Published in final edited form as:

Metallomics. 2016 September 1; 8(9): 1012–1022. doi:10.1039/c6mt00079g.

Copper, Zinc and Calcium: Imaging and Quantification in Anterior Pituitary Secretory Granules

Mathilde L. Bonnemaïson^{1,%}, Megan E. Duffy^{2,&}, Richard E. Mains³, Stefan Vogt⁴, Betty A. Eipper^{1,*}, and Martina Ralle^{2,*}

¹Department of Molecular Biology and Biophysics, UConn Health, Farmington, CT 06030

²Department of Molecular and Medical Genetics, Oregon Health & Sciences University, Portland, OR 97239

³Department of Neuroscience, UConn Health, Farmington, CT 06030

⁴X-ray Science Division, Advanced Photon Source, Argonne National Laboratory, Argonne, IL 60439

Abstract

The anterior pituitary is specialized for the synthesis, storage and release of peptide hormones. The activation of inactive peptide hormone precursors requires a specific set of proteases and other post-translational processing enzymes. High levels of peptidylglycine α -amidating monooxygenase (PAM), an essential peptide processing enzyme, occur in the anterior pituitary. PAM, which converts glycine-extended peptides into amidated products, requires copper and zinc to support its two catalytic activities and calcium for structure. We used X-ray fluorescence microscopy on rat pituitary sections and inductively coupled plasma mass spectrometry on subcellular fractions prepared from rat anterior pituitary to localize and quantify copper, zinc and calcium. X-ray fluorescence microscopy indicated that the calcium concentration in pituitary tissue was about 2.5 mM, 10-times more than zinc and 50-times more than copper. Although no higher than cytosolic levels, secretory granule levels of copper exceeded PAM levels by a factor of 10. Atp7a, which transports copper into the lumen of the secretory pathway, was enriched in endosomes and Golgi, not in secretory granules. If Atp7a transfers copper directly to PAM, this pH-dependent process is likely to occur in endosomes.

Introduction

Several metals play a role in anterior pituitary secretory granule formation. The aggregation and storage of growth hormone and prolactin in somatotrope and lactotrope secretory granules, respectively, is facilitated by zinc¹⁻³. Metals also act as cofactors for each of the enzymatic reactions necessary for peptide hormone biogenesis, a process that begins as precursors and processing enzymes move from the lumen of the endoplasmic reticulum, into

*Co-corresponding authors; to whom correspondence should be addressed at: Betty Eipper, UConn Health, Department of Molecular Biology and Biophysics, 263 Farmington Avenue, Farmington, CT 06030-3401; eipper@uchc.edu; Martina Ralle, OHSU, Department of Molecular and Medical Genetics, Mail Code L103, 3181 SW Sam Jackson Park Road, Portland, OR 97239, ralle@ohsu.edu.

%Current address: Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE

&Current address: School of Oceanography, University of Washington, Seattle, WA

the Golgi complex and then into immature secretory granules ⁴. The luminal environment in the trans-Golgi network (TGN) promotes secretory granule protein aggregation, a process thought to facilitate the sorting of granule cargo from other luminal proteins ⁴. The prohormone convertases which cleave the precursors are calcium-dependent subtilisin-like endopeptidases ^{5, 6}. Carboxypeptidase E, which removes basic amino acids from the C-termini of many intermediates, requires zinc ⁷. One of the final steps in the sequence of post-translational modifications that yields bioactive product peptides is amidation, which requires copper, zinc and calcium. While the major anterior pituitary hormones (growth hormone, prolactin, adrenocorticotrophic hormone, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone) are not amidated ⁸, these same cells produce precursors whose amidated product peptides (substance P, neuropeptide Y, vasoactive intestinal peptide, galanin, GnRH, TRH, pyroglutamyl-glutamyl-proline-amide) function as autocrine and paracrine agents after secretion ⁹.

Amidation requires the conversion of peptidylglycine intermediates into α -amidated products and is catalyzed by peptidylglycine α -amidating monooxygenase (PAM). Lack of PAM is embryonic lethal in mice, and *Drosophila* lacking *phm* do not survive the larval stage ^{10, 11}. Acting sequentially, the two catalytic core domains of PAM, peptidylglycine α -hydroxylating monooxygenase (PHM) and peptidyl- α -hydroxyglycine α -amidating lyase (PAL), produce an amidated peptide and glyoxylate ¹² (Figure 1A). PHM requires copper, oxygen and ascorbate while PAL requires zinc for catalysis and calcium for structure ¹³⁻¹⁶. Copper is the only metal that restores monooxygenase activity to PAM after metal chelation ¹⁷ and is the only metal ion present in the PHM crystal ¹⁸. Removal of zinc and calcium from PAL requires chelators, but copper is lost from the active site of PHM during its purification. After granule exocytosis, PAM deposited on the plasma membrane undergoes endocytosis and can be returned to secretory granules or degraded in lysosomes.

Although multiple metals are required for anterior pituitary secretory granule formation and function, little is known about their concentrations or subcellular distributions in this tissue. X-ray fluorescence microscopy was used to determine levels of multiple metals in the anterior, intermediate and neural lobes of the adult rat pituitary. Differential centrifugation and sucrose density gradient centrifugation were used to prepare fractions enriched in secretory granules or cytosol. Using inductively coupled plasma mass spectrometry (ICP-MS), levels of calcium, zinc and copper were quantified in each fraction. Quantitative Western blot analysis was used to compare levels of PAM in secretory granules to levels of the three metals it requires. Recent studies suggesting signaling roles for labile cytosolic copper pools ^{19, 20}, redox-regulated flux of copper into the secretory pathway ²¹ and the direct transfer of copper from Atp7a to its target luminal cuproenzymes ²² require better knowledge of copper levels in specific subcellular organelles.

Results

X-ray fluorescence microscopy evaluation of metal distribution in rat pituitary gland

Adult rat pituitaries were frozen in cryoprotectant; 5 μ m sections were stained with hematoxylin and eosin for orientation using visible light microscopy (Figure 1B). The anterior lobe (yellow arrow) can be easily distinguished from the neural (blue arrow) and

intermediate (red arrow) lobes in the center. The anterior pituitary is composed primarily of peptide producing cells (chromophils; purple) filled with secretory granules. The intermediate lobe, a thin but homogeneous layer of proopiomelanocortin producing melanotrope, is separated from the anterior lobe by the remnants of Rathke's pouch (green arrow). The neural lobe is composed of the secretory granule filled axonal terminals of neurons whose cell bodies are situated in the hypothalamus, along with specialized glial cells called pituicytes.

X-ray fluorescence microscopy was performed on 10 μm unstained sections and was used to determine the concentration of several elements in the anterior, intermediate and neural lobes (Figure 1C; Table 1). Overall, sulfur and iron were more concentrated in the neural lobe than in the anterior and intermediate lobes, while phosphorus, calcium, copper and zinc were present at similar concentrations in all three regions (Table 1). Phosphorus was the most abundant element, with an average concentration of $100 \pm 20 \text{ mM}$, reflecting its presence in inorganic phosphate as well as ATP and nucleic acids. Sulfur was also prevalent, with concentrations of 50-83 mM in the anterior and intermediate lobes and $113 \pm 5 \text{ mM}$ in the neural lobe. The concentrations of calcium, iron and zinc in all three lobes were in the low mM range. The least prevalent metal was copper, present at about 45-140 μM in all three lobes.

The three metals required by PAM (copper, zinc and calcium) were present at vastly different levels (Table 1). Copper levels were about five times lower than zinc and fifty times lower than calcium in all lobes of the pituitary gland. Since the catalytic domains of PAM only have access to luminal metals, the subcellular distribution of these three metals was explored using differential centrifugation.

Fractionation of rat anterior pituitary by differential centrifugation

In order to maintain subcellular organelle integrity, rat anterior pituitaries were homogenized in isotonic buffer before differential centrifugation (Figure 2A). Poorly homogenized tissue and nuclei were discarded by centrifugation at $800 \times g$ for 5 min. Approximately 40% of the total protein was recovered in the P2 fraction, which was expected to contain secretory granules, and 40% in the cytosol (S4, Figure 2B).

Western blot analysis of established organelle marker proteins was used to characterize the fractions obtained. Antisera to prolactin (PRL) and growth hormone (GH), the major anterior pituitary hormones, and to γ_3 -melanocyte stimulating hormone (γ_3 -MSH), a product of proopiomelanocortin (POMC) processing in corticotropes, were used to identify secretory granules. Equal amounts of protein from each fraction were analyzed. Both PRL and GH were enriched in the P2 and P3 fractions (Figure 3A). The γ_3 -MSH antibody identified POMC and two major POMC cleavage products (18K fragment and γ_3 -MSH) in the P3 fraction. Corticotropes, the cells that produce POMC, produce smaller secretory granules than somatotropes and lactotropes^{23, 24}. Importantly, relatively little PRL, GH or γ_3 -MSH was recovered in the S4 (cytosolic) fraction, indicating that secretory granule integrity was not compromised during homogenization.

PAM, which spans the membrane of secretory granules, endosomes and the TGN, has been identified in multiple subcellular fractions²⁵. In Sprague-Dawley rats, the major isoforms expressed are PAM-1 Bb, PAM-2 Bb and PAM-3 (Figure 1A)⁹. Within secretory granules, the PAM-1 Bb isoform can be cleaved between PHM and PAL, generating soluble PHM and PAL membrane (PALm). Soluble 45 kDa PHM has served as a useful marker for secretory granules (Figure 3B); 45 kDa PHM was most prevalent in the P3 fraction. Membrane PAM (PAM-1 Bb and PAM-2 Bb) resides in secretory granules, the trans-Golgi network and in endosomes and was prevalent in each P fraction and absent from the S4 (cytosol) fraction, as expected. The enrichment of PHM and γ_3 -MSH in the P3 fraction is consistent with the presence of smaller secretory granules (Figure 3B).

We next used antisera to proteins known to reside in other organelles to determine whether they had been successfully depleted from the P2 and P3 fractions. Clathrin, which forms coats on vesicles budding from multiple organelles, was recovered from P4 and S4 (Figure 3C); clathrin is absent from mature²⁶ secretory granules and was largely absent from P2 and P3. Antisera to GM130, a soluble protein that binds to Golgi membranes²⁷ and calreticulin, a soluble luminal protein, were used to identify the Golgi complex and endoplasmic reticulum, respectively. GM130 was fairly evenly distributed between the P2, P3 and P4 fractions while calreticulin was somewhat enriched in the P3 fraction (Figure 3C). Carboxypeptidase D, a metalloprotease that processes proteins in the TGN and cycles through the endocytic compartment²⁸⁻³⁰ was enriched in P4, consistent with the presence of endosomes.

Since Atp7a is known to play an essential role in the delivery of copper to the PHM domain of PAM, which functions in the lumen of the secretory pathway, its subcellular localization was determined. Atp7a was not enriched in P2, the secretory granule enriched fraction. Atp7a traverses the endocytic pathway and, like carboxypeptidase D, was enriched in P4 (Figure 3C). The fact that Atp7a and intact membrane PAM were both found in this endosome-enriched fraction means that the two proteins could interact in this compartment. The direct interaction of PAM with Atp7a is an essential feature of the hypothesis that pH- and copper-dependent conformational switches in a luminal loop of Atp7a and in the Cu_H electron transfer site of PHM facilitate luminal copper transfer from Atp7a to PHM²².

As expected, given the length and speed of the centrifugation step used to produce P4, essentially no CPD or Atp7a was recovered from the S4 (cytosol) fraction. With substantially more protein recovered in the P2 fraction than in the P3 fraction (Figure 2B) and more calreticulin, CPD and Atp7a recovered in the P3 fraction than in the P2 fraction (Figure 3C), we focused our efforts on further enrichment of secretory granules from the P2 fraction.

Analysis of copper, zinc and calcium content after differential centrifugation

ICP-MS analysis of the resuspended pellets (P2-P4) and the cytosolic fraction (S4) indicated that the cytosol contained over half of the total copper and zinc present in the anterior pituitary (Figure 4A). In contrast, less than 10% of the total calcium was recovered from the cytosolic fraction; over 60% of the total calcium was recovered from the P2 fraction (Figure 4A). When normalized to the amount of protein in each fraction ($\mu\text{g metal/mg protein}$) it

was clear that copper levels did not vary greatly between the particulate fractions and the cytosol (Figure 4B). Zinc levels in the P2 fraction were approximately twice those of copper (Figure 4B). In contrast, calcium levels in each particulate fraction greatly exceeded cytosolic calcium levels and calcium was about 50 to 100-fold more prevalent in the P2 and P3 fractions than copper (Figure 4B).

Secretory granule enrichment by sucrose density gradient fractionation

To further enrich the secretory granules, the P2 pellet was gently resuspended and loaded onto the top of a non-linear sucrose density gradient, which was centrifuged long enough to allow organelles to move through the gradient until they reached the concentration of sucrose matching their density (Figure 2A). Fractions were collected from the top of the gradient (low density) to the bottom (high density). As for the P fractions obtained by differential centrifugation, the presence of secretory granules and contaminating organelles was evaluated by Western blot analysis of appropriate markers after SDS-PAGE (Figure 5).

Secretory granules were localized to fractions 11 to 14 using antisera to PRL and to the PHM domain of PAM (Figure 5A). Antisera to growth hormone and to γ ₃-MSH confirmed the enrichment of secretory granules in these fractions (not shown). The small amount of PRL and PHM recovered from the top of the gradient indicated that very few secretory granules were lysed during this fractionation step. GM130 was again used to identify Golgi membranes, which were concentrated in fractions 7 to 9 (Figure 5B). As expected, much of the small amount of Atp7a recovered in P2 co-localized with the Golgi marker, GM130, after sucrose gradient centrifugation. Intact membrane PAM (PAM-1 Bb and PAM-2 Bb) was present in fractions 4-6, which were previously shown to contain endosomes³¹ and in the Golgi-enriched fractions, identifying another site at which PAM and Atp7a might interact directly. Calreticulin, the endoplasmic reticulum marker, was recovered from fractions 10 to 15 (Figure 5B). The small amount of clathrin recovered in the P2 fraction accumulated at the top of the gradient, indicating that its association with membranes had been lost during the fractionation (Figure 5B).

Although depleted of endosomal and Golgi markers, the secretory granule enriched fractions (11 to 14) are clearly not devoid of endoplasmic reticulum. The abundance of secretory granules in these fractions was verified using transmission electron microscopy after negative staining. Secretory granules were identified as round structures filled with electron dense material (Figure 5D); on average, the secretory granules had a diameter of 280 ± 30 nm. Similar experiments were done with P3, revealing a similar distribution of organelles.

Copper, zinc and calcium content of secretory granule enriched fractions

The concentrations of copper, zinc and calcium in each fraction of the sucrose gradient separation of P2 were measured by ICP-MS (Figure 6). The high concentrations of sucrose present at the bottom of the gradient contributed a significant amount of background metal to the analysis. To correct for this, blank gradients were analyzed so that background levels could be subtracted (Figure 6). Copper and calcium concentrations were highest in the secretory granule enriched fractions near the bottom of the gradient (Figure 6A and C).

Significant levels of zinc were present in the fractions enriched in endosomes (fraction 4 – 6) and in the secretory granule enriched fractions (Figure 6B).

Copper levels in the granule enriched fractions were approximately 100-fold lower than calcium levels; zinc levels were approximately twice as high as copper levels (Figure 6A-C). Expressed on a per protein basis, the copper and calcium concentrations in the granule enriched fractions were about double the copper and calcium concentrations in the P2 fraction loaded onto the sucrose gradient. To our surprise, the concentration of copper in the secretory granule enriched fractions ($\mu\text{g Cu/gm protein}$) was almost identical to the concentration of copper in the cytosol. In contrast, calcium was concentrated in the granule enriched fractions.

We next estimated the concentration of these metals in intact secretory granules. The protein concentration in secretory granules is thought to be about $\approx 150 \text{ mg/ml}$ ^{32, 33}; based on this estimate, the concentration of copper in the granules would be about $20\text{-}50 \mu\text{M}$ and the concentrations of zinc and calcium would be $160 \mu\text{M}$ and 30 mM , respectively. For copper, these values for the secretory granule enriched fractions ($\approx 20 \mu\text{g Cu/g protein}$) are the same as established numbers for synaptosomes and whole brain³⁴.

In order to estimate the molar ratio of copper to PAM in the granule enriched fractions, we included purified PAM in a western blot of the secretory granule enriched fractions (Figure 6D). Dilutions of purified recombinant PAM 820s allowed us to generate a standard curve. The total amount of PAM protein (intact + processed) in fractions 11 - 14 ranged between 20 and 55 nM (Figure 6E). The ratio of the molar concentration of copper to the molar concentration of PAM protein was then calculated. The values obtained for each individual fraction were averaged and two separate P2 gradients were analyzed. If most of the copper and most of the PAM protein in these fractions is contained in secretory granules, the concentration of copper exceeds that of PAM by approximately 10-fold. Unlike other cuproenzymes, the PHM domain of PAM does not bind copper with extremely high affinity [$K_D \sim 0.06 \mu\text{M}$]¹³.

Discussion

Heterogeneity within the rat anterior pituitary

Cells have developed tightly regulated systems to import, compartmentalize, store and export metals³⁵. We focused on PAM, an essential secretory pathway cuproenzyme that also requires zinc and calcium. Our aim was to utilize XFM to quantify metal levels in the different regions of the pituitary and then combine subcellular fractionation with ICP-MS to quantify levels of these three metals in well characterized subcellular fractions, including secretory granules.

Using differential centrifugation, we observed that GH was enriched in P2, γ_3 -MSH was enriched in P3 and PRL was almost evenly distributed between P2 and P3. The anterior pituitary contains five major cell types and each peptide producing cell can be distinguished by its morphology and the size of its secretory granules²³. The concentration of the different metals in anterior pituitary secretory granules may be cell type specific; previous studies

demonstrated cell type specific expression of PAM in pituitary ³⁶. In addition, heterogeneity in secretory granule content from the same cell has been observed in corticotrope and somatotrope tumor cell lines, suggesting that copper amounts within secretory granules of the same cell may vary ²⁴.

Copper is not concentrated in secretory granule enriched fractions

Like PAM, several cuproenzymes function in the secretory pathway and extracellularly; in contrast, cytochrome c oxidase functions in mitochondria and Cu/Zn-dependent superoxide dismutase 1 in cytosol. These environments are fundamentally very different. After entry into cells via Ctr1, copper binds to cytosolic chaperones, which deliver the metal to its next destination. Copper targeted to the secretory pathway binds to Atox1, which transfers the metal to Atp7a, the P-type ATPase that delivers it into the lumen of the secretory pathway ³⁴. Recent studies indicate that this transport process is facilitated with more reducing cytosolic conditions ²¹. Most of the copper measurements available in the literature report total copper levels. Based on the presence of amidated product, PAM function is first detected in the trans-most cisterna of the Golgi complex ³⁷, but levels of PAM are highest in secretory granules, where its product peptides are stored. Since the anterior pituitary provides a rich source of secretory granules, the concentration of copper could be determined in this compartment.

To our surprise, our data indicated that secretory granule copper concentrations were not substantially higher than the average copper concentration in the pituitary. Similarly, a study of SH-SY5Y neuroblastoma cells reported very similar copper levels in whole cell lysates and in fractions enriched in microsomes ²¹. One of the only earlier studies analyzing purified secretory granules reported levels of copper in adrenal chromaffin granules (0.6 nmol Cu/mg chromaffin granule protein) ³⁸ that are very similar to the levels found here for pituitary granules. Analysis of purified secretory granules from a variety of tissues indicates that hormone accounts for a very large fraction of their protein content, which is estimated at about 150 mg/ ml ^{32, 33}; the Coomassie stained gel shown in Fig. 5C certainly supports this conclusion. Using this protein concentration for secretory granules and our protein measurements for the gradient fractions, the concentrations measured for PAM and copper in these fractions need to be multiplied by a factor of ~100 to estimate the concentrations of PAM and copper in granules. Thus our data suggest that PAM levels in granules are 2.5 to 5.5 μ M while copper levels are 25 to 62 μ M, 10-fold higher (Fig. 6E). Estimates of PAM and copper levels based on granule diameter yielded similar numbers.

The dissociation constant for Cu(II) binding to what is thought to be the Cu_H site of PHM is about 0.06 μ M; copper must bind to both the Cu_H and Cu_M sites and redox cycles between Cu(I) and Cu(II) ¹³. Since luminal pH, redox status and the presence of other proteins and small molecules that might bind copper need to be taken into account, it is not clear whether the Cu_H and Cu_M sites of PAM are both saturated when PAM is localized to secretory granules containing this concentration of copper. Interestingly, no luminal copper chaperones have been identified, leaving it unclear how secretory granule proteins are protected from copper toxicity ^{31, 39}. If there are no copper chaperones in the lumen of the secretory pathway, copper delivery may be the key step. Using a neuroblastoma cell line,

data supporting the hypothesis that cytosolic copper binding proteins serve as a reservoir of copper that can be delivered by Atp7a when needed were recently reported ²¹.

Very little Atp7a is localized to pituitary secretory granules. Instead, most of the Atp7a and a significant amount of the intact PAM protein are localized to endosomes; the amount of endocytic material obtained precluded accurate assessment of copper levels. Data demonstrating the pH-dependent transfer of copper from a histidine- and methionine-rich luminal loop connecting transmembrane domains 1 and 2 of Atp7a to the active site of PAM have been reported ^{22, 40}. A direct transfer of this type could occur when PAM and Atp7a traverse the trans-Golgi network or in an endocytic compartment.

A better quantitative understanding of the subcellular localization of metals such as copper is needed. Menkes and Wilson diseases are the best described pathologies linked with impaired copper homeostasis. Other pathologies have also been associated with mutations in ATP7A; occipital horn syndrome is caused by a change in splicing and a reduction in functional ATP7A ⁴¹. ATP7A mutations that affect protein trafficking have been associated with distal motor neuropathy ^{42, 43}. MEDNIK (Mental Retardation Enteropathy Deafness Peripheral Neuropathy Ichtyosis Keratoderma) is caused by lack of the σ 1A (AP1S1) small subunit of adaptor protein 1 (AP-1), a cytosolic protein complex involved in post-Golgi trafficking and secretory granule biogenesis ^{44, 45}. This syndrome combines characteristics of both Menkes and Wilson disease, presumably because ATP7A trafficking is perturbed in cells with reduced AP-1 function ^{46, 47}.

Zinc is essential in secretory granule formation and peptide hormone signaling

Zinc deficiencies can lead to an inability to thrive, impaired brain development, diarrhea, skin inflammation and alopecia. Some of these symptoms are directly associated with impaired pituitary function. Members of the zinc transporter family are divided into two groups: ZIPs (Zrt/Irt-like protein [Slc39a]) are responsible for zinc uptake and ZnTs (cation diffusion facilitator family proteins [Slc30a]) are responsible for its export ⁴⁸. ZIPs have been observed in the plasma membrane, Golgi and endosomal/lysosomal membranes, while ZnTs were described in secretory granules, secretory vesicles, Golgi and endosomal/lysosomal membranes. While secretory granule levels of zinc were at least twice as high as copper levels and far in excess of PAM levels, they were substantially lower than cytosolic zinc levels. Both growth hormone and prolactin require zinc for proper sorting into secretory granules ^{1, 2}. ZnT5 (Slc30a5), the major zinc transporter of the regulated secretory pathway in the anterior pituitary, is necessary for growth hormone secretion ⁴⁹. A reduction in zinc availability can lead to an inability to secrete growth hormone properly and can impair development and growth.

Unlike copper, zinc was enriched in the endosome-rich P4 fraction and in gradient fractions enriched in endosomes. The zinc transporter, ZnT2, is expressed in the pituitary gland and was reported to protect cells from zinc toxicity by accumulating excess zinc in endosomal and lysosomal compartments ^{50, 51}. While copper is essential for the activity of only a dozen or so enzymes, almost 10% of the eukaryotic proteome interacts with zinc.

Calcium: a metal with multiple functions

Calcium plays an essential role in a wide variety of processes including endoplasmic reticulum stress, oxidative stress, and regulated secretion. Effective calcium signaling requires its sequestration and controlled appearance in the cytosol. Consistent with this, calcium levels in all three particulate fractions greatly exceeded cytosolic levels. Calcium levels in the *trans*-Golgi network, site of secretory granule formation, are estimated at 1 to 10 mM, and are thought to stimulate secretory granule protein aggregation, promoting entry into the regulated secretory pathway⁵²⁻⁵⁴. The presence of high levels of calcium at the bottom of the sucrose gradients reflects its presence in secretory granules and in endoplasmic reticulum. In order to compare calcium levels in the endoplasmic reticulum to those in secretory granules, additional fractionation steps are needed.

Material and Methods

X-ray fluorescence microscopy (XFM)

Rat pituitaries were collected and frozen in Tissue-Tek® O.C.T. Compound (VWR; Radnor, PA) and stored at -80°C until sectioning. For XFM imaging 10 µm sections were cut using a cryostat and allowed to air-dry before imaging. XFM was carried out as described previously⁵⁵. Briefly, XFM scans (excitation energy, 10 keV) were recorded on beamline 2-IDE at the Advanced Photon Source (Argonne National Laboratory, Argonne IL). On-the-fly using scanning with nominal pixel sizes between 300 nm × 300 nm and 5 µm × 5 µm (Fig. 1C) and 50 msec dwell time per point was used in data collection. Quantitation was carried out using an X-ray thin film standard (AXO Desden GmbH, Germany) that was measured separately. Regions of interest were defined and quantified in the program MAPS⁵⁶. For each sample several high resolution scans (500 nm × 500 nm pixel size) were recorded, the elemental concentrations determined, and the values averaged. The averaged values were used to determine the concentration ranges as listed in Table 1 from 4 animals. Adjacent sections were cut and stained with hematoxylin and eosin to ensure appropriate morphology and identify regions of interest for XFM scanning.

Inductively coupled plasma mass spectrometry (ICP-MS)

Tubes used for ICP-MS were incubated in 1% HNO₃ (trace metal grade, Fisher Scientific) for at least 24 hours. Samples were digested with 50% HNO₃ (trace metal grade, Fisher Scientific) in pre-rinsed 15 ml conical tubes at room temperature overnight. The digested samples were brought to a total volume of 500 µL with 1% HNO₃, mixed by vortexing, and centrifuged at 3500 rcf for 3 minutes to remove debris. ICP-MS analysis was carried out as described previously³⁴. Homogenization buffer and sucrose solutions were also analyzed to determine background contributions.

Differential centrifugation and subcellular fractionation

Anterior pituitaries from adult male Sprague-Dawley rats or mouse pituitaries were homogenized at 4°C in 10 volumes (w/v) of homogenization buffer: 0.32 M sucrose in 10 mM Tris-HCl, pH 7.4, containing 34 mg/ml phenylmethylsulfonyl fluoride, 16 mg/ml benzamidine, 2 mg/ml leupeptin, and 10 mg/ml lima bean trypsin inhibitor (inhibitor mix)

using six strokes of a motor-driven glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was subjected to differential centrifugation at 4°C. The homogenate was first centrifuged for 5 minutes at 800 g yielding a pellet (P1) containing nuclei and cell debris. The supernatant (S1) was centrifuged at 4,000 g for 17 minutes to obtain a pellet (P2) and a supernatant (S2), which was then centrifuged at 10,000 g for 15 minutes generating a pellet (P3), and a supernatant (S3). Lastly, S3 was centrifuged at 350,000 g for 15 minutes and provided another pellet (P4) and a cytosolic fraction (S4). Each pellet was resuspended in 225 µL of homogenization buffer and either kept at -80°C for further analysis or loaded on top of a non-linear sucrose gradient. Sucrose solutions were always made fresh in 10 mM Tris-HCl, pH 7.4, containing inhibitor mix. P2 was resuspended in homogenization buffer and loaded onto a discontinuous sucrose density gradient composed (bottom to top) of: 200 µL 2M sucrose, 350 µL of 1.6M sucrose, 350 µL 1.4M sucrose, 350 µL 1.2M sucrose, 200 µL 1M sucrose, 200 µL 0.8M sucrose, 200 µL 0.4M sucrose, all in homogenization buffer. After centrifugation at $120,000 \times g$ in a TLS-55 rotor for two hours at 4°C, aliquots of 150 µL were collected from top to bottom and kept at -80°C for further analysis.

Negative staining and transmission electron microscopy

Fractions 12 and 13 (100 µL) from the P2 sucrose gradient fractionation were combined and 200 µL of 10 mM HEPES, pH 7.4 containing protease inhibitors. The sample was centrifuged at 10,000 g for 15 minutes at 4°C. The supernatant was discarded and 200 µL of 2.5% glutaraldehyde in 10 mM HEPES, pH 7.4 was added on top of the pellet. The sample was then centrifuged at 10,000 g for 10 minutes at 4°C. The pellet was resuspended in 100 µL of 10 mM HEPES, pH 7.4 and used for negative staining. 4-5 µL of sample were added on top of a copper mesh grid. After waiting for 1 minute for the sample to diffuse across the grid, excess sample was removed using a filter paper. Uranyl acetate solution (4-5 µL of 1% to 3% uranyl acetate dissolved in water) was added on top. After allowing the uranyl acetate to diffuse for 1 minute, excess salt was removed with filter paper. The sample was then imaged using a Hitachi H-7650 Transmission Electron Microscope.

Purification of PAM 820s

A CHO-DG44 cell line stably expressing pCIS-PAM3- Pro- Gly 820s was grown in serum-free medium in CellMax artificial capillary cartridges (Spectrum laboratories, CA, USA; Cartridge #400-023) ⁵⁷. Spent medium was collected (three times per week or daily, depending on density) and stored frozen after addition of PMSF. Secreted proteins were concentrated by addition of solid (NH₄)₂SO₄. PAM820s was purified on a DEAE-Sepharose FF column (20 mm × 150 mm) equilibrated with 20 mM NaTES (pH 7.0) and eluted with a 150 ml gradient to 0.5M NaCl in the same buffer followed by gel filtration on a Superose 6 10/300GL column (25 × 350 mm) equilibrated with 20 mM NaTES, 150 mM NaCl (pH 7.0). The purity and homogeneity of the protein was evaluated by SDS-PAGE (Bio-Rad Criterion TGX gels, 4-15% gradient) followed by staining with Coomassie Brilliant Blue R250.

Acknowledgments

This work was supported by NIH DK-32949. ICPMS measurements were performed by the OHSU Elemental Analysis Core with partial support from the NIH core grant S10RR025512 (MR). MR was supported by

GM090016. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

References

1. Cunningham BC, Mulkerrin MG, Wells JA. Dimerization of human growth hormone by zinc. *Science*. 1991; 253:545–548. [PubMed: 1907025]
2. Sankoorikal BJ, Zhu YL, Hodsdon ME, Lolis E, Dannies PS. Aggregation of human wild-type and H27A-prolactin in cells and in solution: roles of Zn(2+), Cu(2+), and pH. *Endocrinology*. 2002; 143:1302–1309. [PubMed: 11897686]
3. Suckale J, Solimena M. The insulin secretory granule as a signaling hub. *Trends Endocrinol Metab*. 2010; 21:599–609. [PubMed: 20609596]
4. Bonnemaison ML, Eipper BA, Mains RE. Role of adaptor proteins in secretory granule biogenesis and maturation. *Front Endocrinol (Lausanne)*. 2013; 4:101. [PubMed: 23966980]
5. Blanco EH, Peinado JR, Martin MG, Lindberg I. Biochemical and cell biological properties of the human prohormone convertase 1/3 Ser357Gly mutation: a PC1/3 hypermorph. *Endocrinology*. 2014; 155:3434–3447. [PubMed: 24932808]
6. Seidah NG, Sadr MS, Chretien M, Mbikay M. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. *J Biol Chem*. 2013; 288:21473–21481. [PubMed: 23775089]
7. Greene D, Das B, Fricker LD. Regulation of carboxypeptidase E. Effect of pH, temperature and Co2+ on kinetic parameters of substrate hydrolysis. *Biochem J*. 1992; 285 (Pt 2):613–618. [PubMed: 1637350]
8. Yeung CM, Chan CB, Leung PS, Cheng CH. Cells of the anterior pituitary. *Int J Biochem Cell Biol*. 2006; 38:1441–1449. [PubMed: 16621669]
9. El Meskini R, Mains RE, Eipper BA. Cell type-specific metabolism of peptidylglycine alpha-amidating monooxygenase in anterior pituitary. *Endocrinology*. 2000; 141:3020–3034. [PubMed: 10919291]
10. Czyzyk TA, Ning Y, Hsu MS, Peng B, Mains RE, Eipper BA, Pintar JE. Deletion of peptide amidation enzymatic activity leads to edema and embryonic lethality in the mouse. *Dev Biol*. 2005; 287:301–313. [PubMed: 16225857]
11. Jiang N, Kolhekar AS, Jacobs PS, Mains RE, Eipper BA, Taghert PH. PHM is required for normal developmental transitions and for biosynthesis of secretory peptides in *Drosophila*. *Dev Biol*. 2000; 226:118–136. [PubMed: 10993678]
12. Bousquet-Moore D, Mains RE, Eipper BA. Peptidylglycine alpha-amidating monooxygenase and copper: a gene-nutrient interaction critical to nervous system function. *J Neurosci Res*. 2010; 88:2535–2545. [PubMed: 20648645]
13. Bell J, El Meskini R, D'Amato D, Mains RE, Eipper BA. Mechanistic investigation of peptidylglycine alpha-hydroxylating monooxygenase via intrinsic tryptophan fluorescence and mutagenesis. *Biochemistry*. 2003; 42:7133–7142. [PubMed: 12795609]
14. Chufan EE, De M, Eipper BA, Mains RE, Amzel LM. Amidation of bioactive peptides: the structure of the lyase domain of the amidating enzyme. *Structure*. 2009; 17:965–973. [PubMed: 19604476]
15. Prigge ST, Eipper BA, Mains RE, Amzel LM. Dioxygen binds end-on to mononuclear copper in a precatalytic enzyme complex. *Science*. 2004; 304:864–867. [PubMed: 15131304]
16. Prigge ST, Kolhekar AS, Eipper BA, Mains RE, Amzel LM. Amidation of bioactive peptides: the structure of peptidylglycine alpha-hydroxylating monooxygenase. *Science*. 1997; 278:1300–1305. [PubMed: 9360928]
17. Eipper BA, Mains RE, Glembotski CC. Identification in pituitary tissue of a peptide alpha-amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. *Proc Natl Acad Sci U S A*. 1983; 80:5144–5148. [PubMed: 6576381]

18. Prigge ST, Mains RE, Eipper BA, Amzel LM. New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *Cell Mol Life Sci.* 2000; 57:1236–1259. [PubMed: 11028916]
19. Turski ML, Brady DC, Kim HJ, Kim BE, Nose Y, Counter CM, Winge DR, Thiele DJ. A novel role for copper in Ras/mitogen-activated protein kinase signaling. *Mol Cell Biol.* 2012; 32:1284–1295. [PubMed: 22290441]
20. Dodani SC, Firl A, Chan J, Nam CI, Aron AT, Onak CS, Chang CJ. Copper is an endogenous modulator of neural circuit spontaneous activity. *Proc Natl Acad Sci USA.* 2014; 111:16280–16285. [PubMed: 25378701]
21. Hatori Y, Yan Y, Schmidt K, Furukawa E, Hasan NM, Yang N, Liu CN, Sockanathan S, Lutsenko S. Neuronal differentiation is associated with a redox-regulated increase of copper flow to the secretory pathway. *Nature Comm.* 2016; 7:10640.
22. Kline CD, Gambill BF, Mayfield M, Lutsenko S, Blackburn NJ. pH-regulated metal–ligand switching in the HM loop of ATP7A: a new paradigm for metal transfer chemistry. *Metallomics.* 2016; doi: 10.1039/c6mt00062b
23. Hopkins CR, Farquhar MG. Hormone secretion by cells dissociated from rat anterior pituitaries. *J Cell Biol.* 1973; 59:276–303. [PubMed: 4375681]
24. Sobota JA, Ferraro F, Back N, Eipper BA, Mains RE. Not all secretory granules are created equal: Partitioning of soluble content proteins. *Mol Biol Cell.* 2006; 17:5038–5052. [PubMed: 17005911]
25. Milgram SL, Kho ST, Martin GV, Mains RE, Eipper BA. Localization of integral membrane peptidylglycine alpha-amidating monooxygenase in neuroendocrine cells. *J Cell Sci.* 1997; 110 (Pt 6):695–706. [PubMed: 9099944]
26. Farquhar MG, Palade GE. The Golgi Apparatus (Complex) - (1954-1981) - from Artifact to Center Stage. *J Cell Biol.* 1981; 91:77–103. [PubMed: 6271793]
27. Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, Slusarewicz P, Kreis TE, Warren G. Characterization of a cis-Golgi matrix protein, GM130. *J Cell Biol.* 1995; 131:1715–1726. [PubMed: 8557739]
28. Varlamov O, Wu F, Shields D, Fricker LD. Biosynthesis and packaging of carboxypeptidase D into nascent secretory vesicles in pituitary cell lines. *J Biol Chem.* 1999; 274:14040–14045. [PubMed: 10318817]
29. Varlamov O, Fricker LD. Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the trans-Golgi network and recycling from the cell surface. *J Cell Sci.* 1998; 111 (Pt 7):877–885. [PubMed: 9490632]
30. Varlamov O, Eng FJ, Novikova EG, Fricker LD. Localization of metallocarboxypeptidase D in AtT-20 cells. Potential role in prohormone processing. *J Biol Chem.* 1999; 274:14759–14767. [PubMed: 10329672]
31. Oyarce AM, Eipper BA. Identification of subcellular compartments containing peptidylglycine alpha-amidating monooxygenase in rat anterior pituitary. *J Cell Sci.* 1995; 108 (Pt 1):287–297. [PubMed: 7738106]
32. Arvan P, Castle D. Protein sorting and secretion granule formation in regulated secretory cells. *Trends Cell Biol.* 1992; 2:327–331. [PubMed: 14731510]
33. Hutton JC, Penn EJ, Peshavaria M. Low-molecular-weight constituents of isolated insulin-secretory granules. Bivalent cations, adenine nucleotides and inorganic phosphate. *Biochem J.* 1983; 210:297–305. [PubMed: 6344863]
34. Gaier ED, Eipper BA, Mains RE. Copper signaling in the mammalian nervous system: synaptic effects. *J Neurosci Res.* 2013; 91:2–19. [PubMed: 23115049]
35. Malinouski M, Hasan NM, Zhang Y, Seravalli J, Lin J, Avanesov A, Lutsenko S, Gladyshev VN. Genome-wide RNAi ionomics screen reveals new genes and regulation of human trace element metabolism. *Nat Commun.* 2014; 5:3301. [PubMed: 24522796]
36. El Meskini R, Mains RE, Eipper BA. Cell type-specific metabolism of peptidylglycine alpha-amidating monooxygenase in anterior pituitary. *Endocrinology.* 2000; 141:3020–3034. [PubMed: 10919291]

37. Schnabel E, Mains RE, Farquhar MG. Proteolytic processing of pro-ACTH/endorphin begins in the Golgi complex of pituitary corticotropes and AtT-20 cells. *Mol Endocrinol*. 1989; 3:1223–1235. [PubMed: 2550814]
38. Phillips JH, Allison YP, Morris SJ. The distribution of calcium, magnesium, copper and iron in the bovine adrenal medulla. *Neuroscience*. 1977; 2:147–152. [PubMed: 917272]
39. El Meskini R, Culotta VC, Mains RE, Eipper BA. Supplying copper to the cuproenzyme peptidylglycine alpha-amidating monooxygenase. *J Biol Chem*. 2003; 278:12278–12284. [PubMed: 12529325]
40. Otoikhian A, Barry AN, Mayfield M, Nilges M, Lutsenko S, Blackburn NJ. Luminal loop M672-P707 of the Menkes protein (ATP7A) transfers copper to peptidylglycine monooxygenase. *J Am Chem Soc*. 2012; 134:10458–10468. [PubMed: 22577880]
41. Kaler SG, Gallo LK, Proud VK, Percy AK, Mark Y, Segal NA, Goldstein DS, Holmes CS, Gahl WA. Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. *Nat Genet*. 1994; 8:195–202. [PubMed: 7842019]
42. Kennerson ML, Nicholson GA, Kaler SG, Kowalski B, Mercer JF, Tang J, Llanos RM, Chu S, Takata RI, Speck-Martins CE, Baets J, Almeida-Souza L, Fischer D, Timmerman V, Taylor PE, Scherer SS, Ferguson TA, Bird TD, De Jonghe P, Feely SM, Shy ME, Garbern JY. Missense mutations in the copper transporter gene ATP7A cause X-linked distal hereditary motor neuropathy. *Am J Hum Genet*. 2010; 86:343–352. [PubMed: 20170900]
43. Yi L, Donsante A, Kennerson ML, Mercer JF, Garbern JY, Kaler SG. Altered intracellular localization and valosin-containing protein (p97 VCP) interaction underlie ATP7A-related distal motor neuropathy. *Hum Mol Genet*. 2012; 21:1794–1807. [PubMed: 22210628]
44. Montpetit A, Cote S, Brustein E, Drouin CA, Lapointe L, Boudreau M, Meloche C, Drouin R, Hudson TJ, Drapeau P, Cossette P. Disruption of AP1S1, causing a novel neurocutaneous syndrome, perturbs development of the skin and spinal cord. *PLoS Genet*. 2008; 4:e1000296. [PubMed: 19057675]
45. Bonnemaison M, Back N, Lin Y, Bonifacino JS, Mains R, Eipper B. AP-1A controls secretory granule biogenesis and trafficking of membrane secretory granule proteins. *Traffic*. 2014; 15:1099–1121. [PubMed: 25040637]
46. Holloway ZG, Velayos-Baeza A, Howell GJ, Levecque C, Ponnambalam S, Sztul E, Monaco AP. Trafficking of the Menkes copper transporter ATP7A is regulated by clathrin-, AP-2-, AP-1-, and Rab22-dependent steps. *Mol Biol Cell*. 2013; 24:1735–1748. S1731–1738. [PubMed: 23596324]
47. Martinelli D, Travaglini L, Drouin CA, Ceballos-Picot I, Rizza T, Bertini E, Carrozzo R, Petrini S, de Lonlay P, El Hachem M, Hubert L, Montpetit A, Torre G, Dionisi-Vici C. MEDNIK syndrome: a novel defect of copper metabolism treatable by zinc acetate therapy. *Brain*. 2013; 136:872–881. [PubMed: 23423674]
48. Kambe T. An overview of a wide range of functions of ZnT and Zip zinc transporters in the secretory pathway. *Biosci Biotechnol Biochem*. 2011; 75:1036–1043. [PubMed: 21670538]
49. Petkovic V, Mileta MC, Eble A, Fluck CE, Mullis PE. Alteration of ZnT5-mediated zinc import into the early secretory pathway affects the secretion of growth hormone from rat pituitary cells. *Horm Res Paediatr*. 2014; 82:245–251. [PubMed: 25196974]
50. Palmiter RD, Cole TB, Findley SD. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. *EMBO J*. 1996; 15:1784–1791. [PubMed: 8617223]
51. Zhong ML, Chi ZH, Shan ZY, Teng WP, Wang ZY. Widespread expression of zinc transporter ZnT (SLC30) family members in mouse endocrine cells. *Histochem Cell Biol*. 2012; 138:605–616. [PubMed: 22673841]
52. Chanat E, Huttner WB. Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. *J Cell Biol*. 1991; 115:1505–1519. [PubMed: 1757460]
53. Colomer V, Kicska GA, Rindler MJ. Secretory granule content proteins and the luminal domains of granule membrane proteins aggregate in vitro at mildly acidic pH. *J Biol Chem*. 1996; 271:48–55. [PubMed: 8550606]
54. Gerdes HH, Rosa P, Phillips E, Baeuerle PA, Frank R, Argos P, Huttner WB. The primary structure of human secretogranin II, a widespread tyrosine-sulfated secretory granule protein that exhibits

low pH- and calcium-induced aggregation. J Biol Chem. 1989; 264:12009–12015. [PubMed: 2745426]

55. Ohrvik H, Nose Y, Wood LK, Kim BE, Gleber SC, Ralle M, Thiele DJ. Ctr2 regulates biogenesis of a cleaved form of mammalian Ctr1 metal transporter lacking the copper- and cisplatin-binding ecto-domain. Proc Natl Acad Sci U S A. 2013; 110:E4279–4288. [PubMed: 24167251]
56. Vogt S. Maps: A set of software tools for analysis and visualization of 3D X-ray fluorescent datasets. Journal De Physique IV. 2003; 104:635–638.
57. Kolhekar AS, Quon AS, Berard CA, Mains RE, Eipper BA. Post-translational N-glycosylation of a truncated form of a peptide processing enzyme. J Biol Chem. 1998; 273:23012–23018. [PubMed: 9722525]
58. Milgram SL, Mains RE. Differential effects of temperature blockade on the proteolytic processing of three secretory granule-associated proteins. J Cell Sci. 1994; 107 (Pt 3):737–745. [PubMed: 8006087]

Figure 1. X-ray Fluorescence Microscopy (XFM) analysis of metals in the pituitary)

A. Left, diagrams of PAM-1 Bb (PAM-1), PAM-2 Bb (PAM-2) and PAM-3, major PAM proteins expressed in the anterior pituitary⁹. The striped boxes represent the signal peptide. PHM and PAL core domains are shown in pink and gray, respectively. The transmembrane domain (TMD) and cytosolic domain (CD) are located at the C-terminus of the protein. In PAM-1, PHM and PAL domains are separated by exon 16 (16). Right, reactions catalyzed by PAM. The rest of the peptide substrate is represented by P in a green circle.

B. Top, Hematoxylin and eosin staining (H&E) showing the anterior (yellow arrows), intermediate (red arrow), and neural lobes (blue arrow) of a 5 μm thick transverse section. Rathke's pouch is indicated by the green arrow. Bottom, Brightfield image of a 10 μm thick transverse section observed by X-ray fluorescence microscopy (XFM). The white rectangle depicts the area scanned with XFM. **C.** XFM image of the depicted area in B, showing the elemental distribution for phosphorus (P), sulfur (S), calcium (Ca), iron (Fe), copper (Cu) and zinc (Zn). The maximum elemental density for each element is indicated above each map in μg/cm². To optimize contrast the maximum displayed densities for Ca, Fe, and Cu were adjusted. Therefore, the maximum density for these elements is displayed as a range. Two boxes were drawn in each lobe to determine the concentration of each element measured. Data were further filtered by gauss 2 to decrease pixilation. Scale bar for each map is 100 μm, false color bar below the elemental maps indicates relative elemental densities.

Figure 2. Differential centrifugation

A. Subcellular fractionation protocol. After differential centrifugation, fractions enriched in secretory granules (P2 and P3) were resuspended and subjected to equilibrium centrifugation on sucrose gradients; 150 μ L fractions were collected from the Top (#1) to the Bottom (#15) of the tube.

B. Protein recovery after each centrifugation step was > 95% (n = 3).

Figure 3. Distribution of organelle-specific marker proteins following differential centrifugation

A. Prolactin (PRL) and growth hormone (GH), the two major anterior pituitary hormones, and proopiomelanocortin (POMC) products were localized in order to identify fractions enriched in secretory granules. A diagram illustrates POMC with some of its cleaved products and γ_3 -MSH antibody specificity below the blots; the fragment corresponding to γ_3 -MSH is shown in a green box. The red, blue and gray boxes correspond to joining peptide, adrenocorticotropin and β -lipotropin, respectively. The 18 kDa fragment observed in P3 corresponds to a POMC product that contains γ_3 -MSH and joining peptide.

B. Antisera to Peptidylglycine α -Amidating Monooxygenase (PAM), an integral membrane protein whose cleavage products are found in the *trans*-Golgi network, secretory granules and endosomes, was localized using an antibody to the PHM domain⁵⁸. Diagrams below the blot illustrate PAM products and PHM antibody specificity. The asterisk indicates soluble PAM proteins generated from membrane isoforms and from PAM-3. **C.** Organelle markers examined included the heavy chain of clathrin (clathrin coated vesicles and cytosol), GM130 (*cis*-Golgi complex), calreticulin (endoplasmic reticulum), carboxypeptidase D (CPD) (endosomes and secretory granules) and Atp7a (*trans*-Golgi network and endosomes).

Figure 4. Levels of copper, zinc and calcium in P fractions and S4 (Cytosol)

Metal concentrations in each P fraction were measured by ICP-MS. Data from two independent fractionations of five adult male rat anterior pituitaries were averaged; error bars show the range. **A.** The percentage of the total copper, zinc and calcium recovered in each fraction is shown. **B.** The ratio of copper, zinc or calcium to the concentration of protein in each fraction is shown; data for calcium and zinc were plotted using the right y-axis; data for copper were plotted using the left y-axis.

Figure 5. Sucrose gradient fractionation of P2 fraction

The P2 pellet was suspended in homogenization buffer and loaded onto a sucrose density gradient; Fraction #15 was the bottom of the gradient. **A.** Secretory granules were localized using antibodies to prolactin (PRL) and PHM; soluble PHM, which accumulates in secretory granules, was recovered in fractions #11 to #14. **B.** Golgi markers, Atp7a and GM130, were both localized to fractions #7 to #9, identifying them as Golgi/TGN-enriched. Calreticulin, a marker for the endoplasmic reticulum was recovered in fractions #10 to #15. Clathrin heavy chain was recovered from the top of the gradient. SGs, secretory granules; ER, endoplasmic reticulum; Endo, endosomes. **C.** Coomassie stain of PVDF membrane after Western blot analysis; the major bands near 20 kDa are primarily GH and PRL, leading to the estimate that these hormones are the major proteins in the SG fractions. **D.** Negative staining and transmission electron microscopy were performed after pelleting the granules; aliquots of the two fractions that were pooled were used to verify the presence of granules.

Figure 6. Quantification of copper, zinc and calcium in secretory granule enriched gradient fractions

Levels of copper (**A**), zinc (**B**), and calcium (**C**) in each sucrose gradient fraction were measured by ICP-MS. Analysis of a gradient that contained buffer, but no sample, allowed subtraction of metal contributed by the increasing amounts of sucrose in the gradient fractions. Levels of all three metals peaked in the granule-enriched fractions. Similar data were obtained from an independent analysis of 5 adult rat anterior pituitaries. SGs, secretory granules; ER, endoplasmic reticulum; Endo, endosomes. (**D**) PAM levels in fractions #11 to #14 were measured by Western Blot using known amounts of purified PAM 820s (PAM-1 truncated at residue 820). (**E**) Quantification of PAM concentration in fractions #11 to #14 is shown. Data from two independent fractionations of five adult male rat anterior pituitaries were averaged; error bars show the range.

Table 1
X-ray fluorescence microscopy analysis of metals in the pituitary

Lobe→	Anterior	Intermediate	Neural
Element↓			
P, mM	110 (±12)	110 (±9)	87 (±7)
S, mM	57 (±6)	50 (±6)	80 (±30)
Ca, mM	4.1 (±0.7)	5 (±1)	2.6 (±0.8)
Fe, µM	700 (±400)	400 (±200)	900 (±400)
Cu, µM	150 (±30)	137 (±5)	130 (±9)
Zn, µM	480 (±20)	430 (±4)	340 (±10)

Concentrations: millimolar for P, S and Ca; micromolar for Fe, Cu, and Zn. Sections were 10 µm thick and the density used was 1.035 g/cm³. Data shown are the mean (±sem) for 3 determinations using tissue from P12 to adult rats.