Central amygdala PKC-δ+ neurons mediate the influence of multiple anorexigenic signals

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Feeding can be inhibited by multiple cues, including those associated with satiety, sickness or unpalatable food. How such anorexigenic signals inhibit feeding at the neural circuit level is not completely understood. Although some inhibitory circuits have been identified, it is not yet clear whether distinct anorexigenic influences are processed in a convergent or parallel manner. The amygdala central nucleus (CEA) has been implicated in feeding control, but its role is controversial. The lateral subdivision of CEA (CEl) contains a subpopulation of GABAergic neurons that are marked by protein kinase C-δ (PKC-δ). We found that CEl PKC-δ+ neurons in mice were activated by diverse anorexigenic signals in vivo, were required for the inhibition of feeding by such signals and strongly suppressed food intake when activated. They received presynaptic inputs from anatomically distributed neurons activated by different anorexigenic agents. Our data suggest that CEl PKC-δ+ neurons constitute an important node that mediates the influence of multiple anorexigenic signals.

RESULTS
CEl PKC-δ+ neurons are activated by diverse anorexigenic signals
PKC-δ labels ~50% of CEl GABAergic neurons20. To investigate the involvement of CEl PKC-δ+ neurons in the regulation of feeding, we first monitored c-Fos expression after intraperitoneal injection of several known anorexigenic signals: cholecystokinin (CCK), which mimics satiety23, lithium chloride (LiCl), which induces nausea and visceral malaise24, and lipopolysaccharide (LPS), which triggers a wide range of inflammatory and sickness responses25,26. Double immunostaining for c-Fos and PKC-δ revealed that these anorexigenic agents induced substantial c-Fos expression in CEl PKC-δ+ neurons and that PKC-δ marked a sizeable fraction of c-Fos+ neurons activated by CCK or LiCl (but not LPS; Fig. 1 and Supplementary Fig. 1a). Notably, almost 80% of Foxa+ neurons activated by CCK in CEl expressed PKC-δ (Supplementary Fig. 1a).

Because CCK has been suggested to be a mediator of satiety23, we investigated whether CEl PKC-δ+ neurons become activated during satiation. Indeed, we observed a substantial c-Fos induction in CEl PKC-δ+ neurons 3 h after ad libitum feeding by mice that had been food-deprived almost 80% of Fos+ neurons activated by CCK in CEl expressed PKC-δ (Supplementary Fig. 1a).

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PKC-δ+ neuronal activity is required for anorexigenic influences

We next investigated whether the activity of CEi PKC-δ+ neurons is required for feeding inhibition by the anorexigenic agents that activated these cells. To do this, we pharmacogenetically inhibited CEi PKC-δ+ neurons using an inhibitory DREADD GPCR (hM4Di) that is activated by the pharmacologically inert ligand clozapine N-oxide (CNO)27. Selective expression of hM4Di in these neurons was achieved using a transgenic mouse line in which Cre recombinase is specifically expressed in PKC-δ+ neurons20. Because PKC-δ+ is expressed in multiple brain regions20, we restricted expression of hM4Di to CEi via intracranial stereotaxic injection of a Cre-dependent adeno-associated virus (AAV) encoding hM4Di (AAV8-hSyn-DIO-hM4Di-mCherry)28. Electrophysiological analysis in acute amygdala slices29 confirmed that CNO inhibited spiking in hM4Di-mCherry–expressing PKC-δ+ neurons (Fig. 2a).

Anorexigenic agents such as CCK, LiCl and LPS strongly inhibit feeding in food-deprived mice. Because these agents activate PKC-δ+ neurons, we tested whether pharmacogenetic inhibition of PKC-δ+ neuronal activity could overcome the effect of these anorexigenic agents. c-Fos induction by CCK, LiCl and LPS in PKC-δ+ neurons expressing hM4Di was substantially reduced by CNO co-administration (Supplementary Fig. 2a,b), indicating that pharmacogenetic inhibition of these neurons was effective in vivo. Notably, inhibition of these neurons rescued the effect of CCK to inhibit feeding in food-deprived animals (Fig. 2b). Food intake (measured 20 min after drug administration) was restored to a level statistically indistinguishable (unpaired t test, P = 0.37) from control mice that did not receive CCK (Fig. 2b). Administration of saline rather than CNO to CCK-treated hM4Di-expressing mice failed to rescue feeding (Fig. 2b). Similarly, CNO failed to rescue feeding in CCK-treated mice expressing humanized Renilla GFP (hrGFP)28 rather than hM4Di in CEi PKC-δ+ neurons (Fig. 2b). Pharmacogenetic inhibition of CEi PKC-δ+ neurons also overcame the anorexigenic effect of LiCl, whereas feeding inhibition induced by LPS was unaffected (Fig. 2b). This `rank-order potency' of rescue from anorexia (CCK>LiCl>LPS) mirrored the rank order potency of c-Fos induction in CEi PKC-δ+ neurons (Fig. 1d,e). Pharmacogenetic silencing of CEi PKC-δ+ neurons did not increase food intake in animals deprived of food for 24 h in the absence of anorexigenic drugs (Fig. 2b), indicating a true epistatic interaction with the drugs rather than an independent compensating effect to promote feeding.

To extend these observations, we asked whether hM4Di/CNO-mediated silencing of CEi PKC-δ+ neurons could overcome the suppression of feeding by bitter tastants in food-deprived mice. Indeed, the addition of quinine to food pellets substantially reduced food intake, and this reduction was reversed by CNO administration to mice expressing hM4Di in PKC-δ+ neurons (Fig. 2c). However, pharmacogenetic silencing of PKC-δ+ neurons did not reduce sensitivity to bitter tastants, as determined using quantitative lickometer assays (Supplementary Fig. 3a,b). This suggests that these neurons gate the intake of potentially toxic food resources rather than controlling gustatory sensitivity or discrimination. Taken together, these data
suggest that CEIC PKC-δ+ neurons mediate feeding inhibition by many (although not all) anorexigenic agents.

Silencing CEIC PKC-δ+ neurons increases feeding in satiated mice
Given that the satiety signal CCK, as well as re-feeding of food-deprived mice to satiety, induced c-Fos in CEIC PKC-δ+ neurons (Fig. 1a–d–g), we asked whether silencing CEIC PKC-δ+ neurons would increase food intake in fed mice. Indeed, CNO injection caused a robust (−2-fold) and significant increase (unpaired t-test, *P = 0.045, n.s. = not significant; **P < 0.01, ***P < 0.001) in food intake in fed mice expressing hrGFP (n = 13 animals) or hM4Di (n = 14 animals) in CEIC PKC-δ+ neurons. Box plots show mean (+), median, quartiles (boxes) and range (whiskers). Unpaired t-test showed a significant effect of silencing. (d) Food intake in CNO-treated fed mice expressing hrGFP (n = 13 animals) or hM4Di (n = 14 animals) in CEIC PKC-δ+ neurons. The time-resolved manipulations afforded by optogenetic silencing of PKC-δ+ neurons allowed us to examine the role of these neurons during re-feeding of food-deprived mice. eNpHR3.0-mediated silencing significantly increased (unpaired t-test, *P = 0.0048) the number of feeding bouts, but not the average bout duration, and caused a trend to a decreased latency to feed (Fig. 3e–h). Cumulative bout analysis (Fig. 3i) indicated that mice with silenced PKC-δ+ neurons took longer to reach plateau than controls (T0.5(eNpHR) = 8.2 min, T0.5(hrGFP) = 4.2 min), suggesting that they achieved satiety more slowly. Together, these data indicate that the normal kinetics of feeding reduction as animals reach satiety requires PKC-δ+ neuronal activity.

Activation of CEIC PKC-δ+ neurons inhibits feeding
The foregoing loss-of-function data raised the question of whether artificial activation of CEIC PKC-δ+ neurons would suffice to inhibit food intake. To test this, we expressed ChR2 in CEIC PKC-δ+ neurons using a Cre-dependent AAV (AAV2-EF1α-DIO-Chr2-EYFP)31. Whole-cell patch-clamp recordings in CEA brain slices from virus-injected PKC-δ-Cre mice confirmed that 473-nm light pulses triggered robust spiking in PKC-δ+ neurons (Fig. 4a).

Figure 2 Activity of CEIC PKC-δ+ neurons is required for the influence of anorexigenic agents. (a) Cell-attached slice recording from a CEIC PKC-δ+ neuron expressing hM4Di-mCherry that was silenced by bath application of CNO. DIC, infrared differential interference contrast image. (b) Food intake in 24-h fasted animals after administration of different anorexigenic agents. The number of animals in each condition is indicated in the bars. Data are presented as mean ± s.e.m. Two-way ANOVA with post hoc Bonferroni t-tests showed a significant effect of CNO silencing (saline, F1,24 = 0.363, *P = 0.55; CCK, F1,31 = 11.7, **P = 0.0018; LPS, F1,32 = 4.50, *P = 0.042; LPS, F1,32 = 0.001, **P = 0.98). (c) Intake of control versus quinine-laced food in 24-h fasted animals expressing hrGFP (n = 5 animals) or hM4Di (n = 5 animals) after intraperitoneal injection of CNO. Note that addition of quinine (bitter) inhibited food intake in controls. Data are presented as mean ± s.e.m. Two-way ANOVA (F1,16 = 9.91, *P = 0.0062) with post hoc Bonferroni t-test showed a significant effect of silencing. (d) Food intake in 24-h fasted animals after administration of different anorexigenic agents. The number of animals in each condition is indicated in the bars. Data are presented as mean ± s.e.m. Two-way ANOVA with post hoc Bonferroni t-tests showed a significant effect of CNO silencing (saline, F1,24 = 0.363, *P = 0.55; CCK, F1,31 = 11.7, **P = 0.0018; LPS, F1,32 = 4.50, *P = 0.042; LPS, F1,32 = 0.001, **P = 0.98). (e) Intake of control versus quinine-laced food in 24-h fasted animals expressing hrGFP (n = 5 animals) or hM4Di (n = 5 animals) after intraperitoneal injection of CNO. Note that addition of quinine (bitter) inhibited food intake in controls. Data are presented as mean ± s.e.m. Two-way ANOVA (F1,16 = 9.91, *P = 0.0062) with post hoc Bonferroni t-test showed a significant effect of silencing. (d) Food intake in CNO-treated fed mice expressing hrGFP (n = 13 animals) or hM4Di (n = 14 animals) in CEIC PKC-δ+ neurons. Box plots show mean (+), median, quartiles (boxes) and range (whiskers). Unpaired t-test showed a significant effect of silencing. (d) Food intake in CNO-treated fed mice expressing hrGFP (n = 13 animals) or hM4Di (n = 14 animals) in CEIC PKC-δ+ neurons. Box plots show mean (+), median, quartiles (boxes) and range (whiskers). Unpaired t-test showed a significant effect of silencing.
Bilateral optogenetic activation of CEi PKC-δ neurons in vivo at 5 Hz (Online Methods), a spiking rate comparable with that measured for these neurons in vivo\(^{30,32}\), strongly inhibited food intake during a 20-min test in mice that were deprived of food for 24 h (Fig. 4b). Normal feeding resumed after the offset of photostimulation (Fig. 4b).

A similar inhibition was observed in fed mice (Fig. 4c), whereas controls expressing hrGFP in PKC-δ neurons showed no effect (Fig. 4b,c). Photoactivation of PKC-δ neurons also interrupted ongoing feeding in the home cage within a few seconds following the onset of photostimulation (Fig. 4d,e and Supplementary Movie 1). Activation also transiently inhibited drinking in water-deprived mice, although this effect was modest and, in contrast with the effect on food intake, was only observed during the first few minutes of the 20-min test (Supplementary Fig. 3c–g). Notably, feeding inhibition by PKC-δ neuron activation was not rescued when drinking water was provided (Supplementary Fig. 3h), suggesting that the suppression of food intake is not a result of increased thirst.

To gain more insight into the inhibitory effect of CEi PKC-δ neurons on feeding, we performed a time-resolved analysis of feeding in mice that were deprived of food for 24 h. This analysis revealed that activation of CEi PKC-δ neurons increased the latency to approach the food (approach defined as the time at which the animal's nose touched the food pellet; Fig. 4f,g), and decreased the number of approaches (Fig. 4f,h). Thus, activation of CEi PKC-δ neurons inhibited both the appetitive and consummatory phases of feeding behavior.
Notably, mating behavior toward a female intruder mouse was not affected (Supplementary Fig. 3), suggesting that PKC-δ+ neurons do not inhibit approach to any appetitive stimulus, but specifically inhibit approach to food resources.

**Activation of CEI PKC-δ+ neurons does not increase anxiety**

The CEA is well known for promoting anxiety and fear\(^\text{33–35}\). This raised the possibility that activation of CEI PKC-δ+ neurons could inhibit feeding indirectly by promoting anxiety or fear. Several lines of evidence suggest that this is not the case. First, in three different assays of anxiety, including the elevated plus maze, open field test and light-dark box test, optogenetic activation of CEI PKC-δ+ neurons was anxiolytic rather than anxiogenic (Fig. 5a–c). Second, the inhibition of feeding caused by optogenetic activation of CEI PKC-δ+ neurons was not reversed by the anxiolytic drug diazepam (DZP; Fig. 5d), although DZP did increase food intake in control mice (Fig. 5e). Third, activation of CEI PKC-δ+ neurons did not promote freezing or reduced locomotion (Supplementary Fig. 4a,b). Finally, activation did not produce conditioned place aversion, a measure of discomfort or unpleasantness\(^\text{36}\) (Supplementary Fig. 4c–e). These data suggest that the anorexie effect of CEI PKC-δ+ neuronal activation is unlikely to be a result of increased fear or anxiety.

To investigate whether CEI PKC-δ+ neuron activation might inhibit feeding by promoting visceral malaise, we asked whether it could serve as an unconditional stimulus (US) in conditioned taste aversion (CTA) assays. Stimuli such as LiCl that cause visceral malaise are able to act as a US in such assays\(^\text{24}\). However, activation of PKC-δ+ neurons did not promote CTA, although control LiCl administration to mice expressing hrGFP in CEI PKC-δ+ neurons did serve as an effective US for CTA (Supplementary Fig. 4f). This positive control indicates that expression of virally encoded proteins in these neurons does not interfere with CTA per se. Together, these data suggest that activation of PKC-δ+ neurons does not inhibit feeding by promoting visceral malaise.

**Monosynaptic anorexigenically activated inputs to PKC-δ+ neurons**

The observation that CEI PKC-δ+ neurons are activated by, and required for the effect of, several types of anorexigeneic agents raised the question of which circuits these agents act through. To address this question, we performed Cre-dependent, rabies virus–based monosynaptic retrograde tracing from CEI PKC-δ+ neurons\(^\text{37,38}\), in combination with c-Fos labeling following treatment with anorexigeneic agents (Fig. 6 and Supplementary Figs. 5 and 6). On the one hand, these agents might all act through a final common pathway presynaptic to CEI PKC-δ+ neurons, in which case c-Fos+ neurons in a single location would be retrogradely labeled (Supplementary Fig. 6a); on the other hand, if c-Fos+ neurons in multiple areas were retrogradely labeled, it would suggest convergence from several pathways onto CEI PKC-δ+ neurons (Supplementary Fig. 6a).

These experiments yielded retrogradely labeled (GFP+) cells in CEA itself, as well as in the ipsilateral basolateral amygdala (BLA), insular cortex, lateral parabrachial nucleus (LPB) and several other brain regions (Supplementary Fig. 5). Moreover, different anorexigenic agents activated c-Fos expression (relative to saline-injected controls) in these retrogradely labeled cells in a combinatorial manner: LiCl and quinidine induced c-Fos expression in rabies-GFP+ neurons in all of these areas (LPB, BLA, and insula), whereas CCK induced c-Fos expression in retrogradely labeled neurons in the LPB and BLA, but not in the insula (Fig. 6a–j and Supplementary Figs. 6 and 7). Slice recordings using ChR2-assisted circuit mapping\(^\text{39}\) confirmed that CEI PKC-δ+ neurons receive monosynaptic excitatory inputs from the insula and LPB (Supplementary Fig. 8a–g). Taken together, these data argue against the idea that the upstream neurons activated by these anorexigenic agents converge on a common target presynaptic to CEI PKC-δ+ neurons, instead indicating that they converge on PKC-δ+ neurons themselves (Supplementary Fig. 6a).

A recent study showed that activation of CGRP+ neurons in the LPB inhibits feeding and that this inhibition is mediated by projections to CEI\(^\text{5}\). Cre-dependent monosynaptic retrograde tracing from CEI PKC-δ+ neurons combined with CGRP immunostaining revealed that over 60% of retrogradely labeled LPB neurons expressed CGRP (Fig. 6k). These data suggest that PKC-δ+ neurons are synaptic targets...
of CGRP+ LPB neurons that project to CEl, as well as targets of CGRP− LPB neurons. Consistent with these retrograde tracing experiments, slice recordings revealed that CEI PKC-δ− neurons receive excitatory inputs from the LPB (Supplementary Fig. 8c–g).

Local CEA inhibitory circuits mediate feeding inhibition

As a first step toward investigating the circuit-level mechanisms through which CEI PKC-δ− neurons might exert their inhibitory influence on feeding, we mapped their downstream projections using Cre-dependent fluorescent tracers. These experiments revealed local projections of PKC-δ− neurons in CEA (lateral and medial divisions), as well as longer range projections to the bed nucleus of the stria terminalis (BNST) and the LPB (Supplementary Fig. 8h). Using ChR2-assisted circuit mapping, we confirmed that PKC-δ− CEI neurons mono-synaptically inhibit PKC-δ− CEA neurons (20 of 20 neurons tested evoked inhibitory postsynaptic currents, IPSCs), as well as BNST neurons (IPSCs evoked in 9 of 10 neurons); however, inhibition of LPB neurons was weak (only 1 of 6 neurons showed a small IPSC) (Supplementary Fig. 8i–n).

To determine which of these outputs might contribute to the influence of CEI PKC-δ− neurons on feeding, we first optogenetically activated the projections of these neurons to the LPB and BNST. No inhibition of feeding was observed (n = 5 mice tested for each projection; data not shown). To determine whether intra-amygdalal inhibition is involved, we bilaterally infused the GABA_A receptor antagonist bicuculline into CEa while optogenetically activating PKC-δ− neurons (Fig. 7a). Amygdala acute slice recordings revealed that bicuculline blocked IPSCs in PKC-δ− neurons evoked by optogenetic activation of PKC-δ− neurons (Fig. 7b). Photostimulation-induced inhibition of food intake was blocked by bicuculline infusion (Fig. 7c), suggesting that local GABAergic signaling in CEa is required for the inhibitory influence of GABAergic PKC-δ− neurons on feeding.

These results prompted us to investigate the intra-amygdalal targets through which PKC-δ− neurons might inhibit feeding. As described earlier, inhibition of these neurons using hM4Di not only reduced c-Fos induction in PKC-δ− cells by anorexigenic signals, but concurrently increased c-Fos expression in CEI PKC-δ− neurons (Supplementary Fig. 2). These data, and the fact that CEI PKC-δ− neurons monosynaptically inhibit CEI PKC-δ− neurons (20, 32) (Fig. 7b), raised the possibility that the latter neurons exert an influence on feeding opposite to that of PKC-δ− neurons. If so, then inhibition of these PKC-δ− neurons might inhibit feeding, similar to the effect of activating CEI PKC-δ− neurons.

To test this hypothesis, we used a ‘Cre-out’ strategy in which PKC-δ−Cre mice were injected in CEa with an AAV in which Cre recombines excise, rather than activates, the eNpHR3.0 coding sequence (Fig. 7d). In this way, PKC-δ− neurons in CEa, rather than PKC-δ− neurons, should express eNpHR3.0 (Fig. 7d). Slice recordings confirmed photostimulation-dependent silencing of PKC-δ− neurons, whereas PKC-δ− neurons were not obviously affected (Fig. 7d) and Online Methods). Food intake in mice deprived of food for 24 h was partially, but significantly (paired t test, P = 0.0030), inhibited after silencing PKC-δ− neurons in vivo (Fig. 7e) in a manner that scaled with the level of eNpHR3.0 expression in CEa (Supplementary Fig. 9). These data support the idea that CEI PKC-δ− neurons exert a net positive influence on feeding, in contradistinction to PKC-δ− neurons.

To extend this result, we asked whether activation of PKC-δ− neurons might, conversely, increase food intake. To do this, we first activated genetically defined subsets of CEI PKC-δ− neurons using two different Cre lines: Tac2-Cre (obtained from the Allen Institute for Brain Science; http://connectivity.brain-map.org/transgenic/experiment/131034318) and CRF-Cre (of which 50 and 70% are PKC-δ−, respectively; Supplementary Fig. 10a–c). However optogenetic activation of these neurons yielded neither increased nor decreased feeding (Supplementary Fig. 10d–g). To activate other populations of CEI PKC-δ− neurons that might not be covered by these Cre lines, we expressed ChR2 in them using the same Cre-out approach described earlier for eNpHR3.0 (Fig. 7f). Activation of these PKC-δ− neurons did not increase food intake in 24-h fasted mice or in fed mice (Fig. 7g,h). This may reflect a ceiling effect on feeding or incomplete...
elimination of ChR2 expression from PKC-δ neurons that exert a counteracting influence.

Given that inhibition of PKC-δ neurons likewise failed to increase food intake in 24-h fasted mice, but reversed the inhibitory effect of some anorexigenic agents (Fig. 2b), we asked whether activation of PKC-δ neurons would similarly attenuate the effects of anorexigenic agents. Indeed, optogenetic activation of CEA PKC-δ neurons significantly rescued (unpaired t test, P = 0.014, CCK + control versus CCK + activation) the inhibition of feeding by CCK, although complete recovery of food intake to control levels was not achieved (Fig. 7i). Inhibition of feeding by LiCl was not significantly rescued (food intake: control, 0.018 ± 0.003 g; activation, 0.028 ± 0.010 g; unpaired t test, P = 0.40). These data suggest that the influence of at least one anorexigenic signal (CCK) may be exerted, at least in part, through inhibition of CEA PKC-δ neurons.

DISCUSSION
CEI PKC-δ+ and PKC-δ− neurons exert opposing influences on feeding
Although the CEA has been implicated in the control of feeding, prior results have been contradictory, likely reflecting the cellular heterogeneity of this structure and the inadequate specificity of the perturbational methods used. We found that PKC-δ neurons in CEI have a central role in mediating the inhibitory influence on feeding of diverse anorexigenic signals, as well as in satiety, and can exert a marked and rapid inhibitory influence on feeding when activated. We also found that some CEA PKC-δ neurons may exert an opposite-direction influence on feeding (although the resolution of our method did not allow us to distinguish whether these neurons are present in CEI, CEm or both). Furthermore, both populations mediated the influence of some anorexigenic agents to suppress food intake, but in opposite directions: PKC-δ− neurons must be active, whereas PKC-δ+ neurons must be inhibited, to achieve complete suppression of feeding. These functional data are consistent with our observation that anorexigenic agents increased c-Fos expression in CEI PKC-δ+ neurons and reduced its expression in PKC-δ− neurons (Supplementary Fig. 2). The presence of intermingled populations of neurons in CEA with opposing influences on feeding may explain previous inconsistencies in the literature.

CEI PKC-δ+ neurons as a locus for multiple anorexigenic influences
CEI PKC-δ+ neurons were activated by multiple anorexigenic signals, as well as by bitter tastants and satiety. Inhibition of these neurons impaired the influence of some, but not all, anorexigenic agents tested (CCK and LiCl, but not LPS, which induces a wide range of inflammatory and sickness responses). It also blocked the influence of quinine, a bitter tastant, to inhibit feeding and increased the time to satiation in food-deprived animals. Taken together, these data suggest that PKC-δ+ neurons mediate the effects of multiple (but clearly not all) inhibitory influences on feeding. This finding raises the question of whether these influences converge on a final common input to PKC-δ− neurons or act through multiple, parallel circuits that converge at the level of these neurons themselves. Our combined c-Fos and monosynaptic retrograde tracing data argue against the former and in favor of the latter possibility. However, further studies will be required to determine whether each of these upstream inputs acts through the same or through different PKC-δ− neurons.

A recent study has shown that LPB CGRP+ neurons inhibit feeding via projections to CEI. Our data indicate that ~60% of LPB inputs to PKC-δ− neurons were derived from CGRP+ neurons. Whether the CEI PKC-δ− neurons that receive input from CGRP+ neurons are activated by anorexigenic agents and mediate feeding inhibition, and whether inputs from CGRP+ LPB neurons also inhibit feeding via inputs to these neurons, are unclear and remain to be investigated. We note, moreover, that LPB CGRP+ neurons mediate the anorexigenic influences of LiCl and LPS, but not CCKδ, whereas CEI PKC-δ− neurons mediate the inhibitory influences of CCK and LiCl, but not LPS. This suggests that other inputs to PKC-δ− neurons may mediate the inhibitory influence of CCK and that the LPB represents only one of multiple anorexigenic pathways that converge on these CEI neurons. Notably, our ChR2-assisted circuit-mapping studies revealed that most, if not all, of the upstream inputs to PKC-δ− neurons that we have tested are excitatory. The fact that these CEI neurons are GABAergic therefore suggests that they may represent the first inhibitory relay in the central processing of inhibitory influences on food intake.

CEI PKC-δ+ neurons, feeding and fear
The CEA is thought to be involved in promoting a variety of aversive states, including fear, anxiety and visceral malaise. Because fear and anxiety reduce appetitive behaviors, including feeding, it is possible that activation of CEI PKC-δ+ neurons might inhibit feeding indirectly by promoting such aversive states. However, using multiple behavioral assays of fear and anxiety, we found no evidence that activation of CEI PKC-δ+ neurons, under conditions that inhibit feeding, made mice more fearful or anxious; on the contrary, the influence of their activation was anxiolytic. These data are consistent with previous studies indicating that CEI PKC-δ+ neurons exert an inhibitory influence on conditioned fear. Moreover, innate fear, as expressed by unconditional freezing, was not evoked by optogenetic activation of PKC-δ+ neurons at frequencies that inhibit feeding (however, stimulation at threefold higher frequencies did promote freezing).

Finally, although fear and anxiety inhibit not only feeding, but also other appetitive behaviors such as mating, activation of CEI PKC-δ+ neurons, in contrast, had no inhibitory effect on mating.

These data notwithstanding, we cannot exclude the possibility that the optogenetic activation of PKC-δ+ neurons inhibits feeding indirectly via an influence on some other behavioral or motivational state that in turn reduces food intake. For example, the CEA is also involved in autonomic processes such as the control of heart rate and blood pressure, and we have not ruled out that the effect of CEI PKC-δ+ neuron activation to inhibit feeding may involve some of these physiological influences. Nevertheless, the fact that PKC-δ− neurons are activated by anorexigenic drugs in vivo, and, notably, that inhibiting these neurons reduces the anorexigenic influence of some of these drugs, makes it more likely, in our opinion, that these neurons are involved in mediating the influence of certain anorexigenic signals.

The relationship between the CEI PKC-δ− neurons involved in the control of feeding and those involved in the regulation of conditioned fear remains to be established. Our c-Fos data indicate that ~20–25% of PKC-δ− neurons are activated by anorexigenic drugs (Supplementary Fig. 1). This 20–25% may represent a deterministic subset of the PKC-δ− population. In other words, there may be different subsets of CEI PKC-δ− neurons that regulate the expression of conditioned fear and the influence of anorexigenic agents, respectively. Our previous study provided evidence of molecular heterogeneity in the CEI PKC-δ− population; for example, 40% of these neurons express the neuropeptide enkephalin, whereas 65% express the oxytocin receptor. Whether such heterogeneity is relevant to the control of fear versus feeding is not clear. Alternatively, a common or overlapping set of PKC-δ− neurons in CEI may be involved in promoting aversive states, as well as by bitter tastants and satiety.
in both fear and in feeding according to their state of activity and/or the functional networks in which they participate. Further studies will clearly be required to resolve these alternatives.

**How do CEl PKC-δ+ neurons inhibit feeding?**

The neural circuits through which CEl PKC-δ+ neurons exert their inhibitory influence on feeding remain to be established. Because feeding is promoted by hypothalamic structures such as the arcuate nucleus (Arc) and lateral hypothalamus (LH), it seems likely that direct or indirect projections from CEA to these structures are involved in the inhibition of feeding. Whether these projections derive from CEl PKC-δ+ neurons or from other CEA populations that are targets of PKC-δ+ neurons is not yet clear. It has recently been shown that GABAergic neurons in BNST promote feeding via projections to the LH. Because CEl PKC-δ+ neurons project to and make inhibitory synapses in the BNST, it is attractive to think that these GABAergic neurons might suppress feeding via inhibition of LH-projecting BNST neurons. However, our attempts to inhibit feeding by stimulating CEl PKC-δ+ neuron projections to the BNST were unsuccessful, although we cannot rule out technical reasons for this negative result (for example, insufficient levels of ChR2 expression in projections).

Although other extra-amygdalar projections of CEl PKC-δ+ neurons (http://connectivity.brain-map.org/) may be involved in feeding suppression, these neurons also make extensive projections in the amygdala. We found that local infusion of bicuculline in CEA could block feeding inhibition caused by optogenetic activation of PKC-δ+ neurons. This epistasis suggests that intra-amygdalar GABAergic synapses made by PKC-δ+ neurons are required for their anorexigenic function, although we cannot rule out an indirect effect as a result of dis-inhibition of other neighboring amygdala populations. Whether these synapses inhibit amygdalar subpopulations that make long-range (direct or indirect) projections to feeding centers or rather minimize local feedback inhibition onto PKC-δ+ neurons (for example, from CEl PKC-δ- neuron), is not clear and will require further study.

Our results identify a population of central amygdala GABAergic neurons that are important for mediating the influence of signals that inhibit feeding behavior. The fact that optogenetic activation of these neurons strongly reduced food intake further suggests that these cells could provide a target for therapeutic interventions to treat obesity, anorexia or other eating disorders.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

H.C. designed and performed the experiments and wrote the manuscript. W.H. generated BAC constructs for PKC-δ- transgenic mice. T.E.A. made the virus constructs for Cre-oe1 ChR2 and Cre-out eNpHR. D.J.A. contributed to experimental design and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

All experimental protocols were conducted according to US National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the California Institute of Technology.

Mice. Male mice were housed on a 12-h light (7 a.m.–dark 7 p.m.) cycle with ad libitum access to water and rodent chow (Lab Diet, 5001) unless otherwise noted. Male mice were used for all of the behavioral experiments, and male and a few female mice were used in brain slice electrophysiology. Ai14 Cre-reporter mice45 have been described previously.

Virus. All the AAV viruses were produced at the Gene Therapy Center Vector Core in the University of North Carolina at Chapel Hill. Rabies virus was purchased from Salk Gene Transfer, Targeting and Therapeutics Core. Behavioral experiments were usually performed 4 weeks after virus injection. For rabies virus tracing, AAV1-EF1α-FLEX-TVaMCherry and AAV1-Ca-FLEX-RG were mixed at a ratio of 1:4 and then injected in CEA. About 3 weeks later, EnvA G-deleted Rabies-GFP were injected in the same place. Mice were perfused 5–6 d later and their brains were sectioned to check the monosynaptic upstream neurons. Each virus and its serotype are indicated in the main text.

Animal surgery. Mice survival surgeries were performed as described previously20. Briefly, 2–4-month-old mice were deeply anesthetized with 5% isoflurane (vol/vol) in oxygen and kept at 1.5% isoflurane during surgery. Surgery was performed with a stereotaxic frame (Kopf). Viral injection coordinates (in mm, midline, Bregma, dorsal surface): CEA (±2.85, −1.40, −4.72), LPB (±1.50, −5.30, −3.40), LH (±1.20, −1.55, −5.20), ventral BNST (±1.00, +0.00, −4.70), insula (±3.90, +0.05, −4.00). Ferrule fibers were implanted −0.5 mm above the injection sites. After ferrule fiber implanted, dental cement (Metabond) was used to anchor the fiber to the skull. For behavioral experiments that require drug infusion, guide cannulas (26 G, PlasticsOne) were implanted −0.8 mm above the injection sites. Mice were single housed after surgery. At least 3–4 weeks were allowed for mice recovery and viral expression after surgery.

In vivo photostimulation. Several lasers (Shanghai DreamLaser: 473 nm, 50 mW; 593 nm, 50 mW; Crystal Laser: 445 nm, 50 mW; 473 nm, 50 mW; 561 nm, 50 mW) were used to deliver light. An Accupulser Signal Generator (World Precision Instruments, SYS-A310) was used to control the frequency and pulse width of the laser light. Light was delivered to the brain through an optic fiber (200-µm-diameter core, NA 0.22, Doric Lenses) connected with the implanted ferrule fiber by a zirconium sleeve. The light power in the brain regions varied as 7.5−8 mW mm−2. At 5-Hz, 10-ms pulse width light was used in all the optogenetic activation experiments. 5-Hz light stimulation was used because PKC-δ neurons show an average firing rate of 3–5 Hz in in vivo recordings42. CEA neurons fires at a rate of less than 5 Hz at innocuous stimulus and fire more than 10 Hz in response to noxious stimuli20, and PKC-δ neurons can be triggered robustly to fire action potentials by ≤10-Hz light pulses, as shown by whole-cell patch-clamp slice recordings45.

Continuous light illumination in the brain over a long time generates heat48, so pulsed light was used for optogenetic silencing during the 20-min feeding test. And because PKC-δ neurons do not have rebound firing after the light is off as tested in slice recording (Fig. 3a), a protocol of alternating 20-s light on and 10 s light off was used to silence CII PKC-δ neurons (Fig. 3b). Because some types of PKC-δ neurons have rebound firing after hyperpolarization20, a different protocol of 20-Hz, 20-ms light pulse (calibrated light power in CEA, −2 mW mm−2) was used to silence PKC-δ neurons (Fig. 7d). As tested in brain slices, neurons expressing eNPHR3.0 can be hyperpolarized by different wavelengths (we tested 445 nm, 473 nm, 561 nm, 593 nm and 633 nm); given that mice eat normally with 20-Hz, 20-ms, 593-nm light pulses at a power less than 10 mW mm−2 (food intake in mice with light: 0.29 ± 0.03 g; food intake in mice without light: 0.30 ± 0.02 g; t-test, P = 0.78), we used the food intake when mice were implanted with optic fiber, but received no light, as control (Fig. 7e).

Pharmacology. CNO (Enzo Life Sciences-Biomol, BML-NS105-0005) were freshly dissolved in injection saline (9 g l−1 NaCl) and intraperitoneally injected at 5 mg per kg of body weight for HM4Di silencing or 2 mg per kg for hM3Dq activation. Other compounds used for intraperitoneal injection: CCK (5 µg per kg, Tocris), Diazepam (1 or 2 mg per kg, Hospira), LiCl (150 mg per kg, Sigma, prepared in 150 mM) and LPS (0.1 mg per kg, Sigma, L4516-1MG). Behavioral tests were usually performed 20–30 min after drug injection.

Feeding behavior. For 24-h fasted feeding test, mice were food-deprived the day before test. Mice were briefly anesthetized with isoflurane and coupled with optic fibers. 15 min after recovery, mice were introduced into a novel empty cage with a regular food pellet (Lab Diet, 5001), and allowed for feeding for 20 min. The weight of the food pellet, including the food debris left in the cage floor after test, was measured to calculate the food intake. For fed feeding test, mice were not food deprived before feeding test. For optogenetic experiments, the light was started just before the mice were introduced into the testing cage. The feeding behavior was videotaped and manually analyzed. For pharmacogenetic experiments, CNO was injected 20 min before the feeding test. All of the feeding tests were performed between 2 p.m. and 7 p.m. Bitter food was prepared by immersing regular food pellets in 10 mM quinine (Sigma) solution for 10 min and dried overnight, and the control taste food was prepared by immersing the food pellets in dH2O for 10 min and dried overnight. In the conditioned taste aversion experiment (Supplementary Fig. 4f), bacon taste food pellets (Newco Distributors, 10 mg tablet) were used as a novel taste food.

Intraoral infusion. A standard ball-tipped gavage needle was put just in the mouth and ~0.2 ml water was slowly infused in ~15 s. To induce c-Fos expression by bitter taster, mice were orally infused with ~0.2 ml 10 mM quinine solution for 15 s; animals infused with water or 0.5 M sucrose solution were used as controls. Mice were perfused 1–1.5 h after infusion.

Anxiety test. Standard elevated plus maze, open field and light/dark box tests were used. A platform (74 cm above the floor) with two opposing open arms (30 × 5 cm) and two opposing closed arms (30 × 5 × 14 cm) was used as elevated plus maze. Mice were placed into the center of the plus and their behavior was tracked for 5 min. A square plastic box (50 × 50 × 30 cm, a 25 × 25 cm square center was defined as center in analysis) was used as open box. Mice were placed individually in the center of the box, and their behavior was tracked for 10 min. A plastic box consisting of a dark (black with cover, 16 × 30 cm) and a bright (white without cover, 34 × 50 cm) compartment, which are connected by a central opening (7 × 14 cm) at the floor level, was used as a light/dark box. Mice were placed individually in the center of the light area. The behavior was tracked for 10 min. All the behaviors were videotaped and analyzed offline with Ethovision.

Place preference test. Place preference tests were performed with a three-chamber system described previously48, in which Chamber A and Chamber B have different visual and tactile cues by having distinct walls and floors, while the center chamber is a neutral plastic enclosure. Mice were allowed to explore all three chambers for 15 min on day 1, and restricted in either chamber A or B for 20 min with light delivered on day 2. Mice were then allowed to explore all three chambers for 15 min on day 4. The behavior was videotaped and analyzed offline with Ethovision.

Behavioral tests were performed by an investigator with knowledge of the identity of the experimental groups versus control groups.

Immunohistochemistry and histology. All mice after behavioral test were perfused and checked for virus expression and implanted fiber or cannula location. For immunofluorescent staining, mice were transcardially perfused with 20 ml phosphate-buffered saline (PBS) followed by 20 ml 4% paraformaldehyde (wt/vol) in PBS. For cryosection staining, brains were dissected out and immersed in 15% sucrose (wt/vol) overnight. Cryosections of 30-µm thickness were processed. For the vibratome sectioning, brains were removed and postfixed in 4% paraformaldehyde overnight, then the sections were cut with a vibratome (Leica, VT1000S) at 100 µm thickness. Sections were stained with primary antibody at 4 °C overnight in a blocking solution containing 1% BSA or 5% donkey serum and 0.5% Triton X-100 (wt/vol). After three 10-min washes in PBS, standard Alexa Fluor secondary antibodies (Invitrogen, 1:250; A21141 and A21206 for PKC-δ, and A11057 for c-Fos) were used at 25 °C for 1 h. Sections were then washed three times for 10 min each in PBS and mounted in Fluo
Gel (17985-10; Electron Microscopy Sciences, with DAPI) and viewed under an Olympus confocal microscope. Primary antibodies used: mouse antibody to PKC-δ (BD Biosciences, 610398, 1:500), rabbit antibody to CGRP (Bachem, T4032, 1:500), goat antibody to c-Fos (Santa Cruz Biotech, sc-52-G, 1:250).

Electrophysiological slice recordings. Mouse brain slice was prepared as described20. In brief, mouse coronal sections of 250-µm thickness were cut with a vibratome (Leica, VT1000S), using ice-cold glycerol-based ACSF containing 252 mM glycerol, 1.6 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, 11 mM glucose, oxygenated in carbogen (95% O2 balanced with CO2) for at least 15 min before use. Slices were recovered for at least 1 h at 32 °C and then kept at 25 °C in regular ACSF containing 126 mM NaCl, 1.6 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, 11 mM glucose, oxygenated with carbogen. The fluorescence of the cells was detected by a fluorescence video microscopy (Olympus BX51). Whole-cell voltage, current-clamp or cell-attached recordings were performed with a MultiClamp 700B amplifier and Digidata 1440A (Molecular Devices). The patch pipette with a resistance of 5-8 MΩ was filled with an intracellular solution containing 135 mM potassium gluconate, 5 mM EGTA, 0.5 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 2 mM MgATP and 0.1 mM GTP, pH 7.2, 290–300 mOsm. Data were sampled at 10 kHz, filtered at 3 kHz and analyzed with pCLAMP10 software.

For photostimulation in brain slice, several lasers (Shanghai DreamLaser, 473 nm, 50 mW; 593 nm, 50 mW; Crystal Laser, 561 nm, 50 mW) were used to deliver pulsed light or continuous light, the typical power at the specimen ranges from 0.1 to 10 mW mm−2, as measured with a photodiode. In ChR2-assisted circuit-mapping experiments, 2-ms light pulses were used to trigger IPSCs or EPSCs, and a 0.1 µs 0.1 mV was applied at the same time of light trigger to help identify the start of light pulse.

Statistics. Data are represented as means ± s.e.m. or box-and-whister plots. P values were calculated by paired (within subjects comparisons) or un-paired (between subjects comparisons) Student’s t test, and a value of smaller than 0.05 was consider significant. For comparisons across more than two groups, data were analyzed using one-way ANOVA and adjusted with the Bonferroni’s correction. For data with more than one independent variable, two-way ANOVA was used. Data were analyzed with GraphPad Prism Software.

A Supplementary Methods Checklist is available.

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