Molecular diversity of corticotropin-releasing hormone mRNA-containing neurons in the hypothalamus

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Abstract

Hormonal responses to acute stress rely on the rapid induction of corticotropin-releasing hormone (CRH) production in the mammalian hypothalamus, with subsequent instructive steps culminating in corticosterone release at the periphery. Hypothalamic CRH neurons in the paraventricular nucleus of the hypothalamus are therefore considered as ‘stress neurons’. However, significant morphological and functional diversity among neurons that can transiently produce CRH in other hypothalamic nuclei has been proposed, particularly as histochemical and molecular biology evidence associates CRH to both GABA and glutamate neurotransmission. Here, we review recent advances through single-cell RNA sequencing and circuit mapping to suggest that CRH production reflects a state switch in hypothalamic neurons and thus confers functional competence rather than being an identity mark of phenotypically segregated neurons. We show that CRH mRNA transcripts can therefore be seen in GABAergic, glutamatergic and dopaminergic neuronal contingents in the hypothalamus. We then distinguish ‘stress neurons’ of the paraventricular nucleus that constitutively express secretagogin, a Ca^{2+} sensor critical for the stimulus-driven assembly of the molecular machinery underpinning the fast regulated exocytosis of CRH at the median eminence. Cumulatively, we infer that CRH neurons are functionally and molecularly more diverse than previously thought.

Key Words

- calcium-binding proteins
- neuronal heterogeneity
- single-cell RNA-sequencing
- stress

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Introduction

The introduction of molecular methods in the neurobiologists’ tool kit has changed many long-held views on the structural and functional organization of the nervous system. One of the most recent additions is the use of single-cell RNA-sequencing (scRNA-seq), which can produce quantitative mRNA counts to predict the origin, lineage segregation, functional diversification and disease susceptibility of any cell type in the nervous system. Taking the power of ‘forward’ (or predictive) transcriptomics to the level of neuroanatomy, connectivity mapping by electrophysiology and genetic models, one can expect a sea-change of organizational rules that have

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hitherto been applied on neuronal identity and function (Shapiro *et al*. 2013, Romanov *et al*. 2015, Zeisel *et al*. 2015). Alternatively, the use of 'reverse' transcriptomics can validate many datasets that were generated using alternative tools. This is a particularly exciting prospect in neurobiology where sometimes mm-to-cm-scale distances exist between synaptic release sites and the somata of relevant projection neurons. Thus, functional transcriptomics at projection sites might be validated by scRNA-seq of somatic compartments (Romanov *et al*. 2015), which contain all mRNAs for the protein domain sustaining the regulated release of any neurotransmitter or neuroactive modulator. Here, we will review recent data on corticotropin-releasing hormone (CRH) *mRNA*-containing neurons in the mammalian hypothalamus to describe their unexpected molecular heterogeneity. By relying on a combination of novel experimental tools, we suggest that Crh expression can occur in glutamatergic, GABAergic and dopaminergic neurons and is a descriptor of specific 'working modes' and that the co-localization of CRH with secretagogin is a defining hallmark of 'stress on' CRH neurons in the paraventricular nucleus of the hypothalamus (PVN).

Organization of the hypothalamus–pituitary–adrenal axis: from systems to molecules

The body's reaction to environmental stressors, including physical and psychosocial factors (McEwen 2007), is orchestrated by sequential hormone release in the hypothalamus–pituitary–adrenal (HPA) axis (Herman *et al*. 2003). HPA activation triggers corticosteroid release from the cortex of the adrenal gland to mobilize energy utilization, thus leading to integrative-protective responses to noxious conditions (Sapolsky *et al*. 2000, McEwen 2007). The first hierarchical level in the HPA axis is the release of CRH (Spiess *et al*. 1981, Vale *et al*. 1981) from the terminals of parvocellular hypothalamic neurons into hypophyseal portal vessels (Green & Harris 1947) at the median eminence (Swanson *et al*. 1983). According to the classical definition, the peripheral stress response then progresses through the secretion of adrenocorticotropic hormone (ACTH) (Mains *et al*. 1977, Roberts & Herbert 1977, Nakanishi *et al*. 1979) from the anterior lobe of the pituitary. Finally, ACTH induces steroidogenesis in, and corticosteroid release from, the adrenal cortex (Munck *et al*. 1984, McEwen & Stellar 1993). Corticosteroid action is then executed through glucocorticoid receptors (Gustafsson *et al*. 1987), encoded by the *Nr3c1* gene (McEwen 2012), with ensuing changes in cellular states ('cell-state switch') tuned to overcome metabolic compromise.

Parvocellular neurons are broadly viewed as the origins of CRH for stress-induced, fast release and are situated in the PVN (Merchenthaler *et al*. 1983, Swanson *et al*. 1983, 1986, Alonso *et al*. 1986, Ceccatelli *et al*. 1989*b*, Swanson 1991). The PVN itself showcases the complexity of neuronal organization in the hypothalamus: it contains at least eight subdivisions (Swanson & Kuypers 1980, Simmons & Swanson 2009) with the tight spatial concentration of magnocellular and parvocellular neuroendocrine secretory motor neurons (that is, cells that release bioactive peptides into the portal circulation through release sites located outside the blood–brain barrier), neurons projecting to the brainstem and locally projecting hypothalamic neurons (Daftary *et al*. 1998, Krashes *et al*. 2014). Oxytocin and vasopressin (Du Vigneaud 1954) are produced in magnocellular neurons projecting directly to the posterior pituitary (Swaab *et al*. 1975, Rhodes *et al*. 1981). In contrast, parvocellular neurons are a kaleidoscope of neuroendocrine modalities, often co-releasing several neuropeptides and fast neurotransmitters in an 'on-demand' manner (Swanson *et al*. 1986, Ceccatelli *et al*. 1989*a*,*b*). Among parvocellular PVN neurons, CRH cells, besides projecting to the median eminence, also form axon collaterals toward other intrahypothalamic nuclei (e.g., lateral hypothalamus), thus increasing integrative capacity through the coincident control of neuronal circuitries and hormonal responses at the pituitary (Fuzesi *et al*. 2016).

Besides CRH at its 'stress center' (Alonso *et al*. 1986), parvocellular neuroendocrine cells can contain 'releasing' and 'release-inhibiting' hormones (e.g., growth hormone-releasing hormone and somatostatin (growth hormone-inhibiting hormone), thyrotropin-releasing hormone, gonadotropin-releasing hormone and dopamine (as prolactin-inhibiting hormone)) to regulate hormone release from the anterior pituitary (classed as growth hormone, thyroid-stimulated hormone, luteinizing and follicle-stimulating hormones and prolactin) (Guillemin 1978, Schally 1978, Ben-Jonathan & Hnasko 2001). Of particular interest in relation to stress, vasopressin potentiates the effect of CRH (Gillies *et al*. 1982, Turkelson *et al*. 1982, Rivier & Vale 1983) and can undergo induced expression in CRH neurons after adrenalectomy (Tramu *et al*. 1983, Kiss *et al*. 1984, Sawchenko *et al*. 1984). As such, these peptides co-exist in the same storage granules in boutons residing in the external layer of the median eminence (Whitnall *et al*. 1985).

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The observation that basal ACTH and corticosterone levels can always be detected in blood (rather than being 'on'/'off' signals) (Girotti *et al.* 2009, Romanov *et al.* 2015) indirectly suggests that CRH is constitutively released in minute amounts to continuously stimulate the production and release of downstream hormones. This concept has broad physiological significance as, for example, pancreatic β cells require glucocorticoids for their survival. Consequently, hypothalamic CRH hypofunction leads to Addison's disease with diabetes being its primary comorbidity (Miller & O'Callaghan 2002, Napier & Pearce 2014, Kahaly & Hansen 2016). There also is a well-established relation of CRH (and corticosteroids) to mood disorders (Gold *et al.* 1995, Reul & Holsboer 2002, Lloyd & Nemeroff 2011, Waters *et al.* 2015): CRH release follows the diurnal cycle with autonomous peaks observed during the early morning hours and late afternoon/evening (Salata *et al.* 1988, Owens *et al.* 1990, Watts *et al.* 2004, Girotti *et al.* 2009). These data suggest that CRH might have more widespread biological roles than previously thought through cell- or tissue-specific expression of its receptors (see below). As such, and beyond regulating the stress response, CRH is increasingly implicated in the regulation of appetite (Drescher *et al.* 1994, Uehara *et al.* 1998).

CRH production in the brain

Beyond its canonical expression site identified as the parvocellular neurons of the PVN (Simmons & Swanson 2009), CRH production is not restricted to the PVN. In the hypothalamus, CRH is also detected in magnocellular cells of the lateral hypothalamus and preoptic area (Alon *et al.* 2009). A critical difficulty to identify the cellular sites of CRH production is its unusually low levels at rest and fast induction during stressful conditions or in particular metabolic states. Therefore, histochemical solutions so far relied on agents blocking the axonal transport machinery to elevate CRH levels in neuronal somata (Merchenthaler *et al.* 1982, Alonso *et al.* 1986) or by experimentally boosting its expression (Ceccatelli *et al.* 1991). In accord with the expansion of amenable molecular genetic tools, e.g. the Cre/lox system, we recently used *Crh*-IRES-Cre-driven green fluorescent protein (GFP) expression to allow the accumulation of

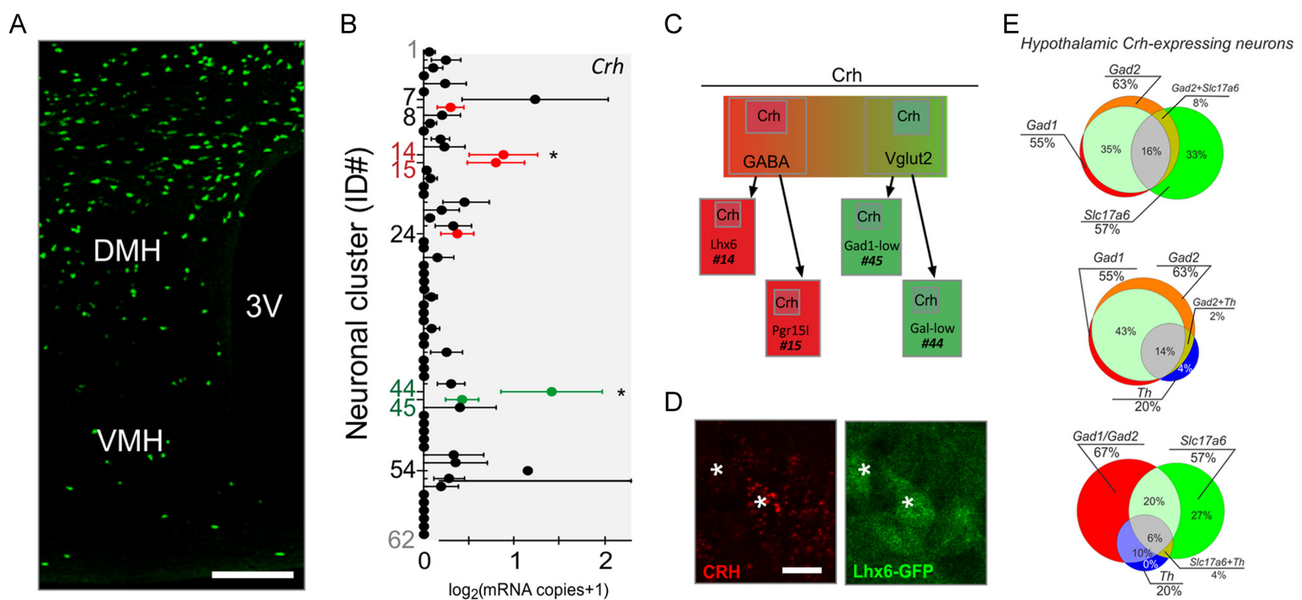

Figure 1

Molecular heterogeneity of Crh⁺ neurons in the hypothalamus. (A) Distribution of GFP⁺ neurons outside the PVN in the hypothalamus of *Crh*-IRES-Cre::GFP mice. (B) Single-cell RNA-seq distinguishes 62 neuronal subclasses along the PVN–arcuate axis of the hypothalamus (Romanov *et al.* 2015). Expression levels (horizontal axis) were plotted as means of $\log_2$ transformed mRNA copy numbers ± S.E.M. Red and green colors identify GABAergic (#14, #15 and #24) and glutamatergic clusters (#44 and #45) of hypothalamic neurons, which express *Crh* mRNA at levels exceeding 2× the S.E.M. **q* < 0.05 (Wilcoxon rank-sum test corrected for multiple testing). (C) Among both GABA and glutamate neurons, subsets are endowed with *Crh* mRNAs. (D) CRH co-exists in *Lhx6*-GFP neurons (asterisks) particularly in the bed nucleus of stria terminalis and preoptic area. (E) Neurotransmitter heterogeneity of *Crh*⁺ neurons. Venn diagrams demonstrate the proportion of dual and triple neuronal phenotypes in GABA/glutamate (top), GABA/dopamine (middle) and GABA/glutamate/dopamine (bottom) neurons. This analysis was performed using a threshold for mRNA expression at a level of ≥2 mRNA transcripts for each gene. Percentage values indicate the proportion of *Crh*⁺ neurons falling into groups and intersections ('dual phenotype' categories). Scale bars = 250 µm (A), 10 µm (D). Adapted, with permission, from Romanov *et al.* (2016).

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GFP in response to episodic *Crh* expression during a cell's life time (Fig. 1A). These results document many GFP+ neurons scattered within the hypothalamus, including its anterior and ventromedial areas. This genetic model suggests, in accord with earlier histochemical studies (Merchenthaler *et al*. 1982, Swanson *et al*. 1983, Keegan *et al*. 1994, Potter *et al*. 1994, Alon *et al*. 2009), that episodic *Crh* mRNA expression might be intrinsic to many hypothalamic neurons. Likewise, *Crh* gene transcription and protein expression were documented in many extrahypothalamic brain areas, including thalamic nuclei, bed nucleus of the stria terminalis, amygdala, medulla oblongata (especially inferior olive), piriform cortex and many neocortical and hippocampal subregions (Merchenthaler *et al*. 1982, Potter *et al*. 1994, Lantos *et al*. 1995, Alon *et al*. 2009, Zeisel *et al*. 2015, Hooper & Maguire 2016). Thus, an essential heterogeneity of CRH neurons emerges with CRH likely being a molecular determinant of functional cellular competence under particular behavioral and metabolic conditions.

Exquisite co-localization studies suggest that CRH-positive(+) neurons could mediate either excitatory or inhibitory neurotransmission in a regionally defined manner. For excitatory CRH+ neurons, those of the bed nucleus of stria terminalis are considered as prime candidates (Lee & Davis 1997, McNally & Akil 2002, Hrabovszky *et al*. 2005). Likewise, CRH+ cells of the central nucleus of the amygdala participate in extended excitatory circuits in which these neurons might potentiate differential responses to tonic/unpredictable vs phasic/predictable stressors is currently under debate (Lee & Davis 1997, Palkovits 2000, McNally & Akil 2002), even including peripheral responses such as the regulation of heart rate (Nijsen *et al*. 2001). In turn, cortical and hippocampal interneurons might also express CRH, which use GABA as inhibitory neurotransmitter (Zeisel *et al*. 2015, Hooper & Maguire 2016). Nevertheless, CRH actions are chiefly diversified at the subcellular level as its two G-protein coupled receptors (with G_s/G_q coupling), CRHR1 (*Crhr1*) and CRHR2 (*Crhr2*) can differentially signal in response to this neuropeptide (Dautzenberg *et al*. 2001, Dautzenberg & Hauger 2002, Aguilera *et al*. 2004, Gutknecht *et al*. 2010).

ScRNA-seq-based subclassification of *Crh* mRNA+ hypothalamic neurons

The classical doctrine of hypothalamic stress induction posits CRH neurons being parvocellular neurosecretory cells situated in the PVN in mammals (Swanson *et al*. 1986, Simmons & Swanson 2009). However, 'why', 'when' and 'for how long' other neurons (GFP+; Fig. 1A) beyond those in the PVN produce CRH and at which biophysical thresholds and synaptic sites release their CRH contents are much less unequivocal. Among the many reasons for this ambiguity at the molecular regulatory levels, we emphasize the rapid induction of CRH production (Watts *et al*. 1999) under certain metabolic demands. This would allow for CRH being re-classified as a permissive cellular feature underscoring functional competence and circuit recruitment rather than a cell-identity mark (we define a cell-identity mark as a molecular feature that determines the cell's function, sets it apart from neighboring cells and whose loss might render the cell functionally incompetent and misplaces it in the molecular taxonomy of cells in a given brain volume. In contrast, functional modality refers to a molecular feature (gene, gene set and combinatorial code) that is only present under certain (patho)physiological conditions yet does not define either its neurotransmitter phenotype or connectivity pattern over long time scales. To test if CRH is an identity mark or a cell-state modality, we analyzed the level of heterogeneity of gene expression defining CRH signaling (that is, *Crh*, *Crhr1*, *Crhr2* and *Crhbp*) in hypothalamic neurons (Fig. 2A)). If so, a combination of novel methods including scRNA-seq and *in situ* gene expression (Stahl *et al*. 2016) profiling could decode the heterogeneity of hypothalamic CRH+ neurons and allow for their subclassification based on the correlated analysis of fast neurotransmitter contents. In addition, one would expect these data be supportive of more conventional approaches, such as the intracerebroventricular injection of colchicine, an axonal transport inhibitor (Ceccatelli *et al*. 1991), which is widely used for the improved detection of neuropeptide expression (and also represents a substantial stress stimulus).

As such, many neuropeptides (beyond CRH in the PVN) are used as specific hallmarks of neuronal identity in the hypothalamus (e.g. neuropeptide Y, oxytocin, agouti-related peptide (AgRP)) (Meister *et al*. 1990, Lantos *et al*. 1995, Aponte *et al*. 2011, Dietrich *et al*. 2015), asserting that expression of a single gene (or at most their circumscribed subset) typifies a neuronal subtype. This knowledge can thus be used to justify recent developments in quantitative mRNA profiling tools, as any single-cell analysis, if correct, would lean toward neuronal identity marks that are stably expressed in a given contingent of neurons. Accordingly, the recent development of mathematical algorithms (*t*-distributed stochastic neighbor embedding (tSNE)

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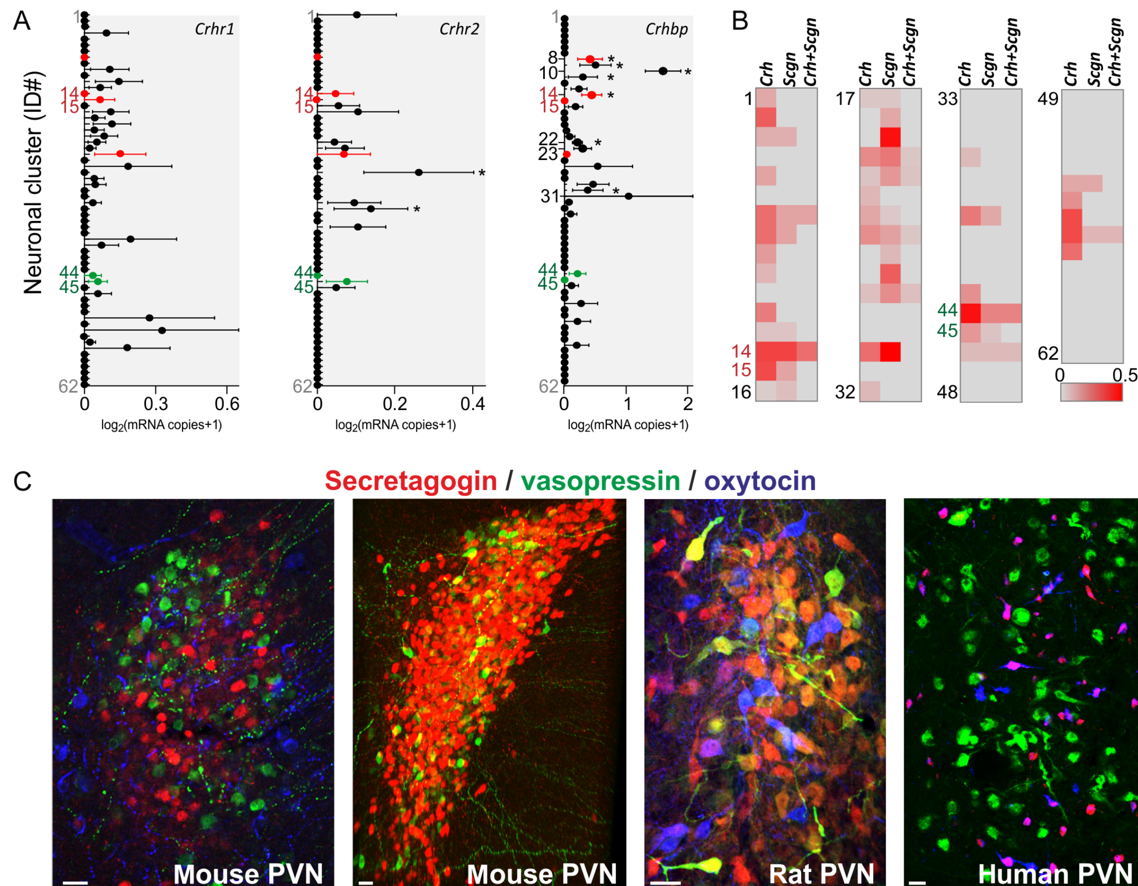

Figure 2

Molecular determinants of CRH signaling in the mouse hypothalamus. (A) Neuronal subclusters expressing CRH receptors (*Crhr1* and *Crhr2*) and CRH-binding protein (*Crhbp*). Note that some cells in clusters #14 (GABA) and #44 (glutamate) can express at least one receptor and binding protein for CRH signaling. Red and green colors identify respective GABAergic and glutamatergic clusters, which express *Crh* mRNA at levels exceeding 2× the S.E.M. Note that significant levels of gene expression were found only for *Crhr2* and *Crhbp* but not for *Crhr1* within the hypothalamus (*$q < 0.05$, Wilcoxon rank-sum test corrected for multiple testing). (B) Secretagogin expression among hypothalamic neurons. Cluster #44 (glutamate) coexpresses both *Scgn* and *Crh* genes, and this cluster localizes to the PVN (Romanov *et al.* 2015). (C) Secretagogin expression in the hypothalamus of laboratory rodents and humans. In mouse, secretagogin co-exists with neither oxytocin nor vasopressin. In contrast, a subset of vasopressin+ and oxytocin+ neurons can co-express secretagogin in rat and human, respectively. Color code: secretagogin (red), vasopressin (green) and oxytocin (blue). Scale bars = 20 µm. (A and B) adapted, with permission, from Romanov *et al.* (2016).

and BackSpin clustering (van der Maaten & Hinton 2008, Islam *et al.* 2014, Macosko *et al.* 2015, Zeisel *et al.* 2015)) allows for the refined analysis of scRNA-seq data on hypothalamic neurons (Romanov *et al.* 2015, 2016). This approach confirmed that many neuropeptide mRNAs (*Hcrt*, *Agrp*, *Npy* and *Trh*) are indeed neuronal identity marks (Romanov *et al.* 2016) and once ensuing peptides are produced, their 'use-dependent' release will impinge upon a neuronal circuit to determine metabolic output (e.g. AgRP invariably present in a neuronal subset in the arcuate nucleus that controls body weight and metabolic rate (Mizuno *et al.* 2003)). In contrast, *Crh* mRNA expression fails to meet these criteria: *t*-SNE projections demonstrate widespread *Crh* expression in hypothalamic neurons, which lack subtype definition. In accord, BackSpin clustering characterized several neuronal subtypes (including clusters #14, 15, 44 and 45 as per Romanov *et al.* (2016)) that contain fractions of *Crh* mRNA-expressing neurons (Fig. 1B and C). Thus, we conclude that a neuron's ability to produce CRH is a functional modality rather than an identity-defining feature.

If CRH expression is a functionally induced modality, then the diversity of fast neurotransmitter contents in hypothalamic neurons expressing this neuropeptide can also be expected (Fig. 1C). Our detailed analysis of all hypothalamic *Crh* neurons showed their segregation into either GABAergic (that is, *Gad1*, *Gad2* and *Slc32a1* co-expression) or glutamatergic (*Slc17a6*) neurotransmitter phenotypes. As neurotransmitter coexistence appears as a

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unique and relatively common feature of hypothalamic neurons (in 10–20% of cells as coincidently revealed by both Fluidigm C1 and Drop-seq transcriptomics) (Romanov *et al.* 2016), dual GABA/glutamate, dopamine/GABA and dopamine/glutamate neurons can also contain *Crh* mRNA (Fig. 1E). In sum, *Crh*+ neurons encompass several subtypes of GABA and glutamate neurons, reinforcing the concept that episodic CRH production can be associated with multiple network-driven, metabolic or disease-related contexts rather than being a feature of a developmentally coded subset of hypothalamic neurons (that is, phylogeny defined at the transcriptional level).

ScRNA-seq uncovered that CRH neurons residing in the PVN are glutamatergic. This cell cluster can be distinguished by the co-existence of several mRNA marks, including *Tmem59l*, *Fuca1*, *Npr3* and *Arnt2*, and whose existence in the PVN is validated by open-source histochemical databases (see *in situ* hybridization, Allen Brain Atlas/brain-map.org). Meanwhile, GABAergic neurons that can contain CRH co-express *Pgr15l*, a histochemical mark mapped onto the anterior part of the PVN, cells spread around the histochemical boundaries of the PVN and those residing in the dorsomedial hypothalamus (Fig. 1C). Alternatively, GABAergic CRH neurons can co-express LIM homeobox 6 (*Lhx6*) transcription factor and map onto the preoptic area (Fig. 1D). Moreover, a continuum of *Lhx6*+/*Crh*+ neurons extends toward the bed nucleus of stria terminalis, which is an extrahypothalamic area with innate projections to, among other, the PVN where it forms local circuits regulating the stress axis (Choi *et al.* 2007). Based on their transcriptional code, we hypothesize that GABAergic CRH+ neurons, despite their spatial heterogeneity, can originate from the same precursors, migrate toward different (extra)hypothalamic subregions and ultimately be a unified neuronal subtype. An outstanding question based on novel scRNA-seq and localization data is the determination of when (context and mode of operation) and for how long (induction) any neuronal subclass can express physiologically meaningful levels of CRH protein and if their postsynaptic targets are endowed with CRH receptors for the efficient translation of CRH signals into functional outcome.

CRH receptors in the brain

Two genes (*Crhr1* and *Crhr2*) encode receptors to mediate CRH action with their mRNAs detected in many brain regions (Wynn *et al.* 1984, Aguilera *et al.* 2004). *Crhr1* is widely expressed in the neocortex, piriform cortex, olfactory system and cerebellum (Van Pett *et al.* 2000). Most recently, cortical and hippocampal *Crhr1* mRNA expression was also validated by scRNA-seq (Zeisel *et al.* 2015), showing pronounced *Crhr1* gene expression in pyramidal cells in cortical layers 2/3, 4 and 5. These findings contrast those in interneurons, which generally lack *Crhr1* mRNA transcripts. Given that interneurons can instead express CRH itself, the combination of cell-type-specific transcriptomics with circuit mapping allows predictions on local CRH signaling between CRH+ interneurons and *Crhr1*-laden pyramidal cells. *In situ* hybridization demonstrates a more restricted distribution pattern for *Crhr2* mRNA, with its primary concentration in lateral and triangular nuclei of the septum and the amygdaloid complex. Thus, CRHR1 and CRHR2 functions might mediate vastly different biological responses and involve a combination of short- and long-range modes of intercellular signaling.

When analyzing CRH receptor distribution in the hypothalamus, expression levels are relatively low for both *Crhr1* and *Crhr2* with the former being present in arcuate, suprachiasmatic, anterior and dorsomedial hypothalamic nuclei (Van Pett *et al.* 2000), whereas the latter is distributed across anterior periventricular, ventromedial, arcuate and anterior hypothalamic nuclei (Van Pett *et al.* 2000). Notably, stress induces *Crhr1* expression in the PVN, which is a significant feedback station for sustained CRH production and release (Makino *et al.* 1995, Aguilera *et al.* 2004).

Recently, we sought to address the precise cellular distribution of neurons that can respond to CRH as a neuromodulator locally in the hypothalamus (Romanov *et al.* 2016). Although ~10% of hypothalamic neurons express *Crh* mRNA (>1 mRNA copy), *Crhr1* and *Crhr2* were only sparsely present in hypothalamic neurons (4.5% and 2.6% of 898 neurons, respectively) at unexpectedly low copy numbers. The following steps of caution are warranted when considering these data: (i) low levels of *Crhr1* mRNA detection (Hata *et al.* 1993, Sollner *et al.* 1993, Sutton *et al.* 1998, Pang & Sudhof 2010) might lead to false negatives (missed fraction of neurons), (ii) mRNA-to-protein translation and the stability of ensuing receptor complexes and their effectors (Fig. 2A) might diversify the efficacy of signal transduction and (iii) induced expression (Aguilera *et al.* 2004) could grossly affect CRH-responsive hypothalamic neuronal networks. Yet, our comparison of scRNA-seq data from hypothalamic and cortical datasets (Romanov *et al.* 2015, Zeisel *et al.* 2015) produced on identical platforms suggests

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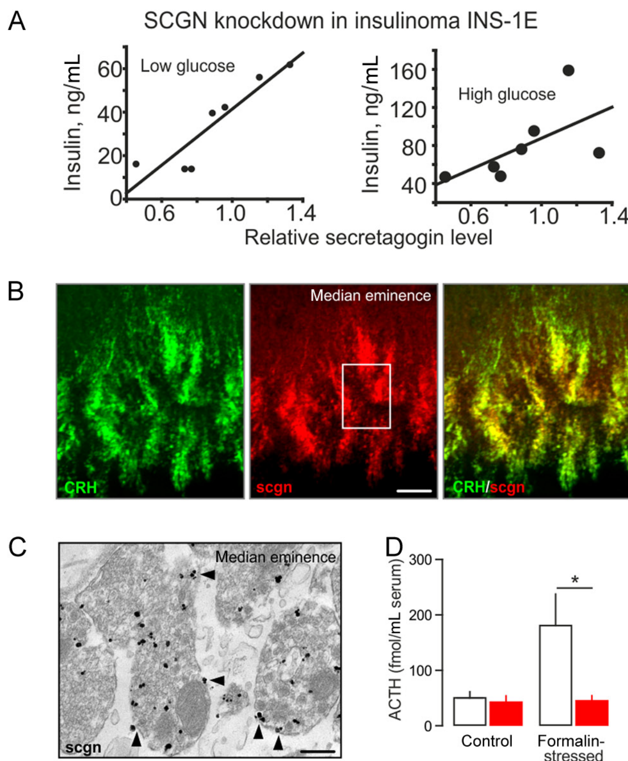
that CRH+ efferents commonly form synapses outside the hypothalamus and/or CRH is mainly released into the ventricular and hypophyseal portal systems. Conversely, the likelihood of local-circuit neuronal ensembles using CRH as neuromodulator at rest is relatively modest. An exception to the above rules might be an autocrine role for CRH in the PVN as these neurons express both *Crh* and *Crhbp* (CRH binding protein) mRNAs with *Crhbp* inactivating CRH action (Dautzenberg & Hauger 2002, Seasholtz *et al.* 2002). Cumulatively, these data suggest a dichotomy of CRH content and CRH-responsive neuronal substrates within the hypothalamus and at extrahypothalamic sites.

Secretagogin controls CRH release at the median eminence

The regulated release of any bioactive peptide within the brain or at the median eminence relies on the coordinated assembly of a soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) complex, allowing for the priming, docking and fusion of neuropeptide-filled large dense-core vesicles (LDCVs) (Hata *et al.* 1993, Sollner *et al.* 1993, Sutton *et al.* 1998, Pang & Sudhof 2010). In the hypothalamus, the exocytosis machinery includes many region- and cell-type-specific proteins, such as CAPS-1 and secretogranins (Miyazaki *et al.* 2011, Tobin *et al.* 2012). Until recently, our understanding of the molecular underpinnings of CRH release was limited. Recently, a focused scRNA-seq study (Romanov *et al.* 2015) in the mouse PVN identified the coincident presence of *Crh*, *Nr3c1* (glucocorticoid receptor, subfamily 3, group C member 1) (van Rossum *et al.* 2004) and secretagogin (*Scgn*) (Romanov *et al.* 2015) (Fig. 2B and C). These data were validated by light and electron microscopy in both the PVN and the median eminence, localizing secretagogin to membrane surfaces at terminals releasing CRH into the hypophyseal portal system (Romanov *et al.* 2015) (Fig. 3B and C), anatomically tying secretagogin to the machinery priming CRH release.

Secretagogin is an appealing subject of study as it is a member of the EF-hand superfamily of Ca^{2+}-binding



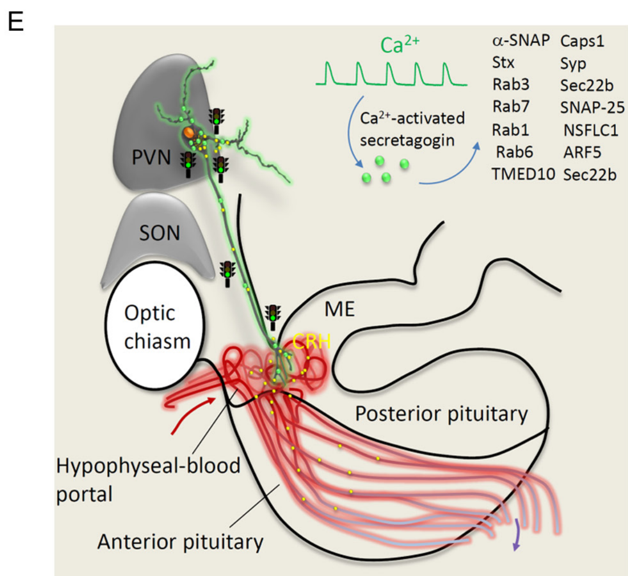


Figure 3

Secretagogin affects hormone secretion. (A) Secretagogin knockdown in INS-1E insulinoma cells limits insulin release. (B and C) Secretagogin co-localizes with CRH at the median eminence (B) and accumulates as a membrane-associated synaptic component at the ultrastructural level (C). (D) Plasma ACTH levels 12 min after formalin (4%) injection into a paw of conscious adult mice. Red color: secretagogin knockdown *in vivo*. Note that secretagogin deletion occludes ACTH release. *, $P<0.05$ vs. formalin-stressed controls. (E) The scheme showing involvement of secretagogin in hypothalamic CRH release, including potential protein-protein interactions. Scale bars = 10 µm (B) and 500 nm (C). Reproduced, with permission, from Romanov *et al.* (2015).

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proteins, expressed in all organ systems typically associated with neuroendocrine cells (Wagner *et al.* 2000, Adolf *et al.* 2007, Alpar *et al.* 2012), including the pituitary, adrenal gland, gut and pancreas (Wagner *et al.* 2000, Hasegawa *et al.* 2013). In the nervous system, secretagogin is localized to neurons populating the neocortex, hippocampus, cerebellum, basal ganglia, rostral migratory stream and olfactory bulb (Attems *et al.* 2008, Mulder *et al.* 2009, 2010, Gati *et al.* 2014). Secretagogin is a Ca^{2+} sensor protein ($[Ca^{2+}]_{0.5}$ is of ~25 µM for secretagogin in physiological salt buffers), which by definition induces protein–protein interactions through conformational change upon Ca^{2+} binding followed by downstream signaling to control discrete cellular functions (Schwaller 2010). Secretagogin's *in vitro* interactome initially included synaptosomal-associated protein 25 kDa (SNAP-25) (Rogstam *et al.* 2007) with the more recent discovery of vesicle cargo, traffic and docking/release proteins (Bauer *et al.* 2011*a*,*b*, Romanov *et al.* 2015). Particularly, the 5th EF-hand domain can interact with cytoskeletal components (microtubules) (Maj *et al.* 2010, Yang *et al.* 2016). Thus, secretagogin possesses basic properties of an integrative sensor that can orchestrate the release of neuropeptide-containing LDCVs.

In the hypothalamus, secretagogin is chiefly expressed in the PVN and arcuate nuclei (Mulder *et al.* 2009, Romanov *et al.* 2015). Even though secretagogin can partially co-exist with vasopressin and oxytocin in some mammals (Fig. 2C), the majority of secretagogin$^+$ neurons lacks any appreciable neuropeptide/hormone co-labeling in resting mice (Romanov *et al.* 2015). This constellation led to the detailed study of secretagogin$^+$ neurons and their molecular interrogation by correlated transcriptomics and proteomics. Notably, converging genetic and histochemical evidence suggests that secretagogin is a constitutive mark of stress-activated CRH$^+$ neurons that gate ACTH release from the pituitary (Romanov *et al.* 2015). Acute silencing of secretagogin expression (by siRNA) *in vivo* and *in vitro* provided critical insights into secretagogin's function (Hasegawa *et al.* 2013, Romanov *et al.* 2015): (i) secretagogin knockdown induces CRH accumulation in the PVN. This suggests that secretagogin deficiency might limit the translocation of CRH to release sites and its Ca^{2+}-dependent release into the portal circulation and (ii) concordantly, ACTH (Cam & Bassett 1983) and corticosterone production are blunted after secretagogin knockdown in animals exposed to acute formalin stress (Romanov *et al.* 2015) (Fig. 3D). These data also provide genetic evidence on a selective parvocellular Ca^{2+}-dependent gatekeeper to the HPA axis whose function underpins a peripheral corticosterone surge from the adrenal cortex (Makara *et al* 1981).

Even though secretagogin is broadly recognized to scale hormone release (Wagner *et al.* 2000, Hasegawa *et al.* 2013, Romanov *et al.* 2015) (Fig. 3A), its exact interacting partners in neurons that might prime axonal transport and docking secretory vesicles remained unknown until recently. Using subtractive proteomics comparing Ca^{2+}-saturated and Ca^{2+}-free conditions and 'reverse' transcriptomics to verify the expression of co-purifying proteins in parvocellular neurons, we have defined protein subfamilies essential for the formation, intracellular traffic, priming and docking of release vesicles (examples: α-SNAP, Rab family members, syntaxins, synaptophysin, TMED10 and CAPS1), and some of them are specific to hypothalamic neurons (Fig. 3E) and release mechanisms (e.g. CAPS-1) (Tobin *et al.* 2012). In accord, ultrastructural data localized secretagogin to large axon terminals at the median eminence and substantiated its association to the readily releasable pool of vesicles, as well as plasmalemmal compartments. Because CRH, as many other peptide hormones released at the median eminence, is neither reused nor recycled after entering the blood stream, we hypothesize that impaired CRH secretion into the portal blood upon genetic secretagogin ablation impairs not one but many regulated Ca^{2+}-sensitive intracellular checkpoints in parvocellular neurons (Fig. 3E). Thus, secretagogin qualifies as a molecular determinant rate-limiting CRH release.

Conclusions

Here, we collated recent molecular evidence highlighting the unprecedented molecular heterogeneity of hypothalamic neurons. The use of multiparametric experimental paradigms on the backdrop of array technologies provides unique precision and analytical power to distinguish neuronal modalities, let these be graded identity differences or temporal recruitment to neuronal circuits. Besides CRH neurons, novel levels of evidence emerge on the molecular heterogeneity of those affecting food intake (Henry *et al.* 2015), hedonism (amygdala), multisensory processing (e.g. association of vision and smell) (Macosko *et al.* 2015, Saraiva *et al.* 2015) and conscious representation of metabolic demands (cortex) (Zeisel *et al.* 2015). Thus, an unexpected and rapid expansion of concepts substantially refining and sometimes revising decade-long theories might be

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expected, allowing multimodal integration of information flow and refinement at extra- and intrahypothalamic circuits. The advent of deciphering the molecular heterogeneity of CRH neurons already produced novel insights into permissive checkpoints of the release of this hormone, as well as the parsing of probable subclasses of CRH neurons. Accordingly, CRH belongs primarily to 'functional modality' rather than 'cell-identity' marks. Future studies dissecting the upstream control of CRH neurons classically not considered 'stress effectors' in the hypothalamus, the metabolic contexts prompting their CRH production and if CRH receptors are coincidently expressed at synaptically wired circuit components and if receptor-level switches in decoding the physiological gain of CRH signaling exists will undoubtedly fuel the establishment of novel organizing principles of hypothalamic neuronal diversity.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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