BNSTov by administration of CNO did not affect restraint-induced body weight loss [post hoc Bonferroni *t* test, in BNSTov_AAV-DIO-hM4D(Gi) groups after RS, CNO vs. saline, $p = .861$]. However, inactivation of PKCδ neurons in the CeA by administration of CNO led to attenuation of restraint-induced body weight loss [post hoc Bonferroni *t* test, in CeA_AAV-DIO-hM4D(Gi) groups after RS, CNO vs. saline, $p = .001$]. $n = 9$–14 mice per group, males (blue dots) and females (pink dots) are combined because of no sex difference for body weight loss in response to acute 2-hour restraint. $^{*}p < .05$; $^{**}p < .001$. **(G)** Inactivation of PKCδ neurons in the CeA or in BNSTov did not affect CORT elevation induced by 2 hours of acute RS. Three-way analysis of variance showed significant stress effect ($F_{1,80} = 841.668$, $p < .001$) and sex effect ($F_{1,80} = 138.9$, $p < .001$). However, CORT elevation examined immediately after 2-hour restraint was not different between PKCδ-cre mice with AAV9-hSyn-DIO-hM4D(Gi)-mCherry injection at the CeA and BNSTov or treated with saline and CNO ($F_{3,80} = 1.313$, $p = .276$). AC, anterior commissure; ARS, acute restraint stress; BLA, basolateral amygdala; BNSTov, oval nuclei of bed nucleus of stria terminalis; BNSTv, BNST ventral; CeA, central amygdala; CNO, clozapine *N*-oxide; CORT, cortisol/corticosterone; CRH, corticotropin-releasing hormone; CRS, chronic restraint stress; IHC, immunohistochemistry; ir, immunoreaction; ip, intraperitoneal; ISH, in situ hybridization; LPBn, lateral parabrachial nucleus; lv, lateral ventricle; mRNA, messenger RNA; NRS, no restraint stress; ns, not significant; opt, optic tract; PACAP, pituitary adenylate cyclase-activating polypeptide; PKCδ, protein kinase C delta type; PVN, paraventricular hypothalamic nucleus; RS, restraint stress; 3V, third ventricle; WT, wild-type.

trigger behavioral stress-like responses in rodents. The potential roles of both frontocorticohypothalamic and parabrachioamygdalar PACAP-containing pathways in response to psychogenic stress uncovered here may be relevant to human brain processing of stress in well-known PACAP-dependent disorders, including posttraumatic stress disorder (43).

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ARTICLE INFORMATION

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