Brief Communication

MOLECULAR METABOLISM

Dissecting a disynaptic central amygdala-parasubthalamic nucleus neural circuit that mediates cholecystokinin-induced eating suppression

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ABSTRACT

Objective: Cholecystokinin (CCK) plays a critical role in regulating eating and metabolism. Previous studies have mapped a multi-synapse neural pathway from the vagus nerve to the central nucleus of the amygdala (CEA) that mediates the anorexigenic effect of CCK. However, the neural circuit downstream of the CEA is still unknown due to the complexity of the neurons in the CEA. Here we sought to determine this circuit using a novel approach.

Methods: It has been established that a specific population of CEA neurons, marked by protein kinase C-delta (PKC-δ), mediates the anorexigenic effect of CCK by inhibiting other CEA inhibitory neurons. Taking advantage of this circuit, we dissected the neural circuit using a unique approach based on the idea that neurons downstream of the CEA should be disinhibited by $CEA^{PKC\text{-}\delta+}$ neurons while being activated by CCK. We also used optogenetic assisted electrophysiology circuit mapping and *in vivo* chemogenetic manipulation methods to determine the circuit structure and function.

Results: We found that neurons in the parasubthalamic nucleus (PSTh) are activated by the activation of $CEA^{PKC\text{-}\delta+}$ neurons and by the peripheral administration of CCK. We demonstrated that $CEA^{PKC\text{-}\delta+}$ neurons inhibit the PSTh-projecting CEA neurons; accordingly, the PSTh neurons can be disynaptically disinhibited or "activated" by $CEA^{PKC\text{-}\delta+}$ neurons. Finally, we showed that chemogenetic silencing of the PSTh neurons effectively attenuates the eating suppression induced by CCK.

Conclusions: Our results identified a disynaptic CEA-PSTh neural circuit that mediates the anorexigenic effect of CCK and thus provide an important neural mechanism of how CCK suppresses eating.

Keywords Cholecystokinin; Central amygdala; Parasubthalamic nucleus; Neural circuits; Food intake; Anorexia; Satiation

1. INTRODUCTION

Cholecystokinin (CCK) is a peptide secreted from the gastrointestinal tract during a meal to induce satiation, suppress food intake, and regulate metabolism [1—3]. Based on a wide variety of tests of the "physiological range" of CCK, it was concluded that "premeal intraperitoneal (IP) doses $\leq$5 μg/kg CCK-8 elicit satiation in the absence of side effects, whereas doses $\geq$10 μg/kg CCK-8 are aphysiological" (see summary by Nori Geary [4] and others [5,6]). The satiation effect of CCK, independent of nausea or malaise, has been confirmed extensively in both human and animal studies [3,7]; nonetheless, chronic administration of CCK causes CCK tolerance and compensation eating, and most drugs based on CCK or CCK receptors failed to control body weight [8—12]. If we could identify the brain circuits that regulate the anorexigenic effect of CCK, we could control appetite, and potentially body weight, by instead directly targeting the circuits that control eating behaviors. However, the neural mechanism regulating the eating-suppressing effect of CCK remains unclear.

Peripheral administration of CCK activates neurons in numerous brain regions, but the circuit organization of these brain regions is unclear, and many of these regions may not even be involved in the regulation of eating [13—20]. Thus far, one established neural axis for CCK-mediated appetite control starts from the vagus nerve, which excites neurons in the brainstem nucleus tractus solitarii (NTS), then excites neurons in parabrachial nucleus (PBN), and finally excites the neurons in the central amygdala nucleus (CEA) (Figure 1A) [21—33]. Lesions of

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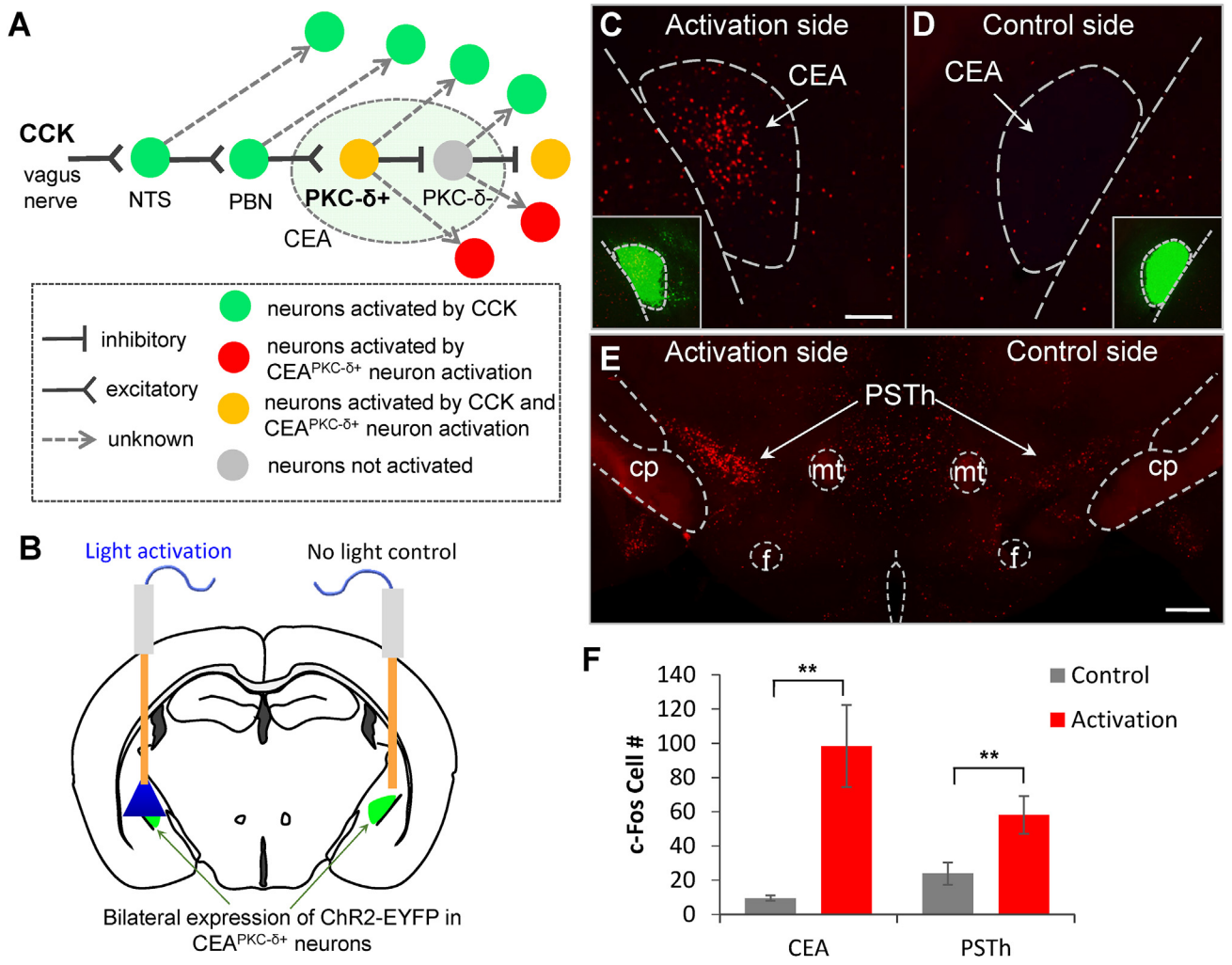

Figure 1: Neurons activated by the activation of CEA$^{PKC\text{-}\delta+}$ neurons. (A) Diagram illustrating the neural pathway that mediates CCK-induced eating suppression and the strategy to identify the common brain regions that are activated by the activation of CEA$^{PKC\text{-}\delta+}$ neurons and by CCK. (B) Diagram illustrating the unilateral optogenetic activation of the CEA$^{PKC\text{-}\delta+}$ neurons. (C–D) Optogenetic activation induces massive c-Fos expression (each red dot is immunostaining of an activated neuron) in the CEA received light (left). The control side (right) has little c-Fos expression in CEA. Inset, location of ChR2-EYFP in CEA. Bar, 200 μm. (E) Representative histological staining of c-Fos in PSTh. Left, activation side. Right, control side. cp, cerebral peduncle; f, fornix; mt, mammillo-thalamic tract. Bar, 200 μm. (F) Quantification of the number of c-Fos positive neurons in each PSTh and LPB brain section. $^{**}p < 0.01$, *t*-test, n = 4–6 mice in each group. Data displayed as mean ± SEM.

the upstream neurons will block the activation of the downstream neurons and attenuate the eating suppression induced by CCK. Importantly, almost all neurons in the CEA are gamma aminobutyric acid-expressing (GABAergic) inhibitory neurons [34,35] and represent the first node of inhibition along this neural axis. Although CEA neurons are activated by CCK or refeeding after fasting [13,36,37], a general CEA lesion yields small or no effect on food intake [38,39]. This lack of effect is likely due to the cellular complexity of CEA [35,40], in which different types of neurons with disparate or even opposing functions in eating are intermingled and complicated circuit structure is involved [41–44]. Due to these complexities, the brain region downstream of the CEA that regulates CCK-induced eating suppression remains unknown.

Our previous study found that a specific population of CEA neurons, marked by the expression of protein kinase C-delta (PKC-δ) and occupying ~50% of the CEA neurons, is required for the appetite suppression of CCK [41]. The eating inhibition elicited by CCK can be largely blocked by chemogenetic silencing of the CEA$^{PKC\text{-}\delta+}$ neurons, and activation of the CEA$^{PKC\text{-}\delta+}$ neurons is sufficient to suppress food intake. Importantly, a thorough survey of CEA neurons labeled by distinct genetic markers revealed that the PKC-δ+ neurons in the lateral part of CEA are the only population in the CEA that is preferentially activated by CCK [42]. It has also been demonstrated that CEA$^{PKC\text{-}\delta+}$ neurons suppress eating by inhibiting local GABAergic PKC-δ negative neurons (CEA$^{PKC\text{-}\delta-}$) [41–44]. The genetic identification of the CEA$^{PKC\text{-}\delta+}$ neurons thus provides a unique tool to determine the neurons downstream of the CEA that mediate the eating-suppressing effect of CCK. Here we dissected the circuit using a unique approach that combines the circuitry mapping of CEA$^{PKC\text{-}\delta+}$ neurons with c-Fos mapping after CCK administration. We identified a previously understudied brain region in the posterior edge of the lateral hypothalamus area (LHA) that regulates the anorexigenic effect of CCK; it is called the parasubthalamic nucleus (PSTh).

2. METHODS AND MATERIALS

2.1. Animals

All animal care and studies were conducted according to the guidelines of US National Institutes of Health for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona. The PKC-δ-Cre mice were originally generated in Dr. David Anderson's lab at the California Institute of Technology [35] and maintained by crossing with wild-type C57BL/6 mice from the Charles River Laboratory. All mice were housed on a 12-hour light cycle with ad libitum access to water and rodent chow unless placed on a food restriction schedule for fasted eating experiments. The genotype of transgenic PKC-δ-Cre mice offspring is identified by PCR on genomic tail DNA. Both wild-type and PKC-δ-Cre offspring were used in this study. Since we did not observe any significant difference between male and female mice in our experiments (Supp. Figures 7A and 8A), unless indicated, we analyzed the data by combining approximately the same number of male and female mice throughout the study.

2.2. Stereotaxic animal surgery and viruses

Survival surgeries and ferrule fiber implantation were performed as previously described [45]. Injection coordinates (in mm) relative to midline, bregma, and skull surface at bregma: CEA (±2.85, −1.40, −4.75), PSTh (±1.10, −2.30, −4.80). AAV2-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA and AAV2-EF1a-DIO-EYFP-WPRE-pA, AAV2-CaMKIIa-hChR2(H134R)-EYFP were originally generated in Dr. Karl Deisseroth's lab and purchased from the University of North Carolina at Chapel Hill Vector Core. pAAV-CaMKKIIa-hM4D(Gi)-mCherry (AAV5) was originally generated in Dr. Bryan Roth's lab and purchased from Addgene. Two hundred nanoliters of virus suspension was stereotaxically injected 3−4 weeks before the experiment. 50 nl Cholera toxin B subunit (CTB) (Invitrogen C34776) was stereotaxically injected one week before the experiments.

2.3. Optogenetics

Optogenetic activation was performed as previously described [41]. During the stimulation, the activation of CEA$^{PKC\text{-}\delta+}$ neurons was performed without food.

2.4. Pharmacology

For hM4Di chemogenetic silencing, clozapine-N-oxide (CNO) (Enzo life science-Biomol, BML-NS105-0005) was freshly dissolved in saline (0.9% NaCl) to a concentration of 1 mg/ml and IP injected at 5 mg/kg one hour before the eating test. CNO used in slice electrophysiology was applied to the bath at a concentration of 1 μM. CCK-8S (Bachem 4033010) was dissolved in saline to a final concentration of 1 μg/ml and IP injected at a 5 μg/kg dose immediately before the food intake tests. Saline with comparable volume was injected as vehicle control.

2.5. Food intake measurement

Mice were habituated to the experimenter handling and testing environment for one week, 20 min per day, before the test. For the fasted eating test, mice were food-deprived, with ad libitum water, 24 h before testing. Mice were introduced into a clean, empty testing cage with a pre-weighed regular chow food pellet (NIH-31, Zeigler Bros, Inc.) and allowed to feed for 90 min, measuring remaining food every 30 min. Net food intake was calculated by weighing the leftover food pellet and crumbs. For the fed eating test, mice were not food deprived prior to the testing, and the same food intake test was performed.

2.6. Immunohistochemistry and histology

Immunostaining and histology analyses were performed as previously described [41]. Primary antibodies used included the following: rabbit anti-PKC-δ (Abcam, ab182126, 1:1000), goat anti-c-Fos (Santa Cruz Biotech, sc-52-G, 1:500), and rabbit anti-c-Fos (Santa Cruz Biotech, sc-52, 1:500). Secondary antibodies used included the following: Alexa Fluor 488 donkey anti-rabbit IgG (Jackson Immuno Research Inc. 711-545-152, 1:250), Alexa Fluor 594 donkey anti-goat IgG (Jackson Immuno Research Inc. 705-585-003, 1:250), Alexa Fluor 594 donkey anti-rabbit IgG (Jackson Immuno Research Inc. 705-585-152, 1:250).

2.7. Electrophysiological slice recordings

The brain slice electrophysiology recording and analysis were performed as previously described [41].

2.8. Statistical analysis

Data represent mean ± s.e.m. The unpaired Student's *t*-test was used to compare two groups, and two-way ANOVA with post hoc-tests were used for data with more than one independent variable. A p value < 0.05 was considered significant. Data were analyzed with GraphPad Prism Software.

3. RESULTS

3.1. Neurons in multiple brain regions are activated by optogenetic activation of CEA$^{PKC\text{-}\delta+}$ neurons

Because CEA$^{PKC\text{-}\delta+}$ neurons suppress eating by inhibiting CEA$^{PKC\text{-}\delta-}$ neurons [41—44], neurons downstream of CEA$^{PKC\text{-}\delta-}$ neurons involved in eating suppression should be disinhibited (i.e., "activated") by the activation of CEA$^{PKC\text{-}\delta+}$ neurons (Figure 1A). However, CEA$^{PKC\text{-}\delta+}$ neurons are also involved in non-CCK-mediated eating suppression such as bitter taste [41], suggesting some neurons activated by the CEA$^{PKC\text{-}\delta+}$ neuron activation may not be involved in CCK's effect. To determine the brain regions that are downstream of CEA in regulating the anorexigenic effect of CCK, we reasoned that neurons in these brain regions should be activated by both the activation of CEA$^{PKC\text{-}\delta+}$ neurons and the IP injection of CCK (Figure 1A).

To systematically identify the brain regions activated by the stimulation of CEA$^{PKC\text{-}\delta+}$ neurons, we bilaterally injected the adeno-associated virus (AAV) carrying Cre-dependent ChR2-EYFP into the CEA of PKC-δ-Cre transgenic mice and implanted optic ferrule fibers above the CEA. Three to four weeks after the mice recovery and viral expression, we delivered blue light to activate the CEA$^{PKC\text{-}\delta+}$ neurons on one side of the brain (473 nm, 5 Hz, 10 ms pulse width, 20 min, ~5 mW at the fiber tip, a protocol previously used to induce eating suppression but that does not increase anxiety or freezing [41]). Because CEA neurons do not have contralateral projections [41,46], the neurons disynaptically disinhibited by CEA$^{PKC\text{-}\delta+}$ neurons for eating should be on the ipsilateral side of the activated CEA$^{PKC\text{-}\delta+}$ neurons. Thus, we used the contralateral side as control, in which we also expressed ChR2-EYFP and implanted ferrule fiber but did not deliver light (Figure 1B). We then perfused the mice, 90 min after light stimulation, and dissected the brains for immunostaining of c-Fos, a cellular marker widely used to indicate neural activation [47]. The delivery of light alone (<10 mW at the tip, <1 h) in mice expressing EYFP control does not affect mouse behaviors or cause significant c-Fos expression [41,48]. As expected, we found that c-Fos expression was dramatically increased in the CEA following CEA$^{PKC\text{-}\delta+}$ neuron activation (Figure 1C, D, F). We found that the most robust increase in c-Fos expression was in the PSTh region (Figure 1E, F, and Supp. Figures 2 and 3). We also found significant increases of c-Fos

expression in the PBN and the reticular nucleus, and modest increases in several other brain regions (Supp. Figures 1, 4 and 5).

3.2. PSTh is a common brain region activated by the activation of CEA$^{PKC\text{-}\delta+}$ neurons and by CCK

To identify the common brain regions activated by both the stimulation of CEA$^{PKC\text{-}\delta+}$ neurons and the peripheral administration of CCK, we performed c-Fos immunostaining on mouse brains 90 min after IP injection of CCK (5 μg/kg) or saline control. Consistent with previous c-Fos studies following CCK injection [13–18,49], we found significant c-Fos induction in various brain regions, including the CEA, PSTh, and PVH (Figure 2, and Supp. Figures 3, 5). After careful comparison of the c-Fos expression following IP injection of CCK to that following the activation of CEA$^{PKC\text{-}\delta+}$ neurons, we found that the expression spatial distribution in PSTh is similar and robust in these two conditions, suggesting the PSTh is a common brain region activated by both the CEA$^{PKC\text{-}\delta+}$ neuron activation and CCK injection. It has been demonstrated that PSTh receives inputs from the CEA [50–52]; however, in mice expressing ChR2-EYFP in CEA$^{PKC\text{-}\delta+}$ neurons, we did not find strong EYFP-expressing axon terminals in this region (Supp. Figure 2), nor did we observe any light-triggered postsynaptic response when we performed electrophysiology recordings on cells in this area or the surrounding LHA region [41]. On the other hand, it has been determined that CEA$^{PKC\text{-}\delta+}$ neurons send monosynaptic inhibition to almost all other CEA neurons, including both the lateral/capsular and the medial parts [35,41,44]. These results suggest that neurons in the PSTh might be disynaptically disinhibited by the activation of CEA$^{PKC\text{-}\delta+}$ neurons.

3.3. Neurons in the PSTh are disynaptically innervated by CEA$^{PKC\text{-}\delta+}$ neurons

To test if the PSTh contains neurons immediately downstream of CEA$^{PKC\text{-}\delta-}$ neurons and 2nd-order downstream of CEA$^{PKC\text{-}\delta+}$ neurons, we expressed ChR2 in CEA$^{PKC\text{-}\delta+}$ neurons by injecting Cre-dependent AAV-ChR2-EYFP in the CEA of PKC-δ-Cre mice and injected a retrograde neuronal tracer cholera toxin B subunit (CTB) conjugated with red Alexa Fluor into the PSTh of the same animal. Histology analysis showed that less than 3% of the CEA$^{PKC\text{-}\delta+}$ neurons are positive for CTB labeling, and ~6% of the CTB-labeled cells are PKC-δ neurons (Figure 3A–E), suggesting these are two largely different populations. We then performed ChR2-assisted circuit mapping [35,53] to test if the CEA neurons that project to the PSTh receive monosynaptic inhibition from CEA$^{PKC\text{-}\delta+}$ neurons (Figure 3F). Consistent with previous results [35], there is no spontaneous firing of the CEA neurons observed in slice recording. We performed whole-cell patch clamp recordings on the CTB-labeled CEA neurons while using a blue light pulse that covers the whole CEA region for the light stimulation. We did not observe any light-triggered action potentials or ChR2 currents in these CTB-labeled CEA neurons, suggesting they are CEA$^{PKC\text{-}\delta-}$ neurons. Under a −40 mV voltage-clamp mode, we observed a robust inhibitory postsynaptic current (IPSC) induced by the light pulse. This IPSC was blocked by the GABAA receptor antagonist picrotoxin (Figure 3G). The latency from the start of the light pulse to the beginning of the IPSC was less than 5 ms (3.7 ± 0.2 ms), suggesting a monosynaptic connection. Taken together, these studies demonstrated that CEA$^{PKC\text{-}\delta+}$ neurons send monosynaptic inhibition to PSTh-projecting CEA$^{PKC\text{-}\delta-}$ neurons.

3.4. Neurons in the PSTh regulate the eating suppression induced by CCK

As demonstrated, neurons in PSTh are activated not only by CCK but also disynaptically disinhibited by CEA$^{PKC\text{-}\delta+}$ neurons, silencing of which can largely block the eating suppression induced by CCK. This suggests the activity of PSTh neurons might also be required for the eating suppression induced by CCK. To test this hypothesis, we

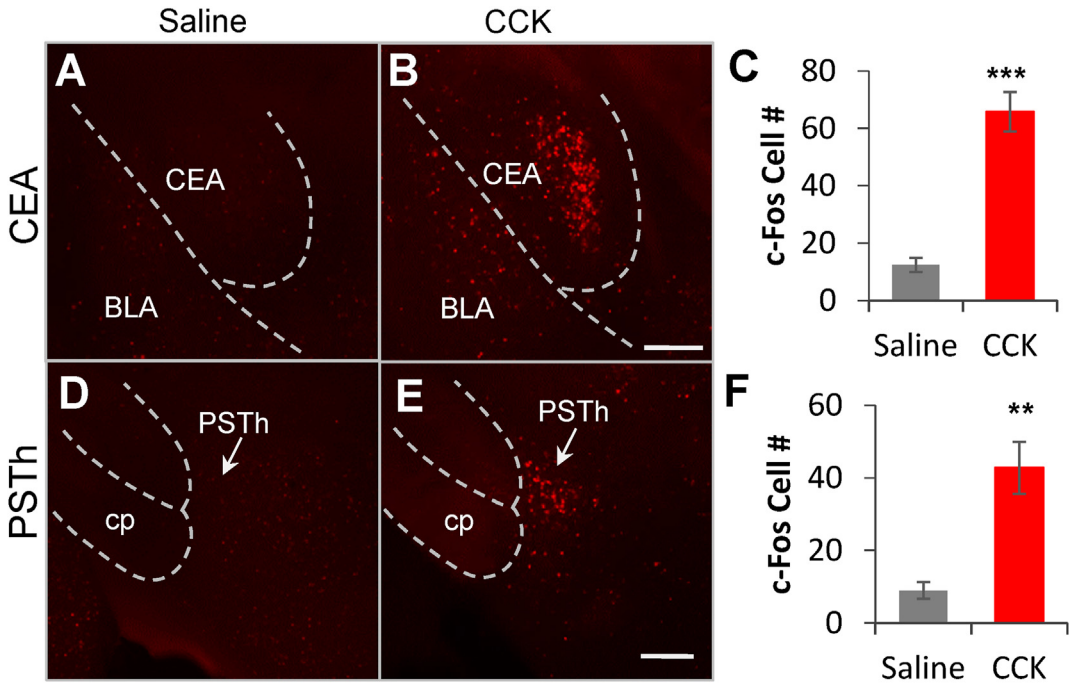

Figure 2: CEA and PSTh neurons activated by IP injection of CCK. (A–C) Representative c-Fos immunostaining (A, B) and quantification (C) in the CEA of mice IP injected with saline (A) or CCK (B). BLA: basal lateral amygdala nucleus. Unpaired *t* test, n = 5 mice in each group. ***p < 0.001. Data displayed as mean ± SEM. Bar, 200 μm. (D–F) Representative c-Fos immunostaining (D, E) and quantification (F) in the PSTh of mice IP injected with saline (D) or CCK (E). Unpaired *t* test, n = 5 mice in each group, **p < 0.01. Data displayed as mean ± SEM. Bar, 200 μm.

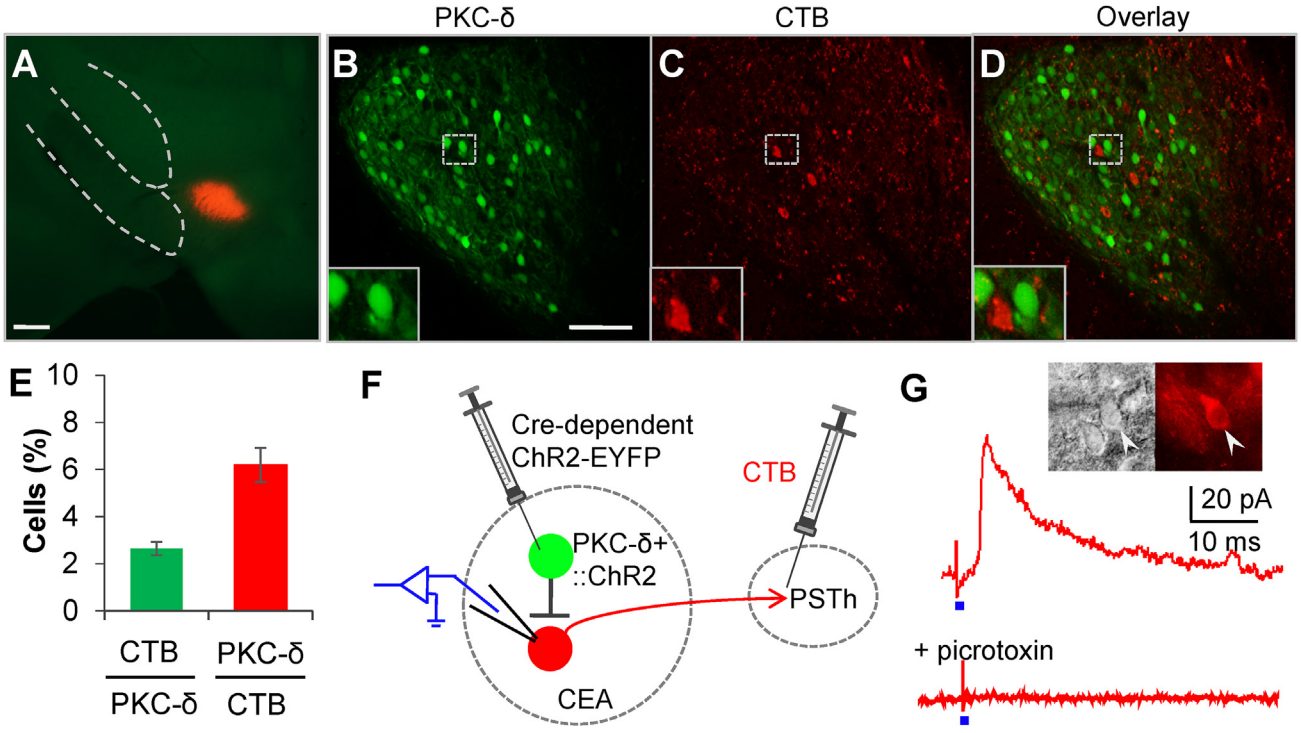

Figure 3: PSTh-projecting CEA$^{PKC\text{-}\delta-}$ neurons are monosynaptically inhibited by CEA$^{PKC\text{-}\delta+}$ neurons. (A) Representative fluorescent images showing the location of the CTB (red) injected in the PSTh. Green color is the overexpressed background to reveal brain structure. Bar, 200 μm. (B—E) Representative images (B—D) and quantification (E) showing cells retrogradely labeled by CTB (red) in the CEA and expression of EYFP in CEA$^{PKC\text{-}\delta+}$ neurons (green). Insets, enlargement of the boxed areas. Bar, 100 μm. Data displayed as mean ± SEM. (F) The circuit diagram shows recordings on PSTh-projecting CEA$^{PKC\text{-}\delta-}$ neurons (Red). The CEA$^{PKC\text{-}\delta+}$ neuron expressing ChR2 is in green. (G) Top: A representative whole-cell voltage-clamp recording trace shows a light-triggered monosynaptic IPSC in CTB-expressing cell. Bottom: a representative recording trace from the same cell shows that IPSC is blocked by bath application of picrotoxin (100 μM). Inset, A CEA$^{PKC\text{-}\delta-}$ neuron expressing red CTB is visualized in the live brain slice. The blue dots indicate 2 ms light pulses. IPSC amplitude, 35 ± 12 pA. IPSC latency, 3.7 ± 0.2 ms. n = 9 CTB-labeled cells from three animals; all 9 cells tested show light triggered IPSCs. Data displayed as mean ± SEM.

delivered a bilateral injection of AAV expressing hM4Di-mCherry into the PSTh of wild-type mice. After allowing three to four weeks for mice recovery and viral expression, we fasted the mice ~24 h and measured their food intake following IP injection of CCK (5 μg/kg), CCK plus CNO (5 mg/kg), CNO, or saline control. The expression of hM4Di-mCherry in the PSTh was confirmed by histology after the experiments (Figure 4A). Whole cell current-clamp recordings in brain slice confirmed that the firing of the hM4Di-expressing PSTh neurons can be suppressed by CNO (Figure 4B). We found that silencing of PSTh neurons can significantly attenuate the eating suppression caused by CCK (Figure 4C, and Supp. Figure 7B). Interestingly, the food intake in mice injected with CNO plus CCK was not significantly different from that in mice injected with CNO alone (Figure 4C), suggesting that silencing PSTh neurons can largely block the eating suppression induced by CCK. Similar to silencing of CEA$^{PKC\text{-}\delta+}$ neurons, we did not observe any difference in food intake between the mice injected with saline and those injected with CNO (Figure 4C), indicating that silencing of PSTh neurons does not affect food intake under normal conditions. These effects are consistent with the previous reported effect of silencing CEA$^{PKC\text{-}\delta+}$ neurons, which prevented CCK-induced eating suppression but did not induce hyperphagia [41]. To further confirm that silencing PSTh neurons does not affect food intake, we also measured the food intake in fed mice. Again, there was no significant difference in food intake after silencing PSTh neurons (Figure 4D, and Supp. Figure 8B). We did not detect any difference between male and female mice in these manipulations (Supp. Figures 7A and 8A), suggesting that PSTh neurons function similarly for eating in male and female mice.

4. DISCUSSION

Circuit mapping with c-Fos or other immediate early genes after CCK administration has successfully identified many brain regions that play an important role in eating regulation [13,19,20]. To understand how these brain regions cooperate to control eating in health and disease, it is necessary to clarify how neurons in these brain regions are organized and how they form functional circuits. However, it is difficult to identify the brain regions in which CCK causes neural inhibition with the simple c-Fos mapping method and even more difficult to determine the circuit organization downstream of these inhibited neurons. While almost all neurons in the CEA are GABAergic inhibitory neurons, the function of CEA neurons are heterogenous and can even play opposing roles in eating [41—44]. Activation of CEA$^{PKC\text{-}\delta+}$ neurons suppresses food intake [41], but activation of a CEA$^{PKC\text{-}\delta-}$ population expressing serotonin receptor 2a (Htr2a) increases food intake [44]. Furthermore, activation of all CEA neurons by activating the excitatory inputs from insula also suppresses eating [43,54]. Thus, previous studies have successfully established a neural axis from the vagus nerve to the NTS, to the PBN, and to the CEA, but failed to identify the circuits downstream of CEA neurons that mediate the effect of CCK. Using the unique genetic marker-labeled CEA$^{PKC\text{-}\delta+}$ neurons that regulate the anorexigenic effect of CCK, here we demonstrated that we can

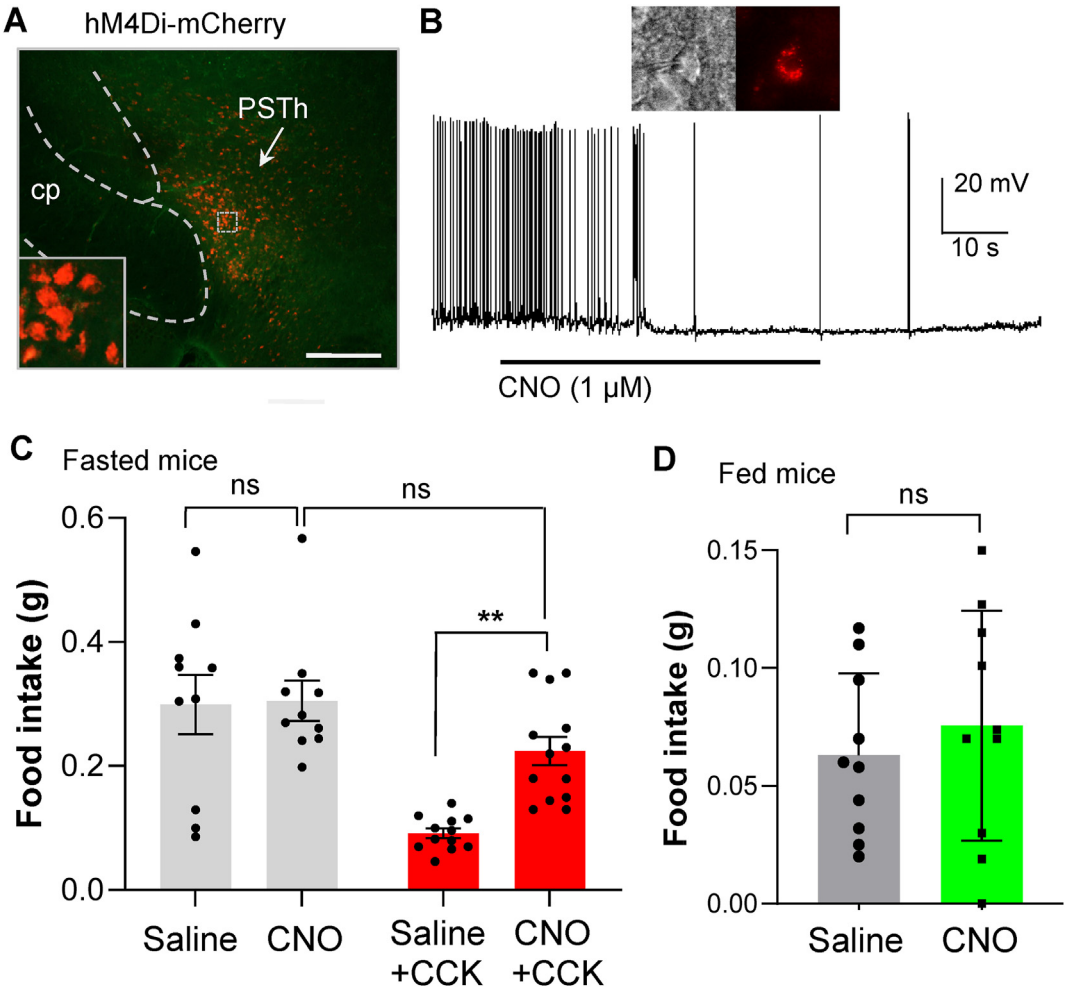

Figure 4: Chemogenetic silencing of PSTh neurons attenuates CCK-induced eating suppression. (A) A representative image shows expression of hM4Di-mCherry in PSTh. Green is background color to help identify the location. The inset shows enlargement of the boxed area. Bar, 200 μm. (B) A representative whole-cell current-clamp recording trace shows PSTh neurons expressing hM4Di-mCherry was silenced by bath application of CNO (1 μM). Inset, a PSTh neuron expressing hM4Di-mCherry is identified by its red fluorescence in live brain slice. (C) Silencing PSTh neurons increases the amount of food intake (measured in a feeding period of 30 min, mice were fasted 20–24 h before the test). Mice in all groups express hM4Di-mCherry in PSTh. CNO (5 mg/kg) was IP injected around 60 min before the test, and CCK (5 μg/kg) was injected immediately before the test; both injections have a saline injection as control. $^{**}p < 0.01$, two-way ANOVA with post hoc Bonferroni *t* test. n = 10–13 mice in each group. Data displayed as mean ± SEM. (D) Silencing PSTh neuron does not affect the food intake in fed mice. Unpaired *t* test. N = 10 mice in each group. Data displayed as mean ± SEM.

determine the downstream neural circuits by combining the c-Fos mapping of these neurons and CCK. Similar approaches and strategies could also be applied to determine many other neural circuits where inhibitory neurons are involved and to unravel the neural pathways of many other physiologically important agents [45].

4.1. Neural pathway of CCK-mediated eating suppression

Studies on food intake and eating suppression with CCK have established a feed-forward neural axis for CCK's effect consisting of vagus nerve → NTS → PBN → CEA. Here we overcame the above-mentioned difficulty in CEA neurons and extended this axis downstream to $CEA^{PKC\text{-}\delta+}$ neurons → $CEA^{PKC\text{-}\delta-}$ neurons → PSTh neurons. These results are consistent with several recent studies which showed that PSTh neurons receives from CEA and might play a role in regulating eating behaviors [50–52]. It should be noted that $CEA^{PKC\text{-}\delta+}$ neurons also send projection to inhibit neurons in the bed nucleus of the stria terminalis (BNST) (Supp. Figure 6 and [41]). We observed a sparse labeling of BNST neurons after injection of CTB in the PSTh (Supp. Figure 6); however, as optogenetic activation of the terminals in BNST projected from $CEA^{PKC\text{-}\delta+}$ neurons did not suppress food intake, this pathway is unlikely to play a major role in mediating the CCK-induced eating suppression. Our results also suggest that the actual neural mechanism for eating control might be much more complicated than this simple feed-forward circuit. For example, while PBN neurons send excitatory innervation to the CEA, they also receive a strong projection from $CEA^{PKC\text{-}\delta-}$ neurons and a very weak projection from $CEA^{PKC\text{-}\delta+}$ neurons (Supp. Figure 4 and [26,41,44]). Moreover, neurons in the PBN also receive excitatory innervation from PSTh [55,56]. Consistent with this circuitry, we found that activation of the $CEA^{PKC\text{-}\delta+}$ neurons induces c-Fos expression in the PBN (Supp. Figure 4). Interestingly, most increased c-Fos was expressed in a PBN region that is not innerved by $CEA^{PKC\text{-}\delta+}$ neurons (Supp. Figure 4), suggesting that a multi-synaptic connection through $CEA^{PKC\text{-}\delta-}$ neurons might also be involved in PBN activation. Whether these activated PBN neurons send feedback excitation to $CEA^{PKC\text{-}\delta+}$ neurons and their relationship to PSTh remain to be determined. Similar feedback also exists between

the CEA and the PSTh. Anatomical studies showed that the PSTh forms mutual connections with many brain regions related to autonomic functions and motivated behaviors [50—52,55,56]. Interestingly, the PSTh also contains neurons activated by the stimulation of $CEA^{PKC\text{-}\delta+}$ neurons as well as neurons that project to the CEA [55]. As neurons in the PSTh are predominantly glutamatergic [56], if the PSTh neurons innervate $CEA^{PKC\text{-}\delta+}$ neurons, it might create a feedback loop that enhances the activity of $CEA^{PKC\text{-}\delta+}$ neurons; conversely, if the PSTh neurons innervate $CEA^{PKC\text{-}\delta-}$ neurons, it might provide a feedback inhibition. Both circumstances might play an important role in appetite control. These extensive loop connections among brain regions in this neural axis are consistent with their function in regulating the homeostasis of energy intake [19].

Another interesting observation revealed by our unilateral activation of the $CEA^{PKC\text{-}\delta+}$ neurons is that PVH neurons are activated bilaterally (Supp. Figure 5). Neurons in the PVH receive direct inputs from the CEA sparsely and indirect inputs through the BNST [57—59]. However, none of these brain regions has been reported to have contralateral projections, and PVH neurons do not form obvious projections to the contralateral PVH either [60]. Therefore, the contralateral PVH should be activated by the $CEA^{PKC\text{-}\delta+}$ neurons through unknown multisynaptic indirect connections. Another possibility is that hormonal signals, such as the corticotropin-releasing hormone (CRH), expressed in this region may mediate the co-activation of bilateral PVH. The PVH is an important node in appetite control and has long been suggested to regulate satiety, but the circuitry mechanism underlying how PVH neurons are activated by CCK is unclear [10,61—63]. The results here suggest that the PVH is an important region further downstream of the CEA to regulate CCK-induced eating suppression. Therefore, a future study of how the PVH receives information from CEA, PSTh, or any other brain region in this neural axis is warranted.

4.2. PSTh neurons in eating control

The PSTh is located in the posterior lateral edge of the LHA [55,64]. Although neurons in the LHA have been widely described in the regulation of eating [61,65—69], the role of PSTh neurons in eating is still relatively understudied [56]. Neurons in the PSTh are activated by CCK, refeeding after fasting, and weight-lowering drugs [37,51,70,71]. Unlike $CEA^{PKC\text{-}\delta+}$ neurons, PSTh neurons are not activated by bitter taste [72], suggesting PSTh neurons might have a more specific function in eating than $CEA^{PKC\text{-}\delta+}$ neurons and explaining why the approach combining $CEA^{PKC\text{-}\delta+}$ neurons with CCK circuit mapping is important in determining the neurons with more specific functions. Consistent with this role, a previous study showed that food intake decreases after terminal activation of the glutamatergic neurons projecting from the PSTh to the paraventricular thalamus (PVT) [73]. To test if activation of PSTh neurons is sufficient to suppress food intake, we performed a pilot experiment using ChR2 optogenetics to activate PSTh neurons. We observed a decrease in food intake (Supp. Figure 9), but also a stimulation-triggered movement problem in these mice (data not shown), which raises the possibility that activation of PSTh neurons or surrounding neurons also controls motor function. Further studies are required to clarify this. Two recent studies manipulating PSTh neurons expressing tachykinin-1 ($PSTh^{Tac1}$) found that silencing $PSTh^{Tac1}$ neurons can increase licking when animals were in neophobia or lipopolysaccharide-induced sickness [52], and activation of $PSTh^{Tac1}$ neurons can reduce liquid diet intake [74]. However, the latter study did not report whether activation of $PSTh^{Tac1}$ neurons affects movement. The latter study also showed that the $PSTh^{Tac1}$ population contains only a subset of the CCK-activated PSTh neurons; accordingly, silencing $PSTh^{Tac1}$ neurons only partially prevented the food intake-suppressing effect of CCK, suggesting a more complete silencing of PSTh neurons is needed in determining this function of PSTh. These studies further confirm that neurons in the PSTh play an important role in regulating eating behavior and the anorexigenic effect of CCK.

5. CONCLUSIONS

In summary, we demonstrated that a novel approach using genetically defined neurons is a powerful tool in dissecting complicated neural circuits with a specific function. We identified a disynaptic neural circuit from $CEA^{PKC\text{-}\delta+}$ neurons to $CEA^{PKC\text{-}\delta-}$ neurons to PSTh neurons that plays an important role in regulating CCK-mediated eating suppression. This study also revealed an important function for a relatively understudied region of the PSTh and provided a circuit mechanism of how CCK suppresses food intake.

AUTHOR CONTRIBUTIONS

H.C. conceived the project, M.R.S., Y.W., and H.C. designed the experiments and collected and analyzed the data. T.S.C. performed pilot PSTh silencing and CCK experiments, and W.I.S. and M.B.S. provided essential research help and training. C.F. performed immunohistochemistry, managed the mice colony, genotyping, and characterization. H.C. wrote the manuscript with the help of all authors.

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CONFLICT OF INTEREST

No competing interests of any authors or persons related to this research are declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101443.

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