

A secretagogen locus of the mammalian hypothalamus controls stress hormone release

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Abstract

A hierarchical hormonal cascade along the hypothalamic-pituitary-adrenal axis orchestrates bodily responses to stress. Although corticotropin-releasing hormone (CRH), produced by parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) and released into the portal circulation at the median eminence, is known to prime downstream hormone release, the molecular mechanism regulating phasic CRH release remains poorly understood. Here, we find a cohort of parvocellular cells interspersed with magnocellular PVN neurons expressing secretagogen. Single-cell transcriptome analysis combined with protein interactome profiling identifies secretagogen neurons as a distinct CRH-releasing neuron population reliant on secretagogen's Ca^{2+} sensor properties and protein interactions with the vesicular traffic and exocytosis release machineries to liberate this key hypothalamic releasing hormone. Pharmacological tools combined with RNA interference demonstrate that secretagogen's loss of function occludes adreno-corticotrophic hormone release from the pituitary and lowers peripheral corticosterone levels in response to acute stress. Cumulatively, these data define a novel secretagogen neuronal locus and molecular axis underpinning stress responsiveness.

Keywords acute stress; Ca^{2+} sensor; HPA axis; vesicular release

Subject Categories Neuroscience

DOI 10.15252/embj.201488977 | Received 14 May 2014 | Revised 7 October 2014 | Accepted 21 October 2014 | Published online 27 November 2014

The EMBO Journal (2015) 34: 36–54

Introduction

Stress is the body's reaction to environmental conditions or noxious challenges. Fast adaptive responses to stress are reliant on a hierarchical hormonal cascade that adjusts and adapts to metabolic processes at the periphery (Selye & Fortier, 1949; Baxter & Forsham, 1972) to maximize energy expenditure in order to avoid adverse environments (Sapolsky *et al*, 1986; Swanson, 1991; Korte, 2001; de Kloet *et al*, 2005; McEwen, 2007). Stress hormone release is controlled by the hypothalamic-pituitary-adrenal (HPA) axis whose anatomical organization, allowing dynamic hormonal integration at the ligand and receptor levels and via extensive feedback and amplification loops, is well established (Kiss *et al*, 1984; Makino *et al*, 2002; Simmons & Swanson, 2009; Tasker & Herman, 2011). In contrast, the localization and molecular identity of many neurosecretory neurons in rodents (particularly mouse) and humans are less well characterized, partly due to the rapid, phase-locked nature of the synthesis and release of stress hormones, limiting their usefulness as cellular markers (Swanson & Simmons, 1989; Meister *et al*, 1990). In this context, the neuronal sites producing

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corticotropin-releasing hormone (CRH) (Spiess *et al*, 1981; Vale *et al*, 1981) in the paraventricular nucleus (PVN) of the hypothalamus (Swanson *et al*, 1986; Swanson & Simmons, 1989; Herman *et al*, 1992; Sawchenko *et al*, 1993), as well as rate-limiting steps of CRH release into the hypophyseal portal circulation at the median eminence, are of particular significance since CRH liberates adrenocorticotrophic hormone (ACTH) as the initial step of the peripheral stress response (Rivier & Vale, 1983; Kovacs & Sawchenko, 1996). Nevertheless, detailed molecular underpinnings of CRH release remain to be defined.

The release of arginine-vasopressin (AVP) and oxytocin from magnocellular neurosecretory cells (Gainer *et al*, 2002), and of combinations of releasing and inhibitory hormones and other neuropeptides from parvocellular neurons (Swanson & Sawchenko, 1983), requires Ca^{2+} -dependent quantal exocytosis of dense-core vesicles in response to afferent stimulation or direct metabolic challenges, entraining bursts of neuronal excitation in the PVN (Dutton & Dyball, 1979; Cazalis *et al*, 1985; Carafoli *et al*, 2001; Petersen *et al*, 2005; Dayanithi *et al*, 2012). The priming of vesicular release is regulated by a multi-protein complex sequentially recruiting Ca^{2+} sensors, SNARE proteins and the docking machinery embedded in the presynaptic terminal (Hata *et al*, 1993; Sollner *et al*, 1993; Sutton *et al*, 1998; Pang & Sudhof, 2010). Ca^{2+} sensors are proteins that undergo conformational changes upon Ca^{2+} binding, sculpting inter-molecular interactions with downstream effectors to propagate action potential-dependent release events (Brose *et al*, 1992; Sudhof & Rothman, 2009). As such, calbindin-D28k and calretinin were histochemically associated with magnocellular neurosecretory cells (Sanchez *et al*, 1992; Arai *et al*, 1993). In contrast, neuron-specific Ca^{2+} sensors were neither anatomically localized to nor functionally characterized in parvocellular hypothalamic neurons.

Recent neuroanatomical mapping places secretagogin, an EF-hand Ca^{2+} -binding protein (Wagner *et al*, 2000), into the PVN and adjacent pre-autonomic areas (Mulder *et al*, 2009; Maj *et al*, 2012). Despite the unexpected abundance of neurons expressing secretagogin mRNA and protein (secretagogin⁺ neurons) in the mammalian hypothalamus, their phenotype, cumulatively defined by their fast neurotransmitter, neuropeptide and/or hormone contents, remains unknown. Moreover, and even if the proximity of secretagogin⁺ neurons to the ventricular system along the longitudinal axis of the forebrain might indirectly infer a conserved role in secretory processes (Mulder *et al*, 2009), secretagogin's role in the nervous system remains elusive.

Here, we sought to address whether secretagogin marks a hitherto undescribed neurosecretory locus in the PVN. We have combined systems neuroanatomy, neurophysiology, single-cell transcriptomics and proteomics to unequivocally define secretagogin⁺ neurons as a cohort of CRH-expressing parvocellular cells, partly interspersed within magnocellular domains of the PVN, and to show that this protein is a Ca^{2+} sensor modulating CRH release. By exploiting a genetic approach, we show that secretagogin-mediated CRH secretion is indispensable to prime ACTH release into the general circulation upon acute noxious stress (Makara *et al*, 1981). Cumulatively, our results define a key molecular mechanism gating the HPA axis upon stress-induced activation of CRH release from a molecularly distinct subtype of neuroendocrine parvocellular neurons in the hypothalamus.

Results

Secretagogin⁺ neurons in the hypothalamic paraventricular nucleus

Recent neuroanatomical studies have identified abundant secretagogin expression in the hypothalamus (Mulder *et al*, 2009; Maj *et al*, 2012). Yet neither the detailed topology of secretagogin⁺ neurons nor their functional significance in neuroendocrine control is known. Herein, we localized secretagogin⁺ neurons, in descending order and along the longitudinal axis of the adult mouse brain, to paraventricular (PVN) = arcuate » periventricular, dorsomedial and ventromedial hypothalamic nuclei (Fig 1A–A₃ and B). A dense secretagogin⁺ cell group was found in the dorsolateral PVN, adjacent to and partly interspersed with magnocellular oxytocin⁺ and AVP⁺ magnocellular neurons, whose axons primarily descend into the posterior pituitary for the transient storage and “on-demand” release of these hormones (Bargmann, 1969; Gainer *et al*, 2002). Clearly, oxytocin, AVP and secretagogin segregated to non-overlapping cell cohorts (Fig 1B and B₁). We extended this analysis to show that the supraoptic nucleus, an alternative site for oxytocin⁺ and AVP⁺ magnocellular neurons to reside (Swanson & Sawchenko, 1983), lacked appreciable secretagogin expression (Fig 1B₂). These data suggest that secretagogin⁺ neurons belong to a discrete group of neurons interspersed with but not identical to oxytocin⁺ or AVP⁺ magnocellular neurons.

Next, we employed quantitative morphometry of secretagogin⁺ neurons to cytoarchitecturally distinguish them from their oxytocin⁺ or AVP⁺ counterparts. Secretagogin⁺ soma were significantly ($P < 0.05$) smaller in diameter than (Fig 1B₃), but equally round (Fig 1B₄) as those of either oxytocin⁺ or AVP⁺ neurons, suggesting parvocellular identity (Swanson & Sawchenko, 1983). Nevertheless, secretagogin⁺ axons occasionally terminated around blood vessels in the posterior pituitary and formed Herring body-like specializations that at points contained moderate levels of oxytocin (Fig 1C–D₃). Cumulatively, these data suggest that secretagogin is broadly expressed in the PVN in a molecularly distinct neuronal contingent. Here, we focused on deciphering the identity and function of small-diameter “parvocellular-like” PVN neurons.

Secretagogin typifies a novel subclass of parvocellular neurons in the PVN

Protein localization at the subcellular level is a reliable predictor of protein function. Our high-resolution laser-scanning analysis (Fig 1B and B₁) suggested that secretagogin, a predicted cytosolic protein (Birkenkamp-Demtroder *et al*, 2005), might be distributed ubiquitously, including dendrites and axons. We confirmed this notion at the ultrastructural level by pre-embedding immunoelectron microscopy (Fig 2A–C). In the PVN, silver-intensified immunogold particles, revealing secretagogin's localization, were detected in the cytoplasm (Fig 2A), particularly in submembranous compartments or in likely contact with the plasmalemma (Fig 2A₁), and along the endoplasmic reticulum (Fig 2A₂). Likewise, secretagogin was localized to dendrite shafts, often along with their inner membrane surface (Fig 2B). Moreover, we demonstrated secretagogin immunoreactivity in close proximity to the plasmalemma in nerve endings and possibly to dense-core vesicles (Fig 2C).

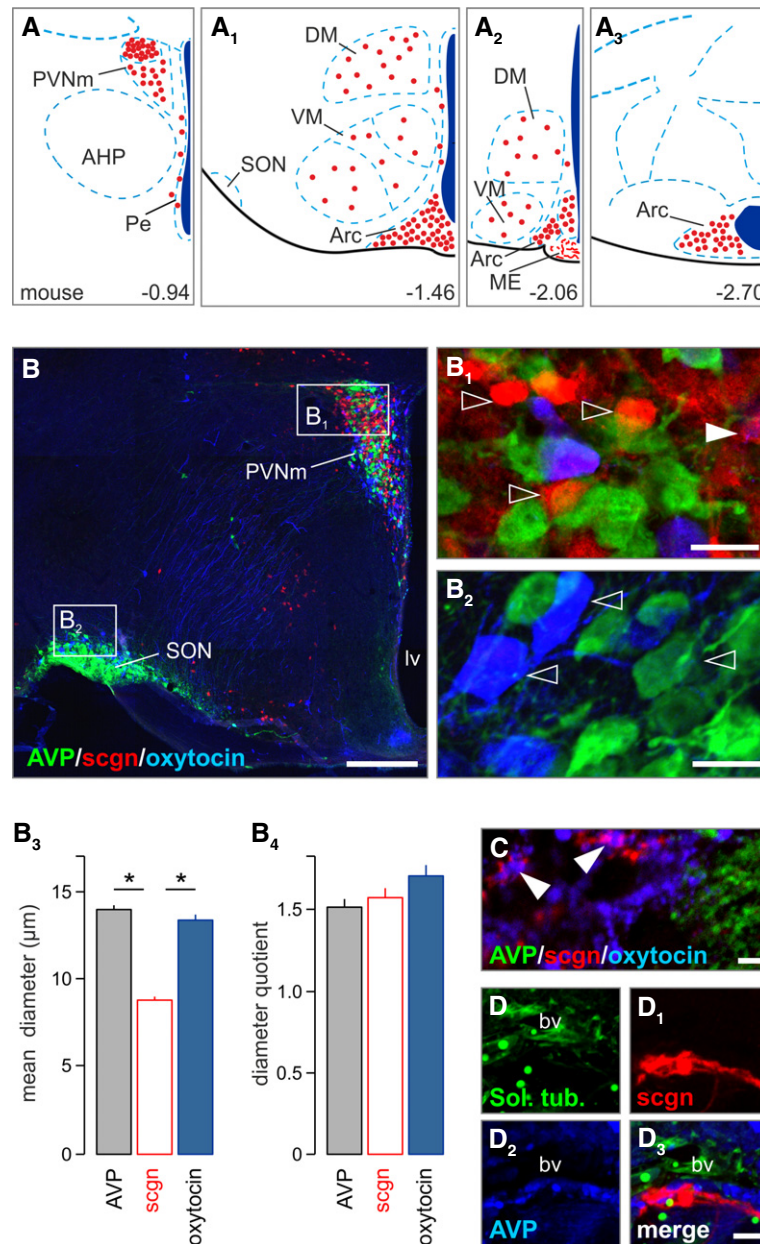


Figure 1. Secretagogin locus in the paraventricular nucleus of the hypothalamus.

A–A₃ Secretagogin⁺ neurons populate the paraventricular, dorsolateral, ventromedial, periventricular and arcuate nuclei of the mouse hypothalamus (Paxinos & Franklin, 2001). Red circles denote the localization of neuronal perikarya and their relative densities. AHP, anterior hypothalamus; Arc, arcuate nucleus; DM, dorsomedial nucleus; ME, median eminence; Pe, periventricular nucleus; PVN, paraventricular nucleus, PVNm, magnocellular part; SON, supraoptic nucleus; VM, ventromedial nucleus.

B–B₂ Largely non-overlapping distribution of AVP⁺, oxytocin⁺ and secretagogin⁺ (scgn⁺) neurons in the magnocellular PVN. The mouse supraoptic nucleus (SON) harbored vasopressin⁺ and oxytocin⁺ but not secretagogin⁺ neurons. Open arrowheads pinpoint single-labeled neurons. Solid arrowhead denotes AVP/secretagogin dual-labeling. lv, lateral ventricle; PVNm, magnocellular part of the paraventricular nucleus; scgn, secretagogin.

B₃, B₄ Secretagogin⁺ neurons had smaller somatic diameters than AVP⁺ or oxytocin⁺ neurons yet without a difference in their diameter quotient, a measure of ovoid profiles (**P* < 0.05, Student's *t*-test).

C–D₃ Terminal-like profiles in the posterior pituitary (arrowheads in C) suggesting that secretagogin can co-exist, even if infrequently with oxytocin. *Solanum tuberosum* lectin (Sol. tub.) was used to identify blood vessels.

Data information: Scale bars: 100 µm (B), 10 µm (B₁, B₂), 2 µm (C–D₃)

Quantitative analysis of gold-intensified silver particles in the PVN (soma, dendrites) and the median eminence (axonal nerve endings) demonstrated that the majority of secretagogin associates to (endo-)

membranes in neurons (that is, a given particle was < 50 nm from the nearest membrane surface; Fig 2D). Nevertheless, we found an opposite configuration in secretagogin distribution when comparing

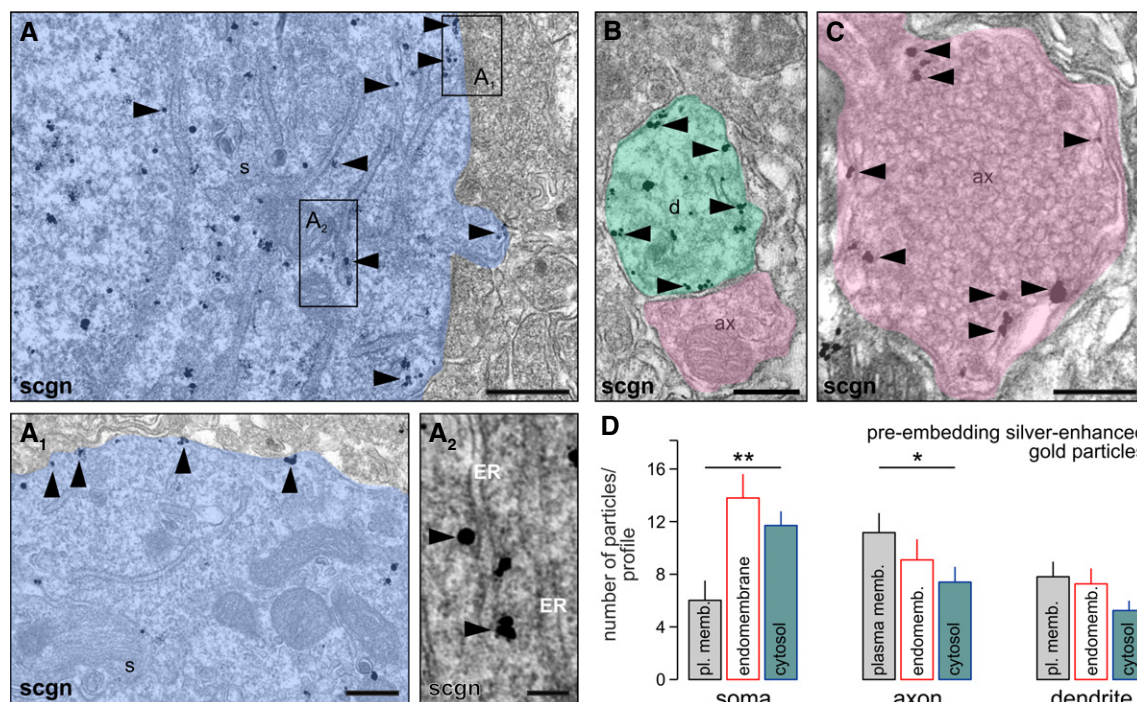


Figure 2. Ultrastructural analysis suggests the prevalence of membrane-bound secretagogin.

A Secretagogin (scgn) distribution at the ultrastructural level as revealed by pre-embedding silver-enhanced immunogold labeling. Secretagogin (arrowheads) was localized to membranous organelles in the perikarya (A), particularly the plasmalemma (A₁) and endoplasmic reticulum (ER) in neuronal soma (s; A₂). Open rectangles in (A) denote the location of insets. Semi-transparent shading is used to visually dissociate subcellular compartments in (A–C).
 B Pre-embedding secretagogin labeling (arrowheads) was also seen in dendrite (d) segments.
 C In axo-dendritic terminals (ax), secretagogin was closely associated with synaptic vesicles along the plasmalemma (arrowheads).
 D Quantitative analysis of subcellular secretagogin distribution upon electron microscopy detection of silver-enhanced gold particles. Particles were considered as membrane bound when they were at < 50 nm of a membrane (plasma membrane or endomembrane; i.e., secretory vesicle, Golgi or endoplasmic reticulum). In the soma of PVN neurons, significantly more particles were found in the cytosol as along the plasma membrane (***P* < 0.01). In contrast, membrane association predominated in axonal nerve endings in the median eminence (**P* < 0.05). Note that a significant proportion of particles was found adjacent to endomembranes in all subcellular compartments studied.

Data information: Scale bars, 1 μ m (A), 500 nm (B, C), 200 nm (A₁, A₂).

particle numbers on the plasmalemma and in the cytosol in neuronal soma and axons: In neuronal soma, the majority of secretagogin labeling appeared intracellular (Fig 2D). In contrast, secretagogin localization along the plasma membrane of nerve endings outweighed cytosolic secretagogin content. These data suggest that secretagogin might play a role in controlling hormone or neuro-peptide release.

Next, we have taken a neurophysiological approach to gain insights into the biophysical properties of secretagogin⁺ neurons and distinguish these from magnocellular, parvocellular or pre-autonomic PVN neurons using their properties to generate action potentials (APs), as well as 16 additional parameters (Supplementary Table S1) as classification criteria (Luther & Tasker, 2000; Luther *et al*, 2002). In rat, magnocellular neurons are characterized by a delay in generating the first AP upon current stimulation after pre-hyperpolarization (type I) due to a relatively high amplitude of their A-type currents (Luther & Tasker, 2000; Lee *et al*, 2012). In contrast, AP delay is atypical for parvocellular neurons (type II) (Luther & Tasker, 2000; Lee *et al*, 2012). Pre-autonomic neurons generate low threshold spikes and bursts given the activity of voltage-gated T-type Ca²⁺ channels (Stern,

2001; Lee *et al*, 2008). In whole-cell patch-clamp experiments using *ex vivo* hypothalamus slice preparations, we have analyzed > 75 neurons in the PVN and pre-autonomic areas of juvenile mice (postnatal days 21–28). Using unified current- and voltage-clamp protocols, we designed novel classification criteria for mouse PVN neurons (Fig 3), distinguishing three primary neuron types, which could then be clustered into six subtypes (Fig 3E). In particular, type I neurons were reminiscent of magnocellular neurons from rat, including delayed AP generation after pre-hyperpolarization and high-amplitude A-type-like currents (Fig 3A–A₃ and D). This neuron population could be divided into *Ia* and *Ib* subgroups, based on outward current properties in recording conditions inactivating A-type channels (Supplementary Fig S1). *Ia* neurons generated outward currents typical for slowly activated delayed-rectifying K⁺ channels (Fig 3D, Supplementary Fig S1). Meanwhile, *Ib* neurons exhibited transient, fast-activated currents upon depolarization (from –40 mV; Supplementary Fig S1). *Post hoc* immunohistochemistry defined biocytin-filled magnocellular neurons as exclusively belonging to the *Ia* group.

Neurons that had histochemically been identified as secretagogin positive (Fig 3B–B₂) primarily belonged to type II mouse parvocellular

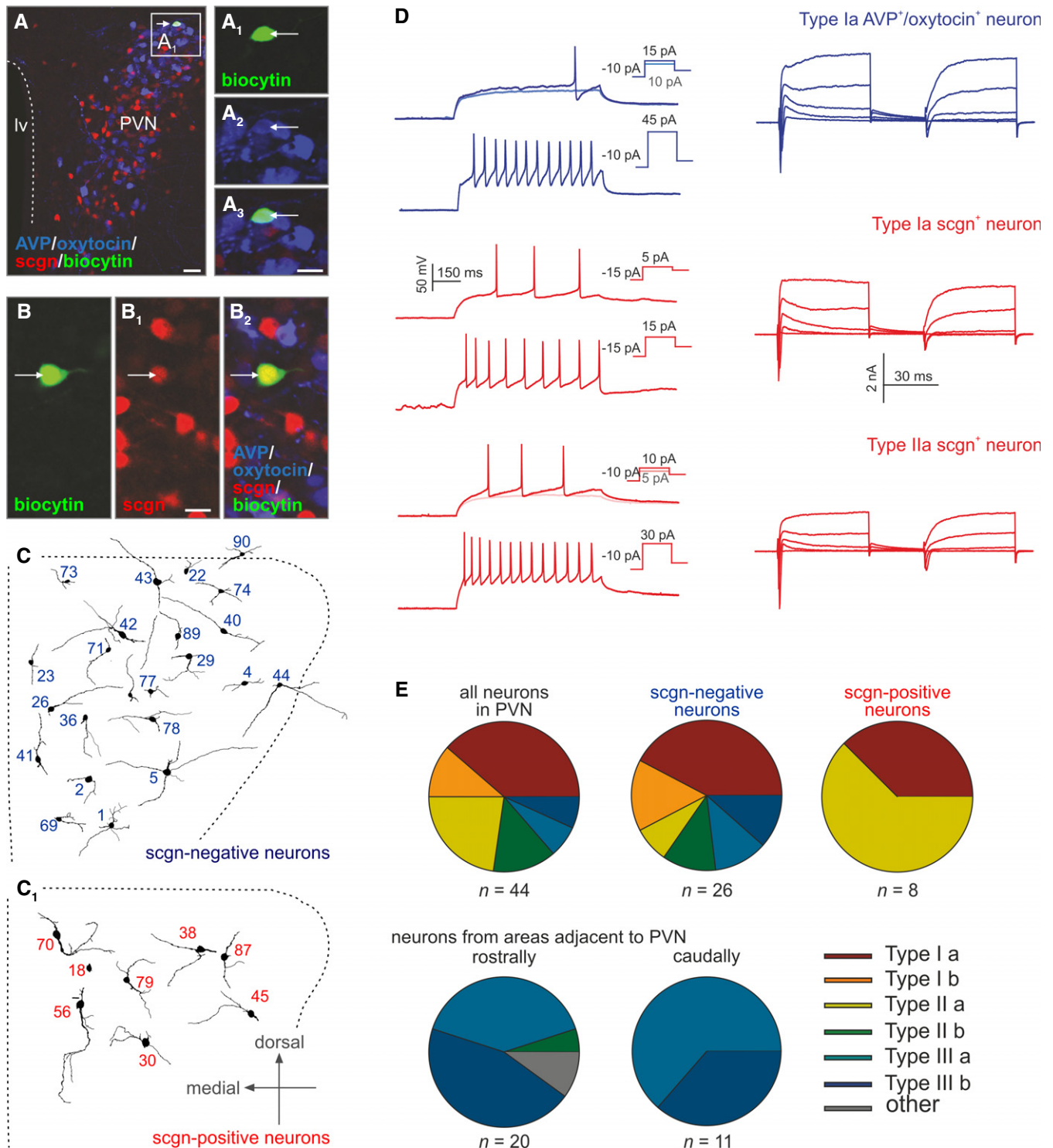


Figure 3. Electrophysiological classification of secretagogin⁺ parvocellular neurons.

A–A₃ Biocytin-filled (arrow) neuron immunonegative for secretagogin yet containing AVP/oxytocin (a mixture of magnocellular markers was used in triple-labeling experiments). Scale bars: 50 μ m (A), 8 μ m (A₃). lv, lateral ventricle; PVN, paraventricular nucleus.

B–B₂ Secretagogin⁺ biocytin-filled neuron (arrow) lacking AVP/oxytocin immunosignal. Scale bars: 8 μ m.

C, C₁ Dendritic reconstruction of secretagogin⁻ and secretagogin⁺ neurons at their actual location in the paraventricular nucleus of the hypothalamus.

D Electrophysiological characteristics of magnocellular and parvocellular neurons. The waveform of repetitive action potential firing is shown on the left, while differential channel characteristics are depicted on the right. Examples of secretagogin⁺ neurons are shown in red.

E Pie diagrams showing the grouping of neurons in the PVN (upper row) and in adjacent pre-autonomic areas (lower row) cumulatively based on electrophysiological criteria listed in Supplementary Table S1. Secretagogin⁺ neurons typically belonged to Ia and IIa subtypes.

neurons and were equivalent in biophysical properties to those described in rat (Lee *et al*, 2012) or mouse (Wamsteeker Cusulin *et al*, 2013). Using the classification criteria introduced for type I neurons above, the type II cohort was subdivided as *Ila* and *Iib* neurons (Supplementary Fig S1). Nevertheless, we also identified some secretagogin⁺ neurons with AP signatures similar but not identical to magnocellular cells (Fig 3D and E), reinforcing our hypothesis on immunohistochemically undetectable AVP and/or oxytocin levels and introducing a novel scale of molecular heterogeneity among magnocellular PVN neurons.

Lastly, type III neurons were secretagogin negative(−) low threshold and produced spike bursts upon somatic current injections (Supplementary Fig S1). These pre-autonomic cells, likewise sub-clustered as *IIla* and *IIlb* (Fig 3E), therefore were excluded as being secretagogin⁺ neurons. Overall, these data suggest that the majority of secretagogin⁺ neurons in the cluster were parvocellular cells in the PVN.

Secretagogin⁺ parvocellular neurons express CRH

Parvocellular neurons in the PVN and other hypothalamic areas are diverse as to their neurochemical phenotypes (Swanson & Sawchenko, 1983; Everitt *et al*, 1986; Swanson *et al*, 1986), with their majority producing releasing or release-inhibiting hormones (Guillemin, 1978; Schally, 1978) that regulate trophic hormone secretion in the anterior pituitary via the *rete mirabile* of the hypothalamo-hypophyseal portal system, a concept originally described by Harris (1972). Considering that somatic neuropeptide and hormone detection is often difficult and relies on the permanent blockade of the anterograde axonal transport machinery (Cortes *et al*, 1990), we applied unbiased clustering analysis to single-cell transcriptome data (Tsafirir *et al*, 2005; Islam *et al*, 2014) generated from 130 cells dissociated from the mouse PVN. Overall, oxytocin, AVP, somatostatin, GABA and glutamate neurons exhibited discrete mRNA profiles in the PVN (“hot spots” in Fig 4A and A₁; GEO accession number GSE63093, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63093>). In contrast, secretagogin⁺ neurons, as well as CRH⁺ and thyrotropin-releasing hormone (TRH)⁺ neurons, did not form separate clusters.

Next, we focused on which RNAs for hormones, neuropeptides and/or low-molecular-weight neurotransmitters were present in secretagogin⁺ neurons. We demonstrated the transcriptome identity of these neurons by analyzing the absolute number of mRNA transcripts from 151 cells dispersed from the mouse PVN (Fig 4A and A₁). We first tested the abundance of AVP, oxytocin, CRH and TRH gene transcripts (Fig 4A and A₁) and show their broad variations, with particular reference to AVP and oxytocin mRNAs (that is, variations at 3–4 orders of magnitude; Supplementary Fig S2A). We defined a GABAergic hypothalamic contingent of ~30%, expressing Gad1 and/or Gad2 encoding respective glutamic acid decarboxylase 65 and 67 kDa (GAD65/GAD67) isoforms, as well as the vesicular GABA transporter (Slc32A1; Fig 4A). Tyrosine hydroxylase and vesicular glutamate transporter 2 mRNAs were present in ~15 and ~10% of the threshold-adjusted sample, respectively.

We then restricted our analysis of baseline mRNA expression values for each gene by ranking from minimum to maximum expression values (Supplementary Fig S2A) to increase homogeneity

(Fig 4A₁). As such, this cell population comprised 7.3% oxytocin⁺ neurons (*n* = 11), 6.0% AVP⁺ neurons (*n* = 9), 4.6% TRH⁺ neurons (*n* = 7) and 6.6% CRH⁺ neurons (*n* = 10). When dissecting the presence of gene transcripts of key pro-neuropeptides, our data from 151 cells revealed distinct somatostatin (*n* = 15 with high level), galanin (*n* = 11), cholecystokinin (*n* = 5), tachykinin 1 (*n* = 9), cocaine- and amphetamine-regulated transcript (CART; *n* = 24), neurotensin S (*n* = 2), VGF nerve growth factor-inducible protein (*n* = 21), calcitonin gene-related peptide (*n* = 4), neuromedin B (*n* = 4), neuromedin S (*n* = 2), natriuretic peptide C (*n* = 2) and adenylate cyclase activating polypeptide 1 (Adcyap1; *n* = 2). Of these, somatostatin, galanin, cholecystokinin, neurotensin S and CART were expressed at mRNA copy numbers exceeding 100 per cell, while other peptides were found expressed at low or moderate copy numbers. mRNA transcripts for neuropeptide Y, neuromedin U, neuropeptide B and pro-opiomelanocortin were infrequently present. The co-existence of fast neurotransmitters (GABA, dopamine, glutamate) with hormones and neuropeptides allowed for a refined molecular classification of the PVN and associated hypothalamic areas (Fig 4A and A₁).

Lastly, we focused on secretagogin mRNA expression (Fig 4A and A₁; GEO accession number GSE63093, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63093>). From a total of 151 cells, we identified nine cells with secretagogin mRNA transcripts. Secretagogin⁺ cells in the sampling cohort lacked AVP, oxytocin, TRH, galanin, cholecystokinin, neurotensin S, calcitonin, neuromedin S, natriuretic peptide C and Adcyap1 mRNAs. In contrast, some secretagogin⁺ cells contained somatostatin, Tac1, CART, neuromedin B, neuromedin U, neuropeptide Y and/or neuropeptide B mRNA transcripts (Fig 4A and B). Secretagogin⁺ cells abundantly carried CRH mRNA transcripts and those of the promiscuous glucocorticoid receptor Nr3c1 (Fuxe *et al*, 1985) (Pearson's correlation coefficient = 0.399, *P* < 0.001), the latter being involved in the stress-induced feedback regulation of CRH synthesis (Aguilera *et al*, 2007).

mRNA may not be translated into appreciable amounts of protein. Therefore, it was imperative to histochemically verify the neurochemical identity of secretagogin⁺ neurons. First, we used a reporter mouse line expressing GFP under the control of the CRH promoter (Alon *et al*, 2009) to reveal the abundant presence of CRH⁺/secretagogin⁺ neurons in the PVN (Fig 4C and C₁). We then applied colchicine *in vivo* to provoke neuropeptide accumulation in somatic domains in parvocellular neurons (Fig 4D–I₂) (Cortes *et al*, 1990). Indeed, secretagogin was found chiefly co-expressed with CRH in PVN in both neuronal soma and local axon collaterals (Fig 4D–E₃) and, less frequently, with tyrosine hydroxylase (ventrolateral arcuate nucleus; Fig 4F–F₃), GABA (dorsomedial arcuate nucleus; Fig 4G–G₂), somatostatin (Fig 4H–H₃) and galanin (Fig 4I–I₂) with dual-labeled secretory terminals accumulating in the median eminence (Fig 5A–A₂). These data define secretagogin⁺ neurons as a CRH-containing cell cohort.

Secretagogin⁺ neurons are tightly packed in the dorsolateral PVN. Dendritic reconstructions clearly distinguish these cells from magnocellular neurons (Fig 3C and C₁). Nevertheless, we controlled our *in vitro* transcriptome profiling by plating dissociated PVN neurons from newborn mice and assessing their dendritic morphology. Secretagogin⁺ neurons had significantly smaller (*P* < 0.05; Supplementary Fig S2B and C) somatic diameter and more emanating (*P* < 0.05) and exceedingly arborizing (*P* < 0.05) processes than

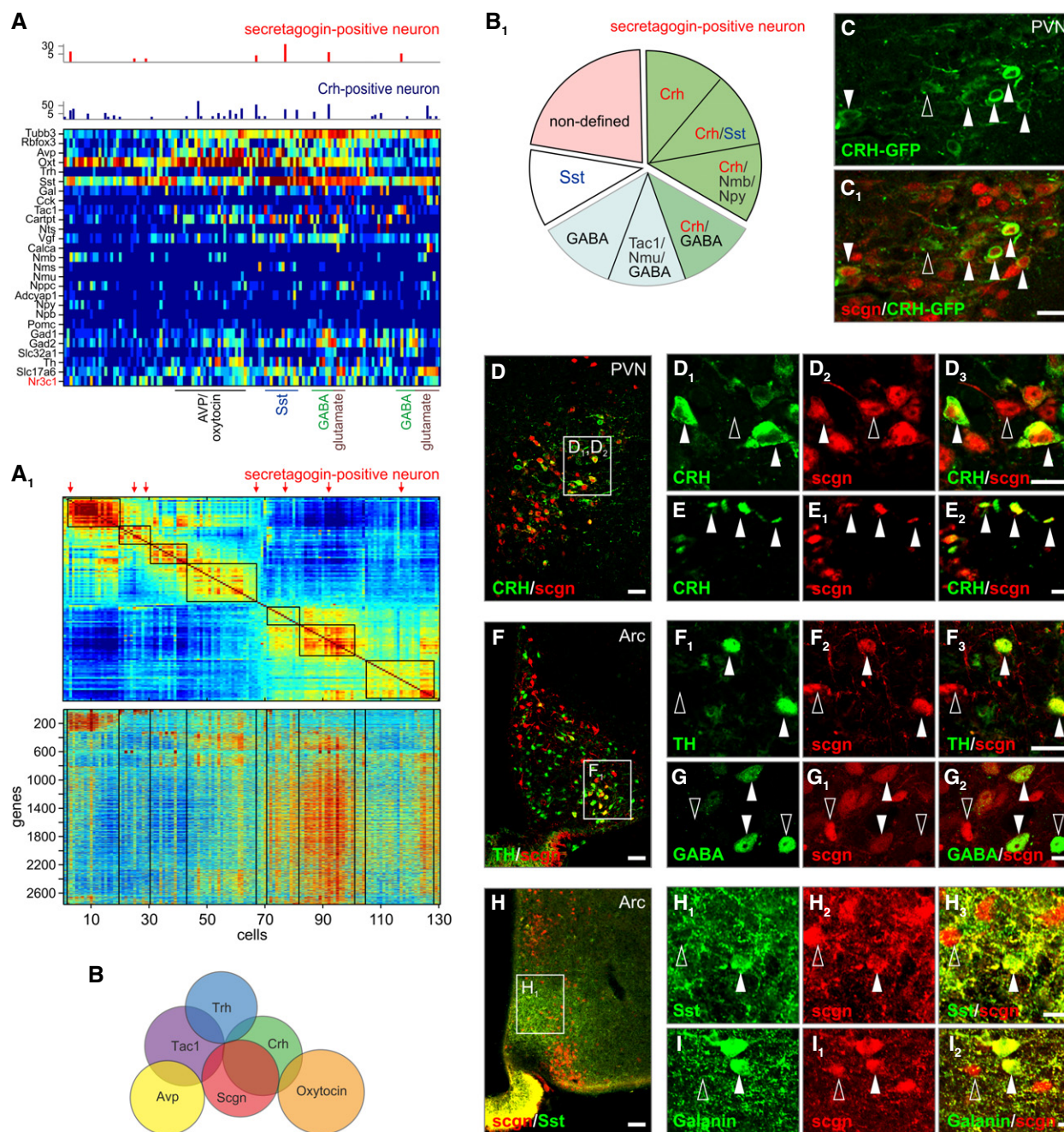


Figure 4. Molecular identity of secretagogin⁺ paraventricular neurons.

Single-cell mRNA transcriptome profiling of dissociated cells from the mouse paraventricular nucleus.

- A** Differential clustering based on secretagogin (Scgn), neuropeptide, hormone and hormone receptor mRNA expression. Secretagogin-expressing(+) neurons typically contained corticotropin-releasing hormone (Crh) and Nr3c1 mRNA transcripts.
- A₁** Clusters of gene transcripts from 130 cells reveal the phenotypic segregation of PVN neurons. Increasing mRNA copy numbers were depicted by a color gradient from deep blue (not detected) to red (high numbers). Secretagogin⁺ neurons are indicated by red arrows.
- B** Venn diagrams depicting the (non-)overlapping relationships of select mRNA transcripts used to molecularly define PVN neurons.
- B₁** Cumulative ratio of phenotypic diversity among secretagogin⁺ neurons. Note that Crh and/or GABA (derived from Gad1, Gad2 and Slc32a1 mRNA expression) co-existed with secretagogin in parvocellular neurons.
- C, C₁** Secretagogin co-localization with GFP in the PVN (solid arrowheads) of adult CRH-GFP (BAC) reporter mice (Alon et al, 2009). Open arrowhead pinpoints a single-labeled neuron.
- D–I₃** We found secretagogin (scgn) co-expressed with CRH in both neuronal soma (solid arrowheads; D₁–D₃) and axon terminal-like specializations (arrowheads; E–E₂) in the PVN. Likewise, secretagogin⁺ neurons harbored, yet infrequently, tyrosine hydroxylase (TH; solid arrowheads; F–F₃), GABA (G–G₂), somatostatin (Sst; H–H₃) and galanin (I–I₂). Open rectangles denote the general location of insets. Open arrowheads indicate the lack of co-localization.

Data information: Scale bars: 40 μ m (D, F, H), 25 μ m (C₁), 10 μ m (D₃, F₃, G₂, H₃), 4 μ m (E₂, I₂).

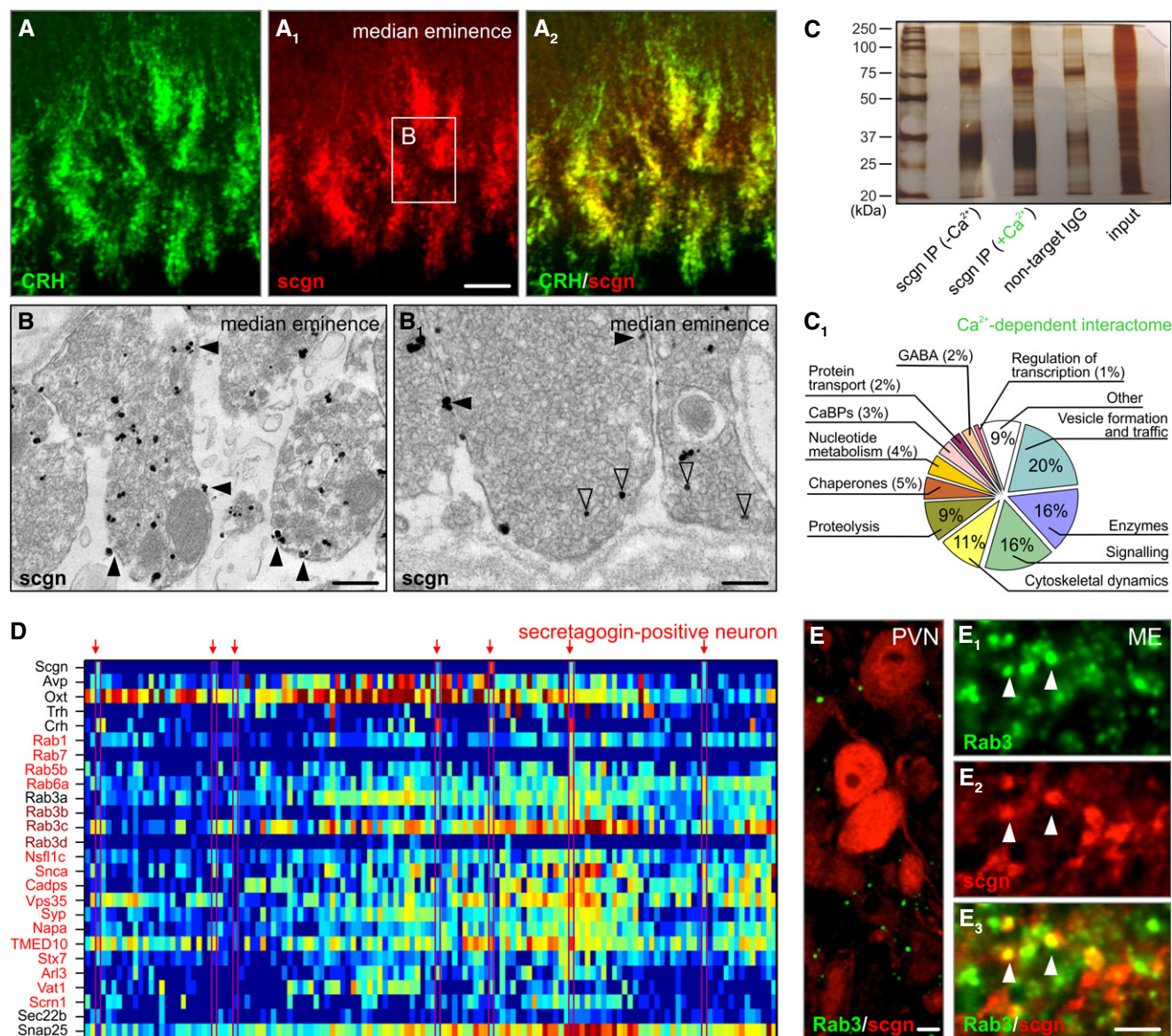


Figure 5. Secretagogin is a Ca²⁺ sensor protein.

A–A₂ Secretagogin co-existed in the majority of CRH⁺ nerve endings in the median eminence (ME). Open rectangle denotes the general location of (B, B₁).
 B, B₁ Large axon terminals in the median eminence were immunopositive for secretagogin with silver-intensified immunogold particles (open arrowheads) associated with axonal membrane and dense-core vesicles (see Fig 1D for quantitative data). Solid arrowheads denote silver deposit particles proximal to the plasmalemma.
 C Immunoprecipitation using an anti-secretagogin antibody in Ca²⁺-free and Ca²⁺-loaded conditions was subtractively used to decipher the Ca²⁺-dependent protein interactions. Silver-stained gel is shown.
 C₁ Ontology classification of the 99 protein hits based on primary function assignments. Unbiased MALDI-TOF proteomics was used to identify interacting proteins. The most abundant hits (Supplementary Table S3 is referred to for details on individual proteins) are proteins implicated in vesicle fusion, trafficking, transport and formation and the regulation of vesicle exocytosis. "Other" refers to a group of proteins without known function.
 D Single-cell transcriptomics was used to validate the above proteome data by clustering mRNA transcripts encoding proteins that underpin vesicular release processes (red color labels highest mRNA abundance, whereas dark blue color indicates the lack of mRNA expression).
 E–E₃ Rab3, a family of vesicular fusion and transport proteins (Schluter et al, 2006), was found co-localized with secretagogin (arrowheads) in the median eminence. Neuronal soma in the PVN lacked appreciable co-localization. Note that neither our MALDI-TOF analysis nor our histochemical probing allowed the precise identification of individual Rab3C-E family members.

Data information: Scale bars: 10 μ m (A₁, E₃), 7 μ m (E), 500 nm (B), 150 nm (B₁).

AVP⁺ neurons. In addition, the majority were secretagogin⁺/AVP[−] neurons. Yet a small secretagogin⁺ subpopulation co-expressed AVP, with their somatic diameters and dendrite complexities reminiscent of AVP⁺/secretagogin[−] neurons (Supplementary Fig S2B

and C), lending further support to our *in vivo* findings (Fig 1). Ca²⁺-imaging experiments after KCl stimulation showed that parvocellular-like secretagogin⁺ neurons clearly segregated from their AVP⁺ or AVP⁺/secretagogin⁺ counterparts (Supplementary Fig S2D).

Moreover, secretagogen⁺ neurons responded differentially to excitatory stimuli, including NMDA combined with glycine ($P < 0.01$), kainate ($P < 0.001$) and GABA (Haam *et al*, 2012) ($P < 0.001$; Supplementary Fig S3A–D₁). These data not only demonstrate that secretagogen⁺ parvocellular neurons represent a molecularly and functionally distinct class of PVN neurons but also imply that secretagogen, a Ca²⁺-binding protein presumed associated with the SNARE machinery *in vitro* (Rogstam *et al*, 2007; Bauer *et al*, 2011), might sculpt their responsiveness and/or affect CRH release. However, direct *in vivo* evidence discerning Ca²⁺ “buffer” versus “sensor” roles for this protein remained elusive.

Secretagogen is a Ca²⁺ sensor expressed in the median eminence

Secretagogen⁺ neurons exhibited low-amplitude Ca²⁺ responses to depolarizing stimuli (Supplementary Fig S2D), as compared to AVP⁺/secretagogen[−] neurons *in vitro*. Therefore, we first tested whether secretagogen acts as a Ca²⁺ buffer. Upon transfecting human SH-SY5Y neuroblastoma cells with a plasmid encoding human secretagogen, we estimated their Ca²⁺ buffer capacity by population-wide (> 100 cells/experiment) Ca²⁺ imaging experiments in combination with *post hoc* assessment of neurochemical properties (Supplementary Fig S4A). The amplitude of Ca²⁺ responses in secretagogen⁺ neurons was not statistically different from control cells (Supplementary Fig S4B). Next, the relative level of secretagogen, scaled as immunofluorescence intensity (Supplementary Fig S4C), did not correlate with either the basal intracellular Ca²⁺ level or peak amplitudes evoked by carbachol or KCl treatments. Similarly, Ca²⁺ response kinetics failed to significantly correlate with secretagogen immunoreactivity at the level of individual cells (data not shown). Cumulatively, these results support that secretagogen is unlikely to function either as slow or as fast Ca²⁺ buffer, at least *in vitro*.

In view of the negative data on Ca²⁺ buffering, we hypothesized that secretagogen is a Ca²⁺ sensor *in vivo*. This notion is supported by secretagogen's concentration and co-localization with CRH in the median eminence (Fig 5A–A₂), the locus of the first capillary network of the hypothalamic artery that collects releasing hormones produced by neurosecretory cells, including CRH (Harris, 1972; Léránth *et al*, 1983; Swanson & Sawchenko, 1983). In particular, we employed pre-embedding silver-intensified immunogold labeling to show, at the ultrastructural level, that secretagogen is present in axon terminals with frequent association with the plasmalemma, and possibly also with the outer membrane of dense-core vesicles (Fig 5B and B₁; for quantitative data in axons see Fig 2D). The presence of silver precipitates along the inner membrane of the nerve terminal reinforces a potential role for secretagogen as a Ca²⁺ sensor.

Next, we addressed whether secretagogen is a Ca²⁺ sensor to influence neurotransmitter transport and vesicular release by defining its Ca²⁺-dependent interactome using unbiased matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) proteomics (Fig 5C). We deployed a subtractive approach, whereby positive hits were defined as only recruited in the presence of 10 μM Ca²⁺ in the isolation buffers but not recovered under Ca²⁺-free conditions or by non-specific rabbit IgGs. Our hits (97 proteins in Ca²⁺-dependent interactions and 15 proteins without Ca²⁺ dependence (Supplementary Table S3); minimum number of peptide fragments ≥ 2, protein threshold: 99%, peptide threshold: 99%) therefore were

interpreted as recruited upon Ca²⁺ influx (Fig 5C₁, Supplementary Table S3), and chiefly included proteins implicated in: (i) vesicle formation, fusion and traffic; (ii) enzymatic reactions; (iii) signaling; and (iv) cytoskeletal dynamics and axonal transport (Fig 5C₁) (Sudhof, 2004; Schluter *et al*, 2006; Wickner & Schekman, 2008). We also uncovered interactions with other Ca²⁺-binding proteins, glutamate decarboxylase and vesicular GABA transporter (Fig 5C₁), reinforcing our transcriptome analysis. We validated the expression of mRNAs for many of these proteins in secretagogen⁺ neurons by targeted analysis of our single-cell transcriptome data (Fig 5D). This approach revealed the expression of multiple genes of interest involved in vesicular traffic and fusion in secretagogen⁺ parvocellular neurons (Fig 5D). Lastly, we performed immunohistochemistry in rat paraventricular neurons and median eminence with antibodies raised against Rab3 (all isoforms) to visualize secretagogen's frequent co-existence with this family of proteins at the median eminence (Fig 5E–E₃). In sum, these findings suggest that secretagogen is a Ca²⁺ sensor, which can affect CRH release from parvocellular neurons.

Secretagogen controls CRH release *in vitro* and *in vivo*

We first determined whether secretagogen can affect CRH release by its RNAi-mediated knockdown in native PVN neurons (Fig 6A), followed by secretagogen histochemistry and the recovery of CRH in culture supernatants (Fig 6A₁). We found significantly decreased CRH content in the culture media of hypothalamic neurons exposed to secretagogen-directed RNAi (Fig 6A₁). Subsequently, we took the opposite approach and transiently overexpressed secretagogen in an immortalized CRH-containing embryonic mouse hypothalamic cell line (mHypoE-N44). Three days after transfection, mHypoE-N44 cells were stimulated with 15 μM ATP for 20 min, fixed and immunostained for secretagogen (Fig 6B and B₁) with simultaneous intracellular CRH detection (Fig 6B₂). CRH levels negatively correlated with that of secretagogen (Pearson's correlation coefficient = −0.189, $P = 0.016$) upon secretagogen overexpression. In contrast, no significant correlation between cytosolic secretagogen and extracellular CRH content was determined in non-stimulated samples (Pearson's correlation coefficient = 0.0379, $P = 0.56$). We concluded that in mHypoE-N44 cells, secretagogen can facilitate ATP-dependent CRH release without an effect on CRH synthesis *per se*.

Next, we sought evidence that secretagogen knockdown affects CRH release *in vivo* by injecting siRNA (“Accell”) against secretagogen into the lateral ventricle and by performing quantitative double-label histochemistry in serial sections 3 days later. In the anterior PVN, which comprises CRH⁺ parvocellular neurons (Keegan *et al*, 1994), secretagogen knockdown occurred in 20–30% of CRH neurons (Fig 6C and C₁). Since CRH, like other neuropeptides, undergoes axonal transport and Ca²⁺-dependent release after synthesis (Alonso *et al*, 1986), we observed CRH retention in parvocellular neuronal soma in PVN in RNAi-exposed hypothalami ($P < 0.05$; Fig 6C₂, D and D₁). We attributed the lack of change in secretagogen immunoreactivity, when measuring immunofluorescence intensity in individual cells, to the fact that our knockdown approach effectively arrested secretagogen expression in high CRH-expressing neurons, as supported by the relative scarcity of dual-labeled neurons. We considered our RNAi

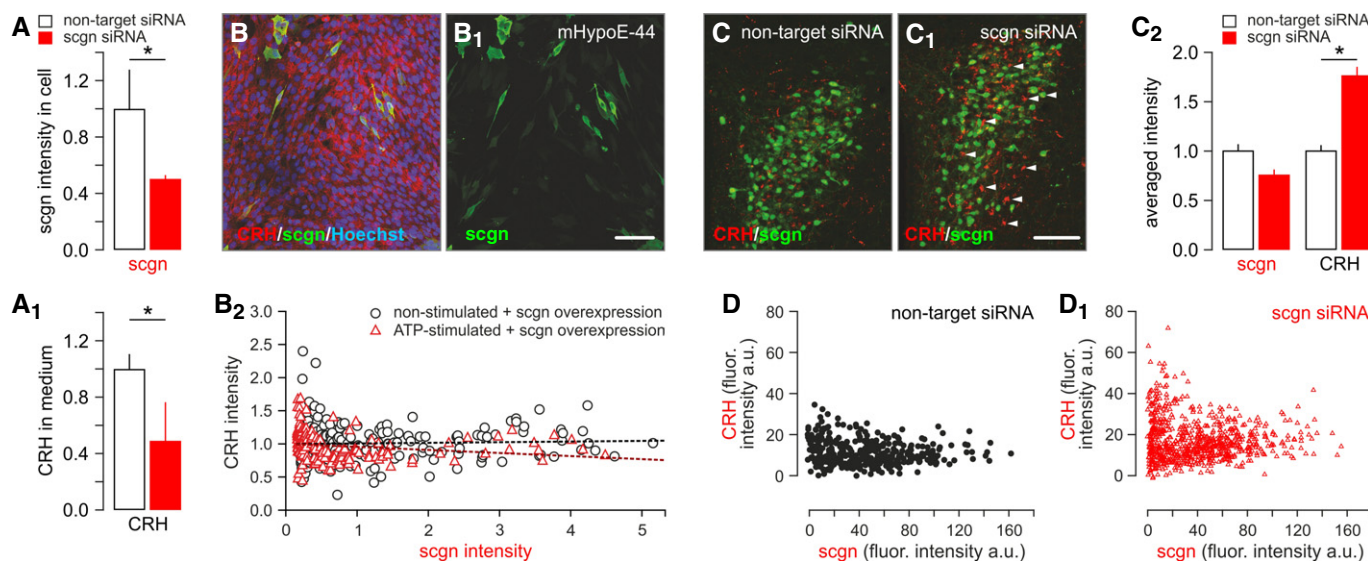


Figure 6. Secretagogen regulates CRH release *in vitro* and *in vivo*.

A, A₁ siRNA-mediated secretagogen (scgn) knockdown in cultured hypothalamic neurons, as indicated by reduced secretagogen immunoreactivity (A) and decreased CRH content in the culture medium (A₁).

B–B₂ Transient overexpression of secretagogen in immortalized CRH-expressing mHypoE-N44 hypothalamic cells significantly reduced CRH immunofluorescence intensity in secretagogen⁺ cell bodies, indirectly supporting enhanced CRH release. All experiments were performed in triplicate. Scale bar: 50 nm.

C, C₁ siRNA-mediated *in vivo* silencing of secretagogen mRNA expression in the PVN provoked somatic CRH accumulation (arrowheads). Scale bar: 150 μ m.

C₂ Quantitative analysis demonstrating significantly increased somatic CRH contents. Note that somatic secretagogen levels remained unchanged, which we interpret as data on a neuronal contingent not affected by siRNA silencing. The lack of secretagogen/CRH co-localization suggests that secretagogen expression fell below detection threshold in many CRH⁺ neurons.

D, D₁ Individual data points show maximal CRH fluorescence intensity (gray scale arbitrary unit (a.u.) expression) in PVN neurons that have low or no secretagogen expression after siRNA infusion.

Data information: Data in (C₂) were normalized to those in non-targeting siRNA controls. **P* < 0.05 versus control.

approach aimed to silencing secretagogen expression specific because neither magnocellular neurosecretory cells nor some neuropeptide systems localized to the PVN were affected upon experimental manipulations (Supplementary Fig S5A–D). Overall, our data suggest that secretagogen might modulate CRH release in the hypothalamus.

Secretagogen gates the CRH–ACTH axis in response to acute stress

Secretagogen's involvement in modulating CRH release *in vitro* and *in vivo* suggests that this Ca²⁺-binding protein can be instrumental to modulate the stress-induced mobilization of the hypothalamic-pituitary-adrenal axis by priming the hierarchical CRH–ACTH cascade that underpins a peripheral corticosterone surge from the adrenal cortex (Swanson & Sawchenko, 1980; Makara *et al*, 1981; Swanson & Simmons, 1989). If so, secretagogen⁺ neurons are assumed to rapidly up-regulate their CRH content and function as “on-neurons” to activate hormonal responses to stress. We addressed this hypothesis by injecting formalin (4%) into one paw (Pacak *et al*, 1995; Naveilhan *et al*, 2001) and detecting activation of the immediate early gene *c-fos* (Ohtori *et al*, 2000) in the PVN 6h later (Fig 7A and B). Quantitative immunohistochemistry revealed that 62 $\pm$ 8% of *c-fos*⁺ neurons co-expressed secretagogen and CRH (Fig 7B₁).

Since secretagogen is a Ca²⁺ sensor protein expressed in CRH⁺ cells recruited by acute stress, it is plausible to assume that its

physiological role is the control of CRH release. To test this hypothesis, we performed secretagogen siRNA knockdown *in vivo* followed by determination of circulating ACTH and corticosterone levels 12 min after evoking formalin stress, coinciding with peak ACTH responses in blood (Cam & Bassett, 1983). Formalin injection in control animals (that is, injected with non-target siRNA 4 days prior) produced significant increase in ACTH content in plasma (Fig 7C). In contrast, infusion of secretagogen-specific siRNA into the lateral ventricle 4 days prior to formalin challenge blunted this ACTH response (*P* < 0.05; Fig 7C). Likewise, the ensuing corticosterone response was significantly reduced (Fig 7D) in mice treated with secretagogen siRNA. In sum, our data suggest that secretagogen is instrumental for CRH release and stress responsiveness.

Discussion

This study provides novel insights into the neurochemical organization of the mammalian hypothalamus. We report that secretagogen in many parvocellular neurons modulates CRH release. Unlike for small neurotransmitters, CRH, as many other neuropeptides/hormones, is not reused or recycled at the locus of its release. Moreover, CRH is transported over considerable distances from its somatic site of synthesis in the PVN to the median eminence (Supplementary Fig S6). Thus, hormonal secretion into the bloodstream is reliant on the coordination of regulated checkpoints,

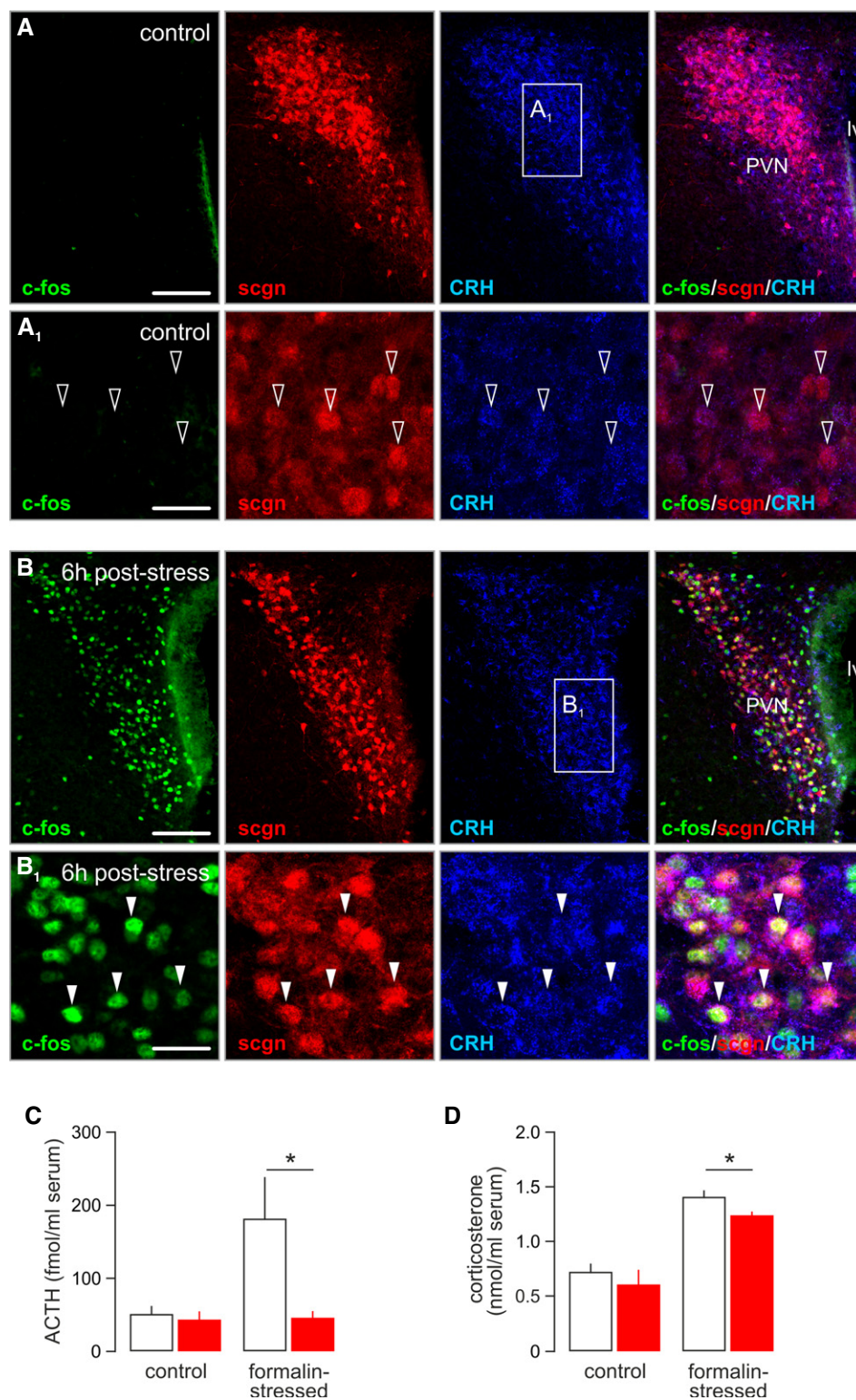


Figure 7. Secretagogin⁺ neurons gate the CRH–ACTH axis in response to acute stress.

A, A₁ Paraventricular secretagogin-immunoreactive (scgn⁺) neurons weakly expressed CRH (open arrowheads) but not c-fos under control conditions. Scale bars: 300 μ m (A), 25 μ m (A₁).

B, B₁ Stress induced by subcutaneous injection of formalin-triggered co-expression of c-fos and CRH in secretagogin⁺ paraventricular neurons (arrowheads). Scale bars: 300 μ m (B), 25 μ m (B₁).

C, D *In vivo* siRNA-mediated silencing of secretagogin expression in the PVN occluded the stress-induced surge of serum ACTH levels (C) and significantly reduced the increase in plasma corticosterone (D). **P* < 0.05 versus control.

which might be either indirectly (vesicle formation and axonal transport; Supplementary Fig S6A) or directly (vesicular exocytosis) affected by secretagogin. From a technical standpoint, our study uses an interactive design of single-cell transcriptome and proteome discovery, verified at the level of systems neurobiology. Yet we did not apply real-time imaging of CRH release upon genetic manipulation of secretagogin expression. Therefore, we cannot entirely exclude that secretagogin, at least in part, primes the release machinery indirectly by recruitment of a multicomponent apparatus rather than directly interacting with its key components (Supplementary Fig S6B). Nevertheless, secretagogin was identified as a Ca^{2+} sensor protein whose proteome-wide interactions include α -SNAP, Rab family members, syntaxins, synaptophysin, TMED10 and CAPS1, many of which were implicated in vesicle formation, trafficking, exo- and endocytosis, and are integral components of synaptic vesicle membranes (Takamori *et al*, 2006; Tobin *et al*, 2012a,b). These interactions are compatible with the predominant association of secretagogin with the plasmalemma and endomembranes in hypothalamic neurons and nerve endings in the median eminence. We hypothesize that differential distribution of secretagogin in axons is due to the fusion of somatic endomembranes into the axonal plasma membrane, thus providing a structural conduit for secretagogin trafficking toward exocytosis release sites. Besides, secretagogin's Ca^{2+} -dependent interactions include molecular motors (kinesin-1, myosin-10), with kinesin-1 known to affect axonal transport (Hirokawa & Takemura, 2005), suggesting that secretagogin can intracellularly modulate the timing and velocity of cargoes moved along the axon and readied for subsequent exocytosis.

Although detailed neuroanatomy studies define at least seven neurochemically distinct sub-regions of the mammalian PVN (Simmons & Swanson, 2009), the classical cytoarchitectonic concept is that dorsolaterally located magnocellular neurons are spatially segregated from the parvocellular cell groups. While parvocellular neurons concentrate between the 3rd ventricle and magnocellular neuron clusters, CRF neurons neighbor magnocellular neurosecretory cells along the ventrolateral extremity of this hypothalamic area (Swanson & Sawchenko, 1980, 1983; Pelletier, 1991). This cytoarchitectonic organization is derived from a string of exquisite neuroanatomical studies spanning the past ~40 years (Kiss *et al*, 1983; Palkovits, 1984; Kita *et al*, 1986; Swanson *et al*, 1986; Swanson & Simmons, 1989; Swanson, 1991; Horvath & Gao, 2005; Maejima *et al*, 2009; Simmons & Swanson, 2009; Vogt *et al*, 2014) and utilizing hormones/neuropeptides to mark neurosecretory cell identity, as well as somatic size measurements as classification criteria (Kiss *et al*, 1991). These classical methods often relied on colchicine-treated or stressed rodents to provide unequivocal phenotypic information on, for example, CRH neurons. Under these conditions, CRH cells co-express additional hormones and peptides, including vasopressin, neurotensin, angiotensin II and enkephalin (Swanson *et al*, 1986; Ceccatelli *et al*, 1989). With regard to fast neurotransmitters, CRH neurons in rat are mainly glutamatergic (Hrabovszky *et al*, 2005) even though a subgroup was shown to be GABAergic (Meister *et al*, 1988). Functionally, the action of amino acid neurotransmitters to modulate pituitary hormone-producing endocrine cells may occur via local axon collaterals within the PVN. Local amino acid transmitter synthesis in, and release from, nerve endings in the external layer of the median eminence may yet

modulate the release of CRH (and other releasing/inhibitory factors) into portal vessels by engaging presynaptic auto- and/or hetero-receptors.

The advent of single-cell transcriptome analysis by RNA sequencing (Shapiro *et al*, 2013) revolutionized insights into cell diversity in many developing (Tang *et al*, 2009) and adult tissues (Islam *et al*, 2011; Kodama *et al*, 2012; Lovatt *et al*, 2014). In the nervous system, transcriptome analysis is predicted to reveal that neuronal diversity is far more complex than initially thought, with many non-classical neuronal sub-types emerging (that is, modalities or combinations of the mRNA landscape overarching several known cell subsets). These might be of particular relevance to specific behavioral contexts, environmental challenges or disease phenotypes. More specifically, in the mammalian hypothalamus, a significant expansion of the phenotypic divergence of functionally distinct neuroendocrine subtypes, carrying mixed phenotypic codes including sub-threshold levels of hormones or neuropeptides, is expected upon unbiased clustering of cellular transcriptomes. This will identify neuroendocrine cell cohorts differentially responding to not one but multiple stimuli. As such, secretagogin⁺ neurons in mouse were found to express mRNA transcripts for CRH, Gad1, Gad2, Slc32a1, Tac1 and somatostatin. This mRNA transcript repertoire confers, as said, a parvocellular origin (Dierckx & Vandesande, 1979; Alonso *et al*, 1986; Ceccatelli *et al*, 1989), yet with a broader mRNA spectrum than for a single classical subtype of parvocellular neuroendocrine cells. These considerations cumulatively have the following inferences: parvocellular neurons express a chief hormone, a neuropeptide. Nevertheless, they maintain the ability to co-express other (secondary) releasable products, including small molecule transmitters such as GABA and glutamate playing auxiliary roles. This allows dynamically switching mode of action upon a range of metabolic stimuli, underpinning fast “on/off” responses. Here, this hypothesis is reflected by the moderate presence of secretagogin in magnocellular neurons and their axon terminals in the posterior pituitary. Moreover, our single-cell transcriptome analysis revealed the coincidence of Scgn, CRH and Nr3c1 mRNA expression. *Nr3c1* encodes a glucocorticoid receptor (nuclear receptor subfamily 3, group C member 1) (van Rossum *et al*, 2004). Nr3c1 can both function as a transcription factor that binds to glucocorticoid response elements in the promoters of glucocorticoid responsive genes to activate their transcription or act as a regulator of other transcription factors. *Nr3c1* mutations manifest as generalized glucocorticoid resistance (Donner *et al*, 2013), characterized by the insensitivity of target tissues to glucocorticoids, resulting in the compensatory activation of the HPA axis and CRH hypersecretion into the systemic circulation. Thus, secretagogin may be the first molecular hinge linking Nr3c1-mediated feedback to CRH release from parvocellular neurons.

Since parvocellular neurons receive local-circuit, as well as long-range afferents (Kiss *et al*, 1984, 1996; Palkovits, 1987), the predominance of single gene products coincides with, and is driven by, the type of excitatory or inhibitory inputs they receive. Considering that hypothalamic neuronal circuitries are prone to synaptic reorganization upon metabolic challenges (Pinto *et al*, 2004; Horvath & Gao, 2005), parvocellular neurons might function as “fast integrators” in hypothalamic neuronal circuitries and can translate synaptic information into hormonal output with high fidelity. This arrangement is compatible with our electrophysiology classification showing that

secretagogin⁺ neurons, although clearly parvocellular, belong to both *Ia* and *Ila* PVN subclasses. In addition, an evolutionary advantage of the broad functional diversity of neuronal contingents in the hypothalamus may be the ability of sustained neural control of secondary metabolism at the periphery, essential for the survival of the organism, even under pathological conditions associated with neuronal loss.

Action potentials represent the temporal code of electrical activity in neurons. Upon the arrival of a depolarization wave, a kaleidoscope of voltage-dependent ion channels regulates ion exchange along the plasma membrane. On the one hand, this influx of Ca²⁺ is to induce signaling events shaping the neurons' transcriptional program to adapt to circuit requirements (Clapham, 2007). On the other hand, Ca²⁺ is the ubiquitous trigger for the quantal release of neurotransmitters and neuromodulators at axon terminals (Sudhof, 2004). In the hypothalamus, intracellular Ca²⁺ signaling regulates AVP and oxytocin release in magnocellular neurons (Dayanithi et al, 2012). Parvocellular neurons are equally dependent on Ca²⁺-dependent mechanisms (Hallbeck et al, 1996). In particular, a Ca²⁺/calmodulin-dependent signaling pathway was reported to modulate the transcription of CRH in neurons (Yamamori et al, 2004). Ca²⁺ waves are transduced into intelligible second messenger codes by Ca²⁺ sensor proteins with low affinity (> 1 μM) in protein–protein interactions. Alternatively, cytosolic Ca²⁺ buffers (Ca²⁺ affinity; < 1 μM) dissipate Ca²⁺ transients by reversibly binding 2–6 Ca²⁺ ions (Andressen et al, 1993). Although a clear separation of these two protein subfamilies is increasingly debated (Schwaller, 2009), a clear evidence for Ca²⁺ sensor function is the Ca²⁺-dependent recruitment of signaling proteins. Here, we used subtractive proteomics to define the Ca²⁺-dependent interactome of secretagogin in native hypothalamic preparations. Our data revealed that secretagogin interacts with protein subfamilies implicated in the formation, intracellular traffic, priming and docking of release vesicles. These findings are not entirely unexpected, since a pull-down analysis using purified secretagogin as bait reported similar results (Bauer et al, 2011). However, this is the first confirmed report of secretagogin's interactome *in situ* in an identified neuronal cohort, which defines cell type-specific protein interactions for this Ca²⁺ sensor. Critically, our single-cell transcriptome data confirmed the presence of mRNAs for the proteins identified by mass spectrometry, providing unbiased support for our functional assays. The above data together with secretagogin's localization in large axon terminals at the median eminence, and its association to the readily releasable pool of vesicles, as well as plasmalemmal compartments in the vicinity of release sites, show that secretagogin is ideally poised to gate neuropeptide secretion. Considering that secretagogin[−] nerve endings also exist in the median eminence, our results also suggest that as yet unknown and additional Ca²⁺-dependent or Ca²⁺-independent sensor proteins might exist to control the release of hormones distinct from CRH, galanin or somatostatin (Fig 5).

A primary drive of molecular neuroscience is the discovery of signaling events' rate-limiting neural processes indispensable for the formation, maintenance and responsiveness of key neural axes. We demonstrated that acute noxious stress increases CRH expression in the PVN, particularly in secretagogin⁺ neurons. A causal relationship between secretagogin expression and CRH release is cumulatively highlighted by gain-of-function and loss-of-function analysis

employing the coincident detection of the intracellular candidate (secretagogin) and the physiological output (CRH in media). Based on our protein interactome profiling, cell biology and neuroanatomy analyses, we propose that genetic impairment of secretagogin expression disrupts the traffic of CRH-laden neurosecretory vesicles toward release sites, as shown by the somatic accumulation of CRH immunoreactivity upon siRNA-mediated secretagogin silencing *in vivo*. Most notably, genetic manipulation of secretagogin availability led to the fallout of stress-induced ACTH elevation in peripheral blood, which is normally driven by the activation of CRH receptors on secretory cells of the anterior pituitary (Wynn et al, 1983). While ACTH expression is a multifactorial mechanism (Armario, 2006), we argue that the blunted ACTH response was due to the experimentally evoked attenuation of CRH release at the level of the median eminence. Nevertheless, corticosterone release in stressed animals was only partially suppressed. This may be due to our coincident ACTH and corticosterone sampling protocol focused on the primary ACTH response, and only capturing a relatively early up-shoot in the plasma corticosterone concentration.

Overall, we show that a spatially segregated parvocellular neuron population produces CRH and regulates its release via a novel Ca²⁺ sensor. Nevertheless, to what extent secretagogin is expressed in other neuron populations, e.g. the arcuate nucleus, and if secretagogin is also involved in the regulated release of many other neuropeptides/hormones remains to be determined. Therapeutic implications of these findings are vast, since they offer novel biomarkers for some of the most common metabolic disorders, and druggable targets for ceasing CRH release particularly in chronic stress, depression and genetic or sporadic forms of generalized glucocorticoid resistance.

Materials and Methods

Animals for histo- and biochemistry

Histochemical, biochemical and *in vivo* RNAi experiments were conducted in male C57BL/6N mice (*n* = 57, 12 weeks of age) and Sprague Dawley rats (*n* = 7; both from Scanbur). CRH-GFP reporter mice (all females, *n* = 3, 19 weeks of age) were generated using bacterial artificial chromosome technology (Alon et al, 2009). Hypothalamic cultures were prepared from mouse pups on postnatal day (P) 2. Animals were housed conventionally (12/12-h light cycle, 25% humidity). Adult mice (*n* = 4) received stereotaxic injections of colchicine (5 μl; Sigma) into the left lateral ventricle under deep anesthesia (5% isoflurane) and survived for an additional 24 h to facilitate somatic neuropeptide accumulation (Cortes et al, 1990). Experimental protocols were in accordance with the European Communities Council Directive (86/609/EEC) and approved by the regional ethical committee (Stockholms Norra Djurförsöksetiska Nämnd; N512/12). Particular effort was directed to minimize the number of animals and their suffering during the experiments.

Tissue preparation, immunohistochemistry and imaging

Wild-type animals were transcardially perfused with a fixative composed of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) that was preceded by a short prerinse with physiological saline (anesthesia: 5% isoflurane). CRH-GFP mice were

perfused with 0.1 M phosphate-buffered saline (PBS; 37°C, pH 7.4) followed by 4% PFA in 0.1 M PBS [37°C (20 ml) switched to ice-cold (50 ml)]. After post-fixation in the same fixative overnight and cryoprotection in 30% sucrose for at least 48 h, 30- or 50- μ m-thick serial sections were cut on a cryostat microtome and processed for multiple immunofluorescence histochemistry according to the published protocols (Mulder *et al*, 2009). Alternatively, perfused brains were cryoprotected in 10% sucrose and sectioned onto SuperFrost⁺ glass slides at 20 μ m thickness.

Free-floating sections were rinsed in PB (pH 7.4) and pre-treated with 0.3% Triton X-100 (in PB) for 1 h at 22–24°C to enhance the penetration of antibodies. Non-specific immunoreactivity was suppressed by incubating our specimens in a cocktail of 5% normal donkey serum (NDS; Jackson), 1% bovine serum albumin (BSA; Sigma) and 0.3% Triton X-100 (Sigma) in PB for 1 h at 22–24°C. Sections were then exposed to select combinations of primary antibodies diluted in PB to which 0.1% NDS and 0.3% Triton X-100 had been added for 16–72 h at 4°C (Supplementary Table S2). After extensive rinsing in PB, immunoreactivities were revealed by carbocyanine (Cy)2, 3 or 5-tagged secondary antibodies raised in donkey (1:200; Jackson; 2 h at 22–24°C). Sections were mounted onto fluorescence-free glass slides and coverslipped with Entellan (in toluene; Merck).

Double labeling of glass-mounted sections with the tyramide signal amplification method (TSA Plus; Perkin-Elmer) combined with the use of Cy2-conjugated secondary antibodies was performed as described (Shi *et al*, 2012). Glass-mounted sections were coverslipped with Aquamount (Dako). Images were acquired on Zeiss 700LSM, 710LSM or 780LSM confocal laser-scanning microscopes with maximal signal separation or spectral scanning. Composite figures were assembled in CorelDraw X5.

Electron microscopy

Male C57Bl6/J mice ($n = 8$) were anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with physiological saline followed by 4% PFA and 0.25% glutaraldehyde in PB for 15 min. After removal of the brain, a diencephalic block was trimmed and cut in 100- μ m frontal sections on a VT1200S vibratome (Leica). Sections containing the anterior hypothalamus were collected and immersed in 4% PFA in PB for an additional 2 h. Specimens were optionally stored in a mixture of 10% glycerol and 25% sucrose at –80°C. For ultrastructural analysis, sections were thawed, rinsed in PB (3 $\times$) and then incubated in 0.1 M Tris-NaCl buffer supplemented with 0.1% Tween-20 (TNT), 1% BSA and 1% glycine for 30 min. This was followed by exposure to anti-secretagogin antibodies [1:2,000 (L.W.), 1:800 (Sigma) in PB-TNT-BSA; Supplementary Table S2] at 4°C overnight. Sections were then rinsed in the above buffer and incubated with goat anti-rabbit F(ab')₂ coupled to 0.8 nm gold particles (1:100; Aurion) in PB-TNT-BSA for 2 h at 22–24°C. After fixation with 1% glutaraldehyde (10 min) and rinsing in water, gold particles were silver-enhanced using the HQ Silver Enhancement Kit (Nanoprobes; 5 min at 22–24°C) prior to post-fixation in 1% OsO₄ for 1 h. Next, sections were flat-embedded (Epoxy resin) between two plastic coverslips. After polymerization, regions of interest were identified under a stereomicroscope, isolated with a razor blade and re-embedded in Epon. Ultra-thin sections (60 nm) perpendicular to the thickness of the vibratome

section were obtained and optionally contrasted by uranyl acetate and lead citrate. Specimens were finally observed by using an H7650 Hitachi electron microscope.

The number of silver-enhanced gold particles in neuronal profiles of three compartments (that is, soma, dendrites and axonal nerve endings) in the PVN and external median eminence was quantified. In every compartment, we determined the distribution of these particles in a membrane-related domain (either the plasma- or an endomembrane) and in a cytosolic domain where no obvious organelle could be detected underneath. For this purpose, we measured the distance between particles and membranes on photomicrographs from $n = 5$ mice and considered a particle membrane bound if its distance was < 50 nm from the closest membrane profile. The number of counted profiles was as follows: soma: plasma membrane = 25, endomembrane = 29 and cytosol = 27; dendrite: plasma membrane = 24, endomembrane = 21 and cytosol = 15; and axon: plasma membrane = 28, endomembrane = 25 and cytosol = 25. Data were expressed as the number of particles per neuronal profile $\pm$ s.e.m.

Patch-clamp electrophysiology

Male C57Bl6/N mice ($n = 22$; 21–28 days) were deeply anesthetized (5% isoflurane) and decapitated. Brains were rapidly removed and immersed in ice-cold pre-oxygenated (95% O₂/5% CO₂) cutting solution containing (in mM) 90 NaCl, 26 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 10 HEPES-NaOH, 5 sodium ascorbate, 5 sodium pyruvate, 0.5 CaCl₂, 8 MgSO₄ and 20 glucose. Subsequently, 300- μ m-thick coronal slices were cut on a vibratome (VT1200S, Leica). Slices encompassing the PVN region were selected and equilibrated in artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 2 CaCl₂ and 2 MgSO₄ at 22–24°C for 1–5 h before recording. Whole-cell recordings in current-clamp or voltage-clamp mode were made by using a HEKA ECP-10USB amplifier and PatchMaster Software (HEKA, Germany). During measurements, slices were continuously perfused with ACSF. The internal pipette solution contained (in mM) 114 potassium gluconate, 6 KCl, 10 HEPES, 5 EGTA, 4 ATP-Mg, 0.3 GTP (pH was adjusted to 7.3 with KOH) and 0.5% biocytin (Sigma) for *post hoc* cell identification. After recordings, brain slices were immersion-fixed with 4% PFA at 4°C overnight. Electrophysiological data were analyzed using Clampfit 10.0 (Molecular Devices) and SigmaPlot (Systat Software Inc.) for the parameters listed in Supplementary Table S1.

Single-cell transcriptome analysis

The PVN region of juvenile mice was isolated from 300- μ m-thick coronal slices under microscopy guidance and dissociated using the Papain Dissociation System (Worthington). Isolated single cells were concentrated by centrifugation to a density of 300–500 cells/ μ l and loaded into a C1-AutoPrep system (Fluidigm) for single-cell capture, lysis, cDNA synthesis and amplification (Islam *et al*, 2014). Each capture site was imaged with 192 cells selected for RNA sequencing on an Illumina HiSeq2000 sequencer. Sequencing data at a depth of 500,000–2,000,000 reads/cell were analyzed (Islam *et al*, 2014). Out of 151 confirmed single cells, 130 cells provided data above a threshold of 10,000 transcript molecules/cell (excluding

repeats and mitochondrial transcripts). Data on molecule counts were next subjected to clustering. In brief, ~2,700 genes were selected based on their expression (average more than one molecule/cell and correlation > 0.5 with other 10 genes). Based on these criteria, the 1D order of cells and genes was performed using the SPIN algorithm (Tsafrir *et al*, 2005).

Immunoprecipitation and unbiased proteomics

Hypothalamic regions containing the PVN were isolated from P7 rats (three animals), divided into left and right parts with one hemisphere incubated in ACSF ("control stimulation"), while another placed in ACSF in which 52.5 mM NaCl was replaced by KCl to depolarize neurons by inducing Ca^{2+} influx ("treatment condition"). After 10 min of incubation, samples were collected in lysis buffer containing 50 mM NaCl, 20 mM HEPES, 10 μM CaCl_2 , 1 mM EDTA, 0.2% Triton X-100 and a cocktail of protease inhibitors (Roche; pH was adjusted to 7.4). Meanwhile, samples treated by depolarizing solution were transferred into the above lysis buffer supplemented with 10 μM CaCl_2 without EDTA. Tissues were homogenized and centrifuged at 18,000 g for 30 min, and supernatants were used in subsequent experiments. After pre-clearance, samples were incubated with rabbit anti-secretagogin primary antibody (1:2,000; provided by L.W.) overnight at 4°C. An aliquot of each sample was exposed to rabbit IgG (Santa Cruz Biotechnology) to control non-specific binding. Subsequently, samples were incubated with GammaBind Plus Sepharose beads (GE Healthcare) for 90 min. After repeated rinses, proteins were eluted with Laemmli buffer and separated on 10% SDS-PAGE under denaturing conditions. After silver in-gel staining, proteins in each lane were identified by matrix-associated laser desorption ionization–time-of-flight mass spectrometry (Proteomics Science Facility at Karolinska Institutet) (Linden *et al*, 2012). The MS/MS data file generated was analyzed using the Mascot algorithm (version 2.5, Matrix Science) against the NCBI database selected for rat (*Rattus norvegicus*) taxonomy, trypsin as the cleavage enzyme, carbamidomethyl as a fixed modification of cysteines and methionine oxidation and then deamidation of glutamines and asparagines as variable modifications (Tortoriello *et al*, 2014). All of the three "pool" mgf files within each treatment were merged before searching. Peptides were selected with a fragment tolerance of 0.1 Da and parent tolerance of 0.05 Da, and with a confidence threshold of > 99%. Proteins were unambiguously identified with 2 or more peptides and a protein threshold of > 99%. Additional data analysis was performed using Scaffold (version 4.3.4).

Cell culture and secretagogin manipulation

Human SH-SY5Y neuroblastoma cells and embryonic mouse hypothalamic cell line N44 (mHypoE-N44; Cedarline) were seeded at a density of 50,000 cells/well on poly-D-lysine (PDL)-coated 24-well plates and cultured in DMEM:Glutamax (Invitrogen) containing 10% fetal bovine serum overnight (without antibiotics). Lipofectamine 2000 (2 μl /well; Invitrogen) was used to transfect both cell lines with a pcDNA3.1 plasmid containing the human *scgn* gene (0.5 μg /well). Forty-eight hours after transfection, the cells were used for Ca^{2+} imaging and *post hoc* immunocytochemistry. mHypoE-N44 cells were routinely serum-starved for 1 day in DMEM:Glutamax also

containing 25 μM forskolin. Subsequently, cells were stimulated by ATP (15 μM) for 20 min before immersion fixation in 4% PFA in PB.

Hypothalami were isolated on ice after decapitation of mice on P2. After enzymatic dissociation, cells were plated at a density of 300,000 cells/well in PDL-coated 24-well plates and grown for 7–8 days *in vitro* (DIV). For Ca^{2+} imaging, 50,000 cells were plated in each well. Cultures were maintained in DMEM/F12 containing B27 supplement (2%), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml; all from Invitrogen). For *post hoc* immunocytochemistry, cells on coverslips were fixed in 4% PFA for 30 min on ice and subsequently immunostained using select combinations of primary antibodies (Supplementary Table S2). Parameters processed for quantitative morphometry of single and dual-labeled cells included: (i) the somatic diameter, (ii) number of neurites, (iii) the maximum length of processes and (iv) the number of neurite branching ("bifurcations"; Supplementary Fig S2C; for analysis procedures see Keimpema *et al*, 2010). For secretagogin knockdown, primary hypothalamic neurons were grown for 4 days *in vitro* followed by exposure to 1 μM Accell secretagogin-specific siRNA (Thermo Fisher) for an additional 3 days. CRH release was determined after adding fresh medium containing 25 μM forskolin (Sigma) and incubation for 18 h by ELISA detection (CusaBio).

Ca^{2+} imaging and quantitative immunocytochemistry

Ca^{2+} responses were measured using Fura-2AM (Invitrogen) as intracellular Ca^{2+} indicator. Ratiometric imaging was performed on a CoolSnap HQ² camera (Photometrics) and analyzed by MetaFluor software (Molecular Devices). After recording, the cells on coverslips were immersed in ice-cold 4% PFA in PB and processed for immunocytochemistry as above. For quantitative immunocytochemistry (fluorescence densitometry), orthogonal z-stacks were captured on an LSM700 laser-scanning microscopy (Zeiss). Fluorescence intensity of single cells was determined by using the Imaris-Pro software (Bitplane) and correlated with corresponding Ca^{2+} responses (Supplementary Figs S3 and S4).

In vivo stress and siRNA-mediated secretagogin knockdown

First, we tested whether formalin stress induces c-fos accumulation in secretagogin⁺ PVN neurons by subcutaneous injection of 4% PFA (50 μl). After 6 h, mice ($n = 2$ /time point) were transcardially perfused as above, and their PVN was processed for multiple immunofluorescence detection of c-fos, secretagogin and CRH (Supplementary Table S2). The number of c-fos⁺, secretagogin⁺ and c-fos⁺/secretagogin⁺ neurons was counted in 8,000–18,000 μm^2 frames superimposed over the PVN (ZEN2010 software, Zeiss). C-fos⁺ nuclei were only counted if their fluorescence intensity exceeded tissue fluorescence by > 4-fold.

Second, the contribution of secretagogin in stress-induced hormone release *in vivo* was assessed in male mice ($n = 24$, 12 weeks of age). Animals ($n = 12$) received stereotaxic injections of either 250 μM SMARTpool Accell Scgn siRNA (1 nmol; Thermo Fisher Scientific) or Accell non-targeting siRNA ($n = 12$) in the left lateral cerebral ventricle (4 μl). A recovery period of 96 h was used before stress induction. Formalin stress was induced by injection of 4% PFA into the left paw. Blood ($n = 24$) was

collected with a lag time of 12 min at the expected peak of circulating ACTH.

Third, the quantitative analysis of secretagogin knockdown efficacy was performed by analyzing the distribution and fluorescence intensity of CRH⁺ neurons in the PVN after *in vivo* knockdown as above with 2 μ l of either 250 μ M SMARTpool Accell Scgn siRNA (0.5 nmol; $n = 3$) or Accell non-targeting siRNA ($n = 3$). After transcardial perfusion and immunohistochemistry, projection images of orthogonal z-stacks (interval depth: 2 μ m, optical thickness: 2 μ m) were analyzed using the maximal intensity projection function of the ZEN2010 software (Zeiss; Fig 6C and C₁).

ACTH concentrations were determined in unextracted plasma samples using a radioimmunoassay (RIA). Synthetic human ACTH1-39 was used as standard and for labeling with ¹²⁵I (I-RB-4, Institute for Isotopes, Budapest, Hungary). An ACTH antibody (no. 8514), directed against the mid-portion of the human ACTH1-39 molecule, was raised in rabbit at the Institute of Experimental Medicine, Hungarian Academy of Sciences (Budapest, Hungary) (Barna & Koenig, 1992). This antibody is highly specific, showing 0.2% cross-reaction with α -MSH and no significant cross-reaction with γ -MSH, CLIP, ACTH11-24, ACTH25-39, ACTH1-14, and ACTH1-19. The intra- and interassay coefficients of variation were 4.7 and 7%, respectively.

Plasma corticosterone was measured from 10 μ l of unextracted plasma with an RIA by use of a specific antiserum (Institute of Experimental Medicine, Hungarian Academy of Sciences) against corticosterone-3-carboxymethyloxim-BSA. An ¹²⁵I-labeled carboxymethyloxim-tyrosine methyl ester derivative was used as tracer (I-RBO-36, Institute for Isotopes, Budapest, Hungary). The corticosterone antibody only crossreacts with other naturally occurring adrenal steroids at < 0.05%, except desoxycorticosterone (1.5%) and progesterone (2.3%). Final dilution of the antibody was 1:40,000. Incubation time was 24 h at 4°C, and a second antibody (anti-rabbit from goat) and 6% polyethylene glycol were used for separation. A calibration curve was prepared from corticosterone (Calbiochem), ranging from 0.27 to 40 pmol/tube. The intra- and interassay coefficients were 12.3 and 15.33%, respectively. All hormone measurements were performed in duplicate.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (version 21.0, SPSS Inc.) and SigmaPlot (Systat Software Inc.). Data were expressed as means $\pm$ s.e.m. A *P*-value of < 0.05 was considered statistically significant and calculated by Student's *t*-test (on independent samples), Mann–Whitney rank-sum test or one-way analysis of variance (ANOVA with Dunn's *post hoc* test), as appropriate.

Supplementary information for this article is available online: <http://emboj.embopress.org>

Acknowledgments

The authors thank T.L. Horvath (Yale School of Medicine) and L. Swanson (University of Southern California) for their critical comments on this manuscript. Lisa E. Pomeranz and Kaamashri N. Latcha (The Rockefeller University) are acknowledged for providing brain tissues from CRH-GFP mice. The expert technical assistance of Mrs. Eva Dobozi Kovacs is greatly appreciated. The authors also thank the Bordeaux Bioimaging Center (CNRS

UMS 3420) for technical support. This work was supported by the Swedish Research Council (T.Ha., T.Hö.), Hjärnfonden (T.Ha.), the Petrus and Augusta Hedlunds Foundation (T.Ha., T.Hö.), the Novo Nordisk Foundation (T.Ha., T.Hö.), Karolinska Institutet (T.Hö.), the Medical University of Vienna (T.Ha.), ANR (France, ANR-10-INBS-04-01, M.L.), the European Commission (PAINCAGE grant, T.Ha.) and the Wellcome Trust (094476/Z/10/Z, equipment grant, C.H.B. and postdoctoral research fellowship, M.F.).

Author contributions

THa conceived the general idea of this study. RAR, AA, M-DZ, HHS, SL, THö and THa designed the experiments. RAR, AA, M-DZ, AZ, ML, MF, SLS, RS, AD, MO, LS, MP, AC and CHB performed the experiments and analyzed the data, JM, HM, MU and LW contributed unique reagents. RAR, AA, THö and THa wrote the manuscript. All authors commented on the manuscript and approved its submission.

Conflict of interest

The authors declare that they have no conflict of interest.

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