1-1-2017

Dense Core Vesicle Heterogeneity in Anterior Pituitary Cells

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Abstract

Peptides, which are packaged in dense core vesicles, are an integral part of the function of the endocrine and neurological systems. The dense core vesicles function as an efficient form of peptide storage prior to regulated exocytosis. Two different dense core specific transmembrane proteins traffic different when comparted to retained prolactin cores, offering evidence of heterogeneity of vesicles within a single cell. By comparing synaptotagmin 1 and 7 distribution in male rat and lactating female lactotrophs, a distinct pattern emerges. Cells that retain prolactin cores after exocytosis correspond with those that contain synaptotagmin 1. This finding is a reversal for previous studies in chromaffin cells supporting the theory that not all dense core vesicles are the same across cell types. These experiments offer evidence of two different levels of heterogeneity in dense core vesicles across cell types and within a single cell. This variation allows cells to have robust regulation over secretion, an necessity in these vital systems.

Document Type
Thesis

Degree Name
M.S.

Department
Biological Sciences

First Advisor
Joseph K. Angleson, Ph.D.

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Keywords
Anterior pituitary, Exocytosis, IA2, Islet antibody 2, Lactotrophs, Phogrin, Synaptotagmin

Subject Categories
Cell Biology | Molecular Biology

Publication Statement

Dense Core Vesicle Heterogeneity in Anterior Pituitary Cells

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A Thesis

Presented to

the Faculty of Natural Sciences and Mathematics

University of Denver

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Kelly Sinak

November 2017

Advisor: Dr. Joseph K Angleson

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Title: Dense Core Vesicle Heterogeneity in Anterior Pituitary Cells
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Peptides, which are packaged in dense core vesicles, are an integral part of the function of the endocrine and neurological systems. The dense core vesicles function as an efficient form of peptide storage prior to regulated exocytosis. Two different dense core specific transmembrane proteins traffic different when comparted to retained prolactin cores, offering evidence of heterogeneity of vesicles within a single cell. By comparing synaptotagmin 1 and 7 distribution in male rat and lactating female lactotrophs, a distinct pattern emerges. Cells that retain prolactin cores after exocytosis correspond with those that contain synaptotagmin 1. This finding is a reversal for previous studies in chromaffin cells supporting the theory that not all dense core vesicles are the same across cell types. These experiments offer evidence of two different levels of heterogeneity in dense core vesicles across cell types and within a single cell. This variation allows cells to have robust regulation over secretion, an necessity in these vital systems.

Acknowledgements

Dr. Joseph Angleson has been an integral part of my success as a graduate student. His guidance, expertise, and sense of humor are greatly appreciated. I would like to thank for committee members, Dr. Eric Boschmann, Dr. Cedric Asensio, Dr. Schuyler van Engelenburg for valuable time, patience, commitment.

As most of my experiments revolved around a microscope, I would like to thank Dr. Erich Kusher and his lab, Dr. Dinah Loerke, and Dr. Yan Qin and her lab for their flexibility and assistance using their various microscopes. Additionally, I would like to thank Dr. Daniel Linesman and his graduate student Liz Ignowski for their assistance.

My graduate student experience has been greatly enriched by the relationships with my colleagues. I am particularly grateful for the encouragement of my lab mates Kristin Dew and Emily Wilkinson.

Last, but not least, I would like to express gratitude to my husband, Paul Della Grotte. His unwavering support and constant encouragement were essential to my success, and for that I am forever grateful.

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Introduction

Development, locomotion, and digestion are a small sample of biological processes that require peptide release. Peptides are released from dense core vesicles, named because of their dense appearance in electron microscopy (EM) images. Dense core vesicles are widely found in endocrine and neuronal cells as a method to efficiently store peptides prior to regulated release. Although, it is not yet understood how dense core vesicles are packaged and synthesized. Peptides, like most proteins, are translated and folded in the ER and transferred to the Golgi. From there, vesicles bud from the trans-Golgi network (TGN) to form immature dense core vesicles and are transported into the cytoplasm to await release. This is a prolific process as a typical lactotroph, a cell in the anterior pituitary which secrete prolactin, contains approximately 30,000 dense core vesicles per cell.

Morphologically not all dense core vesicles appear to be the same within a cell type. Two populations, based on size, has been observed by EM in both mouse chromaffin cells, which are responsible for secreting adrenaline, and rat lactotrophs (Grabner, 2005; Farquhar, 1978). The differences do not end with the size of the vesicle. Endocrine cells have been shown to have multiple populations of dense core vesicles in their cells based on their associated SNARE (SNAP (Soluble NSF Attachment Protein) Receptor) proteins as well (Chieregatti, 2004).

It is logical to assume some tissue-to-tissue and cell type-to-cell type variability with respect to dense core vesicles. After all, there are distinct differences between neurons and beta cells, both of which contain dense core vesicles. Previous work in lactotrophs have shown there are different populations that respond to different physiological stimuli (Christian, 2007). However, it might also be conceivable that heterogeneity exists within an individual cell. This type of variation could be a way for cells to finely tune their secretion of peptides, allowing for more a more controlled regulation of endocrine signaling.

Dense Core Vesicles

Two theories exist that could explain the biogenesis of dense core vesicles: sorting by entry and sorting by retention. Sorting by entry proposes that like peptides aggregate together in the TGN and a homogenous dense core vesicle will bud and be released into the cytoplasm. Carboxypeptidase E (CPE) has been proposed as a spot of aggregation of peptides in the TGN. CPE functions as a convertase in endocrine cells and has been shown to interact with prohormone aggregates. When CPE is knocked out in a cell, no dense core vesicle biogenesis is observed, strengthening the theory of sorting by entry (Cool, 1997). Sorting by retention states that mixed content vesicles bud from the TGN and are sorted into homogenous vesicles by budding off of other peptides. This has been thoroughly studied in beta cells, whose dense core vesicles exclusively include processed insulin. The cleaved C-peptide, which is the remnant of the conversion to the active form of the hormone, has been shown to preferentially bud off of maturing insulin

dense core vesicles. However, not all C-peptide is removed from the dense core vesicle providing credence two both theories (Kuliawat, 1992).

Once synthesized, the vesicles can exist in multiple pools: docked, readily-releasable, or reserve pools (Figure 1). Docked vesicles are present at the plasma membrane and are awaiting a signal for exocytosis. After these vesicles exists the readily-releasable pool, which is a group of vesicles that while not docked are in close proximity to the plasma membrane ready to replenish the docked granules once released. Lastly, there is the reserve pool, which contains all other dense core vesicles present in the cell that are not in either of the other two pools (Sørensen, 2004). This is the largest proportion of the 30,000 dense core vesicles seen in lactotrophs.

Lactotrophs, one of the most abundant cell types in the anterior pituitary, have demonstrated preferential release of newly synthesized dense core vesicles (Smith, 1966). Another phenomenon of these cells is the ability to not release the dense core after undergoing exocytosis. This behavior also been observed in differentiated PC12 cells, a rat derived adrenal cell line (Bauer, 2004A). A classical view of exocytosis would have vesicles fusing with the plasma membrane and releasing of all its contents. However, lactotrophs don't necessarily do that. Some vesicles, after fusion with the plasma membrane, do not release all their contents, they still maintain the dense core of hormone within the vesicle. These retained core vesicles are also preferentially recycled to the plasma membrane for subsequent exocytosis upon a secondary stimulation (Bauer, 2004B). Aside from the ability to retain cores after exocytosis, lactotrophs dense core vesicles also perform more complex exocytic events. One type, multigranular exocytosis,

is defined as the fusion of multiple dense core vesicles prior to fusion with the plasma membrane. This is not common in lactorophs, it's only been observed in blood cells. However, sequential exocytosis, which is the process of a second vesicle fusing with a vesicle already fused to the plasma membrane is quite common in lactotrophs. In fact, sequential exocytosis has been observed in many endocrine cells including: acinar cells, chromaffin cells, and PC12 cells (Kasai, 2016). Sequential events, which occur spontaneously, can be modulated. Increasing intracellular cAMP causes potentiation of sequential events in lactotrophs. However, decreasing cAMP causes lactotrophs to lose the ability to retain the hormone dense core after exocytosis (Angleson, 1999).

Exocytosis

Regulated exocytosis is mediated by a combination of proteins on both the target membrane and the vesicle membrane. These proteins make up the SNARE complex, the required machinery for exocytosis. The prototypic combination of SNARE proteins required for regulated exocytosis includes SNAP-25, syntaxin 1, which are present on the target membrane, and VAMP2, a protein present on the vesicle. SNAP-25, and it's homolog SNAP-23, act as the powerhouse of exocytosis. This zipping together of the cytoplasmic tails of the proteins on the target and vesicle membranes generates enough force to cause the plasma membrane and vesicle membrane to fuse. As these proteins play such an essential role, it is not surprising that a knockout of either protein is lethal in mice (Suh, 2011; Washbourne, 2002). It has been shown that SNAP-25 is responsible for sequential exocytosis in beta cells, whereas SNAP-23 has been shown to have a different role, offering a level of heterogeneity amongst dense core vesicles (Takahashi, 2004;

Kunii, 2016). An additional study in ATt-20 cells, a pituitary derived cell line, also

examined the SNAP proteins. This study showed SNAP-25 was used when cells were

stimulated to secrete , however SNAP-23 was used for basal secretion (Chieregatti,

2004). Regardless of which SNAP protein in used, it will interact with VAMPs, which

are on the vesicle. VAMPs are v̲esicle a̲ssociated m̲embrane p̲roteins required for

docking of the vesicles on the plasma membrane. Additionally, a synaptotagmin is also

present on the vesicle. When the v- and t-SNAREs interact, the vesicle is considered

docked. With an increase in intracellular $Ca2^+$, synaptotagmin senses the change and

removes a protein clamp on the docked vesicles that inhibits premature exocytosis. Once

the clamp is removed, the contents of the vesicle can be released (Südhof, 2013).

Synaptotagmin

Synaptotagmins are vesicle associated proteins used in regulated exocytosis,

however they are not part of the SNARE complex. They are a family of proteins that act

as $Ca2^+$ sensors and function at various points in regulated exocytosis. Synaptotagmins

are proteins that consists of a small transmembrane domain followed by two C2 domains

that bind membranes in a Ca2+ dependent manner. Binding of $Ca2^+$ by the C2 domains

allows synaptotagmin to bind other members of the SNARE complex, an essential step in

regulated exocytosis (Südhof, 2002). One isoform, synaptotagmin 1, has also been

shown to partially penetrate the lipid bilayer after binding with Ca2+, increasing the

curvature of the membrane which is hypothesized to facilitate exocytosis (Chapman,

1998).

There are 13 isoforms of synaptotagmin, divided into multiple classes, each class with a different $Ca2^+$ affinity. Synaptotagamin 1, which is the most commonly found form in brain and endocrine tissue, and synaptotagmin 7, which is widely expressed, are among the most studied isoforms in endocrine cells (Südhof, 2002). Previous studies have shown distinct roles for each of the proteins in chromaffin cells. When cells had a mild increase in intracellular Ca2+, synaptotagmin 7 containing vesicles were favored; whereas, a large change in intracellular Ca2+ selected for synaptotagmin 1 containing vesicles (Rao, 2014). Although not SNARE complex proteins, synaptotagmins play an essential role in $Ca2^+$ regulated exocytosis, yet the loss of synaptotagmins does not have a global effect in an animal. In synaptotagmin 1 knockout mice, ultrafast synaptic fusion was ablated in the forebrain, but no other effects were seen in the mouse (Geppert, 1994). In synaptotagmin 7 knockout mice, only facilitation, a short-term form of enhancement in which each subsequent action potential evokes greater neurotransmitter release, was affected in neurons but no global affects were seen in the animal (Jackman, 2016). These studies give evidence to the plasticity of the exocytic machinery as well as the closely aligned functions of both synaptotagmin isoforms.

Phogrin and IA2

Both IA2 and phogrin are transmembrane proteins that are enzymatically inactive members of the protein tyrosine phosphatase family, yet their exact function is unknown. Islet antibody 2 (IA2) is an autoantigen in diabetes that was identified in 1994 (Rabin, 1994). In the subsequent years, many studies have attempted to identify the function of this dense core specific protein, to no avail. Phogrin (IA2β, phosphatase homologue in

granule insulnoma) is a related protein, which also localizes to endocrine secretary dense core vesicles and has been shown to interact with AP2, offering a role in endocytosis (Wasmeier, 2005). A knockout of phogrin causes a slight defect in insulin secretion and a knockout of IA2 results in a decrease of dense core vesicles in cells (Kubosaki, 2004). Notably, when mice were generated without phogrin or IA2, the females were infertile with a totally abnormal esterus cycle which coincides with a decrease in secretion of leutenizing hormones from the gonadotrophs in the pituitary (Kubosaki, 2006). However, there is no phenotype when only one of the proteins is knocked out.

Although these proteins have an unknown function, when tagged by a fluorescent protein they sort accurately and are often used as markers for exocytosis. When tagged with a pH sensitive fluorophore, the proteins become precise markers for exocytosis as the fluorophore is quenched while in the lumen of the dense core vesicle but becomes unquenched with exposed to the extracellular matrix. These markers are often used in TIRF experiments or as markers for dense core vesicle recycling (Vardjan, 2009; Vo, 2004).

After exocytosis, the vesicle membrane, including transmembrane proteins, much be endocytosed to maintain a constant surface area of the cell. Once endocytosed, vesicles can traffic in multiple areas of a cell. Vesicles can return to the plasma membrane, endosome recycling compartment, the TGN, or they could be shuttled to a late endosome and onto the lysosome for degradation (Bäck, 1993). While retained core vesicles in lactotrophs have been shown to recycle back to immature granules, retained cores in beta cells recycling back to mature granules (Vo, 2004; Bauer, 2004B). These

differences could likely be attributed to cell specific trafficking effects, but they also lend to the idea of heterogeneity of dense core vesicles across various cell types.

To study the inherent heterogeneity of dense core vesicles in lactotrophs, primary tissue from both female and male rats was used. Although lactotroph cell lines exist, the number of dense core vesicles present are disproportionally smaller compared to primary tissue. As this thesis focuses on dense core vesicles, primary tissue was favored over cell lines. Many previous studies have shown variability of dense core vesicles within a cell type. The following experiments will provide evidence that lactotrophs are also heterogeneous as assessed by the various synaptotagmin isoforms present. Additionally, by studying dense core vesicle transmembrane markers, this thesis will also provide evidence of heterogeneity of vesicles within a single cell.
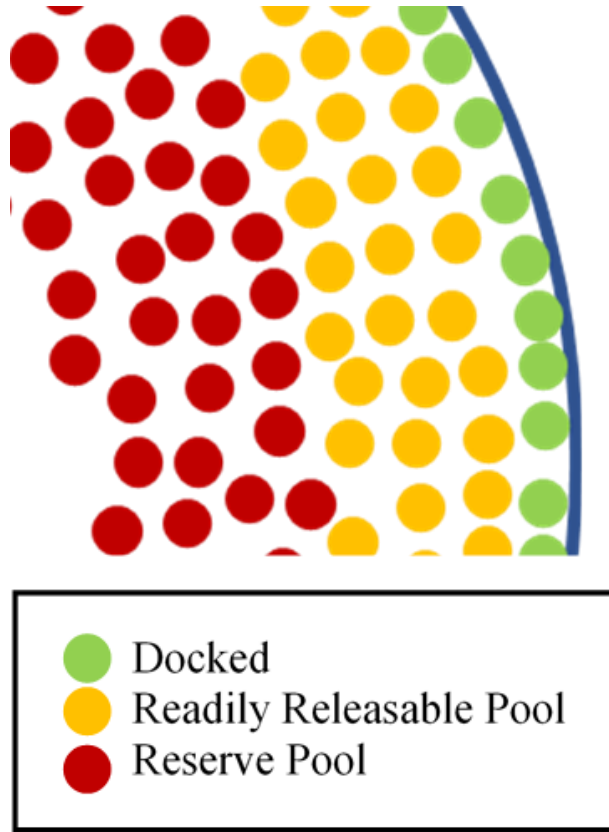
Figure 1 Depiction of dense core vesicle pools in endocrine cells

Materials and Methods

Cell preparation Anterior pituitary cells were obtained from Sprague Dawley male rats (Charles River) or CD female rats (Charles River) via enzymatic dispersion in serum-free media containing collagenase IA (Sigma), dispase II (Sigma), and protease XIV (Sigma) that was placed in a 37°C water bath for 6 minutes the agitated by pipetting up and down 15 times. This was repeated three times. Cells were pelleted and rinsed twice in serum-free media, resuspended in serum-free media with DNase and drawn up into a 28 gauge syringe. The cells were filtered through a 100µm cell sifter, then again through a 40µm or 70µm sifter. Cells were cultured for 48 (male) to 72 (female) hours in suspension wells in DMEM with 10% Newborn Calf Serum (Gibco) and buffered with sodium bicarbonate at 37°C with 5% CO_2. Female cells were cultured in the same media with the addition of 2nM β-estradiol.

Solutions Both stimulatory and basal solutions contained 2mM magnesium chloride, 10mM HEPES, 6mM calcium chloride, and 10mM glucose. Stimulatory solution contained 100mM potassium chloride and 37mM sodium chloride whereas the basal solution contained 5mM potassium chloride and 137mM sodium chloride.

Stimulated Antibody Uptake and Immunocytochemistry Cells were plated into poly-l-lysine (Sigma) coated coverslips and allowed to adhere for at least one hour. Media was removed and replaced with a stimulatory or basal solution containing the

desired antibody and 1% BSA (for prolactin only uptake) or 5% BSA (for phogrin and/or

IA2 uptake) in PBS and incubated at room temperature for a given time. Solutions were

removed and cells were fixed in 4% PFA by incubating a room temperature for 15

minutes and triple rinsed with PBS. If required, cells were then permeabilized with 0.3%

Triton by incubating at room temperature for 10 minutes and triple rinsed in PBS. If

additional primary antibodies were required, there were added at the set concentration in

5% BSA in PBS and incubated in a humifying chamber at 37°C with 5% CO_2 for 90

minutes. Primary antibodies were removed and the cells were triple rinsed with PBS.

Secondary antibodies were added at a given concentration in 5% Donkey Serum in PBS

and cells were incubated at 37°C with 5% CO2 in humidifying chamber for 30 to 45

minutes. Cells were triple rinsed again in PBS and either mounted in VectaShield

(VectraLabs) with DAPI or they were stained using 1:1000 dilution of Draq-7 (Abcam)

in PBS and incubated for 20 minutes in the dark at room temperature prior to mounting in

VectaShield without DAPI. Cells were stored at 4°C until imaged. All stains including

anti-IA2 or anti-Phogrin antibodies were imaged within 24 hours of mounting.

Primary Antibodies		
Antibody	**Source**	**Concentration**
Rabbit anti-prolactin	NIDDK	1:1000
Guinea pig anti-prolactin	NIDDK	1:1000
Guinea pig anti-Phogrin	Bleed 3	1:100
Rabbit anti-IA2	Oreo, bleed 4	1:100
Mouse anti-Synaptotagamin 1	Synaptic Systems	1:500
Rabbit anti-Synaptotagamin 7	Synaptic Systems	1:200

Secondary Antibodies			
Antibody	**Source**	**Fluorophore**	**Concentration**
Donkey anti-Guinea Pig	Biotium	CF568	1:800
Donkey anti-Mouse	Invitrogen	Alexa 488	1:800
Donkey anti-Guinea Pig	Biotium	CF488-A	1:800
Donkey anti-Mouse	Biotium	CF568	1:800
Donkey anti-Guinea Pig	Biotium	CF568	1:800
Donkey anti-Goat	Jackson Immunoresearch	Cy5	1:200
Donkey anti-Mouse	Jackson Immunoresearch	Cy5	1:500
Donkey anti-Rabbit	Life Technologies	Alexa 488	1:800
Donkey anti-Rabbit	Jackson Immunoresearch	CF405S	1:200

Immunofluorescent Imaging Cells were imaged using Nikon Eclipse Ti scope with a Hamatatsu ImagEM X2 C9100- 23b camera with 0.267 µm/pixel with a 60x objective (1.4 N.A.) and a spinning disk which used Micro Manager (NIH). Image stacks were acquired every 300 nm. TIRF images were acquired using a Nikon Eclipse Ti Scope with a Andor iXon3 camera with 0.16 µm/pixel using a 100x objective (1.45 N.A.) using NIS Elements (Nikon).

Image Analysis TIFF stacks were imported into SlideBook 6 (3i Imaging) and thresholding individually. Masks were created manually and data was mined using the Mask Statistics menu in SlideBook. All images shown were edited in FIJI (NIH). All graphs and statistical analysis were performed in Kaleidagraph (Synergy). All data reported at as +/- S.E.M.

FURA Cells were plated onto poly-l-lysine (Sigma) coated imaging dishes and allowed to adhere in media. To load FURA-2, AM (Life Technologies), a basal solution with 500nM FURA was added to the cells. Cells were incubated in the dark at room

temperature for 30 minutes, the FURA-2, AM solution was removed and a basal solution was added to the dish.

FURA Imaging Cells were imaged using a CoolSNAP HQ2 camera with 6.21 µm/pixel using a 40x oil objective (1.3 N.A.). Images were binned (4x4) and cells were alternatively excited by 340 nm and 380 nm light every 500 ms with a 100 ms interval between exposures.

<center>**Results**</center>

Phogrin and IA2

Previous studies in INS-1 cells, a rat derived beta cell line, have shown that phogrin recycles back mature and immature dense core vesicles (Vo, 2004). Although recycling back to an insulin positive compartment would suggest phogrin returns to a mature dense core vesicle, returning to a syntaxin 6 positive compartment indicates phogrin returns to an immature dense core vesicle. Similarly, studies in primary rat lactotrophs showed retained prolactin cores also recycle back to syntaxin 6 positive compartments in the cells, again indicating an association with immature vesicles (Bauer, 2004B). By stimulating male rat lactotrophs in the presence of antibodies for prolactin and phogrin or IA2, exocytoic exposure can be identified and observed. Cells were either fixed immediately or placed in an antibody free basal solution, and chased for different periods of time allowing for the trafficking of the dense core vesicle transmembrane proteins relative to be assessed (Figure 2). Immediately after stimulation (0 minutes) phogrin and IA2 have distinctly different exocytic exposures relative to the retained prolactin cores. Phogrin is punctate and appears like prolactin retained cores whereas IA2 signal is more diffuse. Most of the phogrin signal colocalizes with the retained prolactin cores while there is less colocalization of IA2 and the retained cores (Figure 3A, B).

<center>14</center>

20 minutes after stimulation, there is still a proportion of phogrin and IA2 that colocalize with prolactin; however, there is distinct population of both phogrin and IA2 within the cell. This suggests that phogrin and IA2 can traffic differently than prolactin cores (Figure 2A, B, middle column). At 60 minutes after stimulation, although phogrin is seen associated with the cores, it is significantly less colocalized with the retained prolactin core as seen by phogrin staining in the cytoplasm (Figure 2A, right column, Figure 3A). IA2 does not show a significant change in colocalization from 20 minutes to 60 minutes, as there is still IA2 signal in the cytoplasm as both time points (Figure 2B, middle and right column). When quantified, phogrin has a high colocalization coefficient with prolactin initially after stimulation, and is significantly reduced the longer the vesicles are chased. Conversely, IA2 has a coefficient suggesting near random colocalization that doesn't significantly change over the chase period (Figure 3A, B).

Previous work has shown retained cores are quite stable and remain at the plasma membrane for up to 20 minutes, yet, qualitatively, some phogrin is internalized at 20 minutes (Figure 2A, middle column) (Bauer, 2004B). To assess the time course of phogrin endocytosis, lactotrophs were exposed to anti-phogrin in a stimulatory solution, and chased in a basal solution for 5 minutes. Immediately after stimulation, phogrin does not seem to move away from retained cores (Figure 2A, left column). However, 5 minutes after stimulation, qualitatively there is more phogrin signal inside the cell. Although initial quantitative analysis is not significant, it is clear that there is a migration at 20 minutes based on qualitative images (Figure 4).

Although regulated exocytosis is a $Ca2^+$ dependent process, cAMP also has a role. Previous work in rat lactotrophs shows that increasing cAMP can cause multigranular exocytosis. EM studies, also in rat lactotrophs, have shown that decreasing cAMP causes complete release of the dense core (Angleson, 1999). To determine if phogrin and IA2 are affected similarly, lactotrophs were stimulated in the presence of both phogrin and IA2 antibodies and with various cAMP modulating drugs. When cAMP is at a maximal level through treatment with IBMX and Forskolin, there is an increase in both phogrin and IA2 exocytic exposure when qualitatively compared to a traditional stimulus (Figure 5A, top two rows of images). However, when cAMP levels are decreased, either with bromocriptine, a dopamine agonist, or Rp-cAMP-s, a potent PKA inhibitor, there is decrease in both phogrin and IA2 signal (Figure 5A, third and fourth row of images). Quantifying the intensity of phogrin (Figure 5B) and IA2 (Figure 5C) signal as a measure of the total protein exposed showed a significant decrease in both transmembrane proteins when cAMP is decreased. This demonstrates that phogrin and IA2 are also modulated in a cAMP dependent manner similar to retained prolactin cores.

To assess the immediate exocytic exposure of phogrin and IA2 at the plasma membrane in lactotrophs, cells were again stimulated in the presences of anti-phogrin and anti-IA2 antibodies for 5 minutes and immediately fixed. Using a TIRF microscope allows for imaging only vesicles that are present on the plasma membrane. TIRF allows for the exclusion of light from excited fluorophores within the cell. IA2 shows a very diffuse signal with only a few puncta present. Phogrin was very punctate and had a more robust signal when compared to IA2. When observing the merged image, three different vesicles are seen: those that contain only phogrin, only IA2, or those that contain both

(Figure 6, white arrows). The different exposure patterns offer evidence that there could be different pools of dense core vesicles within an individual cell based on these transmembrane proteins.

Female Lactotroph Behavior

Prolactin, while it has many roles in both males and females, is names for initiation of lactation in females. Even though males do not lactate, a male rat pituitary is approximately 20% lactotrophs (Yeung, 2006). When female rats are lactating, an increase in estrogen causes a massive proliferation of lactotrophs in the anterior pituitary. This proliferation changes the composition of the pituitary from 20% to be approximately 80% lactotrophs. Previous work has shown that in 72-96 hours after lactating stops, which coincides with a sharp decrease in estrogen levels, the anterior pituitary returns to a more male-like composition (Smith, 1966). Within 24 hours after lactation ends, lactotrophs already decreased from 80% to 48% of the total cell population. Because of this, dissociated anterior pituitary cells from females were cultured in a culture containing estrogen to maintain a healthy and distinctly lactating female population of cells.
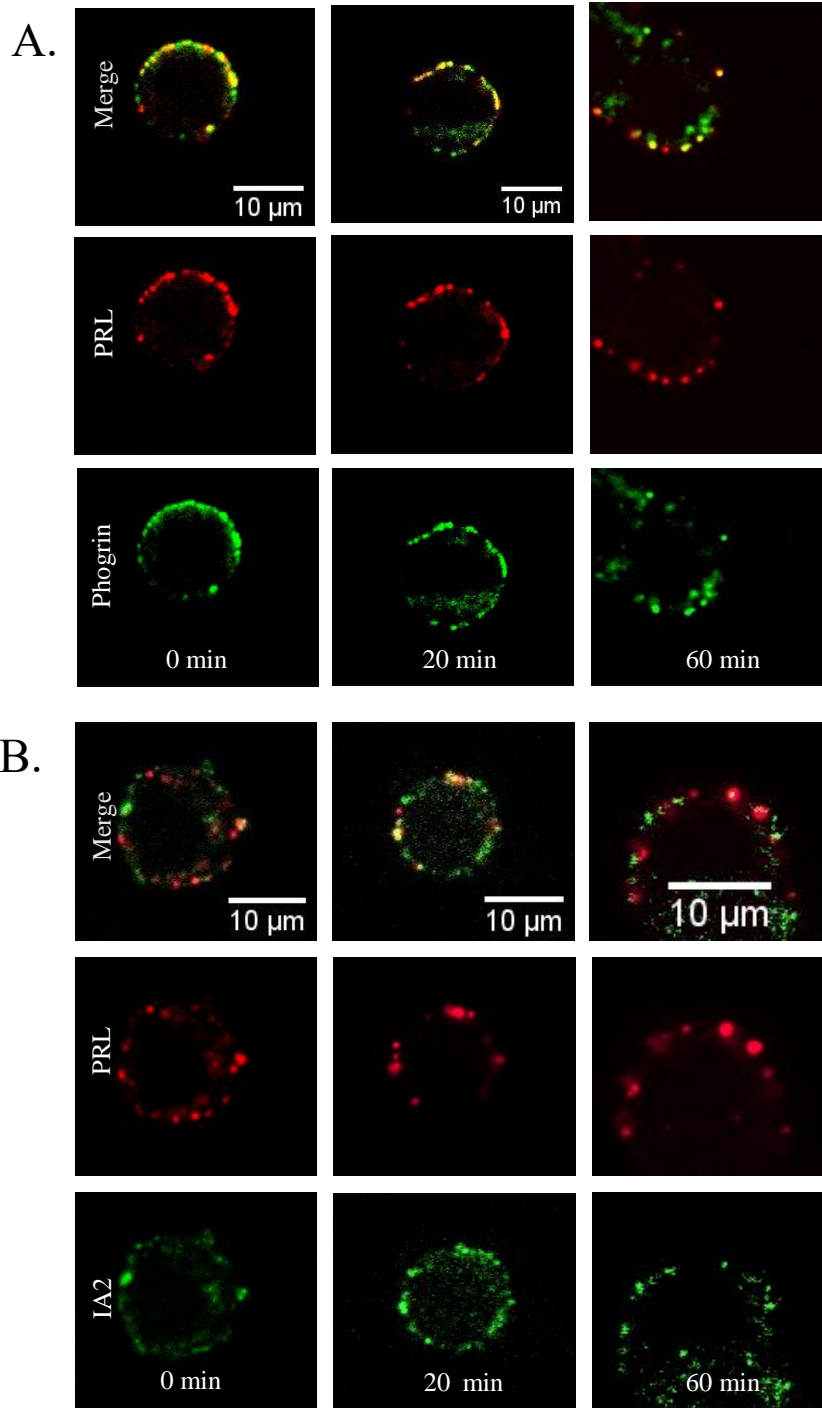
Figure 2 Exocytic exposure of prolactin (PRL) and either phogrin or IA2 in male lactotrophs A. Phogrin and PRL exposure at 0 minutes after stimulation with high K+, or 20 or 60 minutes in basal solution without antibody after stimulation. B. IA2 and PRL exposure 0 minutes after stimulation with high K+, or 20 or 60 minutes in basal solution without antibody after stimulation. Representative equatorial slices shown.
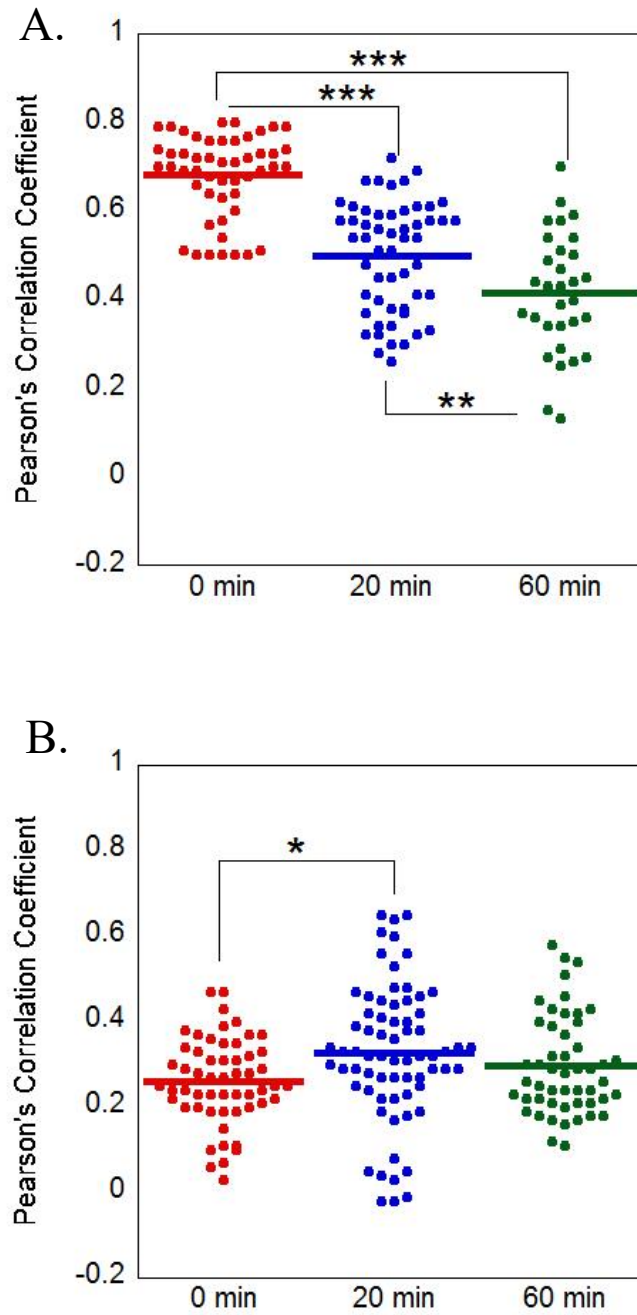
Figure 3 Quantification of equatorial slices of cells shown in Figure 2. Correlation coefficient of transmembrane proteins relative to retained PRL core and different time points A. Phogrin and PRL B. IA2 and PRL. Student's t-test, ***= <0.0001, **= 0.004, *=0.008
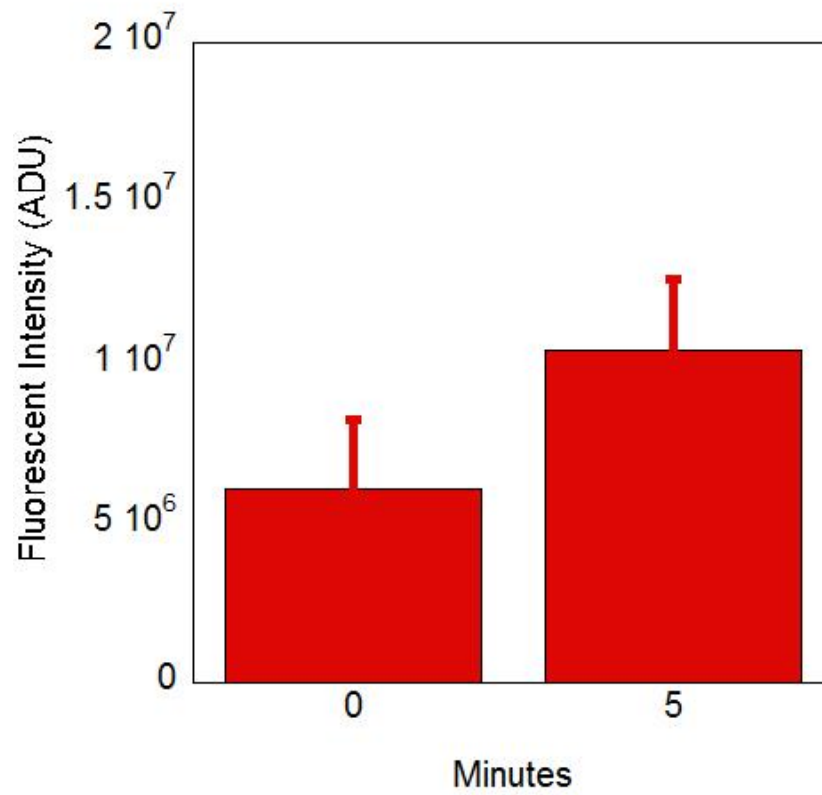
Figure 4 Intensity of phogrin signal representing endocytosed phogrin and 0 minutes of 5 minutes after stimulation with high K+ in the presence of anti-phogrin antibodies.
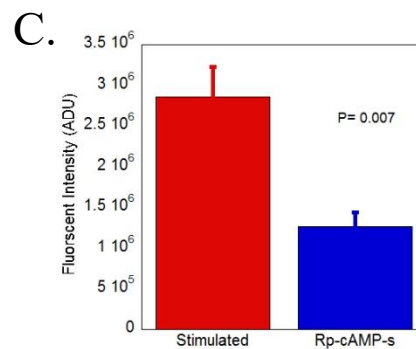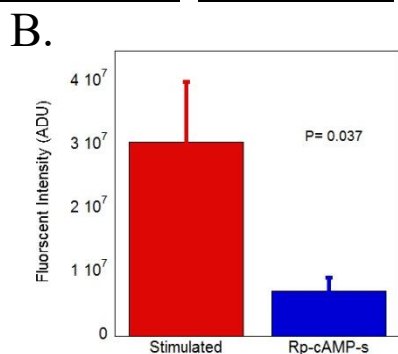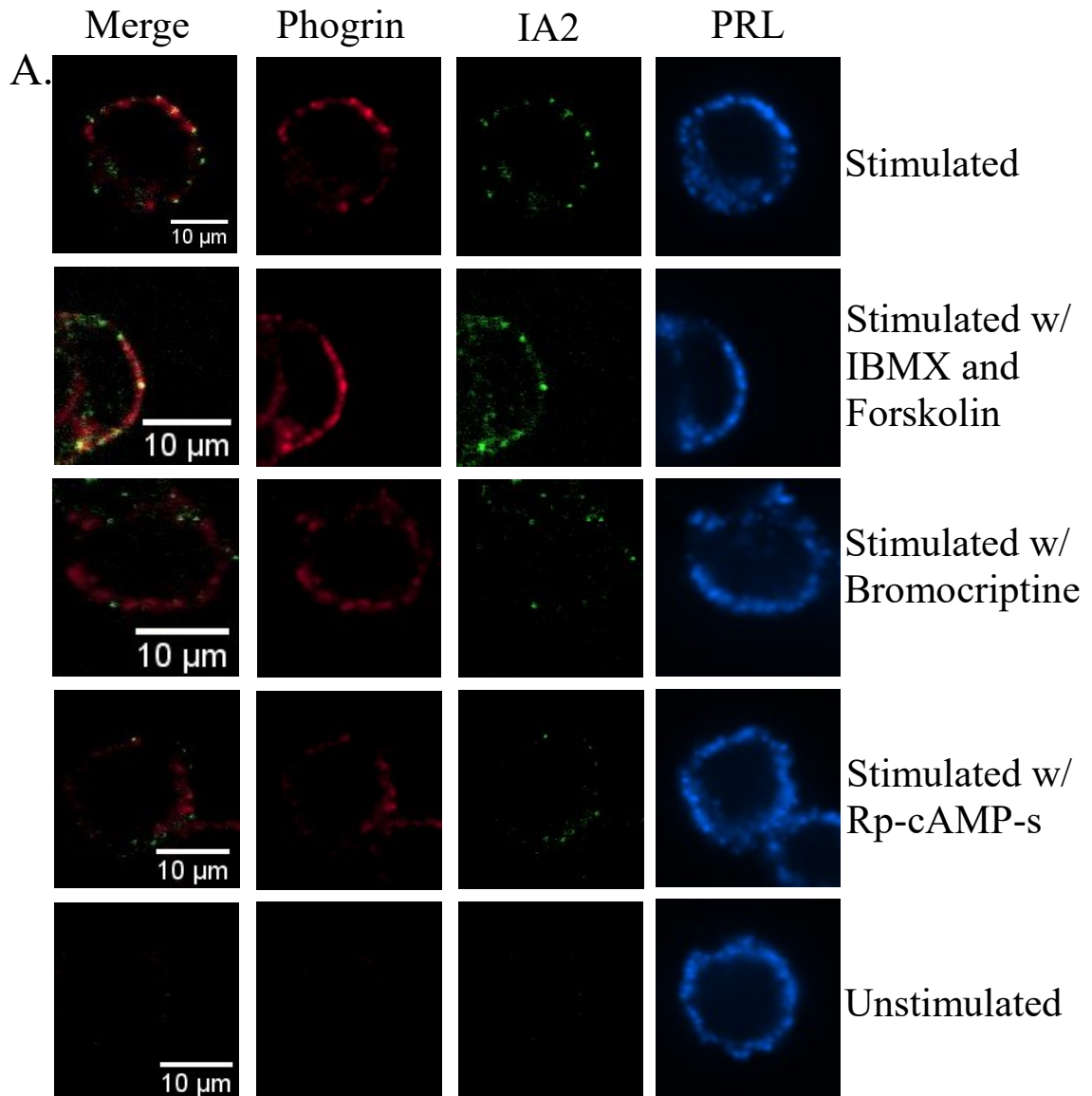
Figure 5 Phogrin Intensity in stimulated lactotrophs with cMAP modulation A. Representative equatorial images of lactotrophs B. Phogrin quantification based on intensity of signal C. IA2 quantification based in signal intensity. P values via Students t-test
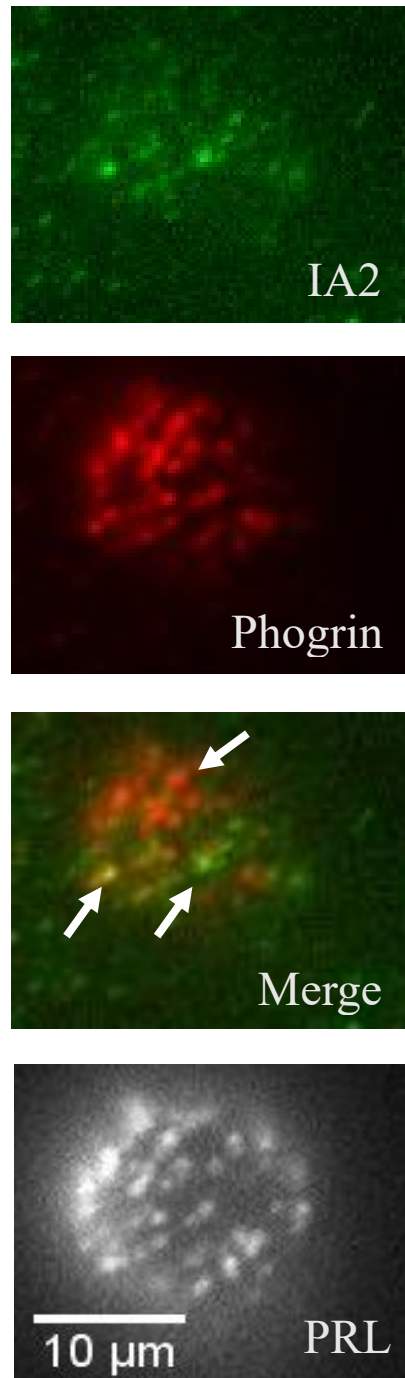
Figure 6 Male lactotrophs were exposed to anti-phogrin and anti-IA2 in a high K+ stimulatory solution and fixed immediately. TIRF images were obtained by looking at events occurring at the coverslip. Arrows indicate the differences in exposure the same lactotroph- some have both, some have one or the other

Synaptotagmins

Many synaptotagmin isoforms have been seen in anterior pituitary including synaptotagmin 1, 4, 7, 9, 10, 11, 12 in both male and female rats. Synaptotagmin 7, a common isoform, has been shown to be enriched in the anterior pituitary in both male and female rats. However, synaptotagmin 1, is expressed at a much lower level in the anterior pituitary of females compared to males (Roper, 2015). Due to this, synaptotagmin 1 and 7 were used as candidates to screen which isoforms were present in primary lactotrophs of male and lactating females. To visualize the different ratios present in male or lactating female lactotrophs, cells were stained for both synaptotagmin isoforms and prolactin (Figure 7). No lactotrophs, regardless of sex, contained only synaptotagmin 1, yet every lactotroph contained synaptotagmin 7, in accordance with previous expression based data. Initial analysis of prolactin vesicles containing synaptotagmin isoforms was not significant. Qualitatively, the prolactin and synaptotagmin 1 staining appeared similar in both sexes. However, synaptotagmin 7, while cytoplasmic in both males and females, it is also retained at the plasma membrane females, which corresponds with previous data in PC12 cells (Sugita, 2001).

57% of male lactotrophs contained both synaptotagmin 1 and 7, compared to 10% of lactating female lactotrophs that contained both isoforms. This result was not initially interesting given that lactotrophs in males and lactating females constitute a distinctly different proportion of the anterior pituitary. However, when compared to the percent of lactotrophs that retain cores, there is a very intriguing similarity: 52% of male lactotrophs and 11% of female lactotrophs retain cores (Table 1). To examine this correlation further,

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female lactotrophs were scored for retaining cores and containing synaptotagmin 1 and 7

or just 7 (Figure 8). When stimulated, 96.3% of synaptotagmin 1 containing female

lactotrophs retained cores, supporting the idea that synaptotagmin 1 containing

lactotrophs could be responsible for the core retention phenomena observed in

lactotrophs.

Lactotrophs in lactating females are responsible for secreting enough prolactin to

sustain the elevated levels required to facilitate lactation. Although some might assume

these lactotrophs are secreting in a constitutive pathway, i.e. not $Ca2^+$ regulated, female

lactotrophs are noisy with random intracellular $Ca2^+$ spikes without extracellular

stimulation. Cells used in these experiments are no different. To assess the physiological

health of the cells, female anterior pituitary cells were cultured for 3 days in media

supplemented with estrogen then loaded with FURA-2AM and placed in a basal solution

to measure resting $Ca2^+$ fluctuations (Figure 9). The first 450 time points of the trace (to

the left of the red dot) show nearly constant $Ca2^+$ spikes; however, when bromocriptine is

added to the solution (the red dot on the x axis), the spikes become much smaller,

indicating smaller oscillations in internal $Ca2^+$. This shows the cells used were healthy

physiologically responsive 72 hours after dissociation when supplemented with estrogen.

Retained Prolactin Cores

Another observation is that retained cores in lactating female lactotrophs were

very rare when compared to the frequency they are seen in male lactotrophs. However,

nearly 85% of lactating female lactotrophs retain cores at basal conditions. To ascertain if

there are any differences between the retained cores, cells were exposed to anti-prolactin

antibody in a stimulatory solution for 5 minutes or a basal solution for 45 minutes. Even though there was no difference in the number of retained cores per cell (Figure 10B), there was a significant difference in the volume of the retained puncta. This is seen both qualitatively and quantitively (Figure10 A, C). Retained cores were approximately 1.5 times larger in basal conditions, and were also seen at the interior of the cell. This could be due to fusion of retained dense cores after endocytosis, which could be in preparation for multigranular exocytosis. Although multigranular exocytosis has not been observed in lactotrophs, the bulk of the studies looked at stimulated lactotrophs. This could coincide with multigranular exocytosis being a common attribute of basal secretion.

Stimulated retained cores in female lactotrophs, although rarer than male, appear similar in qualitive images (compare Figure 2 to Figure 11). Like male lactotrophs female dense core vesicles also contain the transmembrane proteins phogrin and IA2. When stimulated in the presence of anti-prolactin and either anti-phogrin or anti-IA2 antibodies, both transmembrane proteins show distinctly different exocytic exposure patterns relative to the retained cores. Similar to males, phogrin maintained a punctate appearance while IA2 was very diffuse with few puncta observed. These results supports the idea that there is a sub-population of lactating female lactotrophs that retain a more male-like response to stimulus, however, it is a much lower proportion of lactotrophs in the anterior pituitary.
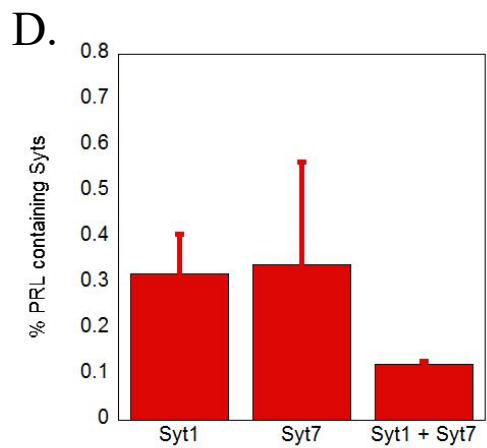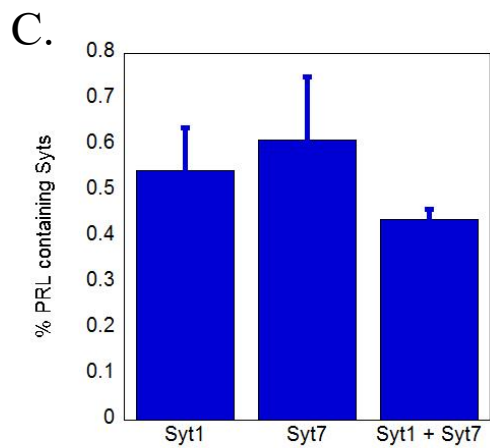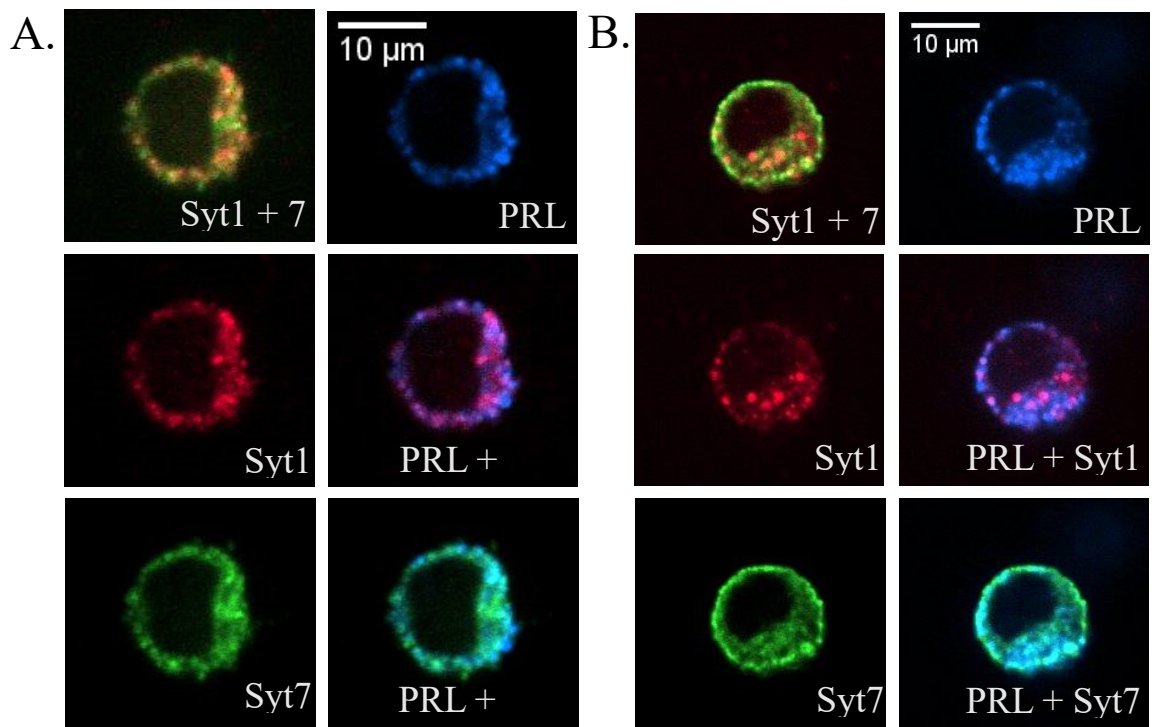
Figure 7 Synaptotagmin 1 (Syt1) and Synaptotagmin 7 (Syt7) staining in lactotrophs A. Representative equatorial images of male lactotrophs containing both isoforms. B. Representative equatorial images of female lactotrophs that contain both isoforms. C. % of prolactin that contains either isoform in males (C) and females (D).

Sex	Only Syt1	Only Syt7	Syt1 + Syt7	Strong Stimulated Core Retention	Basal Core Retention
Male	0.00%	42.86%	57.14%	51.90%	ND
Female	0.00%	89.47%	10.53%	11.38%	84.38%

Table 1 Proportion of rat lactotrophs that contain different synaptotagmin isoforms and retain cores. Data from multiple experiments.

A.

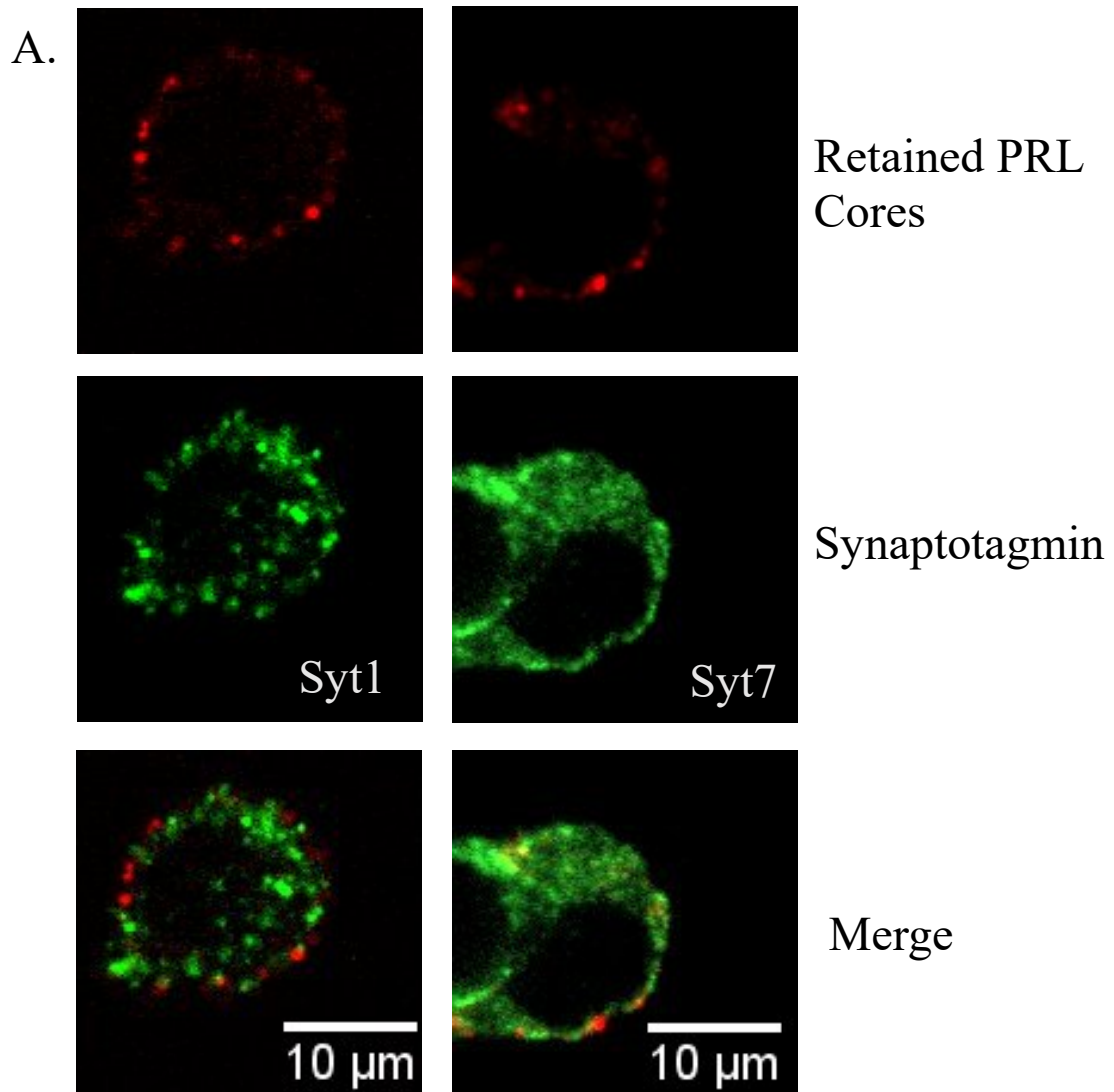**Figure 8** Representative equatorial images of female lactotrophs stimulated with high K+ in the presence of anti-prolactin and immediately fixed and stained for different synaptotagmin isoforms.
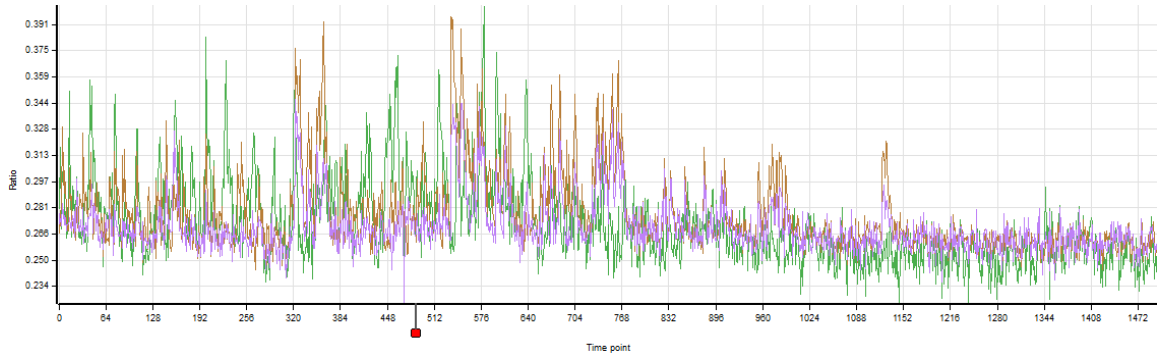
Figure 9 FURA-2, AM trace of female rat lactotrophs showing spontaneous Ca2+ spikes in resting solution. Red dot represents the addition of bromocriptine to the external solution, showing cells are physiologically responsive (n=3).
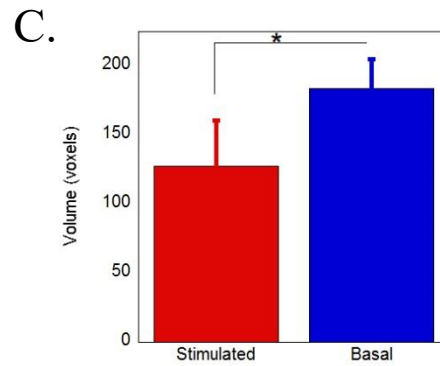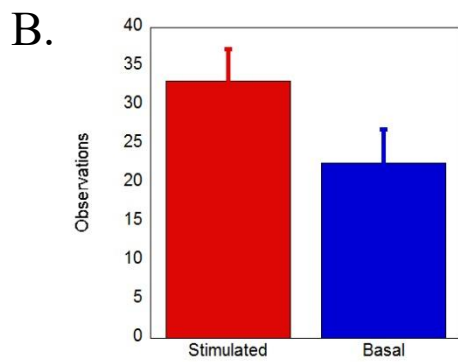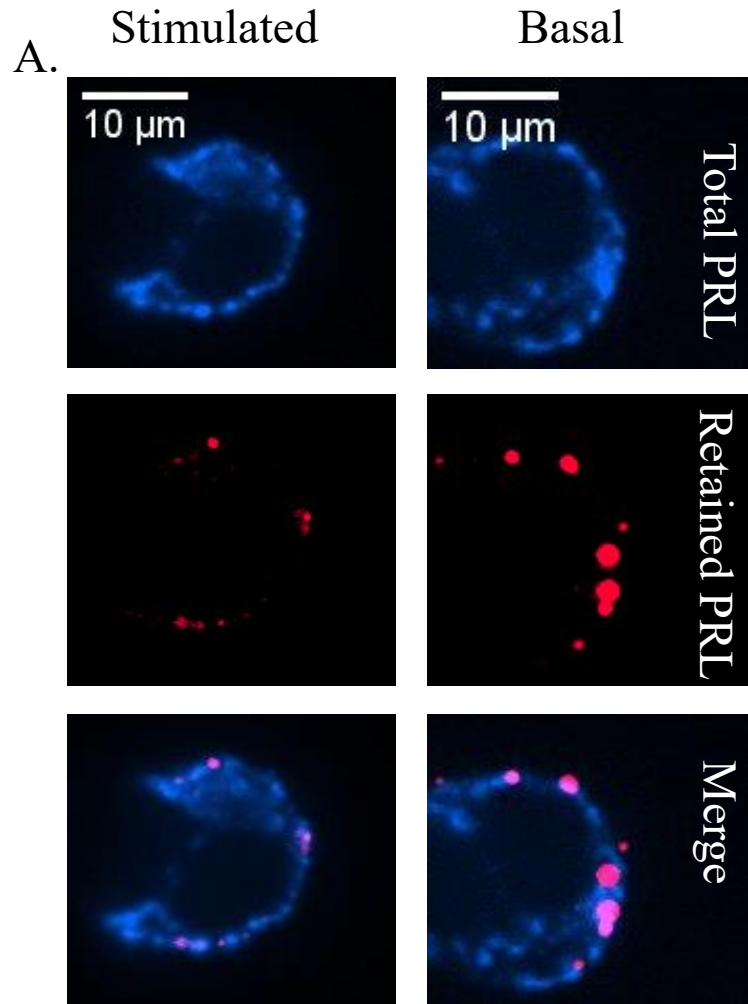
Figure 10 Retained prolactin cores in lactating female rats. A. Representative equatorial images of cells either stimulated with anti-prolactin in high K+ for 5 minutes of in a basal solution for 45 minutes. B. Average number of retained cores per cell. C. Average volume of retained cores. Students t-test, *= <0.001
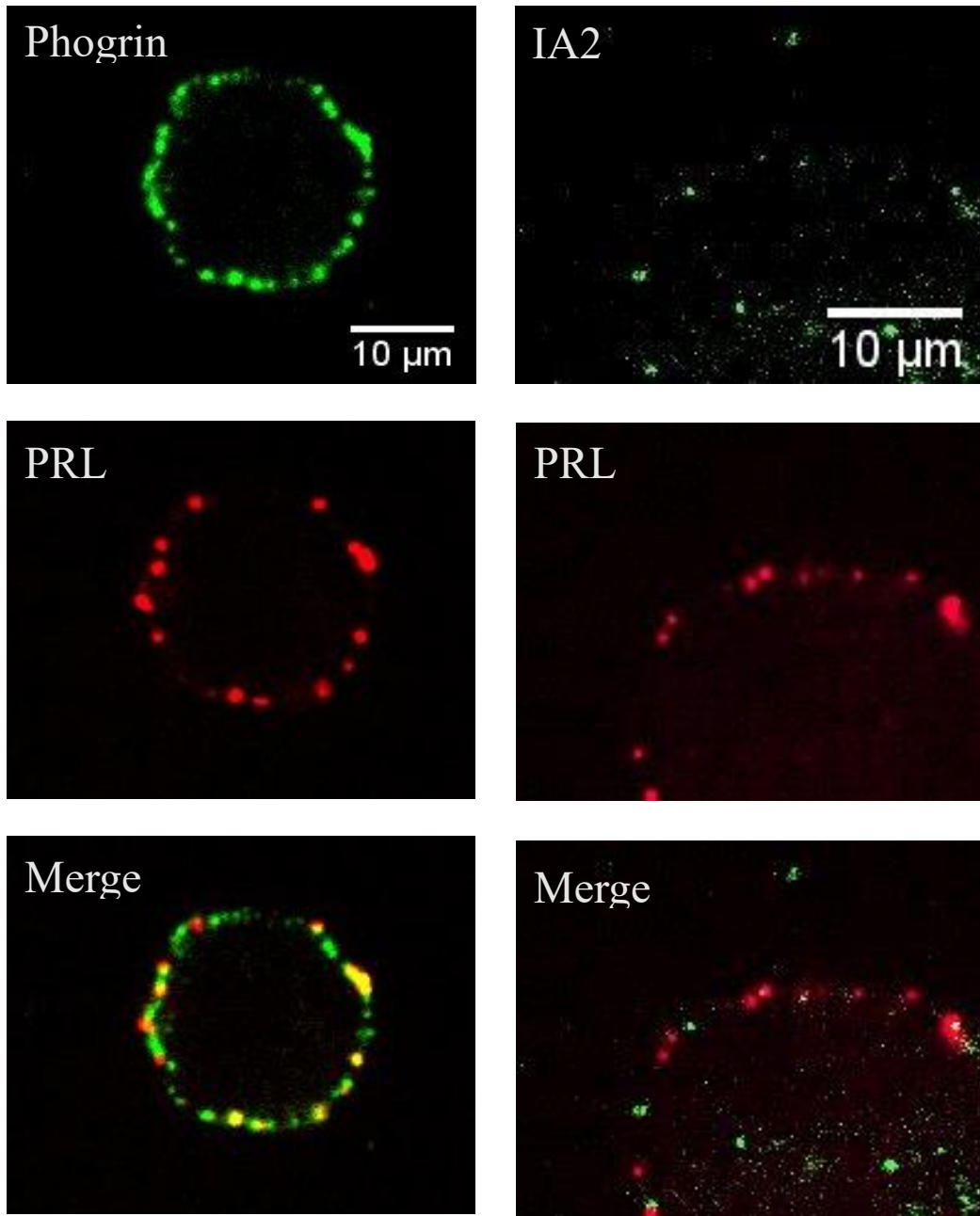
Figure 11 Representative equatorial images of lactating female lactotrophs stimulated in high K+ for 5 minutes in the presence of anti-prolactin and either anti-phogrin or anti-IA2

Discussion

Multiple studies across different cells have provided evidence that heterogeneity exists within single cell type. While this has been accepted, there has not been any considerable evidence to suggest heterogeneity of vesicles within a single cell. By studying dense core vesicle transmembrane and associated proteins, the previous experiments show that difference exist within a lactotroph. Primary tissue was used for all experiments as endogenous levels of proteins could be assayed, providing a more physiologically relevant model without having to rely on overexpression. As shown, cells used were physiological relevant after dissociation. Most experiments involved antibody uptake which has the caveat that the bivalent nature of antibodies could force aggregation and give the false impression of retained protein. However, in these experiments, only high affinity and well characterized antibodies were used mitigating the possibility any retained signal could be an artifact. The change in exposure when physiologically altered also validates the phogrin and IA2 antibodies used. All stimulation on cells was based on an increase in external K+, which although not physiologically relevant, does induce maximal prolactin secretion. Physiological stimuli, such as VIP or TRH, only stimulate a small proportion of lactotrophs, therefore high K^+ was used to stimulate as many lactotrophs as possible.

When qualitatively examining phogrin of IA2 uptake with prolactin immediately after stimulation, the transmembrane proteins appear different. The punctate appearance of phogrin could suggest that it remains in the vesicle with the retained core. Conversely, IA2 showed a diffuse exocytic exposure suggesting IA2 does not remain in the vesicle. This pattern is seen in male and lactating female lactotrophs. As the retained cores traffic into the cell, it is obvious that there are two pools of these transmembrane proteins: one that traffics with the core and one that does not. Of the pool that traffics with the retained hormone core, phogrin has a significant colocalization coefficient while IA2's is nearly a random association. Although this difference could be due to the unknown function of theses proteins, they are both found exclusively in dense core vesicles making this difference unexpected. However, when compared to previous data it seems some phogrin could be trafficking with the retained cores to a syntaxin 6 compartment (Bauer, 2004B). This shows that although both phogrin and IA2 are dense core specific transmembrane proteins, they might be present in different pools of dense core vesicles. Interestingly, not all phogrin seems to traffic with the retained prolactin core, which is stable at the plasma membrane for at least 20 minutes after stimulation. This suggests that some phogrin might be recycled back into the cell to be degraded as it is not needed after exocytosis, or transported to immature dense core vesicles within the cell, similar to its role in beta cells (Vo, 2004).

It is clear that phogrin and IA2 traffic differently with respect to retained cores in lactotrophs. To further examine their differences, trafficking should be examined in the same cell at different time points to see if they colocalize or to what compartment they traffic to- i.e. immature vesicles, the TGN, mature vesicles or lysosomes. These

experiments would be straight forward to complete and could offer more evidence to the idea that phogrin and IA2 act as a marker for different pools of vesicles within the same cell. Also, it would be interesting to repeat the trafficking experiments in other anterior pituitary cells, such as somatotrophs which constitute approximately 50% of anterior pituitary and are responsible for secreting growth hormone. As the other three cell types in the anterior pituitary (corticotrophs, thryrotophs, and gonadotrophs) are all relatively rare it would be difficult to perform extensive experiments in primary tissue. Additionally, using TIRF to examine the exposure of phogrin and IA2 in real-time would also clarify what phogrin and IA2 are doing after vesicles fuse with the plasma membrane. This could confirm the hypothesis that phogrin remains in the vesicles while IA2 mostly diffuses away from the exocytotic site.

Although phogrin and IA2 are not required for exocytosis, as previously assayed in beta cells, the proteins exocytic exposure can be mediated by altering cAMP within the cell. This result is further confirmation of the assay itself while also confirming the presense of IA2 and phogrin within dense core vesicles. Previous studies have shown that maximal cAMP, by treatment with IBMX and forskolin, potentiated sequential exocytic events in lactotrophs (Angleson, 1999). Yet, when this treatment was repeated in this experiment, there was no significant increase in the amount of phogrin exposure. This could possibly be due the inability of the antibody to penetrate the string of vesicles attached to the plasma membrane, or perhaps this shows that vesicles required for vesicle-to-vesicle fusion do not contain phogrin. Although initial quantification of this experiment did not yield significant results with bromocriptine treatment, Rp-cAMP-s, a more potent inhibitor of cAMP production, did show a significant decrease in phogrin

signal. This result is in accordance with what would have been expected as cAMP reduction has been shown to abolish core retention and facilitate complete release of the dense core. These experiments could be expanded upon by using MMQ cells, a rat lactotroph derived cell line, with a pH sensitive tagged phogrin or IA2construct. This could elucidate sequential or multigranular exocytosis as assayed by a change in signal intensity.

Synaptotagmins also offer another level of heterogeneity within lactotrophs. Their function with the SNARE complex is essential for regulated exocytosis, and the multiple isoforms allow for a more refined regulation of hormone release. As prolactin is basally secreted in both male and females, it is logical to assume these dense core vesicles would contain a high affinity $Ca2^+$ sensor, one that would sense a small change in intracellular $Ca2^+$. Synaptotagmins, which are Ca2+ sensors, have been extensively studied in neurons and endocrine cells. Some specific functions have been teased out- synaptotagmin 9 have a sex-specific role in gonadotrophs and synaptotagmin 1 and 7 have distinct $Ca2^+$ dependent roles in chromaffin cells (Roper, 2015; Rao, 2014). These observations seem to translate to lactotrophs in male and lactating female rats. Lactating female rats require elevated levels, significantly higher than basal levels, of prolactin to facilitate lactation (Freeman, 2000). It is logical to assume that lactotrophs in these animals are constantly releasing prolactin via a regulated pathway, yet can be stimulated beyond to secrete more prolactin if required. This coincides with the observation that a small percentage of female lactotrophs contain synaptotagmin 1- the low affinity $Ca2^+$ sensor that responds to strong stimuli- and that most contain only synaptotagmin 7 which is responsible for basal

release allowing for cells to be stimulated to secrete more prolactin above it's basally released levels.

Since these lactotrophs have constant $Ca2^+$ oscillations, it is logical to assume that the cells containing only synaptotagmin 7 are responsible for the basal level of prolactin secretion. To solidify this hypothesis, the same experiments should be repeated to increase the number of cells analyzed. To ascertain if synaptotagmin 1 is responsible for the retained core phenomena in lactotrophs, primary cells could be transduced with adenovirus containing synaptotagmin 1. If all cells transduced retain cores, that would offer compelling evidence that in lactotrophs, synaptotagmin 1 is the responsible for prolactin core retention.

Basal secretion, as measured by prolactin core retention in a non-stimulatory solution, occurs in over 80% of lactating female lactotrophs, and the retained vesicles are significantly larger than the retained cores present after stimulation. This could be due to dense core vesicle fusion after initial exocytosis and endocytosis of retained cores. If multigranular fusion occurs in lactotrophs, this result would coincide with previous results showing retained cored are preferentially used upon subsequent stimulation (Bauer, 2004B). Lactotrophs in a basal solution could be more likely to undergo multigranular exocytosis as a more energetically favorable route of prolactin secretion. It would be interesting to repeat these experiments in male rats to see if the increase in volume of cores is an attribute of all basally secreted prolactin regardless of sex. Traditionally basal prolactin secretion in males has not been of much interest, as it is assumed its role in males is as a growth hormone. However, hyperprolactinemia in males

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can cause erectile dysfunction, infertility and reduced testosterone (Segal, 1976). More recently, hypoprolactinemia has also been shown to be associated with erectile dysfunction, infertility, and reduced function of seminal vesicles in males (Corona, 2014). Perhaps understanding how male lactotrophs basally secrete prolactin could offer insight into the mechanism which could lead to further studies about the function of prolactin in males.

This thesis offers evidence that dense core vesicles are not necessarily a homogenous population. There is evidence that phogrin and IA2 exist in two different populations, those that traffic with the retained core and those that do not. Additionally, different synaptotagmin isoforms have been shown to have different Ca2+ affinities, allowing for different modes of operation in various cell types. By examining different synaptotagmins in lactotrophs, there is a surprising distribution pattern within each sex and between sexes when related to core retention. Based on the exocytic exposure of phogrin and IA2, it is plausible that within a single cell there are different populations of vesicles and based on the presence of different synaptotagmin isoforms it is also plausible that there are differences within a single cell type. This complexity offers cells a highly regulated and tunable process for dense core vesicle secretion showing multifaceted heterogeneity among dense core vesicles in lactotrophs.

References

Angleson, J. K., Cochilla, A. J., Kilic, G., Nussinovitch, I., & Betz, W. J. (1999). Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events. *Nature neuroscience*, *2*(5).

Bäck, N., Soinila, S., & Virtanen, I. (1993). Endocytotic pathways in the melanotroph of the rat pituitary. *The Histochemical journal*, *25*(2), 133-139.

Bauer, R. A., Khera, R. S., Lieber, J. L., & Angleson, J. K. (2004). Recycling of intact dense core vesicles in neurites of NGF-treated PC12 cells. *FEBS letters*, *571*(1-3), 107-111.

Bauer, R. A., Overlease, R. L., Lieber, J. L., & Angleson, J. K. (2004). Retention and stimulus-dependent recycling of dense core vesicle content in neuroendocrine cells. *Journal of cell science*, *117*(11), 2193-2202.

Chapman, E. R., & Davis, A. F. (1998). Direct interaction of a Ca2+-binding loop of synaptotagmin with lipid bilayers. *Journal of Biological Chemistry*, *273*(22), 13995-14001.

Chieregatti, E., Chicka, M. C., Chapman, E. R., & Baldini, G. (2004). SNAP-23 functions in docking/fusion of granules at low Ca2+. *Molecular biology of the cell*, *15*(4), 1918-1930.

Christian, H. C., Chapman, L. P., & Morris, J. F. (2007). Thyrotrophin-Releasing Hormone, Vasoactive Intestinal Peptide, Prolactin-Releasing Peptide and Dopamine Regulation of Prolactin Secretion By Different Lactotroph Morphological Subtypes in the Rat. *Journal of neuroendocrinology*, *19*(8), 605-613.

Cool, D. R., Normant, E., Shen, F. S., Chen, H. C., Pannell, L., Zhang, Y., & Loh, Y. P. (1997). Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpefat mice. *Cell*, *88*(1), 73-83.

Corona, G., Wu, F. C., Rastrelli, G., Lee, D. M., Forti, G., O'connor, D. B., ... & Casanueva, F. F. (2014). Low prolactin is associated with sexual dysfunction and psychological or metabolic disturbances in middle-aged and elderly men: The European Male Aging Study (EMAS). *The journal of sexual medicine*, *11*(1), 240-253.

Farquhar, M. G., Reid, J. J., & Daniell, L. W. (1978). Intracellular transport and packaging of prolactin: a quantitative electron microscope autoradiographic study of mammotrophs dissociated from rat pituitaries. *Endocrinology*, *102*(1), 296-311.

Freeman, M. E., Kanyicska, B., Lerant, A., & Nagy, G. (2000). Prolactin: structure, function, and regulation of secretion. *Physiological reviews*, *80*(4), 1523-1631.

Geppert, M., Goda, Y., Hammer, R. E., Li, C., Rosahl, T. W., Stevens, C. F., & Südhof, T. C. (1994). Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. *Cell*, *79*(4), 717-727.

Grabner, C. P., Price, S. D., Lysakowski, A., & Fox, A. P. (2005). Mouse chromaffin cells have two populations of dense core vesicles. *Journal of neurophysiology*, *94*(3), 2093-2104

Jackman, S. L., Turecek, J., Belinsky, J. E., & Regehr, W. G. (2016). The calcium sensor synaptotagmin 7 is required for synaptic facilitation. *Nature*, *529*(7584), 88.

Kasai, H., Takahashi, N., & Tokumaru, H. (2012). Distinct initial SNARE configurations underlying the diversity of exocytosis. *Physiological Reviews*, *92*(4), 1915-1964.

Kubosaki, A., Gross, S., Miura, J., Saeki, K., Zhu, M., Nakamura, S., ... & Notkins, A. L. (2004). Targeted disruption of the IA-2β gene causes glucose intolerance and impairs insulin secretion but does not prevent the development of diabetes in NOD mice. *Diabetes*, *53*(7), 1684-1691.

Kubosaki, A., Nakamura, S., Clark, A., Morris, J. F., & Notkins, A. L. (2006). Disruption of the transmembrane dense core vesicle proteins IA-2 and IA-2β causes female infertility. *Endocrinology*, *147*(2), 811-815.

Kuliawat, R., & Arvan, P. (1992). Protein targeting via the" constitutive-like" secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. *The Journal of Cell Biology*, *118*(3), 521-529.

Kunii, M., Ohara-Imaizumi, M., Takahashi, N., Kobayashi, M., Kawakami, R., Kondoh, Y., ... & Aoyagi, K. (2016). Opposing roles for SNAP23 in secretion in exocrine and endocrine pancreatic cells. *J Cell Biol*, jcb-201604030.

Rabin, D. U., Pleasic, S. M., Shapiro, J. A., Yoo-Warren, H., Oles, J., Hicks, J. M., ... & Rae, P. M. (1994). Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. *The Journal of Immunology*, *152*(6), 3183-3188.

Rao, T. C., Passmore, D. R., Peleman, A. R., Das, M., Chapman, E. R., & Anantharam, A. (2014). Distinct fusion properties of synaptotagmin-1 and synaptotagmin-7 bearing dense core granules. *Molecular biology of the cell*, *25*(16), 2416-2427.

Roper, L. K., Briguglio, J. S., Evans, C. S., Jackson, M. B., & Chapman, E. R. (2015). Sex-specific regulation of follicle-stimulating hormone secretion by synaptotagmin 9. *Nature communications*, *6*.

Segal, S., Polishuk, W. Z., & Ben-David, M. (1976). Hyperprolactinemic male infertility. *Fertility and sterility*, *27*(12), 1425-1427.

Sørensen, J. B. (2004). Formation, stabilisation and fusion of the readily releasable pool of secretory vesicles. *Pflügers Archiv*, *448*(4), 347-362.

Smith, R. E., & Farquhar, M. G. (1966). Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. *The Journal of cell biology*, *31*(2), 319-347.

Südhof, T. C. (2002). Synaptotagmins: why so many?. *Journal of Biological Chemistry*, *277*(10), 7629-7632.

Südhof, T. C. (2013). A molecular machine for neurotransmitter release: synaptotagmin and beyond. *Nature medicine*, *19*(10), 1227-1231.

Suh, Y. H., Yoshimoto-Furusawa, A., Weih, K. A., Tessarollo, L., Roche, K. W., Mackem, S., & Roche, P. A. (2011). Deletion of SNAP-23 results in pre-implantation embryonic lethality in mice. *PLoS One*, *6*(3), e18444.

Sugita, S., Han, W., Butz, S., Liu, X., Fernández-Chacón, R., Lao, Y., & Südhof, T. C. (2001). Synaptotagmin VII as a plasma membrane Ca 2+ sensor in exocytosis. *Neuron*, *30*(2), 459-473.

Takahashi, N., Hatakeyama, H., Okado, H., Miwa, A., Kishimoto, T., Kojima, T., ... & Kasai, H. (2004). Sequential exocytosis of insulin granules is associated with redistribution of SNAP25. *The Journal of cell biology*, *165*(2), 255-262.

Vardjan, N., Jorgačevski, J., Stenovec, M., Kreft, M., & Zorec, R. (2009). Compound exocytosis in pituitary cells. *Annals of the New York Academy of Sciences*, *1152*(1), 63-75.

Vo, Y. P., Hutton, J. C., & Angleson, J. K. (2004). Recycling of the dense-core vesicle membrane protein phogrin in Min6 β-cells. *Biochemical and biophysical research communications*, *324*(3), 1004-1010.

Washbourne, P., Thompson, P. M., Carta, M., Costa, E. T., Mathews, J. R., Lopez-Benditó, G., ... & Wilson, M. C. (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nature neuroscience*, *5*(1).

Wasmeier, C., Burgos, P. V., Trudeau, T., Davidson, H. W., & Hutton, J. C. (2005). An Extended Tyrosine-Targeting Motif for Endocytosis and Recycling of the Dense-Core Vesicle Membrane Protein Phogrin. *Traffic*, *6*(6), 474-487.

Yeung, C. M., Chan, C. B., Leung, P. S., & Cheng, C. H. (2006). Cells of the anterior pituitary. *The international journal of biochemistry & cell biology*, *38*(9), 1441.