Genetic and Functional Cartography of the Central Amygdala

Matthew T. Soleiman

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Committee:

Richard D. Palmiter (Chair)

David J. Perkel

Charles C. Chavkin

Paul E. M. Phillips

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Matthew T. Soleiman

University of Washington

Abstract

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Matthew T. Soleiman

Chair of the Supervisory Committee:

Professor Richard D. Palmiter

Department of Biochemistry

The central nervous system includes an array of neuronal types classified across multiple features. Genetic dissection of the lateral division of the central amygdala (CeAl) has revealed two functionally distinct neuronal populations expressing the relatively non-overlapping molecular markers, protein kinase C δ (PKCδ) and somatostatin (SOM). Here, I leverage genetic access to neurons expressing the receptor for calcitonin gene-related peptide (CGRPR) to uncover the entire CeAl. Despite rostrocaudal gradients of $PKC\delta^+$ and SOM^+ neurons, $CGRPR^+$ neurons are present across its entire axis. In the caudal CeAl, $CGRPR^+$ neurons coexpress PKCδ and SOM, although to differing extents. Like CGRPR expression, functional activation by satiety recruits the entire CeAl. Hence, these data urge an update to CeAl cell types and their activation. Future monitoring and manipulation of the CeAl must be aware of potential genetic gradients.

Introduction

The central nervous system consists of a diverse collection of neuron types, reflecting differences in gene expression, connectivity, morphology, electrophysiological properties, and perhaps most importantly, function (Luo, Callaway & Svoboda, 2008; Fishell & Heintz, 2013, Seung & Sumbul, 2014). Completely identifying its cell types and their respective functions represents one of the major goals and obstacles of modern neuroscience (BRAIN Working Group Report, 2014). This aim has been aggressively tackled in the neuroanatomical context of the central amygdala (CeA), one of the many inter- and intraconnected nuclei of the larger amygdala structure (Sah et al., 2003; Ehrlich et al., 2009).

The CeA receives direct input from within and outside the amygdala, thereby allowing its potential integration and encoding of a wide array of information (Sah et al., 2003). Primarily composed of GABAergic neurons, the CeA itself can be further subdivided into the lateral capsular, lateral, and medial subdivisions (Allen Mouse Brain Atlas, **Fig 1a**), with neurons of the lateral capsular and later divisions (together the CeAl) inhibiting the medial CeA (CeAm) (Huber et al., 2005; Cassell et al., 1999). CeAm neurons in turn target a number of extra-amygdala populations (LeDoux et al., 1988; Gray et al, 1989; Viviani et al., 2011; **Fig 1b**).

While the CeA was initially thought of as a passive output station for the expression of fear (Samson et al., 2005), the CeAl and its plasticity now seem to actively contribute to its conditioning (Li et al., 2013; Penzo et al., 2014; Han et al., 2015). Within the past several years, two relatively non-overlapping, reciprocally connected CeAl neuronal populations have been shown to be recruited during fear learning and expression – those expressing protein kinase C delta (PKCδ) and the neuropeptide somatostatin (SOM), respectively. $PKC\delta^+$ neurons selectively inhibit vasopressin receptor-expressing CeAm neurons projecting to the ventrolateral periaqueductal grey (PAGvl) (Haubensak et al., 2010; Huber et al., 2005; **Fig 1b**). Because activation of the CeAm-PAGvl circuit is thought to promote defensive fear in the form of immobility or "freezing," inhibition of $PKC\delta^+$ neurons enhances fear conditioning (Haubensak et al., 2010). In contrast, SOM^+ neurons are sufficient for freezing and necessary for fear conditioning/expression, likely due to local inhibition of $PKC\delta^+$ neurons and long-range inhibition of PAGvl neurons (Li et al., 2013; Penzo et al., 2014).

Despite these identified cell types and their microcircuitry, the CeA expresses a large number of other genes, including but not limited to those encoding other neuropeptide transmitters and receptors (**Table 1**; *e.g.* Cassell et al., 1986; Huber et al., 2005). Expression of these other genes does not seem to cleanly segregate into $PKC\delta^+$ or SOM^+ populations however, making classification of CeA cell types using single molecular markers difficult. For example, only about half of neurons expressing *Tac2* (encoding the neuropeptide neurokinin B) express PKCδ, and *vice versa* (Cai et al., 2014). Moreover, proportions of $PKC\delta^+$ neurons coexpress enkephalin (ENK) and corticotropin-releasing hormone (CRH) (Haubensak et al., 2010; Cai et al., 2014), but ENK and CRH are virtually non-overlapping (Day et al., 1999). Beyond the issue of molecular coexpression, CeA cell types have also not been fully related to the region's circuit inputs or outputs (Cai et al., 2014; Penzo et al., 2015; Sah et al., 2003).

Here, I broaden the scope and complexity of CeA cell types using genetic techniques and immunohistochemistry (IHC). Because evolutionarily conserved neurons of the parabrachial nucleus (PBN) expressing calcitonin gene-related peptide (CGRP) densely project to the CeAl (Shimada et al., 1985; Schwaber et al., 1988; de Lacalle & Saper, 2000; Carter et al., 2013), I leveraged genetic labeling of CeAl neurons expressing the $G_{\alpha s}$-coupled receptor for CGRP (CGRPR; encoded by the *Calcrl* locus; Chatterjee et al., 1993) to compare their expression pattern to that of PKCδ, SOM, and *Tac2*$^+$ neurons. Using IHC, I also evaluated coexpression of either PKCδ or SOM by CGRPR$^+$ neurons.

Results

Neuroanatomical and molecular characterization of CeAl CGRPR$^+$ neurons

To gain genetic access to CeAl CGRPR$^+$ neurons, Richard Palmiter engineered knock-in mice in which the *Calcrl* promoter drives expression of Cre recombinase (*Calcrl-IRES-Cre*). To genetically label CGRPR$^+$ neurons, *Calcrl-IRES-Cre* mice were subsequently crossed with Cre-dependent tdTomato reporter mice (henceforth *Calcrl;tdT*). Consistent with *Calcrl* expression in vasculature (Oliver et al., 2002), I detected tdTomato expression in blood vessels throughout the entire brain. Across amygdala nuclei, tdTomato was highly expressed in the CeA, primarily within its lateral capsular and to a lesser extent, its lateral subdivisions. Extremely sparse expression was detectable in the medial subdivision. Somatic CGRPR expression strongly mirrors CGRP innervation, arguing for specific and exhaustive targeting of the downstream targets of PBN CGRP neurons (D'Hanis et al., 2007; Carter et al., 2013). tdTomato was also present in the neighboring striatum (**Fig 1c**). This genetic labeling of CGRPR expression recapitulates previous reports of *Calcrl* mRNA labeling (Oliver et al., 1998) and expression of associated proteins required for ligand affinity and receptor signaling (Oliver et al., 2001; Ma, Chabot et al., 2003).

Upon colabeling for PKCδ, I detected a gradient of its expression along the rostrocaudal axis of the CeAl. Hence, Anna Bowen and I performed a quantitative assessment of genetically labeled CGRPR (using *Calcrl;tdT*) and immunolabeled PKCδ across the entire CeAl. Immunolabeling of PKCδ reproduces its genetic labeling (Cai et al., 2014), allowing for direct comparison of *Calcrl;tdT* and anti-PKCδ. While CGRPR was expressed throughout the entire CeAl, paralleling PBN CGRP innervation, PKCδ was restricted to the caudal CeAl (**Figs 1d,e**). The same pattern has been shown for PKCδ mRNA (the Allen Mouse Brain Atlas). To test whether this gradient expression pattern was unique to PKCδ, I also quantified genetic labeling of SOM$^+$ and *Tac2*$^+$ neurons by crossing *Sst-IRES-Cre* (Li et al., 2013) or *Tac2-Cre* (Mar et al., 2012) mice to tdTomato reporters. Again, both markers exhibited expression gradients, albeit to differing extents. Consistent with overlap of PKCδ and *Tac2* expression (Cai et al., 2014), *Tac2* looked virtually identical in its rostrocaudal expression. However, SOM exhibited a subtler gradient pattern – despite expression peaking caudally, it remained somewhat consistent rostrally. I also qualitatively observed more neurons expressing neurotensin (NTS), a neuropeptide coexpressed by ENK$^+$ neurons (Day et al., 1999), to be concentrated caudally (data not shown). Hence, of the molecular markers examined,

CGRPR is the only one that extends into the rostral CeAl. CRH, dynorphin (DYN), and the oxytocin receptor (OXTR) also seem to be expressed in a graded fashion (Panguluri et al., 2009; Jungling et al., 2015; Haubensak et al., 2010).

It has been previously reported that PKCδ marks ~50% of CeAl GABAergic neurons (Haubensak et al., 2010). Given its gradient however, $CGRPR^+$ neurons significantly exceeded those that were $PKCδ^+$ by 3 fold (PKCδ, 426 $\pm$ 34; CGRPR, 1399 $\pm$ 57; n =3 per group, mean $\pm$ SEM; **Fig 1f**). Hence, PKCδ accounts for a smaller number of CeAl neurons than initially estimated. Moreover, CGRPR was significantly greater than SOM or *Tac2* (SOM, 1149 $\pm$ 98; *Tac2*, 330 $\pm$ 28; n = 3 per group, mean $\pm$ SEM).

To determine the extent to which CGRPR and PKCδ are coexpressed in the caudal CeAl, I immunolabeled for PKCδ in *Calcrl;tdT* brains (**Fig 2a**). Supporting the results of Cai et al. (2014), I found a high percentage of caudal $CGRPR^+$ neurons coexpressed PKCδ and *vice versa* (*e.g.* bregma -1.62mm: ~42% $\pm$ 3% of $CGRPR^+$ neurons coexpressed PKCδ and ~50% $\pm$ 2% of $PKCδ^+$ neurons coexpressed CGRPR; n = 3, mean $\pm$ SEM). More rostrally, the former percentage dropped and the latter percentage increased, reflecting consistent CGRPR expression and low PKCδ expression (**Fig 2b**; *e.g.* bregma -.9mm: ~2% $\pm$ .3% of $CGRPR^+$ neurons coexpressed PKCδ and ~84% $\pm$ 10% neurons coexpressed CGRPR). Neuroanatomically, the $CGRPR^+$ $PKCδ^+$ neurons primarily localized to the lateral capsular CeA, while $CGPR^-$ $PKCδ^+$ neurons were found in the lateral CeA. Averaging across bregma, ~16% of $CGRPR^+$ neurons coexpressed PKCδ, while ~53% of $PKCδ^+$ neurons coexpressed CGRPR (**Fig 2c**). Hence, there is a large population of previously unidentified neurons in the CeAl – *i.e.* those that $CGRPR^+$, but $PKCδ^-$

To similarly detect coexpression of CGRPR and SOM, I immunolabeled for SOM in *Calcrl;tdT* brains (**Fig 2d**). As PKCδ and SOM are almost non-overlapping (~13% of SOM^+ neurons coexpress PKCδ, Li et al., 2013), a small subset of $CGRPR^+$ neurons coexpressed SOM (averaging across bregma: ~6% $\pm$ 1%; n =3, mean $\pm$ SEM). However, almost one-fifth of SOM^+ neurons coexpressed CGRPR (averaging across bregma: ~17% $\pm$ .6%; n =3, mean $\pm$ SEM). Because of the more consistent expression of SOM relative to PKCδ, these percentages were similar as a function of bregma (**Figs 2e,f**). The number of immnolabled SOM^+ neurons however were about one-third of genetically labeled neurons; hence, the calculated percentages may overestimate and underestimate actual overlap. Again, $CGRPR^+$ neurons were located mainly in the lateral capsular CeA, with SOM^+ neurons almost exclusively in the lateral CeA (Cassell et al., 1986). Together, the colabeling experiments demonstrate that some $CGRPR^+$ neurons coexpress PKCδ and SOM, although not to the same extent, and highlight the genetic diversity among CeAl neurons.

Functional recruitment of neurons across the CeAl

Are these genetic gradients of PKCδ and SOM functionally relevant? Testing this using fear conditioning is problematic because $PKCδ^+$ neurons are thought to be inhibited during conditioning (Haubensak et al., 2010). However, CeA neurons are also critically involved in appetite suppression (Carter et al., 2013; Cai et al., 2014), with anorexigenic stimuli activating CeA neurons (Riediger et al., 2004; Ferreira et al., 2006) and in

particular those that are PKCδ$^+$ (Cai et al., 2014). Hence, in collaboration with Anna Bowen, I tested whether physiological satiety indeed selectively activates PKCδ$^+$ neurons of the caudal CeAl, or instead recruits neurons across the entire CeAl.

Using the immediate early gene Fos as a molecular marker for recent neuronal activation, I manipulated the degree of the satiety by either providing mice access to chow *ad libitum* at the onset of the dark phase when they normally eat, or first fasted and then refed them during the same period. Following the feeding assay, mice were perfused and I subsequently colabeled for Fos and PKCδ across the rostrocaudal CeAl.

Refed mice ate twice that of *ad-libitum* controls (**Fig 3a**; refed, 182% ± 10%; *ad-lib*, 75% ± 53%; n =3 per group, mean percentage of *ad-lib* pre-testing baseline ± SEM). In parallel, refed mice had more than 2 fold and significantly more CeAl Fos$^+$ neurons (**Fig 3b**; refed, 110 ± 2 Fos$^+$ neurons; *ad-lib* 47 ± 10 Fos$^+$ neurons; n =3 per group, mean across six sections ± SEM), with the number of Fos$^+$ neurons did not significantly differ for the rostral versus caudal CeAl (**Fig 3c**). However, only ~16% of CeAl Fos$^+$ neurons were PKCδ$^+$ in refed mice, a percentage that did not significantly differ from ~18% colabeled neurons in *ad-lib* controls (**Figs 3d,e**). To address whether this low percentage reflected the PKCδ gradient and activation of rostral CeAl neurons, I calculated these percentages separately for only the caudal CeAl, where PKCδ$^+$ expression is greatest. Even then, they remained similarly low (**Fig 3f**). Hence, satiety recruits neurons across the entire CeAl without selectively activating PKCδ$^+$ neurons more caudally.

Because CGRPR$^+$ neurons are present throughout the entire CeAl, their photostimulation at low frequencies suppresses appetite (Han et al., unpublished), and their upstream inputs are activated by anorexigenic stimuli (Carter et al., 2013), Sung Han and I are now examining whether refeeding more selectively activates CGRPR$^+$ neurons.

CeAl CGRPR$^+$ neurons project locally and to the substantia innominata

To identify the projections of CGRPR$^+$ neuron axons, I coinjected Cre-dependent adeno-associated viruses carrying mCherry (to visualize somata) and Synaptophysin-GFP (Syn-GFP, to visualize putative synapses) into the CeA of *Calcrl-IRES-Cre* mice (n =3). I observed CeAl somata coexpressing cytoplasmic mCherry and more punctate Syn-GFP (**Fig 4a**). I detected projections both within the CeA (in the CeAl and CeAm), and more distally throughout the basal forebrain substantia innominata (SI), with much sparser expression in the bed nucleus of the stria terminalis (**Fig 4b**). This pattern replicates non-cell type-specific anterograde tracing of the lateral capsular CeA (Bourgeais et al., 2001), and is consistent with PKCδ$^+$ neurons (some of which are CGRPR$^+$) inhibiting CeAl and CeAm neurons (Haubensak et al., 2010).

However, despite historical characterization of the PBN and CeA as forming reciprocal connections (Tukeuchi et al., 1982; Fulwiler & Saper, 1984; Moga & Gray, 1985), I failed to find any projections back to the PBN (data not shown). Therefore, it appears there is not a direct reciprocal connection between the two regions, or that the reciprocity does not involve CGRPR$^+$ circuitry.

Discussion

Genetic gradients and their functional relevance

While the CeAl has been genetically dissected into PKCδ$^+$ and SOM$^+$ neurons, I revealed that these cell types only compose a part of the larger region. PKCδ, SOM, and *Tac2*$^+$ neurons primarily occupy the caudal half of the CeAl. In contrast, CGRPR$^+$ neurons are present across its entire rostrocaudal axis. This graded expression pattern does not appear to be unique to these chosen molecular markers. CeA neurons expressing CRH, as causally implicated in pain and general stress (Johnson et al., 2015; Mantsch et al., 2015), are greater in number in the caudal versus the rostral CeA (Panguluri et al., 2009). Based on coexpression, the same is true for DYN$^+$ and OXTR$^+$ neurons (Jungling et al., 2015; Haubensak et al., 2010). Hence, in addition to CGRPR, which markers are expressed rostrally? The Allen Mouse Brain Atlas shows that ENK is expressed in the rostral CeAl. Because PBN CGRP$^+$ neurons make synaptic contacts onto CeA ENK$^+$ neurons (Shimada et al., 1992; Xu et al., 2003), it is possible then that ENK is coexpressed by CGRPR$^+$ neurons throughout the CeAl.

Despite such stark expression gradients, their functional importance remains unclear. Because PKCδ exhibited the most pronounced gradient, and it was reported to selectively mark neuronal activation by anorexigenic stimuli (Cai et al., 2014), I considered the possibility is that they reflect differential activation patterns. However, with respect to physiological satiety, I found activation to encompass the entire rostrocaudal axis. Moreover, even in the caudal CeAl where PKCδ$^+$ neurons are the most numerous, they were not selectively recruited. The discrepancy between my findings and those of Cai et al. (2014) can likely be attributed to a combination of methodological differences, as they performed a short feeding assay without specifically whether it occured at a time when mice normally eat (during the dark phase), and the difficulty in colocalization with PKCδ, whose immunolabeling detects not only cell bodies but also dense microcircuitry.

Complete testing of this hypothesis however requires evaluation of diverse stimuli. Sung Han and I have preliminary data showing that foot shock similarly recruits neurons across the CeAl. Additionally, Xiu et al. (2014) detected spatially segregated activation by foot shock versus morphine, but did not report any rostrocaudal differences. While a large number of PKCδ$^+$ neurons were Fos$^+$ following morphine administration, but not foot shock, the converse was not reported. However, Campos & Palmiter have found that the administration of the satiety hormone cholecystokinin (CCK) selectively activates the caudal CeA (unpublished observation), and Cai et al (2014) report a high overlap between CCK-induced Fos and PKCδ. Indeed, Cai et al. (2014) found that inhibition of PKCδ$^+$ neurons could only rescue appetite suppressed by CCK, but not lithium chloride or lipopolysaccharide – two agents associated with visceral malaise that also activate some PKCδ$^+$ neurons. The PKCδ gradient identified here may explain such behavioral effects.

Because genetic entry into the downstream targets of PBN CGRP neurons revealed the gene expression patterns in the first place, a second possibility is that these gradients mark spatial differences in connectivity, either with respect to circuit inputs and outputs. For instance, neurons of the paraventricular thalamus preferentially innervate

SOM^{+} neurons (Penzo et al., 2015). Monosynaptic retrograde tracing using the modified pseudorabies virus and/or channelrhodopsin-assisted circuit mapping could help test this idea (however, see below).

Finally, the gradients could be remnants of CeA patterning during development. If true, this leaves open the question of why the gradients persist into adulthood. At the very least though, it appears that the rostral CeA is behaviorally relevant, as its preferential lesioning impairs passive avoidance (Coover et al., 1992). Considering the genetic gradients, future monitoring or manipulation of CeAl neuron activity must account for the entire rostrocaudal CeAl to gain a complete picture of cell type-specific activation and to properly test CeAl neuronal function.

CeAl cell types and the issue of coexpression

Beyond the spatial distribution of genetically identified CeAl neurons, I demonstrated that CGRPR expression does not cleanly segregate into either PKCδ^{+} and SOM^{+} subpopulations. Some of PKCδ^{+} and some SOM^{+} neurons coexpress CGRPR. Hence, if these are indeed functionally relevant subpopulations within the CeAl, it appears that upstream PBN CGRP^{+} neurons and consequently CGRP engage both. How recruitment of both PKCδ^{+} and SOM^{+} neurons manifests in terms of their dynamic activity remains to be determined (**Fig 1b**). Importantly, there are rostral CeAl CGRPR^{+} neurons that are neither PKCδ^{+} or SOM^{+}.

Whether PKCδ^{+} and SOM^{+} neurons actually represent functionally relevant subpopulations can be called into question, however. Although the two are considered distinct (Janak & Tye, 2015), ~13% of SOM^{+} neurons coexpress PKCδ (the converse was not reported; Li et al., 2013). This proportion is not *a priori* trivial, as I have reported that <10% PKCδ^{+} neurons are Fos^{+} following refeeding, and significant effects on feeding are seen with their inhibition (Cai et al., 2014). For another example, consider cholinergic neurons of the striatum. Composing ~1% of the total striatal population, they are nonetheless necessary for proper conditioning (Witten et al., 2010).

Small, but potentially non-negligible overlap of PKCδ^{+} and SOM^{+} neurons raises the larger issue of the genetic heterogeneity and coexpression within the CeAl. Some CRH^{+} neurons coexpress PKCδ, and some PKCδ^{+} neurons coexpress ENK (Cai et al., 2014; Haubensak et al., 2010), but CRH^{+} and ENK^{+} neurons are almost non-overlapping (Day et al., 1999). While a large proportion of DYN^{+} neurons coexpress SOM (converse not reported; Jungling et al., 2015), about a third of DYN^{+} neurons coexpress CRH (Merchant et al., 2007). These coexpression profiles are only a portion of those shown (Moga et al., 1985; Shimada et al., 1989; Honkaniemi et al., 1992; Ma, Ye et al., 2003; Loughlin et al., 2006; Haubensak et al., 2010; Reyes et al., 2011). Genetic coexpression poses a difficult problem for identification of discrete cell types, as sought after by the BRAIN Initiative (BRAIN Working Group Report, 2014). This problem is brought to the behavioral level when employing single feature Cre-drivers for manipulation of neuronal activity and testing of behavioral function. When performing gain- or loss-of-function experiments, as long as a sufficient number of relevant neurons are manipulated (likely greater in the loss-of-the function experiment), then the behavior can be causally linked to the manipulated cell type. However, a larger or smaller population of cells, defined by more than one gene, could compose the actual circuit.

Connectivity serves as a second piece of evidence for PKCδ+ and SOM+ neurons being functionally relevant – that is, they have been indirectly shown to mutually inhibit one another. Yet, local synaptic inhibition was compared between genetically labeled and unlabeled cells in either PKCδ- or SOM-targeted knock-in lines, but not tested between genetically labeled neurons using paired recordings (Haubensak et al., 2010; Li et al., 2013). It is possible then that the CeAl is a generally recurrent structure (like the hippocampal CA3 region), with PKCδ+ and SOM+ populations also inhibiting members of their own populations. Even if PKCδ+ and SOM+ neurons are indeed the relevant subpopulations, they must be related to rostral CGRPR+ neurons to gain a complete picture of CeA microcircuitry.

Cell types however, cannot merely be understood in terms of their genetic/molecular expression (Luo, Callaway & Svoboda, 2008). Coexpression can also be related to other neuronal properties, such as morphology and electrophysiological properties. However, these also do not seem to segregate easily. No distinct morphology differences exist among CeA neurons expressing different neuropeptides (Cassell & Gray, 1989). Also, while most PKCδ+ neurons are late-firing neurons (Haubensak et al., 2010), SOM+ neurons consist of several different electrophysiological subtypes (Li et al., 2013). Manipulating neurons on the basis of two or more genes, now possible with Boolean logic technology, may be a first step toward multi-feature characterization of cell types within and outside the CeA (Fenno et al., 2014).

CeAl CGRPR+ circuitry and its behavioral implications

My finding that some CGRPR+ neurons coexpress PKCδ, and project locally and distally to the substantia innominata (SI) has strong implications for how CeAl circuitry controls behavior. First, Cai et al (2014) concluded that PKCδ+ neurons suppress appetite via CeA microcircuitry, as local injection of the $GABA_A$ receptor antagonist bicuculline blocked anorexia induced by PKCδ+ neuron activation. However, I showed that CGRPR+ neurons, some of which are PKCδ+, project to the adjacent SI. This circuit was not appreciated with previous mapping of PKCδ+ projections because a cytoplasmic, rather than a synapse-specific reporter was used (Cai et al., 2014). Because of the approximate distance between the CeA and SI (**Figs 1a, 4b**), it is likely that pharmacological manipulation was not restricted to just the CeA. Fulwiler & Saper (1984) emphasize the close proximity of the two regions, and the likelihood of spillover when attempting to just target the CeA. Therefore, it is possible that the CeAl-SI circuit mediates appetite suppression rather than a local microcircuit.

Further support for this idea comes from close analysis of the logic of the circuitry. Given that PKCδ+ CGRPR+ inhibit PAGvl-projecting CeAm neurons, whose activation normally drives immobility (**Fig 1b**), their activation, whether artificial or via upstream excitatory input, would suppresses immobility (*i.e.* allow for mobility) as well as suppress local microcircuitry. Unless disinhibition of some other circuit targeted by an unidentified CeAl or CeAm population drives anorexia (which appears unlikely, as Cai et al. [2014] manipulated two other predominant cell types in the CeA to no avail), the only remaining candidate circuit is the one to the SI.

Interestingly, I failed to find direct reciprocal connections between the PBN and CeA, at least with respect to the CGRP+- CGRPR+ circuitry. It is possible that direct

reciprocity exists, as including some other CeA-targeting PBN cell type, or the reciprocity is indirect via CeA microcircuitry. Because PKCδ$^+$ neurons were shown to project to the external lateral PBN (Cai et al., 2014), I conclude that PKCδ$^+$ but CGRPR$^-$ neurons project to the PBN (**Fig 5**). This is consistent with localization of this population to the lateral, rather than the lateral capsular CeA (**Fig 2a**), and the lateral CeA selectively targeting the PBN (Petrovich & Swanson, 1997; Jongen-Relo & Amaral, 1998). The lateral CeA-PBN circuit also consists of SOM$^+$, CRH$^+$, and NTS$^+$ neurons (Moga & Gray, 1985).

Taken together with other studies, my data offer revisions to and suggest further testing of the current model of CeA circuitry, accommodating the neglected rostral CeA and the genetic coexpression profiles identified here and elsewhere in the literature. The complexity of cell-type identification within the CeAl likely generalizes to the central nervous system as whole, thereby arguing for more nuanced monitoring, manipulation and ultimately classification of neuron types and their functions.

Methods

Mice

Grouped-housed male and female mice of approximately 2-5 months were used for all studies, as approved by the University of Washington Institutional Animal Care and Use Committee and in accordance with the guidelines set forth by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Calcrl-IRES-Cre mice were made by inserting a 6 kb 5' arm and a 4.1 kb 3' arm (each made by PCR amplification from a BAC clone and extending outward from a site just downstream of the endogenous termination codon) into a targeting vector with IRES-Cre:GFP, frt-flanked SV-Neo (for positive selection), HSV-TK and *Pgk*-DTa (for negative selection). The targeting construct was electroporated in G4 (129/Sv x C57Bl/6 hybrid) embryonic stem cells. Correct gene targeting was detected in 5 of 82 clones by Southern blot using *Bgl*I and a radioactive probe outside the 5' arm. One of the clones gave high percentage chimeras and germline transmission. The SV-Neo gene was removed by a cross with *Gt(ROSA)26Sor-FLP recombinase* and then *Calcrl-IRES-Cre* mice (identified using a 3-primer PCR strategy) were continuously backcrossed to C57Bl/6J mice. *Sst-IRES-Cre* and *Tac2-Cre* mice were obtained from Jackson Laboratory.

Immunohistochemistry and Quantification

For immunolabeling of PKCδ, SOM and Fos, six to seven 30μm (PKCδ/Fos) or 50μm (SOM) sections stored in 1x PBS spanning the rostrocaudal axis of the CeAl (from approximately -0.72mm or -0.9mm to ~ -1.8 mm, separated by 180μm) were processed according to Carter et al (2013). The sectioning procedure for SOM maximized somatic peptide labeling. For primary antibodies, sections were incubated in mouse anti-PKCδ (BD Biosciences, 1:1000), rat anti-SOM (Pierce, 1:250) or rabbit anti-Fos (Cell Signaling, 1:4000). For secondary antibodies used for tdTomato and PKCδ/SOM colabeling, sections were incubated in donkey anti-mouse Cyanine Cy5 (Jackson

ImmunoResearch, 1:500) or anti-rat Cyanine Cy5 (Jackson ImmunoResearch, 1:500) so as to avoid potential tdTomato bleed through. For colabeling of PKCδ and Fos, sections were incubated in donkey anti-mouse Alexa Fluor 488 (Jackson ImmunoResearch, 1:500) and anti-rabbit Alexa Fluor 594 (Jackson ImmunoResearch, 1:500). To track marker expression along the rostrocaudal axis of the CeAl, we took z-stack images of the CeAl unilaterally using a laser scanning confocal microscope (Olympus, FV1200) and performed cell counts and overlap analysis in Adobe Photoshop CS5. For evaluation of relative marker expression (**Fig 1f**), I summed cell counts across the seven sections.

Stereotaxic Surgery
Adeno-associated viruses Cre-dependently (DIO) expressing mCherry or Synaptophysin fused to GFP (driven by the Ef1α promoter) were combined and unilaterally injected (.5 μl) into the CeA of *Calcrl-IRES-Cre* mice (AP: -1.2mm, ML: 2.9mm, DV: 4.9mm) over 2.5 minutes (0.2 μl/min). The DNA plasmid for pAAV DIO mCherry was provided by K. Deisseroth while that of pAAV DIO Syn-GFP was generated in house as described by Carter et al. (2013). All images were acquired using a laser scanning confocal microscope (Olympus, FV1200).

Feeding Assay
Wild-type mice were transferred to a testing room, singly-housed and acclimated for at least 5 days. Prior to testing, we measured *ad-libitum* chow intake over 1.5 hours following the onset of the dark phase for 3 days, the average of which was used as each animal's baseline (**Fig 3a**). The following day, mice either ate *ad-libitum* or were fasted for 24 hours. For testing, chow intake was measured in mice that ate *ad-libitum* or were refed. 1.5 hours later, mice were perfused as described by Carter et al. (2013) to detect Fos induction.

Data analysis and presentation
All data were organized and analyzed using Microsoft Excel. Graphs were created and data were statistically tested using GraphPad Prism, and then imported into Adobe Illustrator CC 2014 for figure construction.

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