Role of SNARE Proteins in Natriuretic Peptide Secretion by the Heart

Nikki Joanne Bulanadi Natividad

A Thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

Graduate Program in Biology

York University

Toronto, Ontario

August 2014

Abstract

The hormones atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are stored and secreted by cardiac myocytes. Recent studies have identified soluble *N*-ethyl-maleimide-sensitive-fusion attachment protein receptors (SNAREs) in the trafficking, docking and fusion of ANP and BNP secretory vesicles. In this study, I characterized the expression profiles of the three SNARE proteins (syntaxin 5A, syntaxin 18, and SNAP29), implicated in constitutive exocytosis, in atrial and ventricular cardiac myocytes. My results suggest that syntaxin 5A, syntaxin 18 and SNAP29 are not important in the constitutive secretion of ANP and may play a role in another protein trafficking pathway. Syntaxin 1A and SNAP25, two SNARE proteins previously characterized in atrial cardiac myocytes, form a complex in adults that has been implicated in the exocytosis of ANP. I investigated the protein expression and promoter activity of these two SNARE proteins in neonatal and adult atrial and ventricular cardiac myocytes. The functional role of syntaxin 1A and SNAP25 was assessed using botulinum neurotoxin C (BoNT/C) and BoNT/A which cleave these SNARE proteins, respectively. Treatment of cardiac myocytes with BoNT/A and BoNT/C suggest that syntaxin 1A and SNAP25 regulate ANP secretion in neonatal cardiac myocytes, albeit low levels. Lastly, I examined the influence of forskolin and phorbol myristate acetate (PMA) on the syntaxin 1A and SNAP25 promoter. The lack of effect of these agents on gene reporter activity suggests that the CRE element in the syntaxin 1A and SNAP25 promoter do not significantly affect transcriptional activity. Overall, my studies demonstrate the developmental changes in SNARE protein expression in the heart, and their potential role in ANP secretion.

Acknowledgements

First and foremost, I would like to thank my mom (Aurea), dad (Ariel) and brother (Jeffrey) for their love and support throughout my entire Master's experience. I would not be the person I am today without them – they are my rock. I would also like to thank Isaiah James Copon, my second pillar of support, for always believing in me and pushing me in the right direction. The late nights and words of encouragement (especially while writing this thesis) will never be forgotten. In addition, I would like to thank the members of the Tsushima lab – Ash Bouramandi (grasshopper), Aryan Abadeh, and Amanda Mohabeer - for the great memories and never-ending support when it came to lab meetings, conferences, and even personal crises. I'd especially like to thank Xiaodong Gao, our lab technician for teaching me protocol after protocol, analyzing data with me, and supporting me and my fellow lab members till no end. I could never be thankful enough for everything she has done. I'd like to personally thank Joe Bacani and Piru Perampalan for keeping me sane the first year of graduate school with coffee breaks and endless conversation. I will never forget the memories we shared. I'd like to thank Andy, who started out with me, for his patience and kindness. I wish him the best of luck in the future. I'd also like to thank Sasan, Aaron and Yaakov for helping replicate experiments and enduring the gruelling process of learning Western Blotting by my side. Michal Sheinis, the newest student in our lab, made a huge impact on my research and personal life. Words will never describe how supportive she has been in terms of helping edit my thesis and providing me with words of wisdom and advice. A special thank you also goes to Gabby Krivdova for her words of positive encouragement while writing this thesis. In addition, I'd like to thank Dr. John McDermott for his knowledgeable input and suggestions towards the growth of my thesis project, as well as Dr. David Hood and Dr. Michael Scheid for allowing me to use their equipment. Lastly, I would like to extend my heartfelt gratitude to my supervisor, mentor, and friend, Dr. Robert Tsushima for seeing my potential; believing in my abilities, supporting my strengths, and helping me improve on my weaknesses. Every student who enters his lab is privileged not only for the research experience they receive, but also for the guidance they are rewarded with, having him as a supervisor. His selflessness goes without saying. A good supervisor helps their students to graduate; a great supervisor helps their students to succeed. He has always looked out for the future of his students, and for that, I will forever be grateful.

Table of Contents

List of Figures

List of Abbreviations

ANGII	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
β-MyHC	β-myosin heavy chain
BNP	Brain natriuretic peptide
BoNT	Botulinum neurotoxin
CaMK	Calcium/Calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CBFHH	Calcium and bicarbonate-free HEPES-based Hanks buffer
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CREB	cAMP-response element binding protein
DMEM	Dulbecco's Modified Eagle Medium
DNP	Dendroaspis natriuretic peptide
ECL	Enhanced chemiluminescence substrate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FBS	Fetal Bovine Serum
FOR	Forskolin
GFP	Green fluorescent protein
GLuc	Gaussia luciferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ITS	Insulin-transferrin-selenium
K_{ATP}	ATP-sensitive potassium channel
K_V	Voltage-gated potassium channel
KRB	Krebs Ringer bicarbonate
mRNA	Messenger ribonucleic acid
NPR	Natriuretic peptide receptor
NEP	Neutral endopeptidase
NPP	Natriuretic peptide pre-cursor
NSF	N-ethyl-maleimide Sensitive Fusion protein
P/S	Penicillin/Streptomycin

PBS	Phosphate buffer saline
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PVDF	Poly-vinylidenedifluoride-plus
RNAPII	RNA polymerase II
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SA-HRP	Streptavidin-horseradish peroxidase
SeAP	Secretory alkaline phosphatase
SEC	Secretory protein
SEM	Standard error of the mean
siRNA	Silencing ribonucleic acid
SM	Sec1/Munc18-like protein receptor
SNAP	Soluble N-ethyl-maleimide-sensitive fusion protein Attachment Protein
SNARE	Soluble N-ethyl-maleimide sensitive fusion protein Attachment protein Receptor
STX	Syntaxin
SUR2A	Sulfonylurea receptor 2A
TBP	TATA-box binding protein
TBST	Tris-buffered saline and Tween 20 mixed buffer
TEMED	Tetra-methyl-ethylenediamine
TeNT	Tetanus neurotoxins
TGF-β	Transforming growth factor-β
TIRF	Total internal reflection fluorescence
TNF-α	Tumor necrosis factor-α
VAMP	Vesicle associated membrane protein

Introduction

1.1 Natriuretic Peptides

1.1.1 Background - Natriuretic Peptides

The observation of granules within atrial cells in the early 1960s laid foundation for the discovery of the heart's endocrine properties (Jamieson *et al.* 1964). Follow-up studies done by de Bold and colleagues (1981) found that these granules were important in salt and water homeostasis. By injecting extracts from the rat atria into the rodent's bloodstream, de Bold and colleagues (1981) were able to stimulate salt excretion. These findings suggested the presence of a peptide important in regulating body fluid and electrolyte balance within the mammalian heart - a function that would have formerly been attributed to the kidney. In 1983, the purified 28-amino acid peptide was sequenced by Flynn and colleagues and pegged the "atrial natriuretic factor". In later years, the protein was re-named "atrial natriuretic peptide", or ANP.

Following the discovery of ANP, two structurally similar peptides, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) were discovered (Sudoh *et al.* 1988, Sudoh *et al.* 1990). Brain natriuretic peptide, originally discovered in the porcine brain, is a 32-amino-acid peptide. It possesses a similar function to ANP in holding both natriuretic and diuretic properties (Mukoyama *et al.* 1991), natriuresis being the excretion of salt and diuresis being the excretion of water. Both ANP and BNP are stored and secreted from granules within the main contractile cells of the heart, cardiac myocytes (Rushkoaho 2003). CNP, on the other hand, holds no natriuretic properties. It possesses two active forms: one which is 22-amino acids in length and the second which is 53-amino acids in length. Its 22-amino acid form is most prevalent in the central nervous system (Levin *et al.* 1998), though CNP is also present in the vascular endothelium, functioning mainly to induce vasodilation and vascular growth (Suga *et al.* 1992).

The common feature among all natriuretic peptides is a cysteine bridge connecting the peptides' 17-amino acid ring (D'Souza *et al.* 2004). Although this segment is conserved amongst the family, the peptide sequence of each hormone varies across species. CNP is found to be the most conserved throughout evolutionary history. ANP is the most homologous across mammals and BNP is the most

variable (Clerico *et al.* 2006) among the three. In addition, two other peptides have also been identified as part of the natriuretic peptide family: DNP - a structurally similar peptide with vasodilative properties, produced by the venom of the green mamba snake (Schweitz *et al.* 1992), and urodilatin, a peptide produced by the kidney through alternative processing of pro-ANP (Schulz-Knappe *et al.*1998). The functional relevance of these peptides during cardiac stress has not been thoroughly investigated.

1.1.2 Function of Natriuretic Peptides

Natriuretic peptides affect a wide variety of organs including the central nervous system, kidney, peripheral vasculature, and adrenal glands. The main receptors at these target organs include: Natriuretic Peptide Receptor – A (NPR-A), NPR-B, and NPR-C.

NPR-A and NPR-B are guanylyl cyclase receptors which convert guanosine triphosphate to the secondary messenger cyclic GMP (cGMP). The increased production of cGMP results in a signalling cascade targeting cGMP-dependent protein kinases (PKGs), cGMP-gated ion channels and cGMP-regulated cyclic nucleotide phosphodiesterases (McGrath *et al.* 2005), which work together to induce the main effects of natriuretic peptides in their target organs. NPR-A is found to mediate most natriuretic effects of both ANP and BNP, with a higher affinity to the former. NPR-B has highest affinity to CNP, followed by ANP and BNP. Both receptors are distributed at the major target organs - the kidney and adrenal glands, with NPR-A receptors focused in the main blood vessels and NPR-B receptors focused in the central nervous system (McGrath *et al.* 2005; Clerico *et al.* 2006).

NPR-C is known as the clearance receptor. In contrast to NPR-A and NPR-B, it contains a fragment in its cytoplasmic portion characteristic of other clearance receptors (McGrath *et al.* 2005). Upon binding, NPR-C is internalized with its natriuretic peptide agonist via endocytosis. The peptide is then degraded in lysosomes and the receptor recycled back to the membrane. This receptor, in synchrony with the protease neutral endopeptidase (NEP), is responsible for the clearance of ANP and BNP. NPR-C is found in most target organs but is most abundant in the kidney and peripheral vasculature (Levin *et al.* 1998; McGrath *et al.* 2005; Clerico *et al.* 2006).

The target organs of ANP and BNP work in synchrony to regulate blood pressure and blood volume, while maintaining proper salt-water balance. The response of these organs to an increase in preload serves as a useful example in demonstration of this property (Figure 1). When an increase in preload is detected via atrial stretch, plasma levels of natriuretic peptides rise resulting in several events including: a decrease in salt and water appetite, an increase in glomerular filtration rate in the kidney, a decrease in aldosterone release from the adrenal glands, and vasodilation in the peripheral vasculature. A decrease in sympathetic activity from the central nervous system also ensues, dampening baroreceptor activity (Levin *et al.*1998, McGrath *et al.* 2005)**.** Natriuretic peptides have also been implicated in growth-suppressing mechanisms. ANP has been found to inhibit hypertrophy and proliferation of vascular smooth muscle cells, while BNP has been found to inhibit myofibroblast formation (Pandey 2005; McGrath *et al.* 2005).

Clerico and colleagues (2006) describe the regulatory effect of natriuretic peptides as a balance between two states: a pro-inflammatory, pro-hypertrophic state in which sodium is retained, and an anti-inflammatory, anti-hypertrophic state in which natriuresis and diuresis are favoured. Natriuretic peptides work in harmony with other systems such as the renin-angiotensin-aldosterone system, the vasopressin hormone system, and potent stimulators such as endothelin to maintain balance between these two states under normal physiological conditions. However, during chronic disease states, the balance is shifted to the pro-hypertrophic state. The difficulty in maintaining such a balanced system may be the reason why natriuretic peptides play such an important role in individuals enduring cases of heart disease and heart failure.

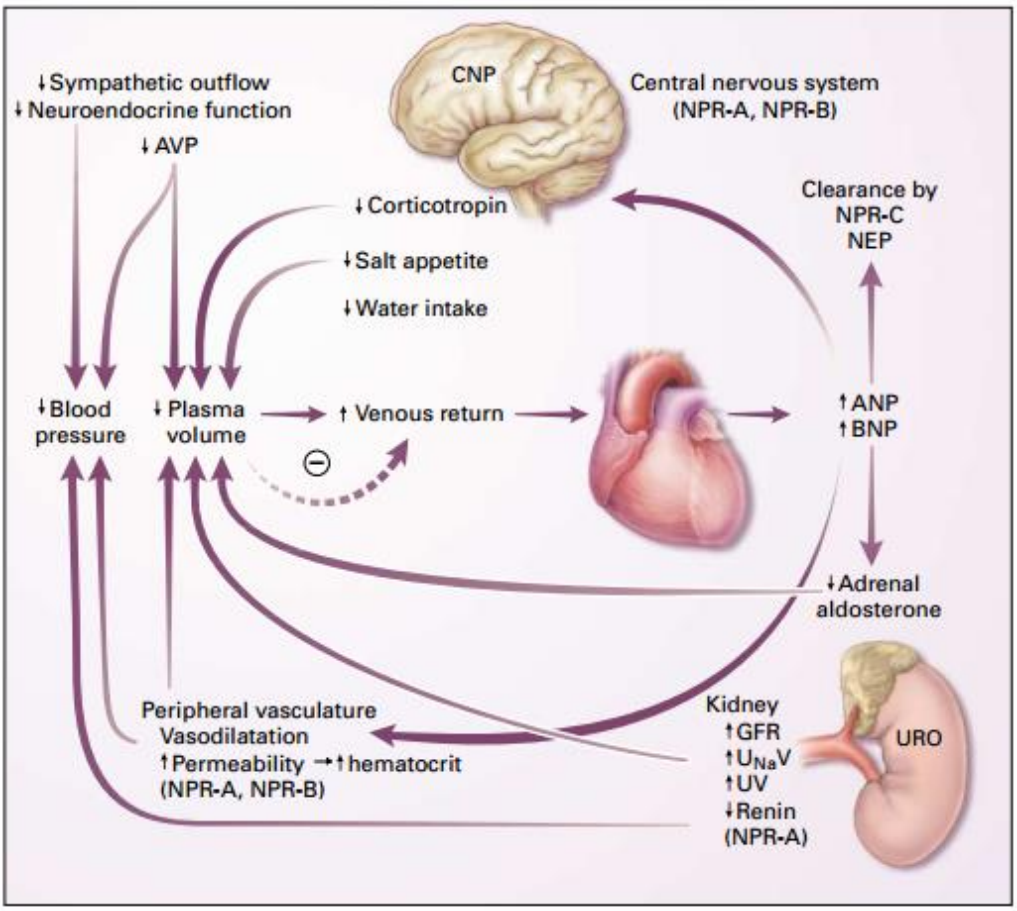


Figure 1. Function of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in target organs. When there is an increase in venous return, the atria of the heart undergo mechanical stretch. This results in an increase in ANP and BNP secretion. Once released, these peptides travel to their target organs eliciting a response that works in homeostasis to combat the original stimulus. In the central nervous system, there is a decrease in salt appetite, in the adrenal cortex – a drop in aldosterone, in the kidney – an increase in glomerular filtration rate, in the peripheral vasculature – vasodilation. These effects come together to decrease blood pressure and plasma volume, ultimately decreasing venous return. *Reproduced with permission from Levin ER, Gardner DG, and Samson WK. Natriuretic peptides. New Engl J Med. 339(5): 321-328. Copyright © (1998), Massachusetts Medical Society.*

1.1.3 Synthesis and Secretion

1.1.3.1 ANP and BNP: Synthesis and Secretion

The genes NPPA and NPPB, originally named “natriuretic peptide precursors” (NPP), encode the proteins ANP and BNP, respectively, and are found on chromosome 1 of humans (1 p 36) and chromosome 5 of rats. The two genes responsible for the two peptides contain three exons, with the peptide-encoding sequence residing on the second exon (McGrath *et al.* 2005). The release of these peptides begins with the transcription and translation of these genes.

The immediate form of ANP following translation is the pre-pro-ANP (1-151) peptide. Pre-pro-ANP is cleaved by signal peptidase in the rough endoplasmic reticulum (ER) to its pro-ANP form (1-126) and stored in intracellular vesicles. Until signalled for release, ANP is stored in this form. Upon stimulation, these vesicles fuse to the membrane and release the protein in its pro-peptide form where it is cleaved by a serine protease, corin, on the plasma membrane of the myocyte. The resulting cleavage forms the N-terminal portion of the peptide, NT-proANP (1-98) and the biologically active form ANP (99-126) (Figure 2a).

The synthesis and secretion of BNP follows a similar pathway: NPPB is transcribed and translated to its pre-pro-BNP (1-134) form, then cleaved to pro-BNP (1-108) by signal peptidase in the rough ER. The underlying difference between the two peptides is that pro-BNP is cleaved to its N-terminus portion (1-76) and its biologically active form BNP (77-108) in the trans-Golgi by the serine protease furin. The biologically active form is then stored in granules for regulated release (Rushkoaho 2003) (Figure 2b). The storage of peptides in granules generally occurs in the atria. In the ventricle however, direct synthesis and secretion of ANP and BNP from their corresponding genes is what typically occurs. Once stimulated for release, granules storing ANP and BNP approach the membrane where they dock, fuse, and release the stored peptides via exocytosis.

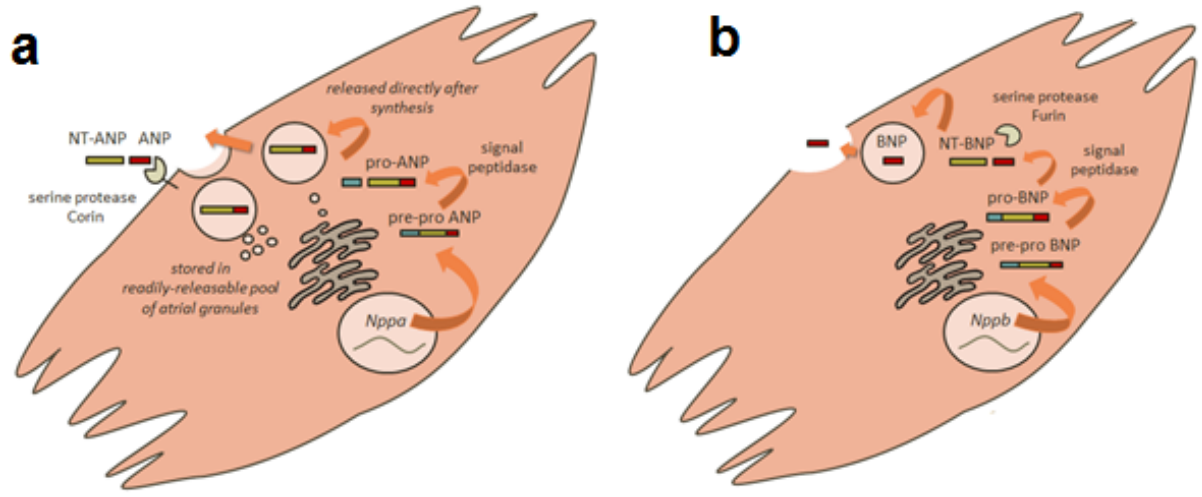


Figure 2. Synthesis and secretion of ANP and BNP in cardiac myocytes. (a) Pre-pro ANP is transcribed and translated from the gene *Nppa*. The N-terminal of pre-pro ANP is then cleaved by signal peptidase in the endoplasmic reticulum (ER) to create pro-ANP which is stored in granules until signalled for release. Once stimulated for release, atrial granules are transported to the plasma membrane where they fuse and release the protein. ANP can either be released from this pool of granules or directly synthesized and secreted. Once the peptide is released, it is cleaved by serine protease corin on the plasma membrane into its biologically active form and it's corresponding N-terminal peptide. **(b)** BNP is processed similarly to ANP. Its pro-BNP peptide, however, is cleaved by serine protease furin at the trans-Golgi to its N-terminus peptide and biologically active form. Both peptides are stored in granules with ANP or directly synthesized and released.

1.1.3.2 Mechanisms of Natriuretic Peptide Secretion

Two methods of natriuretic peptide secretion have been outlined in the literature: constitutive secretion and regulated secretion. Constitutive secretion encompasses direct synthesis and secretion of the peptide wherein release occurs through passive diffusion. Regulated secretion, on the other hand, encompasses the stimulated release of natriuretic peptides via mechanical stretch or neuroendocrine factors such as endothelin-1 (ET-1) and angiotensin II (ANG II) (McGrath and de Bold 2005).

Baseline levels of ANP and BNP are released from atrial cardiac myocytes directly after synthesis. It is hypothesized that a portion of newly synthesized peptide is secreted in small amounts immediately after synthesis, while the other portion is stored in vesicles for future release (McGrath *et al.* 2005). Both hormones are stored together in vesicles, in their pro-peptide forms, until stimulated through hormonal or mechanical means.

Mechanical stimulation, termed "stretch-secretion coupling", is defined as an increase in natriuretic peptide levels caused by atrial or ventricular wall stretch. This phenomenon occurs when a sharp increase in venous return results in an increase in cardiac volume and pressure. In response to this stimulus, pro-ANP and BNP are released from a depletable pool of granules within the atria (McGrath *et al.* 2005). ANP constitutes the major peptide released, while BNP is released at a lower rate. It is suggested that this rate correlates with the ratio of BNP stored with pro-ANP in granules (McGrath and deBold 2005). A subset of BNP peptide, released post-mechanical stimulation may also be directly synthesized and secreted from the atria (Mantymaa *et al.*1993).

“Sub-acute hemodynamic overload”, occurring post-overload, results in an increase in atrial ANP and BNP synthesis in the atria. In the ventricle, however, ANP and BNP mRNA levels do not change. An increase in ANP secretion from the atria is also observed, but because there is no additional release of BNP from the ventricle, an increase in BNP plasma levels is not observed (McGrath *et al.* 2005).

Chronic overload results in direct synthesis and secretion of both ANP and BNP, observed in both atria and ventricle (McGrath *et al.* 2005). The most activity, however, is noted in the ventricle due to the chamber's larger size (Rushkoaho 2003). During normal physiological conditions, the amount of BNP stored is approximately 1-2% that of ANP, within atrial granules (McGrath *et al.* 2005). As a result, during

chronic overload, however, an increase in ANP is immediately observed, followed by the release of BNP after a short time lag. While a large amount of ANP is readily available in storage, BNP must first be synthesized and then secreted, mainly in the ventricle, to display any significant increases in plasma levels. Thus, BNP is known as a better indicator of cardiac disease as an increase in its plasma levels better reflects sustained volume and pressure overload (Rushkoaho 2003).

1.1.3.3 Factors Regulating Natriuretic Peptide Secretion

Endothelin-1, widely known as a strong vasoconstrictor, is the most potent stimulator of atrial natriuretic peptide secretion. When secreted by neighbouring endothelial cells, ET-1 has the ability to induce ANP secretion itself through binding to ETA receptors on cardiac myocytes. In addition, ET-1 has also been shown to augment the release of ANP induced by mechanical stretch. Angiotensin II (ANGII) also stimulates ANP secretion through binding to the AT-1 receptor on cardiac myocytes. In contrast to endothelin, which can stimulate natriuretic peptide on its own, it is not known whether the natriuretic effects of angiotensin II are direct or a result of its ability to increase atrial stretch. Nitric oxide, also released from endothelial cells, is a vasodilator. It opposes the effects of mechanical stretch and endothelin through the inhibition of ANP secretion. The effects of these factors in addition to several others such as adrenomedullin, prostaglandins, vasopressin, catecholamines, and adrenergic agonists are important in the regulation of volume and pressure homeostasis by natriuretic peptides (Thibault, Amiri and Garcia 1999; Dietz 2005). In this study, endothelin-1 is used to stimulate regulated ANP secretion.

1.1.4 Age-Dependent Secretion

1.1.4.1 Fetal Expression of Natriuretic Peptides

The expression of natriuretic peptides in the mammalian fetus surpasses that of the adult, with most transcriptional activity occurring in the ventricle. High fetal levels of ANP and BNP mRNA are found to decrease with development and drop to basal levels in adults. Although mRNA expression patterns

differ between the fetus and adult, ANP and BNP secretion occur in response to the same physiological stimuli: hypoxia, increased volume load, atrial stretch, and vasoconstrictors such as ET-1, ANGII, and phenylephrine. Thus, it is suggested that in both adult and fetal organisms, natriuretic peptides play a similar role in salt and water balance (Clerico *et al.* 2006). In addition, ANP in the human placenta and BNP and CNP in the mouse placenta have been suggested to promote vasodilation through the inhibition of vasoconstrictors and may assist in regulating blood supply to the fetus. It has also been suggested that natriuretic peptides play a role in regulating cardiac myocyte growth (ANP/BNP) and bone growth (CNP) during embryonic development (Cameron and Ellmers 2003). During embryogenesis in mice, ANP and BNP mRNA peak at certain crucial stages of development, indicating that ANP and BNP also play a role in the development of the heart (Cameron and Ellmers 2003).

1.1.4.2 Neonate to Adult: Expression of Natriuretic Peptides

Following birth, there is an age-dependent increase in ANP and BNP secretion levels in both women and men. Past research on healthy humans have found that higher levels of natriuretic peptides are found in women (Wang *et al.* 2002). This finding has also been observed in rodents, where an increase in natriuretic peptide gene expression occurs from neonatal stage to adult (Raizaida *et al.* 2000). This increase may reflect the characteristics an aging heart or occur as a result of a decrease in the clearance rate of natriuretic peptides with age (Clerico *et al.* 2006). Contrary to expression in the fetus, natriuretic peptide secretion post-birth is localized to the atria (Cameron and Ellmers 2003).

1.1.5 Physiological Hypertrophy, Pathological Hypertrophy and Heart Disease

At the cellular level, cardiac hypertrophy refers to an increase in the size of cardiac myocytes as a result of increased protein synthesis. In both physiological and pathological hypertrophy, this increase in cardiac myocyte growth is observed. However, the resulting physiological response to both types of hyertrophy differs between the two.

During physiological hypertrophy, seen in exercise training (physical training, head-out water immersion) or pregnancy, an increase in venous return is sensed via mechanical stretch. The resulting

response is an acute increase in ANP and BNP secretion from stored granules within the atria. Because physiological hypertrophy is reversible and does not result in fibrosis or myocyte apoptosis (Bernardo *et al.* 2010), the response is short-lived. The typical acute response thus occurs wherein both natriuretic peptides work in a homeostatic fashion to combat the original stimulus.

Pathological hypertrophy, however, is observed in disease states such as ischemia, myocardial infarction, and cardiomyopathies. It is often accompanied by myocyte death and fibrosis due to chronic volume and pressure overload (Bernardo *et al.* 2010). In response to pathological hypertrophy, certain fetal genes are re-expressed due to a phenomenon known as "fetal gene reprogramming". These genes include *β-MyHC, α-skeletal actin* as well as *NPPA* and *NPPB,* the genes coding for ANP and BNP. As a result, an increase in ANP and BNP synthesis in the ventricle is observed. The phenomenon of fetal gene reprogramming is characteristic of certain hallmark changes during pathology including cardiac remodeling and reversion to anaerobic metabolism (Rajabi *et al.* 2007). As a result, an increase in natriuretic peptide secretion from ventricular myocytes is observed. As natriuretic peptides are responsible for regulating blood pressure, changes in these peptides, their receptors, or their corresponding genes, may naturally lead to a physiological imbalance, which leads to heart disease in chronic cases. In addition to stimulating natriuretic peptide secretion through chronic wall stretch, pathological hypertrophy also results in the release of neuroendocrine factors such as ET-1, ANGII, and adrenergic receptor agonists which additionally assist in attenuating the original stimuli (Bernardo *et al.* 2010).

Phenylephrine, an α-adrenergic stimulator, and fetal bovine serum (FBS), a media supplement rich with growth factors, have been used in several studies to stimulate hypertrophy *in vitro* (Konhilas and Leinwand 2006; Frey *et al.* 2004; Simpson, McGrath and Savion 1982). These two factors are used in this study to compare two *in vitro* environments - primary myocytes grown at physiological conditions versus primary myocytes undergoing hypertrophy.

1.1.5.1 Fibroblasts

The heart is comprised of four major cell types: cardiac myocytes, fibroblasts, endothelial cells, and smooth muscle cells. In addition to cardiac myocytes, fibroblasts have also been suggested to play a role in natriuretic peptide secretion. These cells are important in the deposition of collagen and scar formation. During hypertrophy, there is a switch in the phenotype of fibroblasts to their active form, myofibroblasts. This switch is stimulated by growth factors such as transforming growth factor beta (TGF-β) or chemokines such as tumor necrosis factor alpha (TNF-α), which are released by neighbouring inflammatory cells in response to injury. Myofibroblasts are found to be more efficient than fibroblasts in creating a collagen framework. It is microfilaments within these cells that interact with extracellular fibronectin to create a stronger extracellular matrix (Baum and Duffy 2011).

Previously it was thought that in non-myocyte cells, the promoter of *NPPA* (the gene encoding ANP) was repressed. As a result, all natriuretic peptide effects on non-myocyte cells were considered paracrine (Cameron *et al.* 2010). However, in 2010, Cameron and colleagues discovered that ANP is secreted from myofibroblasts when stimulated by TGF-β in adult sheep. A similar study done by Tsuruda and colleagues (2012) on adult canines found that stimulation of cardiac fibroblasts with TNF-α resulted in an increase in BNP secretion from fibroblasts. These two studies indicate the importance of fibroblasts in natriuretic peptide secretion during cardiac remodelling. Our current study uses TGF-β to induce myofibroblast formation in order to observe the molecular changes in fibroblasts during hypertrophy.

1.2 SNARE Proteins

1.2.1 SNARE Proteins and SNARE-associated Proteins

The exocytosis of hormones is a complex process requiring a variety of proteins to assist at each step. The two universal components of this process include SNARE (soluble N-ethyl-maleimide-sensitive-fusion-protein attachment protein receptors) proteins and SM (Sec1/Munc18-like) proteins. SNARE-associated proteins such as complexins and synaptotagmin also assist in facilitating SNARE core complex formation and sensing calcium levels during exocytosis (Jahn and Scheller 2006).

SNARE proteins are the key components in vesicular trafficking and exocytosis within the mammalian cell. They are localized to both intracellular granules and target membranes. The characteristic feature of SNARE proteins is a motif consisting of 60-70 amino acid heptad repeats which form an alpha helix. During exocytosis, SNARE proteins on both the vesicle and target membrane interact to form a stable complex facilitating the processes of docking, fusion, and release (Jahn and Scheller 2006). This complex comprises four alpha helices which combine to form a coiled coil (Fasshauer *et al.* 1998; Jahn and Scheller 2006). Originally, SNARE proteins were classified as v-SNAREs and t-SNAREs based on their location on the vesicle or target membrane, respectively. Later, they were classified into two new sub-types: Q-SNAREs and R-SNAREs, as their previous classification did not encompass vesicle-vesicle fusion. This new classification categorizes SNARE proteins by the main amino acid in its SNARE motif that contributes to the central ionic layer of each complex: Q-SNAREs contribute a glutamine residue and R-SNAREs contribute an arginine residue (Fasshauer *et al.* 1998). The most studied SNARE core complex consists of the three SNARE proteins: syntaxin, synaptosomal associated protein-25 (SNAP25) found on the plasma membrane, and vesicle-associated membrane protein (VAMP)/synaptobrevin found on the vesicle. In neuronal cells, syntaxin and VAMP contribute one alpha helix to the complex, while SNAP25 contributes two alpha helices (Chen and Scheller 2001).

SM proteins also assist in the assembly of the SNARE core complex. Munc18, the best characterized SM protein, was originally known as a negative regulator of SNARE core complex formation. This protein binds to the N-terminus of syntaxin 1A on the membrane and interacts with its four alpha helices. One helix is made up of the SNARE motif, while the three remaining helices are made up of the H_{abc} component of the syntaxin 1A gene. The H_{abc} component is unique in that it is able to fold onto syntaxin 1A's SNARE motif creating a "closed" conformation. Later, however, a new model was proposed in which SM proteins *assist* with vesicle fusion by interacting with the four alpha helices from the SNARE core complex, rather than syntaxin 1A alone, "clasping" them together. This "clasp" functions to keep SNARE proteins from moving between the vesicle and membrane during fusion (Shen *et al.* 2007;Sudhof and Rothman 2009). In addition, SM proteins were also suggested to stimulate the mixing of phospholipids at the membrane inducing a change in curvature and creating an ideal environment for

fusion (Carr and Rizo 2010). It should be noted however that SNARE proteins are capable of inducing membrane fusion without the assistance of SM proteins (van den Bogaart *et al.* 2010).

Although SNARE proteins alone can mediate fusion, the fast, regulated release of certain secretions such as neurotransmitter release relies on an influx of calcium. Synaptotagmin-1, a transmembrane protein found on vesicles, functions as a calcium sensor, interacting with both calcium and SNARE proteins during exocytosis. It has two calcium binding sites called C2 domains. When calcium is bound to these domains, synaptotagmin-1 facilitates the assembly of the SNARE core complex through calcium-dependent phospholipid binding and/or calcium-dependent SNARE complex binding. In both scenarios, synaptotagmin binding allows for the formation of a vesicular pore following fusion of the vesicle to membrane (Chapman 2002).

Complexins, another class of regulatory protein, have been found to function alongside synaptotagmin in regulating the formation of the SNARE core complex. Two leading researchers in vesicle trafficking, Rothman and Sudhof, propose different functions for complexins (Brose 2014): Rothman and colleagues suggest that complexins function as a reversible clamp, preventing the SNARE complex from forming until synaptotagmin senses calcium and displaces complexin, allowing for fusion (Giraudo *et al.* 2006). In contrast, Sudhof and colleagues believe complexins to be a co-factor of synaptotagmin, assisting in the priming of SNARE complexes prior to vesicle fusion (Reim *et al.* 2001).

The general assembly of the SNARE core complex is briefly outlined below: Once packaged by the Golgi apparatus, proteins within the cell are trafficked to their main destination via large dense core vesicles. Membrane-targeted proteins are sent to the membrane where the SNARE core complex forms with the assistance of SM proteins, synaptotagmin and complexins. The generally accepted “zipper” model suggests that Q-SNAREs found on the membrane cluster together to form an “acceptor complex”. Once the incoming vesicle approaches, R-SNAREs found on the vesicle interact with Q-SNAREs, each interacting at their SNARE motifs to form a coiled coil alpha-helical complex. This results in the formation of a *trans*-complex, driving the vesicle to closer proximity with the target membrane. In agreement with the “force” model, a mechanical force from the linker region of the SNARE proteins is exerted onto the membrane overcoming the energy barrier required for the vesicle to fuse with the membrane. The result

is the formation of a pore and the exocytotic release of the vesicles' contents. Finally, the SNARE proteins reassemble into a *cis*-complex following exocytosis, in which all SNARE proteins reside on the same side of the membrane (Jahn and Scheller 2006) (Figure 3).

The dissociation and recycling of SNARE proteins post-fusion is facilitated by the ATPase, NSF (N-ethyl maleimide-sensitive factor) and the adaptor proteins, SNAPs (soluble NSF attachment proteins) which bind to the *cis*-SNARE complex, providing the metabolic energy for disassembly. The interaction of NSF and SNAPs with SNARE proteins is what originally inspired the "SNARE" name (Jahn and Scheller 2006).

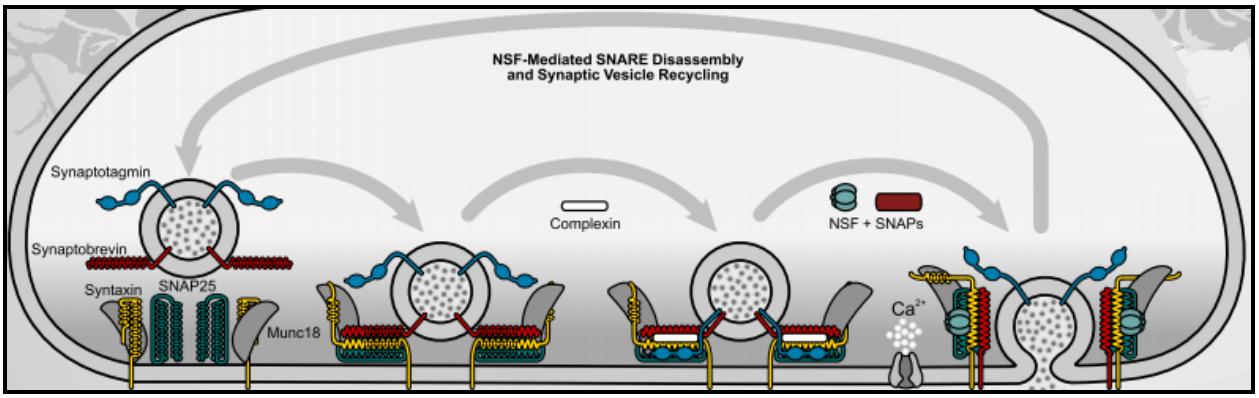


Figure 3. Role of SNARE proteins, SNARE-associated proteins, and SM proteins in neuronal exocytosis.

Before vesicle docking, SM protein Munc18 interacts with SNARE protein syntaxin, keeping it in closed conformation. During neuronal exocytosis, neurotransmitters are packaged in vesicles and transported to the membrane from the Golgi. Upon vesicle approach, Munc18 releases syntaxin. This allows syntaxin, SNAP25 on the membrane, and synaptobrevin/VAMP on the vesicle, to interact, bringing the vesicle closer to the membrane where it docks. When stimulated for release, there is an influx of calcium which is sensed by synaptotagmin. With the help of complexins, synaptotagmin binds to the SNARE proteins facilitating the formation of a trans-SNARE complex. This allows the vesicle to fuse to the membrane creating a pore for neurotransmitters to be released. At this point, the trans-SNARE complex reassembles into a cis-SNARE complex, which is later disassembled by NSF and SNAP proteins. *Reprinted from Brose, Nils. All roads lead to neuroscience. Neuron. 81(4):.723-727. Copyright © (2014), with permission from Elsevier.*

1.2.2 SNARE proteins in the brain

Although the discovery of SNARE proteins in the mammalian system began in the brain, it was the study of vesicular transport in yeast that led to the identification of the SNARE binding partners NSF and SNAP in the brain. In 1979 to 1980, Novick and Schekman identified *sec* genes in yeast that when mutated, resulted in the accumulation of vesicles within its cells. Under the supervision of Dr. James Rothman, the mammalian homologues of the two SEC proteins, SEC18 and SEC17, were identified: N-ethylmaleimide sensitive factor (NSF) and soluble NSF-attachment protein (SNAP) (Block *et al.* 1988; Clary *et al.* 1990). This finding proved crucial to the study of SNARE proteins in the brain as it was due to the abundance of these two proteins in the brain that the discovery of their binding partners, and consequently the first SNARE proteins, were identified. These include: VAMP/synaptobrevin, syntaxin, and SNAP25 (Brose 2014). These proteins were later established as the three main SNARE proteins of the canonical SNARE core complex, well-studied in neurotransmitter release, while NSF and SNAP were found to disassemble the complex post-vesicle fusion (Söllner *et al.* 1993). Synaptobrevin/VAMP was found to localize to synaptic vesicles, while syntaxin and SNAP25 were found to localize to the presynaptic membrane. The relevance of these proteins to membrane fusion was further corroborated through the demonstration that *Clostridial* neurotoxins, which proteolytically cleave specific SNARE proteins, impair neurotransmitter release (Montecucco and Schiavo 1995). Since then, the characterization of SNARE proteins and SNARE-associated proteins have been widely studied using neuronal SNARE proteins as a model.

1.2.2.1 Clostridial Neurotoxins

Clostridial neurotoxins are a family of zinc endopeptidases isolated from the anaerobic bacteria *Clostridium tetani* and *Clostridium botulinum*. These toxins target and cleave specific SNARE proteins involved in neuronal exocytosis, impairing neurotransmitter release. Tetanus neurotoxin (TeNT), produced by *Clostridium tetani,* blocks neurotransmission in the inhibitory interneurons of the spinal cord, while serotypes of botulinum neurotoxins (BoNTs), produced by *Clostridium botulinum,* block

acetylcholine release at the neuromuscular junction. Administering both toxins individually results in paralysis (Montecucco and Schiavo 1995).

When administered, BoNT and TeNT interact with gangliosides on the lipid membrane before binding to an unknown, high affinity receptor, allowing it to be internalized into intracellular vesicles via endocytosis. TeNT travels down the axon to inhibitory interneurons while BoNTs remain in the neuromuscular junction. Once internalized, the inside of the intracellular vesicle undergoes acidification, and the BoNTs within undergo a structural change. The light chain of the BoNT, which contains its zinc endopeptidase, is released from the vesicle. In the cytosol, the light chain proteolitically cleaves its target SNARE (Turton *et al.* 2002). The target proteins are as follows: TeNT, BoNT B, D, F, and G cleave VAMP/synaptobrevin; BoNT A, C and E cleave SNAP25; and BoNT C cleaves syntaxin 1 (Turton *et al.* 2002) (Figure 4). SNARE cleavage has been found to inhibit fusion, but not the binding of vesicles to the presynaptic membrane (Weber *et al.* 1998).

Botulinum toxins have proven to be a crucial tool in characterizing neuronal SNARE proteins. However, they have yet to be used in characterizing SNARE proteins in the heart as this area of research is still in its infancy. In our study, we transfect neonatal atrial and ventricular rat cardiac myocytes with plasmids containing DNA for botulinum toxins A and C. The cleavage sites are as follows: BoNT/A cleaves SNAP25 at Gln177 and Arg176, while BoNT/C cleaves syntaxin 1A at Lys253 and Ala254 (Sutton *et al.* 1998).

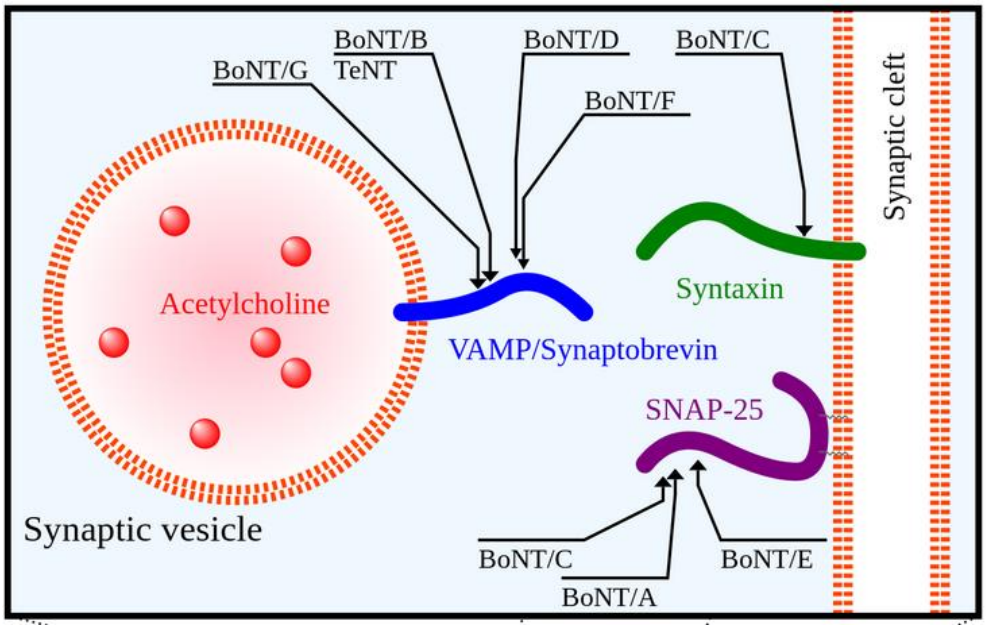


Figure 4. Role of botulinum neurotoxins in the cleavage of SNARE proteins syntaxin, SNAP25, and VAMP/synaptobrevin. The target proteins of botulinum neurotoxins are as follows: syntaxin is cleaved by BoNT/C, SNAP-25 is cleaved by BoNT/C, BoNT/A, and BoNT/E and VAMP/synaptobrevin is cleaved by Tent, BoNT/B, BoNT/G, BoNT/D, and BoNT/F. *Reproduced from: "Presynaptic CNTs targets" by Y tambe - Own work. Licensed under Creative Commons Attribution-Share Alike 3.0 via Wikimedia Commons - http://commons.wikimedia.org/wiki/File:Presynaptic_CNTs_targets.svg#mediaviewer/File:Presynaptic_CNTs_targets.svg. (Accessed on: July 20,2014)*

1.2.3 SNARE proteins in the heart

Formerly, SNARE proteins were most commonly known for regulating the release of neurotransmitters. It was in the last decade that the importance of SNARE proteins in the heart was discovered through the exocytotic release of the hormone, atrial natriuretic peptide. In 2006, Peters and colleagues identified two sets of SNARE complexes involved in regulating exocytosis in rat atrial myocytes - one in neonatal and adult mice and one solely in adults. Using confocal microscopy, they were able to observe that certain SNARE proteins co-localize with ANP granules. The first complex which was found to co-immunoprecipitate with VAMP-2, consisted of synaptotagmin, syntaxin 4 and SNAP23. The second complex which also co-immunoprecipitated with VAMP-2, consisted of synaptotagmin, syntaxin 1, and SNAP25. VAMP-1's association with VAMP-2 was also demonstrated using confocal microscopy. Using cell fractionation, in addition to confocal and fluorescent microscopy, the authors were also able to conclude that these SNARE proteins co-localize with ANP-containing granules (Peters *et al.* 2006). This novel finding suggested that SNARE proteins play a role in ANP secretion.

In 2010, Ferlito and colleagues looked further into the functional significance of syntaxin 4, VAMP-1 and VAMP-2 in terms of ANP secretion. Using siRNA techniques, they knocked down the three SNARE proteins individually resulting in a significant decrease in ANP release after stimulation with endothelin-1. It was noted that although ANP levels decreased, these levels did not fall below baseline. It was concluded that this neonatal complex must play a role in the regulated release of ANP and that constitutive release of ANP must be regulated by a different SNARE complex.

Besides the role of SNARE proteins in exocytosis, SNARE proteins have recently been found to play a role in regulating ATP-sensitive potassium channels (K_{ATP}) as well as voltage gated potassium channels (K_v). In 2011, Chao and colleagues found that syntaxin 1 interacts with cardiac potassium channels in regulating the excitability of the heart. Characteristics of cardiac stress include a decrease in pH (due to an accumulation of acidic by-products from anaerobic glycolysis) as well as a decrease in ATP. The decrease in pH and ATP results in the opening of K_{ATP} channels and could result in arrhythmias of the heart. In response to these stressors, a change in the interaction of syntaxin with SUR2A subunits of K_{ATP} channels was observed. Chao and colleagues proposed that at high ATP levels, K_{ATP} channels

are closed, but during cardiac stress, the ATP:ADP ratio decreases and syntaxin 1A acts on SUR2A by binding to its subunits and opening the channel, allowing potassium to enter. This regulatory role has also been proposed for syntaxin 1A and SNAP25 in relation to other Kv channels.

1.2.4 Transcriptional Control of SNARE Proteins

It was previously shown in rats that syntaxin 1A and SNAP25 protein levels are only expressed in adult atrial cardiac myocytes (Peters *et al.* 2006). However, the transcriptional activity of these proteins has yet to be determined in both the atria and ventricle. The main SNARE proteins examined in this study include: syntaxin 1A and SNAP25.

1.2.4.1 Syntaxin 1A - Overview

Although the promoter region has yet to be characterized, the mRNA transcript of syntaxin 1A has been sequenced in many mammals, specifically, humans and rats. Syntaxin isoforms include but are not limited to: syntaxin 1A and syntaxin 1B. While the different isoforms have several functions in other tissues, syntaxin 1A and 1B complete specific functions in calcium-dependent neurotransmitter release in the brain (Bennett *et al.* 1993).

Syntaxin 1A expression is mostly localized to the brain, but is also present in beta cells of the islets of Langerhans (Lam *et al.* 2005), immune cells such as lymphocytes (Nakamura *et al.* 2003), and more recently in cardiac myocytes (Peters *et al.* 2006). Syntaxin 1A contains a cytoplasmic region in its first 265 residues and a transmembrane domain that makes up the next 266-288 residues (Fernandez *et al.* 1998). The N-terminal domain, spanning from the 28th-144th residue, is suggested to play a crucial role in exocytosis. This domain has many functions including interaction with SM proteins such as Munc 18, blocking the formation of the SNARE core complex itself, and interactions with other regulatory components such as synaptotagmin (Fernandez *et al.* 1998). The many functions of the syntaxin 1A N-terminus are still under investigation.

1.2.4.2 SNAP25 - Overview

The SNAP25 mRNA sequence has been characterized in humans, mice, chicken, zebrafish, *C. elegans*, and *Drosophila*. In the mouse brain, two transcriptional start sites were characterized on the SNAP25 gene, 42 nucleotides apart. Its promoter region, 2073bp upstream of the first transcriptional start

site, contains the following elements: a CRE recognition sequence -11 to -18bp, a TATA box -26bp, three SP1 sites -100bp, three AP-1 binding sites -600bp, and two TG repeats at 0bp and -1490bp. In chicken, however, the SNAP25 promoter contains all elements except for the CRE element (Ryabinin *et al.* 1996).

In terms of development, SNAP25 mRNA is found to be expressed at low levels in both the embryo and the early neonatal stages within the cell bodies of neurons. Expression levels increase during the development of the brain and during synaptogenesis (Bark *et al.* 1995). Oyler and colleagues (1991) studied the brain of adult rodents and found that SNAP25 is synthesized in the cell body of neurons, then transported across the axon until it is ready to be relocated to the presynaptic neuron. Some studies suggest that SNAP25 plays a role in neurite elongation (Kimura *et al.* 2003). While SNAP25 is mostly found in the brain, it is also found at lower levels in other endocrine tissues such as the beta-cells of the pancreas (Ryabinin *et al.* 1996, Sadoul *et al.* 1995).

There are two isoforms of SNAP25, formed through alternative splicing at the fifth exon: SNAP25-a and SNAP25-b. SNAP25-a is localized to neuroendocrine cells and developing axons and it has been suggested that it is responsible for trafficking vesicles containing the components necessary for axonal growth. SNAP25b, on the other hand, is localized to central and peripheral neurons and thought to take part in the canonical function of neurotransmitter release. The presence of two SNAP25 isoforms indicates that post-transcriptional processing may be important in regulating SNAP25's function, whether it be secretion or assisting in the vesicular transport of materials important to the growth of the cell (Bark *et al.* 1995).

1.2.4.3 Transcriptional Activity of SNAP25 in Ovarian Cells

In 2007, Shimada and colleagues looked at the transcriptional activity of SNAP25 in ovarian mouse cells in an attempt to investigate the molecular mechanisms controlling its expression during exocytosis. They transfected granulosa cells with a -1517bp SNAP25 promoter-luciferase reporter, as well as truncated versions of the promoter without certain regulatory regions, to pinpoint the exact regions necessary for activity of SNAP25. By stimulating the transfected granulosa cells with forskolin/phorbol 12-myristate 13-acetate (FOR/PMA), stimulators of cAMP and protein kinase C (PKC) respectively, they

found that the presence of the SP1/SP3 regulatory region, TATA box, and CRE site on the promoter were necessary for SNAP25 promoter activity.

This study was replicated by David Boyce, a former undergraduate thesis student of Dr. Robert Tsushima, in cardiac myocytes. He found that the minimal promoter length for SNAP25 expression in atrial and ventricular cardiac myocytes contains the TATA box, a sequence typically found in housekeeping genes, and the transcriptional element CRE, found to be important in intracellular calcium signalling (Mao *et al.* 2007)(Figure 5). David Boyce postulated that cAMP, stimulated by forskolin, is linked to the calcium-mediated secretion of ANP due its activity upstream of CRE activation. cAMP activates PKA which interacts with calcium response element-binding proteins (CREB) in the nucleus, which ultimately binds to CRE (Vallejo 1994). Previous studies have found that an increase in intracellular Ca^{2+} signalling leads to the increased phosphorylation of CREB through PKC, and increased binding of CRE (Mao *et al.* 2007). Thus, stimulation of both cAMP and PKC in cardiac myocytes should theoretically increase binding of CRE on the SNAP25, and possibly syntaxin 1A promoter, raising its rate of transcription and corresponding protein levels, and ultimately raising ANP secretion levels.

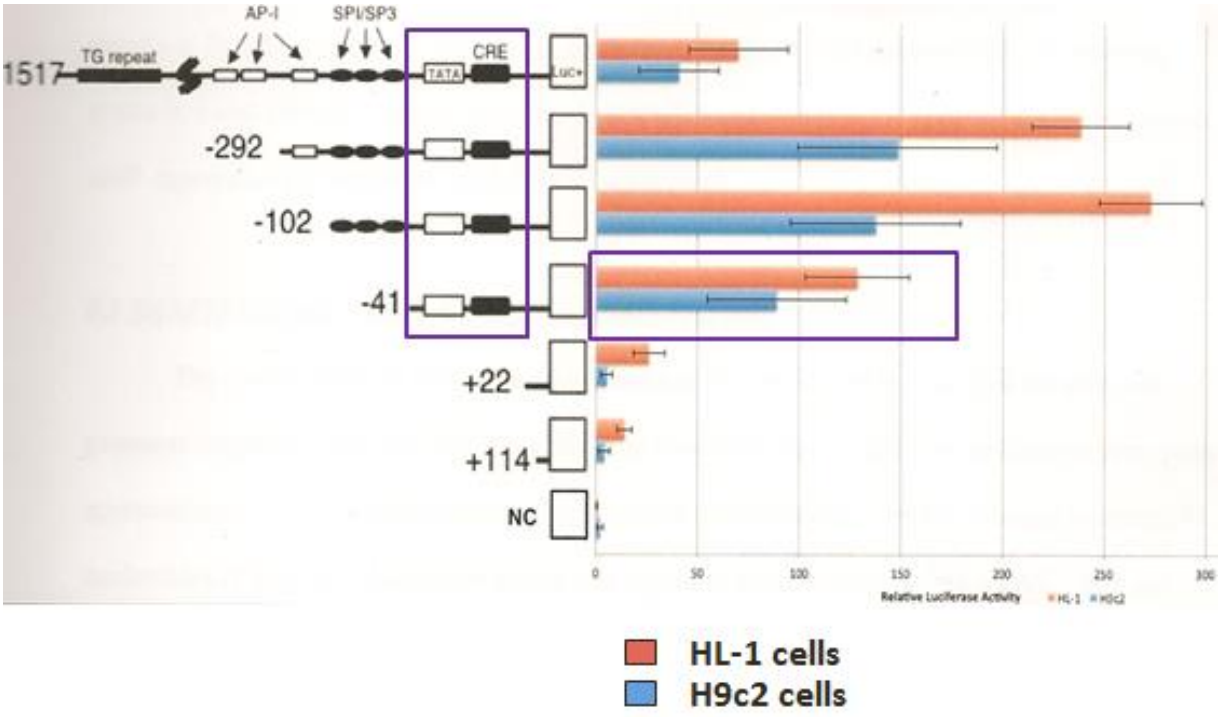


Figure 5. The minimal promoter length required for SNAP25 promoter activity contains a TATA box and the CRE element. HL-1 and H9c2 cell lines, models for atrial and ventricular cardiac myocytes respectively, require a minimum promoter length of -41 kb for transcriptional activity to occur. HL-1 cells display greater promoter activity than H9c2 cells. *Figure adapted from Boyce, D. 2013. Regulation of SNAP-25 promoter activity in cardiac myocytes. Biology Honours Thesis. York University: Toronto, Ontario.*

1.3 SNARE Proteins in Constitutive Secretion

1.3.1 Syntaxin 5A, syntaxin 18 and SNAP29 are important in constitutive secretion within mammalian cells

Despite the vast amount of research done on natriuretic peptides, the mechanisms involved in their secretion are still vague. Although some studies have suggested certain SNARE complexes to be involved in the regulated ANP secretion pathway (Ferlito *et al.*, 2010; Dietz 2005), the specific SNARE proteins involved in the constitutive pathway still remain unknown. Furthermore, the SNARE proteins responsible for BNP secretion have yet to be explored.

In 2010, Gordon and colleagues performed a screen in which they silenced 51 SNARE and SNARE-associated proteins in HeLa cells. These HeLa cells were transfected with a reporter construct containing a GFP-coding region. This reporter construct contains a gene that when synthesized, creates mutant FKBP proteins which form dimers that aggregate at the ER. When the corresponding cell is stimulated with its ligand (AP21998), the dimer solubilizes and can be secreted. The amount of fluorescence was measured using a flow cytometer. The greater the fluorescence, the more dimer has accumulated; the lower the fluorescence, the more constitutive secretion has occurred.

This mass screen identified many SNARE proteins associated with constitutive secretion. Three SNARE proteins were chosen out of the 51 identified to analyze the different components of the vesicle trafficking pathway: syntaxin-5A, syntaxin-18, and SNAP29. Their role in constitutive ANP and BNP secretion is investigated in this study. Previous studies implicate syntaxin 5A in anterograde transport from the ER to Golgi (Dascher *et al.* 1994), syntaxin 18 in retrograde transport from the Golgi to the ER (Iinuma *et al.* 2009), and SNAP29 in post-Golgi transport (Su *et al.* 2001) (Figure 6).

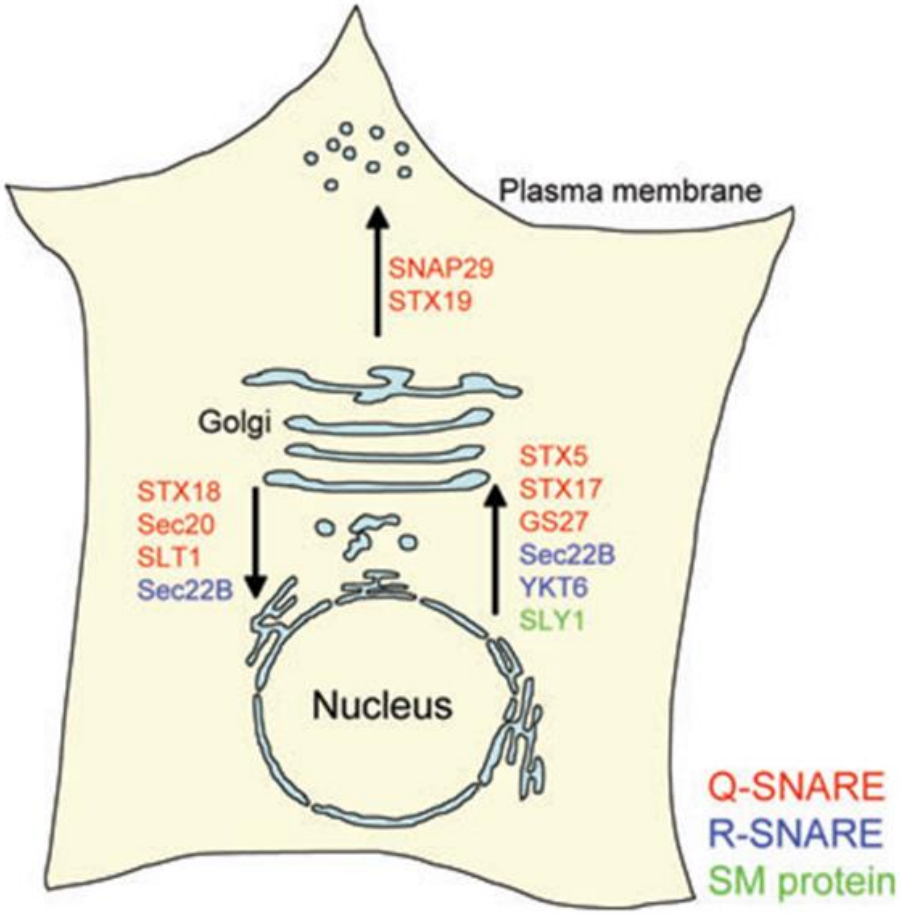


Figure 6. Localization of SNARE and SNARE-associated proteins implicated to play a role in the constitutive secretion pathway. The SNARE proteins indicated above were found by Gordon and colleagues (2010) to be important in constitutive secretion within the mammalian cell. Syntaxin 5 (STX5), syntaxin 18 (STX18) and SNAP29 were chosen for study to represent each segment of the secretory pathway. Syntaxin 5 is found to play a role in anterograde transport of vesicles from the endoplasmic reticulum to the Golgi, syntaxin 18 is found to assist with retrograde transport from the Golgi to the endoplasmic reticulum, and SNAP29 is found to assist with Golgi to plasma membrane transport. *Reprinted from Gordon DE, Bond LM, Sahlender DA, and Peden AA. A targeted siRNA screen to identify SNAREs required for constitutive secretion in mammalian cells. Traffic. 11(9): 1191-1204. Copyright © (2010), John Wiley & Sons, Inc.*

1.4 Hypotheses

1.4.1 Developmental changes in SNARE protein expression in the heart

The first aim of this study is to characterize the age-dependent expression profiles of syntaxin 5A, syntaxin 18, SNAP29, as well as the natriuretic peptides ANP and BNP using neonatal (1 day, 7 day, and 14 day) and adult rat models. As the ventricle is known to be the main site of constitutive secretion (Bloch *et al.* 1986), an increase in the expression of these three SNARE proteins is expected in the ventricle compared to the atria. Fibroblasts as well as 1 day and 7 day neonatal ventricular myocytes, were studied with the goal of observing any differences in SNARE expression across cell types or development.

ANP and BNP plasma levels both increase with age. With this in mind, it is speculated that a greater amount of vesicles are required for the transport of these natriuretic peptides as well as a greater demand for the machinery involved in their exocytosis. As a result, I hypothesize syntaxin 5A and SNAP29 expression levels to increase in both atria and ventricles during development, as there would be a greater need for anterograde transport from the ER to the Golgi and similarly from the Golgi to the cell membrane. Additionally, I would hypothesize syntaxin 18 expression levels to decrease during heart development as less recycling of vesicles and degradation would be required.

Building on this idea, the second aim of this project was to look at the expression of these SNARE proteins in response to pathological hypertrophy. During hypertrophy, there is a significant increase in constitutive natriuretic peptide secretion from the ventricle. This increase is noted in the literature in both cardiac myocytes (McGrath *et al.* 2005, Rushkoaho 2003) as well as fibroblasts (Cameron *et al.* 2000). Our study aims to induce hypertrophy in both these cells and observe whether the expression of these SNARE proteins follow the trends we expect. Consistent with my previous hypothesis, I would expect the previously described SNARE proteins to behave similarly in neonatal ventricular myocytes post-drug treatment. With an increase in ANP and BNP post-drug treatment, I expect to see an increase in syntaxin 5A and SNAP29 protein expression, as well as a decrease in syntaxin 18. I would expect that the trends in expression observed with hypertrophy should be more exaggerated than that observed with age, as the increase seen in ventricular natriuretic peptide synthesis and secretion is much more pronounced.

1.4.2 Syntaxin 1A and SNAP25 expression and promoter activity will be higher in the atria versus ventricle and higher in adults versus neonates.

As previously stated, Peters and colleagues (2006) identified two complexes of SNARE proteins responsible for ANP secretion in atrial myocytes. The first consisted of SNAP23, syntaxin 4, VAMP-1, and VAMP-2. Although a follow-up study was done on this complex, the second complex consisting of syntaxin 1A, SNAP25, VAMP-1 and VAMP-2, did not incite further research. The third aim of this study to characterize the age-dependent expression profiles of syntaxin 1A and SNAP25 in neonatal and adults using immunoblotting, as well as their transcriptional activity using a secreted luciferase assay at 1 day and 7 days of age. Previous work done by Xiaodong Gao in the Tsushima lab characterized syntaxin 1A and SNAP25 protein levels in atrial tissue and found that in addition to being expressed in adults, both proteins were also expressed in neonatal cells and increased with age (Figure 7). Looking at promoter activity in both 1 day and 7 day cells may provide insight into the activity of these SNARE proteins at the neonatal stage. It is peculiar that a second set of SNARE proteins are required in the adult heart when both neonatal and adult hearts already consist of the SNARE protein complex consisting of SNAP23 and syntaxin 4. I hypothesize that with an increase in natriuretic peptide levels seen with age, the additional appearance of a second regulatory complex is required to assist with the increased amount of natriuretic peptide secretion. Both transcriptional activity and protein levels should be reflective of this increase.

The chamber specific expression of syntaxin 1A and SNAP25 was also investigated in this study. Previous work done by Xiaodong Gao in the Tsushima lab characterized these two SNARE proteins in atrial tissue (Figure 7). However, the differences in chamber expression between atrial and ventricular tissue have not been compared on the protein or transcription level. The fourth aim of this project is to characterize the *chamber-specific* expression of syntaxin 1A and SNAP25 protein levels and transcriptional activity in atrial and ventricular tissue and cells. I hypothesize that I will see a bigger increase in expression within the atria, as this is the main site of natriuretic peptide secretion under normal physiological conditions.

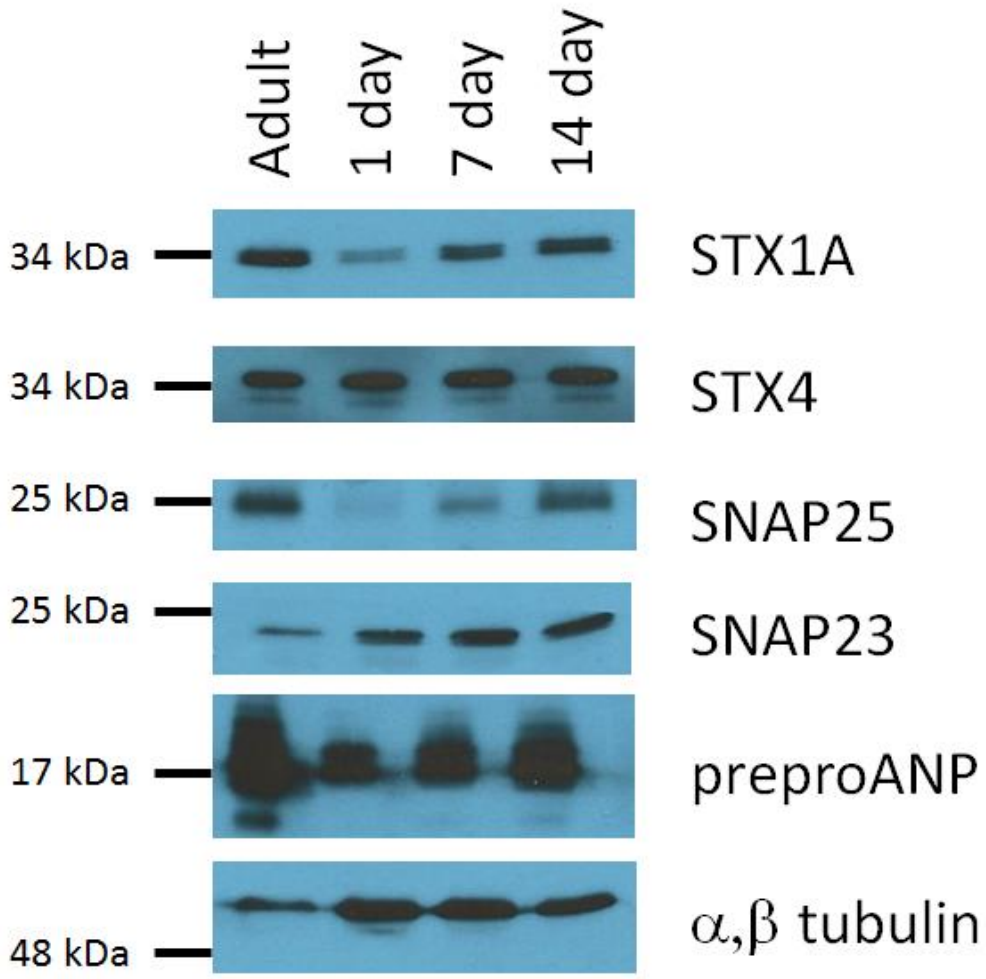


Figure 7. Expression profiles of syntaxin 1A, syntaxin 4, SNAP25, SNAP23, pre-pro-ANP, and α-β-tubulin in 1 day, 7 day, 14 day, and adult atrial tissue probed by Western blot (work conducted by Xiadong Gao, lab technician of Dr. Robert Tsushima's lab). A general increase in syntaxin 1A and SNAP25 protein levels is observed with age. In contrast, there is relatively no change in syntaxin 4 and SNAP23 expression from 1 day to adult rat atrial tissue. The increase in syntaxin 1A and SNAP25 expression corresponds with an increasing trend in protein levels observed in pre-pro-ANP with age.

The age-dependent and chamber-specific protein expression profiles of syntaxin 1A and SNAP25 will be studied using immunoblotting. In terms of transcriptional activity, neonatal atrial and ventricular cardiac myocytes will be transfected with syntaxin 1A and SNAP25 promoters and the secreted luciferase product measured using a luminometer.

Furthermore, I will investigate the functional role of these two SNARE proteins in ANP secretion by transfecting neonatal cardiac myocytes with DNA for botulinum neurotoxin type A and botulinum neurotoxin type C to cleave SNAP25 and syntaxin 1, respectively, in both atrial and ventricular cardiac myocytes. Following this, I will measure ANP secretion after stimulating cells with endothelin-1. Neurotoxin efficiency will be confirmed using Western blot. If these SNARE proteins are indeed important in natriuretic peptide secretion, cleaving these SNARE proteins should result in decreased ANP levels within the media.

1.4.3 Treatment of transfected myocytes with FOR/PMA will increase syntaxin 1A and SNAP25 promoter activity by increasing binding to the CRE promoter element.

Lastly, I endeavoured to support the work of David Boyce in the Tsushima lab by looking into the importance of the CRE element in SNAP25 transcriptional activity. Neonatal cardiac myocytes initially transfected with syntaxin 1A and SNAP25 promoters were treated with forskolin/PMA (FOR/PMA) to increase CREB binding to CRE within the nucleus. Forskolin increases cAMP activity, ultimately leading to downstream CRE binding (Vallejo 1994). PMA stimulates PKC which increases phosphorylation of CREB and also CRE binding (Mao *et al.* 2007). As a result, treatment with FOR/PMA should hypothetically increase binding of CRE on the SNAP25 and possibly syntaxin 1A promoter, raising its corresponding transcriptional activity within the nucleus.

Methods

2.1 Cell Culture & Isolation

2.1.1 Cell lines (HL-1, H9c2, and MIN6)

Cells were plated to 10 mm dishes in their corresponding media. The plating media were as follows: HL-1 media - Claycomb Medium (Cat. #51800C - Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Cat. #12483-020, Life Technologies), 1% penicillin/streptomycin (P/S) (Cat. #15140-122), 1% norepinephrine (NorEpi), and 1% L-glutamine; H9c2 media - F12/Dulbecco Modified Eagle Medium DMEM) (Cat. #319-085-CL - Wisent Inc.) supplemented with 10% FBS, 1% P/S; MIN6 cell media – DMEM (Cat. #319-005-CL - Wisent Inc.) supplemented with 1.7 µL of 14.3M of β-mercaptoethanol (Cat.#M6250-ALDRICH, Sigma-Aldrich), 10% FBS, and 1% P/S. HL-1 dishes and/or flasks were pre-coated with 0.02% gelatin and 200 mL fibronectin 24 hours before plating. Cells were then incubated and maintained at 37°C in 5% CO_2.

2.1.2 Primary cardiac myocytes

All research conducted on animals was approved by York University's Animal Care Committee, and was conducted in accordance to the guidelines of the Canadian Council on Animal Care. Neonatal atrial and ventricular cardiac myocytes were isolated from Sprague-Dawley rats at 1 day, 7 day and 14 days. Hearts were excised and washed in sterilized Ca^{2+}- and bicarbonate-free HEPES-based Hanks (CBFHH) Buffer consisting of 137mM NaCl, 5.36 mM KCl, 0.81 mM $MgSO_4$, 5.55 mM dextrose, 0.44 mM KH_2P0_4, 20.06 mM HEPES, 0.34 mM NaH_2PO_4, pH 7.4. Stock solution was stored at 4°C, diluted, filtered and brought to room temperature before use.

Both atria and ventricle were separated for isolated digestion. Tissues needed for immunoblotting were frozen in liquid nitrogen for homogenization at a later date. Tissues undergoing digestion were minced into small pieces and trypsinized using 300 mg of trypsin 1:250 (Cat. #27250018, Gibco) diluted in 200 mL of CBFHH. Trypsinization comprised of twelve to sixteen five-minute washes in 10 mL of the above-mentioned dilution. All washes took place at 37°C within a water bath. After tissue was completely

digested, the supernatant from each wash was collected and combined with 7 mL FBS in 50 mL tubes until tubes were filled. The enzymatic solution combined with FBS was then spun at 1500 rpm for 5 minutes. The resulting cell pellet was re-suspended in 6 mL of F12/DMEM medium with 10% FBS and 1% P/S. Cells were re-plated to one 10 mm dish per 50 mL tube of solution and incubated for 45 minutes at 37°C. Finally, the cells were combined and spun at 1500 RPM for 5 minutes. The resulting pellet was re-suspended in the media indicated above, re-plated, and maintained at 37°C in 5% CO_2.

2.1.3 Primary fibroblasts

Primary fibroblasts and immortal cell lines were passaged once 80% confluency was reached. Medium was removed and cells were rinsed with phosphate buffer saline (PBS). 2 mL of 0.5% sterilized trypsin was then added to the dish and incubated for 5-10 minutes until cells were dislodged from the plate. The trypsinized cells were then removed and 3 mL of corresponding media combined to neutralize the enzyme. The enzymatic solution was then spun at 1500 rpm on a Thermo Scientific Sorvall Legend T Plus/RT Plus Centrifuge (Thermo Fisher Scientific, Waltham MA, USA) for 5 minutes. Following the spin, the supernatant was removed into a waste beaker. The resulting cell pellet was re-suspended in its corresponding media and re-plated to a new 10 mm dish or flask.

2.1.4 Cell Treatment

2.1.4.1 Probing for syntaxin 5A, syntaxin 18, SNAP29 – Control vs. Hypertrophy-induced cells

24 hours following isolation, 7 day neonatal ventricular cardiac myocytes were treated as follows:

Cardiac Myocytes	
Control Group	DMEM/F12 media supplemented with 1% ITS and 1% P/S
Hypertrophy treatment 1	DMEM/F12 media supplemented with 100 μM phenylephrine
Hypertrophy treatment 2	DMEM/F12 media supplemented with 10% FBS and 1% P/S
Cardiac Fibroblasts	
Control group	DMEM/F12 media supplemented with 10% FBS and 1% P/S
Hypertrophy treatment 1	DMEM/F12 media supplemented with 10% FBS, 1% P/S, and 50 ng/mL transforming growth factor-β (TGF-β)

Cells were treated for 48 hours then harvested to allow sufficient time for drug-treatment to elicit a response.

2.1.4.2 Probing for syntaxin 1A, SNAP25 and pre-pro-ANPs

24 hours following isolation, 1 day and 7 day neonatal atrial and ventricular cardiac myocytes were treated for 48 hours in DMEM/F12 media supplemented with 1% ITS and 1% P/S. Cells were then harvested and used for immunoblotting.

2.2 Tissue Isolation

Adult atria and ventricle tissue were isolated from approximately three month old Sprague-Dawley rats. Similar protocol was followed for the excision of neonatal atrial and ventricular tissue. Rats were anesthetized with AErrane isoflurane gas (Cat. #AHN3637, Baxter) prior to surgery. The desired tissue was then isolated and washed in phosphate buffer saline (PBS). Both atria and ventricle were separated and frozen in liquid nitrogen for future use.

2.3 Tissue Homogenization

Atria or ventricle tissue were cut into small pieces and treated with 100-500µL 1% Triton X-100 lysis buffer. Stock lysis buffer consisted of the following: 10 mL 1% Triton X-100, 1 tablet Complete, Mini, EDTA-free protease inhibitor cocktail (Cat. #04693159001, Roche Diagnostics) and 1 tablet PhosSTOP phosphatase inhibitor cocktail (Cat. #04906837001, Roche Diagnostics). Tissue was further minced in solution and transferred using a 1000 µL pipette to a glass homogenizer. Tissue was disrupted to liquid state. After homogenization, the tissue solution was transferred to an Eppendorff tube with a glass pipette and sonicated for 30 seconds. Tissue lysate was placed on ice for 20 minutes then spun at 120,000 rpm at 4°C. The protein-containing supernatant was collected into separate Eppendorff tubes.

2.4 Cell Harvesting

2.4.1 Using 1% Triton X-100 Lysis Buffer

Medium was removed and cells washed with fresh PBS three times. Cells were then scraped using a rubber scraper in 1 mL PBS per 10 mm dish and collected into Eppendorff tubes. The solution was allowed to spin at 1500 rpm for 5 minutes at room temperature. Following this, the supernatant was removed and the pellet re-suspended in 100 µL of lysis buffer. The resulting cell lysate was then vortexed and placed on ice for 20 minutes. Again, the lysate was spun at 120,000 RPM for 5 minutes at 4°C and the supernatant removed. Samples were stored at -20°C.

2.4.2 Using Sodium Dodecyl Sulfate (SDS)

Media was removed and cells washed with fresh PBS three times. Cells were then scraped with a rubber scraper in 100 µL 2x SDS per 10 mm dish, then transferred and re-suspended in an Eppendorff tube. If cells were harvested from 6-well or 12-well plates, 50 µL 2x SDS was applied. The resulting cell lysate was boiled for 10 minutes, and then spun at 120,000 RPM for 5 minutes at 4°C. The supernatant was then removed and stored at -20°C for future use.

2.5 Bradford Assay

2.5.1 Standard Bradford Assay

Standards were made with 1 µg/µL of bovine serum albumin (BSA) and double distilled water at concentrations of 1 µg/µL, 0.5 µg/µL, 0.25 µg/µL, 0.125 µg/µL, 0.0625 µg/µL, as well as a blank with double distilled water alone. Cell lysates were read at a dilution of 1:10 and tissue lysates read at a dilution of 1:50. 1 mL of Bio-Rad Protein Assay Dye Reagent Concentrate (Cat. #500-0006, Bio-Rad Laboratories) was added to 20 µL of each sample in polystyrene cuvettes. Protein concentrations were then read at 595 nm using a Beckman DU-640 spectrophotometer (Beckman Coulter, Brea CA, USA).

2.5.2 Detergent-compatible (DC) Bradford Assay

Standards were made with 1 µg/µL of BSA and double distilled water at concentrations of 1 µg/µL, 0.5 µg/µL, 0.25 µg/µL, 0.125 µg/µL, 0.0625 µg/µL, as well as a blank with double distilled water alone. Cell lysates were read at a dilution of 1:10. Protein concentrations were determined using a Detergent Compatible (DC) Protein Assay Kit II (Cat. #500-0112, Bio-Rad Laboratories). Reagent S (Cat. #500-0115, Bio-Rad Laboratories) was diluted in Reagent A (Cat. #500-0113, Bio-Rad Laboratories) at 20 µL: 1 mL. 100 µL of this solution and 800 µL of Solution B (Cat. #500-0014, Bio-Rad Laboratories) was added to each sample in polystyrene cuvettes and read at 595 nm on the spectrophotometer mentioned above.

2.6 Western Blotting

Samples were diluted to the desired loading concentration using Triton X-100 lysis buffer (10 mL 1% Triton X-100, 1 tablet Complete, Mini, EDTA-free protease inhibitor cocktail (Cat. #04693159001, Roche Diagnostics) and 1 tablet PhosSTOP phosphatase inhibitor cocktail (Cat. #04906837001, Roche Diagnostics)) and SDS.

10% and 15% polyacrylamide gels were made from solutions consisting of double distilled water, 30% acrylamide mix, 1.5M Tris (pH 8.8), 10% SDS, 10% ammonium persulfate, and TEMED (tetramethylethylenediamine). Proteins were resolved by SDS-PAGE using the Mini Trans-Blot Cell (Cat. #170-3989 - Bio-Rad Laboratories) for 1 hour at 100-150V. 20 µg of sample were loaded alongside a protein ladder. Gels were run in running buffer consisting of 25 mM Tris base, 190 mM glycine, and 0.1% SDS. Following separation, proteins were transferred onto either Immun-Blot polyvinylidenedifluoride-plus (PVDF) membranes (Cat. #162-0177, Bio-Rad Laboratories) for chemiluminescent detection or Immobilon-FL PVDF membranes (Cat. #IPFL00010 - EMD Millipore) for fluorescent detection, for one hour at 100V. Transfer of protein was done in transfer buffer consisting of 25mM Tris base, 192 mM glycine, and 20% methanol. The membrane was then washed in TBST consisting of: 20mM Tris base, 137 mM NaCl and 0.1% Tween 20, three times. One of the two following protocols was then followed:

2.6.1 Antibody Incubation - Chemiluminescent Detection

Post-transfer, the PVDF membrane was exposed to blocking buffer (1 g skim milk powder diluted in 20 mL TBST) for one hour, and then washed three times for five minutes with fresh TBST. Following this, the membrane was washed with primary antibody for another hour. The membrane was again washed three times for five minutes with fresh TBST. Finally, the membrane was probed with the corresponding anti-mouse or anti-rabbit secondary antibody (1:40,000) for one hour. A list of primary and secondary antibodies and their corresponding dilution factor is listed in Appendix A. The probed membrane was again washed three times for five minutes with TBST. Thereafter, 1 mL of ECL (1:1 mixture of Western Lightning Plus-ECL Enhanced Luminol Reagent Plus to Western Lightning Plus-ECL Oxiding Reagent Plus (Cat. #50-904-9326, Thermo Fisher Scientific)) was added to the membrane for one minute.

The membrane was then placed inside a cassette within a polypropylene sheet protector and brought to a dark room for development. A sheet of Diamed Western Blotting Film (Cat. #DIAFILM810, Diamed Lab Supplies) was placed on top of the membrane for the desired exposure time and developed. All dark rooms were exposed to small amounts of red light for visual assistance.

2.6.2 Antibody Incubation - Fluorescent Detection

The membrane was exposed to blocking buffer (1:1 mixture of PBS to Odyssey Blocking Buffer - Cat. #927-40000, LI-COR) for one hour, then washed three times for five minutes with fresh TBST. Following this, the membrane was washed with primary antibody for another hour. The membrane was again washed three times for five minutes with fresh TBST. Finally, the membrane was probed with the corresponding anti-mouse or anti-rabbit secondary antibody (1:10,000) for one hour in light-sensitive containers (Cat. #MTCB1200-7BK - Diamed Lab Supplies). A list of secondary antibodies and their corresponding dilution factor is listed in Appendix A. The probed membrane was washed again with TBST three times. Thereafter, the membrane was placed onto a LI-COR Odyssey B446 Infrared Imaging System (LI-COR, Lincoln NE, USA) connected to Image Studio Software (LI-COR, Lincoln NE, USA). A

picture was scanned of the membrane at 700 nm and 800 nm channels. Exposure levels were adjusted and channels distinguished on Image Studio for a clear picture of the desired bands. All membranes were dried out following development and placed in a transparent sheet for future use.

Densitometry was conducted utilizing the software ImageJ (National Institutes of Health, USA).

2.7 Transfection

Cells were plated to 12-well plates in their corresponding media overnight. If at least 60% confluency was reached, transfection took place. Plasmids containing promoter DNA were prepared at 0.5 µg per 1 mL Opti-Mem media. 2 µL of Lipofectamine 2000 per 1 µg of DNA was added to the mixture and incubated at room temperature for 30 minutes. Before adding Opti-Mem media, media was removed and cells were washed with 1 mL sterile PBS. Cells were then incubated in Opti-Mem containing its corresponding DNA for four hours to allow for sufficient transfection of cells.

After this period, Opti-Mem media was replaced with treatment media for 48 hours to allow the treatment to elicit an effect. Media was replaced every 24 hours. The supernatant of these cells was then collected and frozen at -20°C for future use.

2.7.1 Promoter activity: syntaxin 1A and SNAP25

Cells were transfected with one of three pEZX-PG04 vectors containing the desired promoter sequences: syntaxin 1A (brain) - 1050 bp long (Cat. #MPRM26278-LvPG04, Genecopeia), synaptosomal-associated protein 25 (SNAP25) - 1287 bp long (Cat. # MPRM14574-LvPG04, Genecopeia), or an “empty” vector lacking a promoter sequence. All reporter clones contain Gaussia luciferase (GLuc) downstream of the promoter, and the internal control, secreted alkaline phosphatase (SeAP) downstream of GLuc.

2.7.2 Plasmids - Botulinum neurotoxins

Cells were transfected with plasmid DNA for botulinum neurotoxin A (BoNT/A), botulinum neurotoxin C (BoNT/C), or green fluorescent protein (GFP) as a control to determine transfection efficiency. BoNT/A and BoNT/C cleave SNAP25 and syntaxin 1A, respectively. After 48 hours in treatment media, post-transfection, a secretion assay was done on the cells, and the supernatant collected for enzyme-linked immunosorbent assay (ELISA) analysis.

2.7.3 FOR/PMA Treatment (following transfection)

After incubating with DNA, Opti-Mem media was replaced with DMEM/F12 media supplemented with 1% ITS and 1% P/S, as well as 10uM forskolin and 20 nM phorbol 12-myristate 13-acetate (FOR/PMA) and incubated for 24 hours.

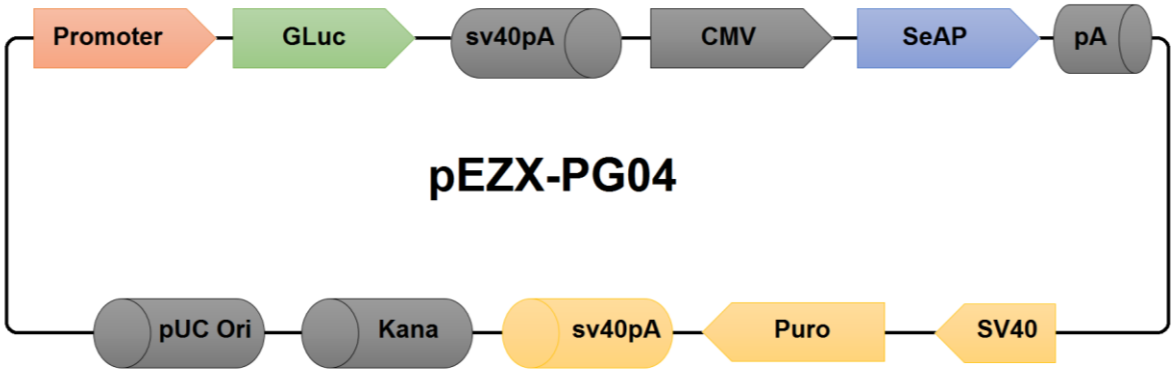


Figure 8. PeZX-PG04 vector containing fused promoter sequences for syntaxin 1A or SNAP25, obtained from Genecopeia. All three vectors obtained from Genecopeia contain Gaussia luciferase (Gluc) immediately downstream of the promoter and secreted alkaline phosphatase (SEAP) downstream of GLuc, acting as an internal control. Promoter activity was measured as a ratio of GLuc/SEAP. An "empty" vector, lacking a promoter sequence, was used a negative control.

2.8 GLuc/SEAP Secreted Luciferase Assay

Samples were thawed from storage at -20°C before use. The supernatant collected from cells transfected with a SNARE protein promoter or negative control clone were analyzed using a Secrete-Pair Dual Luminescence Assay Kit (Cat. #SPDA-D100, Genecopeia). Briefly, a GLuc Assay Working Solution was prepared to dilute the enzyme stimulating luminescence of Gaussia luciferase (Substrate GL-S). This buffer was comprised of a 1:10 dilution of Genecopeia's stock GL-S Buffer in double distilled water. Substrate GL-S was then diluted 1:100 in the buffer, protected from light, and incubated for 25 minutes at room temperature. 10 µL of each sample were then added to luminometer tubes in duplicates and the corresponding samples read using a EG&G Berthold - Lumat LB 9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The luminometer's automatic injector was then primed with the GLuc Assay Working Solution and set at the following parameters: 100 µL of injection, 60 seconds delay, 2 seconds integration, followed by measurement of activity.

Afterward, the collected supernatant was heated to 65°C for 15 minutes, and cooled to 4°C in preparation for SeAP protocol. SeAP Assay Working Solution was prepared to dilute the enzyme stimulating luminescence of SeAP (Substrate AP). This solution was diluted 1:10 using Genecopeia's stock AP Buffer in double distilled water. Substrate AP was diluted 1:100 in ithe buffer, protected from light, and incubated for 5-10 minutes at room temperature. 10 µL of each sample were then added to luminometer tubes and read using the EG&G Berthold - Lumat LB 9507 luminometer. The luminometer's automatic injector was then primed with the SeAP Assay Working Solution and set at the following parameters: 100 µL of injection, 5 minutes delay, 2 seconds integration, followed by measurement of activity.

SeAP was used to remove the impact of transfection efficiency variation across multiple cell types. Thus, the analysis of luciferase readings were graphed as relative lights units (RLU) of GLuc/SEAP.

2.9 ANP Secretion Assay

Myocytes were plated into either 6 well plates or 12 well plates post-isolation. They were then treated for 48 hours in their corresponding media to allow cells to equilibrate with their environment. Cells were washed with PBS and treated with 1 mL of Krebs Ringer Bicarbonate (KRB) buffer (129mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5mM $NaHCO_3$, 10mM HEPES, and 0.1 g of bovine serum albumin, pH 7.4) with or without 30 nM endothelin-1 (ET-1) for 2 hours. ET-1 was used to stimulate ANP secretion in the treated myocytes. Post-treatment, the supernatant was removed and frozen at -20°C.

2.10 ELISA Assay

Supernatant collected from the ANP secretion assay was thawed from -20°C and probed using a pre-coated ELISA kit (Cat. #EK-005-24, Phoenix Pharmaceuticals, Burlingame CA, USA) targeted for atrial natriuretic peptide (1-28 amino acids, specific for rat and mouse). The kit utilizes the principles of a competitive ELISA. The assay is standardized using standard peptide provided by the company and graphed using a four parameter logistic curve. The plate itself is pre-coated with secondary antibody which binds to the F_c component of the kit's primary antibody. The antigen-binding component of the primary antibody (F_{ab}) is then free to bind to either the biotinylated peptide or the targeted peptide, ANP. When the biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP), these samples undergo an observable change in colour from clear to yellow depending on the proportion of biotinylated peptide to targeted peptide bound. Absorbance was read using a DTX 880 Beckman Coulter 96-well plate Multimode Detector (Beckman Coulter, Brea CA, USA) at a wavelength of 450nm. The data obtained was then analyzed using ElisaAnalysis.com's on-line software at: *http://elisaanalysis.com* (Version 3.2) (Leading Technology Group, Australia).

2.11 Statistical Analysis

Each sample is equivalent to experiments done on cells collected from a litter of 15-20 rat pups. Sample size is therefore representative of the amount of litters used in the experiment.

Data was analyzed using Microsoft Excel (Microsoft, Redmond WA, USA) and graphed as mean ± standard error of the mean (SEM). Statistical analysis was conducted using IBM SPSS Statistics (Armonk NY, USA) software using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Significance was generally accepted at $*p<0.05$ and $**p<0.01$.

All statistically analyzed immunoblotting data are representative of at least three separate trials. Each trial of luciferase data is representative of the average of two biological replicates and two technical replicates. Graphed luciferase data is representative of at least three separate trials.

Results

3.1 Characterization of SNARE proteins involved in constitutive secretion

Natriuretic peptides are secreted via a regulated or constitutive pathway. Regulated secretion is stimulated by mechanical stretch and/or stimulation by neuroendocrine factors, while constitutive secretion encompasses the direct synthesis and secretion of the protein. The SNARE proteins involved in the regulated secretion of ANP have been characterized by previous groups (Peters *et al.* 2006; Ferlito *et al.* 2010) but the SNARE proteins involved in constitutive secretion have not yet been identified. Out of the SNARE and SNARE-associated proteins identified to be involved in constitutive secretion by Gordon and colleagues (2010), I chose to characterize the three SNARE proteins syntaxin 5A, syntaxin 18 and SNAP29. These proteins were chosen based on their role in different components of the vesicle trafficking pathway: syntaxin 5a for anterograde transport from the ER to the Golgi, syntaxin 18 for retrograde transport from the Golgi to the ER, and SNAP29 for post-Golgi to membrane transport.

Heart tissue was isolated from neonatal rats at 1 day, 7 days, and 14 days, as well as 3 month old adults and homogenized with lysis buffer containing Triton X-100 detergent. Using Western blot, tissue lysates were probed for each SNARE protein. In atrial tissue, syntaxin 5A levels significantly increased with age from 1 day ($p<0.01$) and 7 day ($p<0.01$) to adult (Figure 9a). In contrast, syntaxin 18 and SNAP29 protein levels significantly decreased from 1 day to adult ($p<0.01$, $p<0.05$) (Figure 9b,c).

Similar expression profiles were observed in ventricle tissue. Syntaxin 5A levels significantly increased in adults ($p<0.01$) relative to 1 day tissue (Figure 10a), syntaxin 18 significantly decreased in 14 day and adult tissue relative to one day ($p<0.05$, $p <0.05$) and 7 day ($p<0.01$,$p<0.01$) neonatal tissue (Figure 10c), and SNAP29 levels remained relatively constant with age (Figure 10e). There were no significant changes in SNARE protein expression from 1 day to 7 day myocytes. Fibroblasts did not express syntaxin 5A, but displayed similar expression to myocytes for syntaxin 18 and SNAP29 (Figure 10b,d,f).

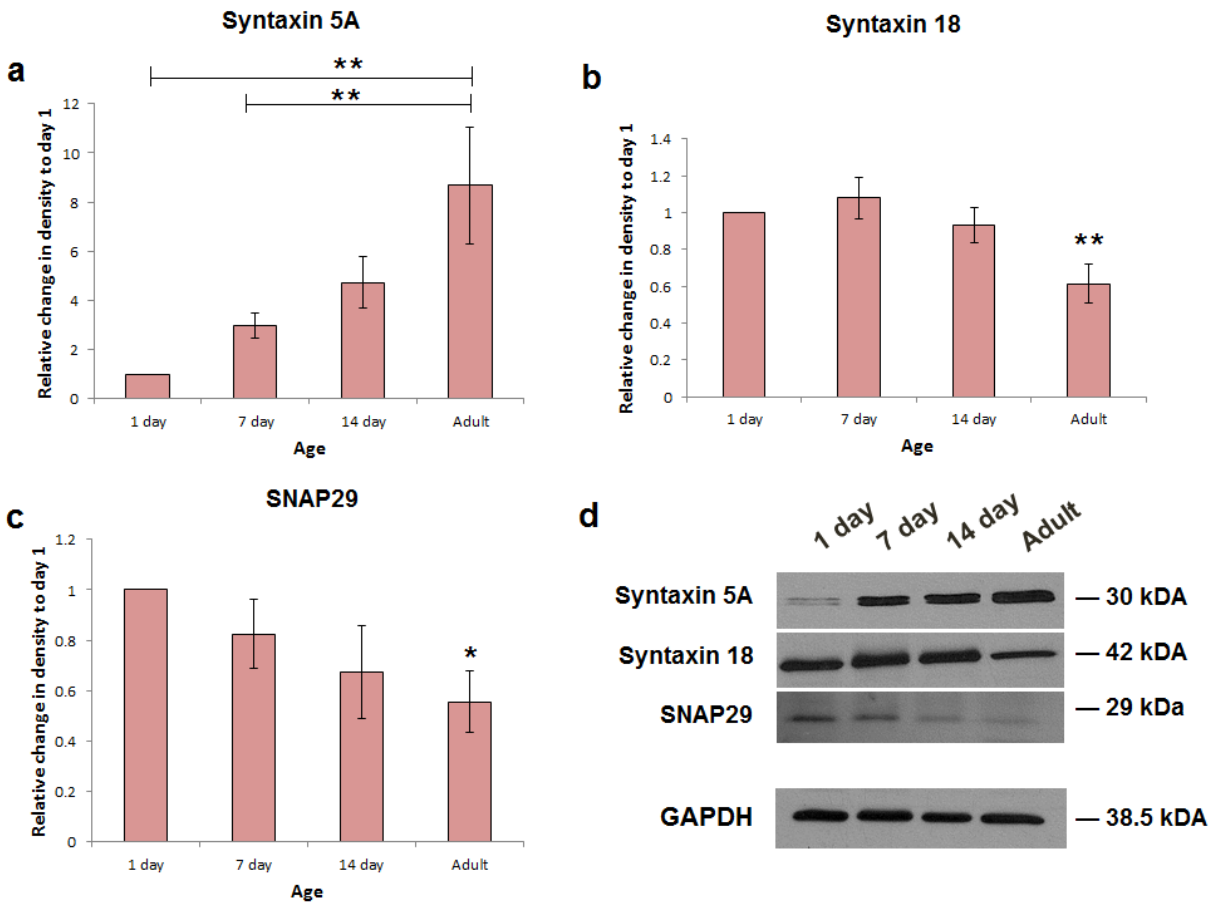


Figure 9. Expression profiles of syntaxin 5A, syntaxin 18, and SNAP29 normalized to 1 day for 1 day, 7 day, 14 day neonatal and adult atrial rat tissue. (a) There is a significant increase in syntaxin 5A protein levels at adult atrial tissue relative to 1 day ($p<0.01$) and 7 day ($p<0.01$) atrial tissue (n=7). **(b)** Syntaxin 18 expression levels decrease in adult atrial tissue relative to 1 day ($p<0.01$) atrial tissue (n=8). **(c)** SNAP29 levels display a significant decrease in adult atrial tissue relative to 1 day ($p<0.05$) atrial tissue (n=4). **(d)** 1 day, 7 day, 14 day and adult atrial tissue probed by Western blot for SNARE proteins Syntaxin 5A, Syntaxin 18, SNAP29 and loading control, GAPDH.

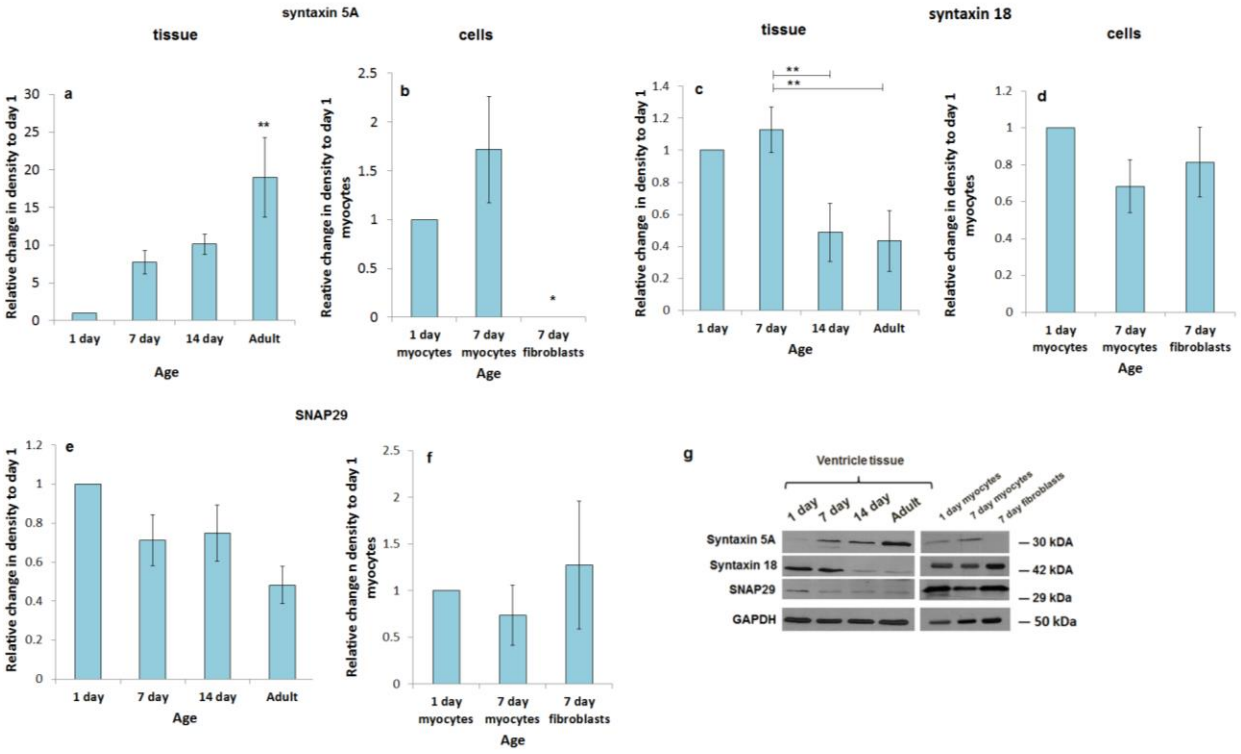


Figure 10. Expression profiles of Syntaxin 5A, Syntaxin 18, and SNAP29 normalized to 1 day for 1 day, 7 day, 14 day neonatal and adult ventricle rat tissue. Cells are normalized to 1 day myocytes for 1 day, 7 day myocytes, and 7 day fibroblasts. (a) There is a significant increase in Syntaxin 5A protein levels in adult ventricle tissue relative to 1 day ($p<0.01$) (n=6). **(b)** Syntaxin 5A levels display a general increase from 1 day to 7 day ventricle myocytes, and is absent in 7 day fibroblasts (n=6). **(c)** There is a significant decrease in Syntaxin 18 protein levels from 7 day ventricle tissue to 14 day ($p<0.01$) and adult ($p<0.01$) ventricle tissue, and 1 day ventricle tissue to 14 day ($p<0.05$) and adult ($p<0.05$) ventricle tissue (n=6). **(d)** Syntaxin 18 levels remain relatively constant between cell types (n=6). **(e)** SNAP29 levels are relatively constant across age (n=6). **(f)** There are no significant changes in SNAP29 levels across cell types (n=6). **(g)** 1 day, 7 day, 14 day neonatal and adult ventricle tissue as well as 1 day, 7 day myocytes, and 7 day fibroblasts probed by Western blot for SNARE proteins syntaxin 5A, syntaxin 18, SNAP29 and loading control, GAPDH.

3.2 Characterization of syntaxin 5A, syntaxin 18 and SNAP29 during hypertrophy

In correspondence with my hypothesis, if syntaxin 5A, syntaxin 18 and SNAP29 are indeed involved in the constitutive secretion pathway, this should be reflective in their response to hypertrophy. As the ventricle is the main site of constitutive secretion, ventricular myocytes and fibroblasts were isolated from trypsinized tissue and incubated for 24 hours. Post-incubation, myocytes were cultured for 48 hours in three conditions: control medium (DMEM/F12, 1% P/S solution, 1% Insulin-Transferrin-Selenium), control medium with 100μM phenylephrine (made in 1 mM ascorbic acid), and control medium with 10% FBS. Phenylephrine, an alpha-adrenergic agonist and fetal bovine serum both stimulate cell growth and/or contain growth factors, inducing hypertrophy in cardiac myocytes. Following treatment, cells were harvested and probed by Western blot. Fibroblasts were cultured for 48 hours in one of two conditions: control medium (DMEM/F12, 1% P/S solution, 10% FBS) or control medium with 50ng/mL of TGF-β. Treatment with TGF-β stimulates the phenotypic change of fibroblasts to myofibroblasts, characteristic of these cells during hypertrophy and cardiac disease.

As expected, 7 day ventricular myocytes displayed a general increase in pre-pro-ANP and pre-pro-BNP expression levels after treatment with phenylephrine or FBS (Figure 11d,e). This homeostatic response is expected as an increase in natriuretic peptide secretion is a natural response to hypertrophy in cardiac myocytes. Surprisingly, a significant decrease in syntaxin 5A ($p<0.05$) (Figure 11) expression was observed post-drug treatment. A slight increase in syntaxin 18 (Figure 11) expression was also observed in cells undergoing FBS-induced hypertrophy. It is worth mentioning that there are clear differences in SNARE protein expression between myocytes treated with FBS versus myocytes treated with phenylephrine, although both were used to induce hypertrophy. Despite the significant increase in pre-pro-ANP ($p<0.05$) and general increase in pre-pro-BNP following both treatments, FBS treated cells generally displayed more pronounced changes in SNARE protein expression. This may be due to the different cellular mechanisms in which phenylephrine and FBS induce cell growth. In terms of SNAP29, expression levels were approximately the same (Figure 11c).

Finally, syntaxin 5A and pre-pro-ANP were absent in fibroblasts in both control and drug treatment. The absence of pre-pro-ANP conflicts with previous studies done by Cameron and colleagues (2000), in which pre-pro-ANP was expressed in sheep cardiac fibroblasts after treatment with TGF-β. Our findings suggest that pre-pro-ANP is not secreted in rat cardiac fibroblasts during hypertrophy (Figure 11d). Pre-pro-BNP is expressed in fibroblasts but does not change with transformation to myofibroblasts (Figure 11e). Although syntaxin 18 and SNAP29 were expressed in fibroblasts, they did not display any significant changes in protein expression between control cells and those treated with TGF-β (Figure 11a,b).

The biologically active form of ANP is cleaved on the membrane of the cardiac myocyte by the serine protease, corin (Rushkoaho 2003). To corroborate that the increase in pre-pro-ANP levels (observed by Western blot) within the cell corresponds with an increase in ANP levels outside the cell, a secretion assay and competitive ELISA were used. 7 day ventricular myocytes were treated with either 1% insulin-transferrin-selenium (ITS) as a control or 10% FBS to induce hypertrophy. Cells were then treated with 30 nM endothelin-1 to stimulate natriuretic peptide secretion. As predicted, an increasing trend in ANP secretion is observed in FBS treated cells (Figure 12), similar to the increase seen in pre-pro-ANP levels by Western blot (Figure 11d). This increase is even more pronounced in cells treated with endothelin-1 (Figure 12).

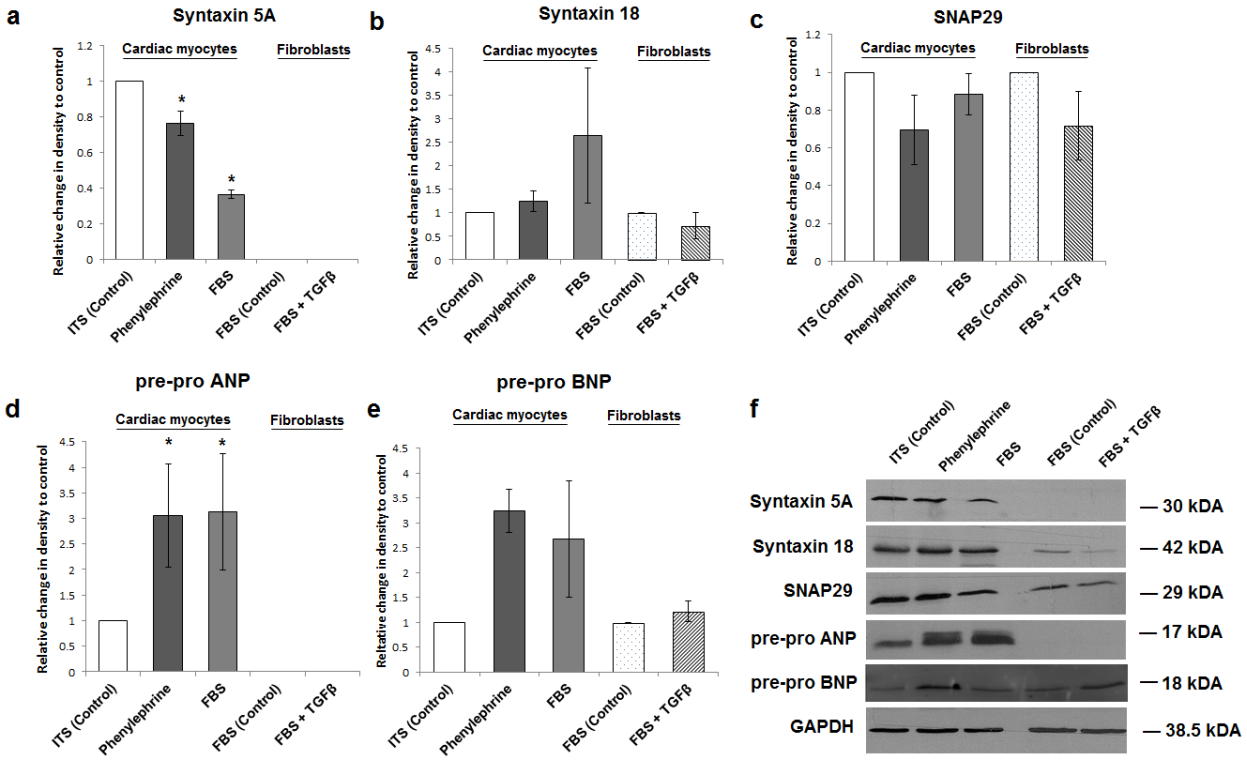


Figure 11. Expression profiles of syntaxin 5A, syntaxin 18, SNAP29, pre-pro ANP, and pre-pro BNP normalized to control groups for 7 day neonatal ventricular rat cardiac myocytes treated with 100μM phenylephrine or 10% fetal bovine serum, and 7 day neonatal ventricular rat cardiac fibroblasts treated with 50 ng/mL TGF-β. Cells were treated for 48 hours with their corresponding treatment (n=3). **(a)** There is a significant decrease in syntaxin 5A levels following treatment with phenylephrine ($p<0.05$) or FBS ($p<0.05$) (n=3). An absence of syntaxin 5A expression is observed in fibroblasts. **(b)** A general increase in syntaxin 18 is observed in FBS-treated myocytes. No significant changes are observed in fibroblasts treated with TGF-β relative to control fibroblasts. **(c)** There are no significant changes in SNAP29 levels between control cells and their corresponding treatments. **(d)** With both FBS ($p<0.05$) and phenylephrine ($p<0.05$) treated myocytes, a significant increase is observed in pre-pro ANP levels (n =3). An absence in expression is displayed in fibroblasts. **(e)** A general increase in pre-pro BNP levels is observed following treatment with phenylephrine or FBS. Pre-pro BNP levels do not change significantly in fibroblasts with TGF-β treatment. **(f)** 7 day ventricular myocytes treated with ITS (control), phenylephrine or FBS and 7 day fibroblasts treated with FBS (control) or FBS and TGF-β probed by Western blot for SNARE proteins syntaxin 5A, syntaxin 18, SNAP29, pre-pro-ANP, pre-pro-BNP and loading control, GAPDH.

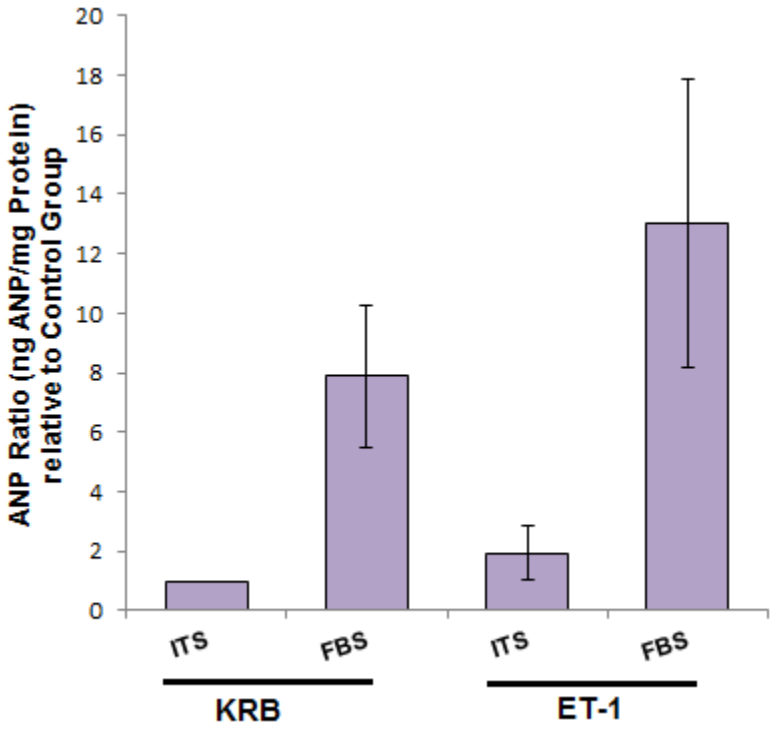


Figure 12. ANP secretion in 7 day control and hypertrophy-induced rat ventricular myocytes treated with Krebs-Heinslett Buffer (KRB) and KRB supplemented with 30 nM endothelin-1 (ET-1). Cells were treated with ET-1 for 48 hours. Myocytes were treated with 1% insulin-transferrin-selenium (ITS) or 10% fetal bovine serum (FBS) to induce hypertrophy (n=3). An increasing trend was seen in both FBS treated groups relative to their corresponding controls. A more pronounced increase was observed in cells treated with ET-1, a potent stimulator of natriuretic peptide secretion.

3.3 Characterization of syntaxin 1A and SNAP25 expression

In 2006, Peters and colleagues were the first to identify the presence of two SNARE complexes in rat atrial myocytes associated with ANP secretion: one found in both neonates and adults, and another found solely in adults, consisting of syntaxin 1A and SNAP25. The presence of a SNARE complex already established in both neonatal and adult myocytes brings into question the importance of a secondary SNARE complex. This said complex must thus have an important age-dependent function that has not yet been brought to light. Before studying the functional role of syntaxin 1A and SNAP25, I first characterized their expression profiles in the atria and ventricle to obtain an idea of their distribution in rat cardiac tissue.

Using immunoblotting, atrial and ventricular myocytes isolated from 1 day and 7 day old rats, as well as tissue isolated from 14 day and adult rats were probed for syntaxin 1A and SNAP25. A general increase was observed in syntaxin 1A protein levels in 14 day and adult atrial tissue relative to 1 day and 7 day myocytes. Syntaxin 1A was also present in the ventricle but did not significantly change (Figure 13a). SNAP25 protein levels were only detected in adult atrial tissue, but not in ventricle tissue (Figure 13b). Pre-pro-ANP was found to significantly increase with age as predicted in the atria, although levels were too low in the lysates of 1 day and 7 day myocytes to be detected via Western blot. In the ventricle, pre-pro-ANP levels were too low to be detected in both cells and tissue (Figure 13c). This is expected as the atrium is the main site of natriuretic peptide synthesis at normal physiological states. These trends were inferred from a sample size of one. As so, these findings would have to be replicated to observe any significant changes.

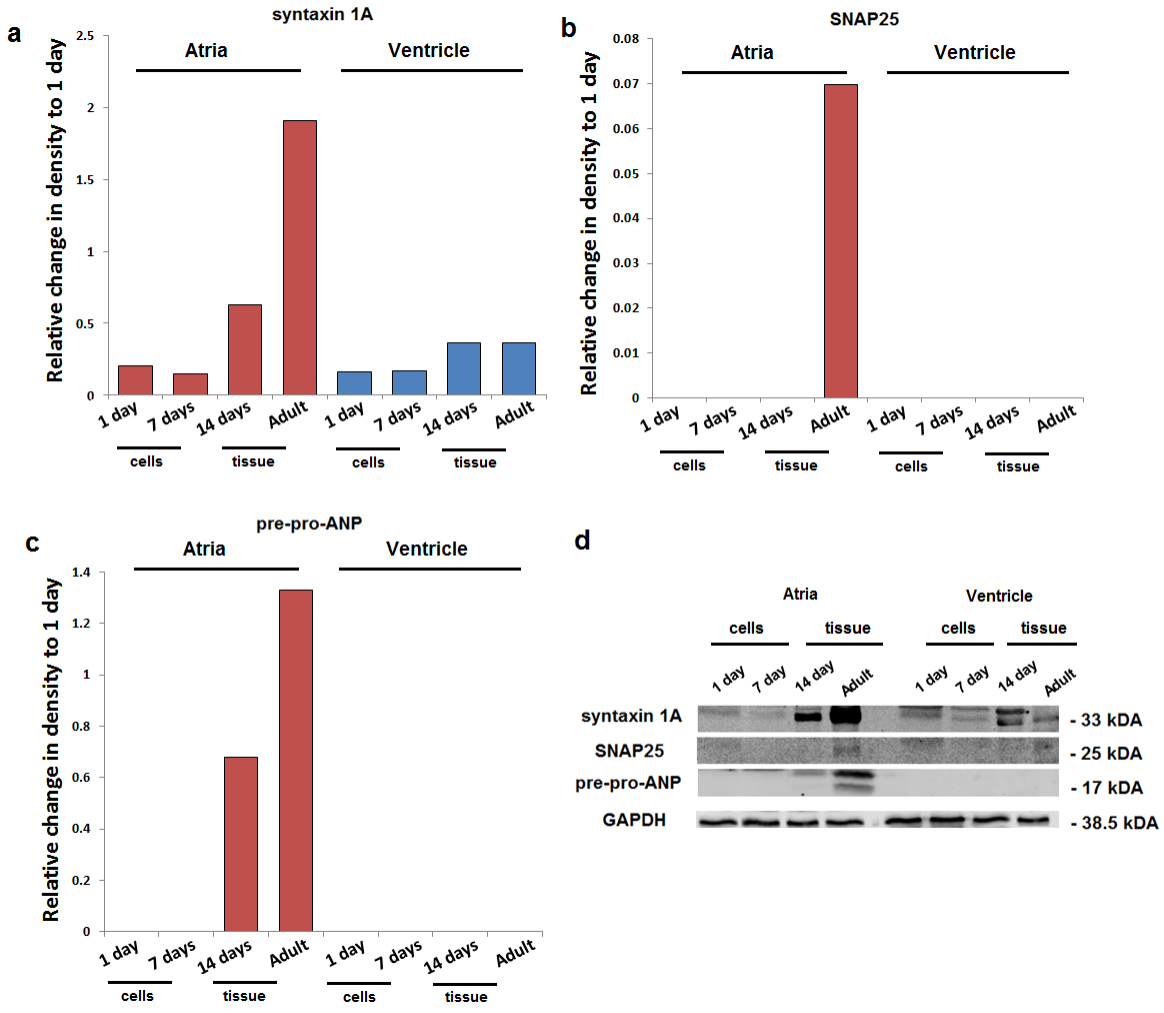


Figure 13. Expression profiles of syntaxin 1A, SNAP25, and pre-pro ANP in atria and ventricle, normalized to 1 day for 1 day and 7 day myocytes, and 14 day and adult rat tissue. (a) There is a general increase in syntaxin 1A protein levels in 14 day and adult atrial and ventricular tissue relative to 1 day and 7 day atrial and ventricular myocytes (n=1). **(b)** SNAP25 protein is observed in adult atrial tissue alone. SNAP25 levels are absent in both ventricular myocytes and ventricle tissue (n=1). **(c)** There is a general increase in pre-pro-ANP levels in 14 day and adult atrial tissue relative to 1 day and 7 day atrial myocytes. Pre-pro-ANP is not observed in ventricle myocytes and tissue (n=1). **(d)** 1 day, 7 day myocytes, and 14 day, adult tissue from both atria and ventricle probed by Western blot for SNARE proteins syntaxin 1A, SNAP25, pre-pro-ANP and loading control, GAPDH (n=1).

3.4 Transcriptional activity of syntaxin 1A and SNAP25 in atrial and ventricular cells

After characterizing the expression profiles of syntaxin 1A and SNAP25, I also endeavoured to look into the transcriptional activity of their corresponding genes. Comparing promoter activity to protein expression profiles may provide insight into the regulation of syntaxin 1A and SNAP25 in both the atria and ventricle. Furthermore, the identification of chamber-specific differences could also be suggestive of their role in natriuretic peptide secretion.

Primary atrial and ventricular myocytes were isolated from 1 day and 7 day neonatal rats and transfected with plasmid DNA containing the two SNARE's corresponding promoters. A secreted luciferase assay was used to measure transcriptional activity. Syntaxin 1A displayed no significant changes in promoter activity between atrial and ventricular myocytes, and across 1 day to 7 day myocytes. There were also no significant changes in SNAP25 promoter activity between atria and ventricle in 7 day myocytes. However, SNAP25 promoter levels in 7 day atrial and ventricular myocytes were significantly lower than 1 day atrial myocytes ($p<0.01$, $p<0.01$). In both atria and ventricle, syntaxin 1A promoter activity doubles that of SNAP25 (Figure 14a).

In addition to primary myocytes, two cell lines were used to study the transcriptional activity of syntaxin 1A and SNAP25: HL-1 cells were used to study promoter activity in atrial cells, while H9c2 cells were used to study promoter activity in ventricular cells. The immortalized HL-1 cell line is obtained from an atrial tumour, while the H9c2 cell line is obtained from the ventricle of the embryonic rat heart (Parameswaran *et al.* 2013). These cell lines are primarily used because they share similar properties and phenotypes to cardiac myocytes.

Transfected HL-1 and H9c2 cells displayed similar trends to their primary cardiac myocyte counterparts in terms of relative syntaxin 1A to SNAP25 activity. Syntaxin 1A displayed two-fold greater promoter activity when compared to SNAP25. In terms of SNARE-specific trends, however, there were no significant changes. No significant differences were observed in syntaxin 1A and SNAP25 promoter activity relative to MIN6 cells (Figure 14b).

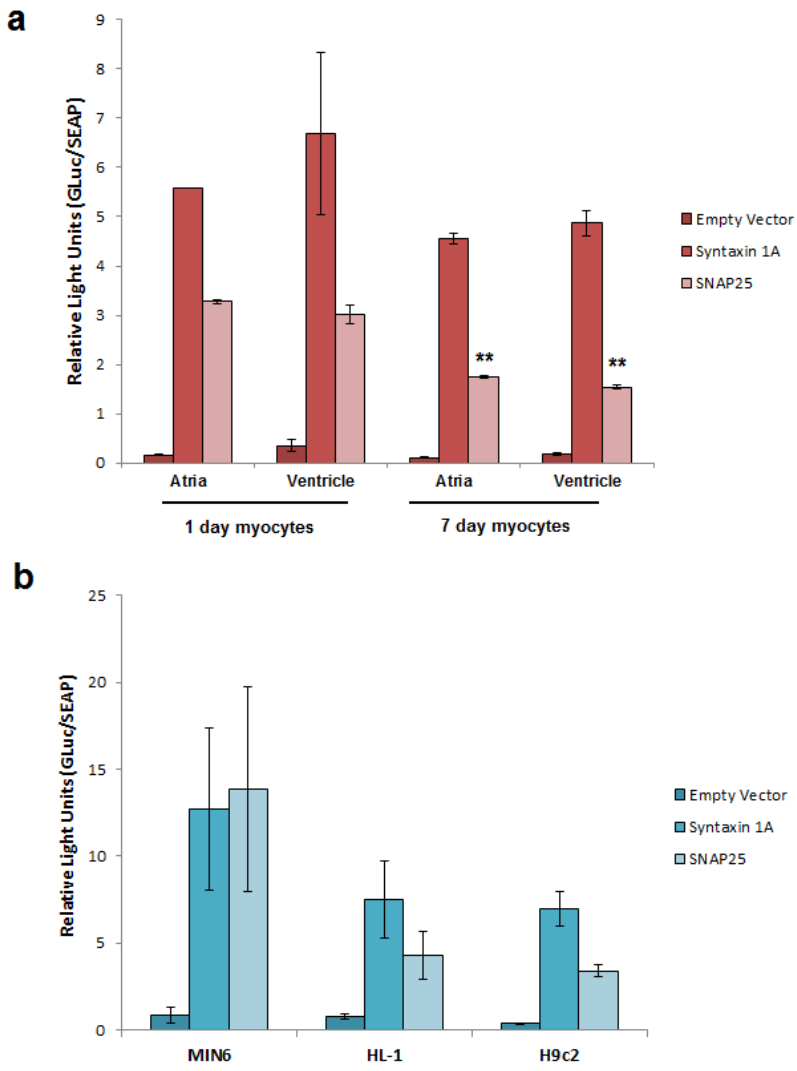


Figure 14. Transcriptional activity of syntaxin 1A and SNAP25 in 1 day and 7 day neonatal atrial and ventricular myocytes, HL-1 and H9c2 cell lines. Expression of syntaxin 1A and SNAP25 was determined using secreted luciferase reporter genes normalized to an empty vector. MIN6 cells were used as a positive control (n=3). In all cells studied, with the exception of MIN6, syntaxin 1A displays greater promoter activity compared to SNAP25. **(a)** There were no significant differences in syntaxin 1A promoter activity between atrial and ventricular myocytes. There were also no significant differences in SNAP25 promoter activity between atria and ventricle. However, SNAP25 promoter levels were significantly lower in both atrial and ventricular myocytes than the corresponding promoter activity in 1 day atrial myocytes ($p<0.01$, $p<0.01$) (n=3).**(b)** Furthermore, no significant differences in syntaxin 1A and SNAP25 are observed across the two cell lines, HL-1 and H9c2 (n=3).Similar promoter activity is observed for both syntaxin 1A and SNAP25, as MIN6 cells used in this study as a positive control.

3.5 Effects of BoNT/A and BoNT/C on SNARE protein expression and ANP secretion

To investigate the functional importance of syntaxin 1A and SNAP25 in atrial natriuretic peptide secretion, botulinum neurotoxins were used to cleave the above-mentioned SNARE proteins. 7 day neonatal atrial and ventricular cardiac myocytes were isolated and transfected with plasmid DNA for BoNT/A and BoNT/C, cleaving SNAP25 and syntaxin 1A, respectively. Cells were stimulated with 30 nM endothelin-1 to increase natriuretic peptide secretion. ANP levels were then measured using ELISA kits and the cell lysate probed using Western Blotting.

As this assay was only conducted once, no significant trends were extracted. The general trends were as follows: Relative to GFP control cells, cells transfected with BoNT/C, which cleaves syntaxin 1A, did not result in decreased ANP levels, nor did this pattern change with treatment of endothelin-1. In contrast, BoNT/A, which cleaves SNAP25, resulted in a decrease in ANP secretion in both atria and ventricle, with the exception of the atria control group. In ventricle cells, this decrease was further pronounced, relative to the GFP control group, in cells treated with endothelin-1 (Figure 15a). Immunoblotting analysis revealed that syntaxin 1A was indeed cleaved by BoNT/C in the atria relative to the control group transfected with GFP alone. In contrast, ventricular cells treated with BoNT/C did not display a decrease in syntaxin 1A levels (Figure 15b). SNAP25 levels were not detectable in 7 day myocytes using immunoblotting. Pre-pro-ANP levels also decreased in both atrial and ventricular myocytes treated with BoNT/A and BoNT/C, relative to the GFP control group (Figure 15c).

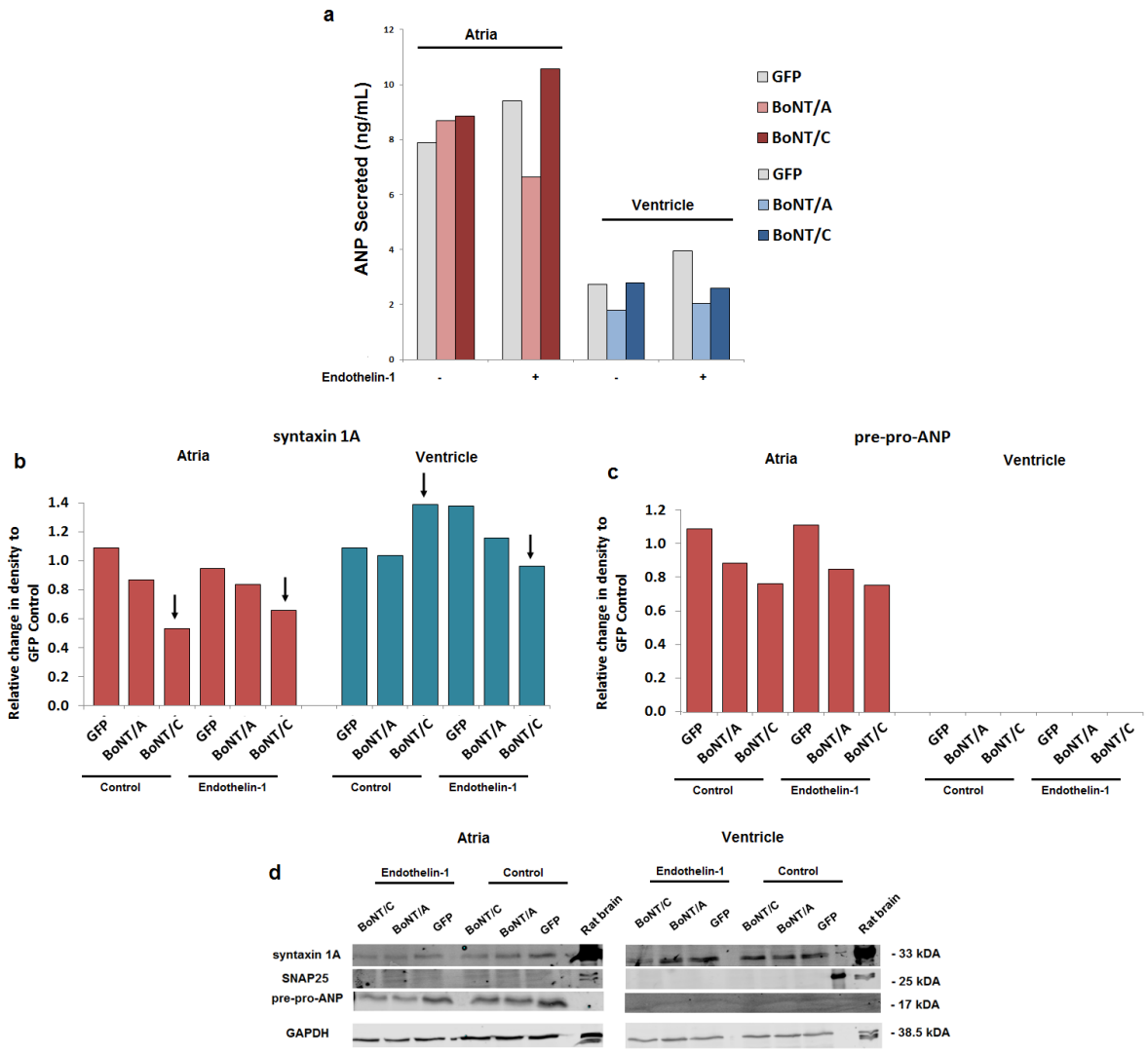


Figure 15. BoNT/A and BoNT/C decrease ANP secretion and pre-pro-ANP levels in 7 day atrial and ventricular rat myocytes. Cells were treated with Krebs-Heinslett Buffer (KRB) or KRB supplemented with 30 nM endothelin-1 (ET-1) for 48 hours (n=1). **(a)** A decrease in ANP secretion is observed in both atrial and ventricular myocytes treated with BoNT/A relative to GFP control cells, excluding atrial myocytes treated with KRB alone. Cells treated with ET-1 display a more pronounced decrease in ANP. No changes are observed in ANP secretion for both atrial and ventricular myocytes treated with BoNT/C relative to GFP control cells. **(b)** A decrease in syntaxin 1A expression is observed in 7 day myocytes treated with BoNT/C relative to GFP control cells. **(c)** Pre-pro-ANP levels decrease in 7 day myocytes treated with BoNT/C and BoNT/A relative to GFP control cells. **(d)** 7 day atrial and ventricular myocytes transfected with GFP, BoNT/A and BoNT/C then treated with KRB or KRB supplemented with ET-1, probed by Western blot for SNARE proteins syntaxin 1A, SNAP25, pre-pro-ANP and loading control, GAPDH (n=1).

3.6 Effect of FOR/PMA treatment on syntaxin 1A and SNAP25 transcriptional activity

As a follow-up to David Boyce, one of the former members of the Tsushima lab, I also investigated the importance of the CRE element in SNAP25 promoter activity. Using truncated promoters, David was able to display that the minimal promoter length for SNAP25 contains the CRE element and TATA box. As a follow-up study, I tested the importance of the CRE element by stimulating 7 day myocytes with 10uM forskolin and 20 nM phorbol 12-myristate 13-acetate (PMA). These compounds stimulate cAMP and PKA respectively, both of which are implicated to increase downstream binding of CREB to CRE. Treatment protocol was replicated from Shimada and colleagues (2007) who used FOR/PMA to stimulate the transcriptional activity of truncated SNAP25 promoters in ovarian cells.

In 7 day atrial and ventricular myocytes, there were no significant changes in syntaxin 1A and SNAP25 transcriptional activity following FOR/PMA treatment. SNAP25 promoter levels were noted to be significantly lower than syntaxin 1A promoter levels in atrial control myocytes. Although conducted on a sample size of three, no remaining significant changes were observed across treatment groups (Figure 16).

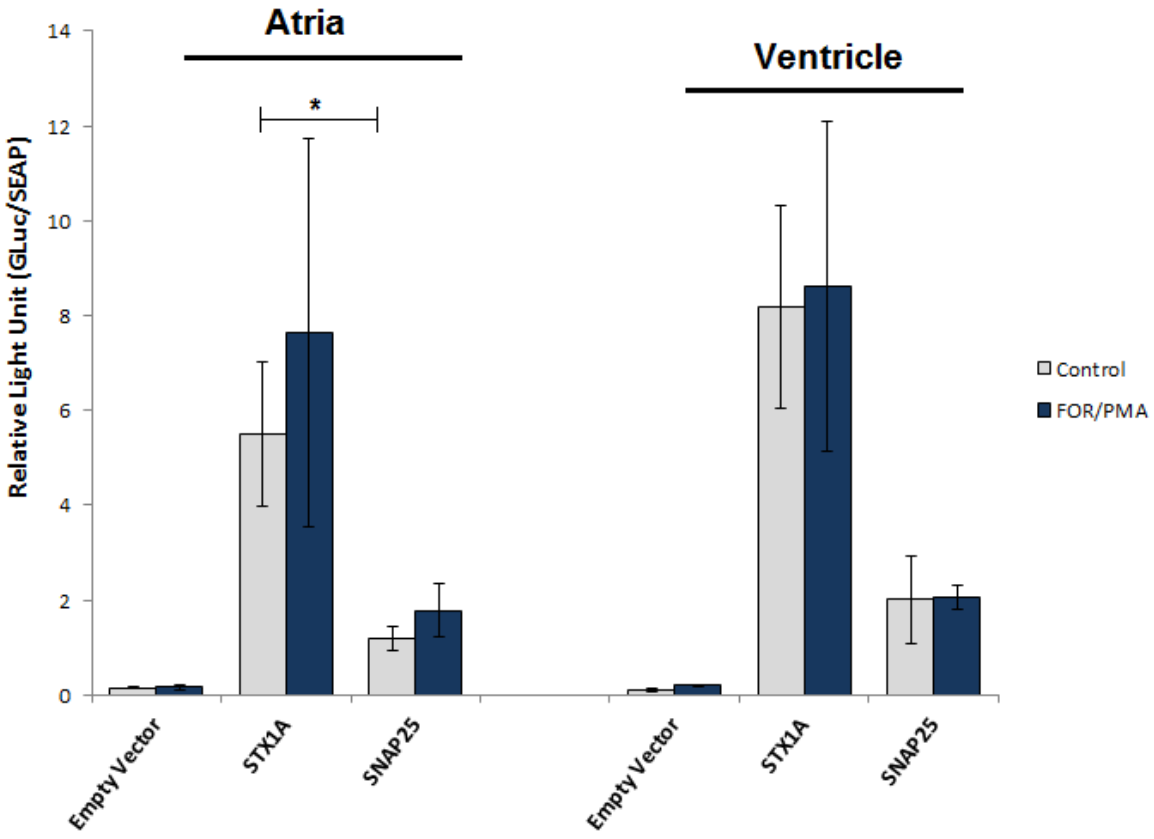


Figure 16. Transcriptional activity of syntaxin 1A (STX1A) and SNAP25 in 7 day ventricular rat myocytes treated with 10 μM forskolin and 20 nM phorbol 12-myristate 13-acetate (FOR/PMA) for 24 hours. SNAP25 promoter levels were noted to be significantly lower than STX1A promoter levels in atrial control myocytes ($p<0.05$) ($n=3$). However, no significant differences were observed in the remaining transfected cells post-treatment or between chambers.

Discussion

4.1 Role of syntaxin 5A, syntaxin 18, and SNAP29 in the constitutive secretion of ANP and BNP

The SNARE proteins I chose to investigate in the constitutive secretion of ANP and BNP were syntaxin 5A, syntaxin 18, and SNAP29. Syntaxin 5A was previously found to be important in the anterograde transport of peptides from the ER to the Golgi (Dascher *et al.* 1994), syntaxin 18 was previously found to be important in retrograde transport from the Golgi to the ER (Iinuma *et al.* 2009), and SNAP29 was previously found to be important for post-Golgi to membrane transport (Su *et al.* 2001).

Characterization of these SNARE proteins in atrial and ventricular tissue reveal expression patterns consistent with my original hypotheses (Figures 9 & 10). I expected that with an increase in pre-pro-ANP and pre-pro-BNP, observed with age, I would see a corresponding increase in syntaxin 5A, due to an increase in anterograde transport, a decrease in syntaxin 18, due to a decrease in retrograde transport, and an increase in SNAP29 due to an increase in vesicle traffic to the membrane. I hypothesized that increased natriuretic peptide secretion would result in the up-regulation of SNARE proteins involved in anterograde transport, as more vesicles would be required for natriuretic peptide secretion. In addition, less vesicle degradation would occur, resulting in decreased expression of the SNARE proteins involved in vesicle recycling and retrograde transport.

An age-dependent increase in syntaxin 5A expression as well as a decrease in syntaxin 18 expression was observed in atrial and ventricular tissue. SNAP29, on the other hand, displayed a decrease in atrial expression and no significant changes in the ventricle (Figures 9 & 10). There were also no significant differences in SNARE protein expression between atria and ventricle. This is contrary to what I would expect as the ventricle is the main site of constitutive natriuretic peptide secretion (Bloch *et al.* 1986). Thus, I would expect to see greater levels of syntaxin 5A and SNAP29, and lower levels of syntaxin 18 in the ventricle. The similarity in protein expression between the two chambers suggests that these three SNARE proteins are not involved in the constitutive secretion of ANP and BNP.

The expression of syntaxin 5A, syntaxin 18, and SNAP29 in ventricular myocytes was similar to that of the ventricle tissue. However, the degree of change in SNARE protein expression from 1 day to 7 day myocytes is small (Figure 10). It is important to keep in mind that the heart is composed of approximately 2/3 cardiac myocytes while the majority of the non-myocyte population consists of cardiac fibroblasts (Banerjee *et al.* 2007), and although the small change in SNARE protein expression does not exemplify the overall trend, this is but only a fraction of the total volume of myocytes in the heart. The absence of syntaxin 5A in fibroblasts suggests that this SNARE is not involved in ANP secretion within neonatal fibroblasts. However, syntaxin 18 and SNAP29 are both present (Figure 10b,d,f).

In our model of heart disease, hypertrophic myocytes and myofibroblasts did not display the exaggerated trends I predicted, but rather the opposite trends. Syntaxin 5A levels decreased, syntaxin 18 levels increased, and SNAP29 levels did not significantly change. These changes correlate with an increase in pre-pro-ANP and pre-pro-BNP in cardiac myocytes (Figure 11). An increase in ANP secretion post-treatment with fetal bovine serum was verified using an ANP-targeted ELISA (Figure 12). During hypertrophy, a phenomenon termed fetal gene reprogramming takes place. As indicated in *section 1.1.5*, this phenomenon is characterized by a switch in energy metabolism from carbohydrates to fats. Genes characteristic of the fetal register are expressed and adult genes are turned off (Rajabi *et al.* 2007). While the molecular mechanisms behind this phenomenon are not completely understood, epigenetics (i.e. methylation), transcriptional regulation, endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) are some of the many factors suggested in regulating the re-expression of these fetal genes (Vo and Hardy 2012).

I propose that the opposite trends in syntaxin 5A, syntaxin 18 and SNAP29 protein expression, observed with fetal bovine serum or phenylephrine treatment, may be due to fetal gene reprogramming. The genes responsible for the normal expression of these SNARE proteins are turning “off" or switching functions as a result of fetal genes turning on (Nakagawa *et al.* 1995). If this is the case and the genes encoding these SNARE proteins are being turned “off”, these genes are likely “adult” genes, suggesting that the traditional expression of these SNARE proteins is not vital to the constitutive secretion of ANP and BNP during fetal bovine serum or phenylephrine-induced hypertrophy.

The reason SNAP29 levels do not change with hypertrophy or age may be because the SNARE protein has multiple trafficking functions. In mast cells, SNAP29 is shown to localize at both the cytosol and membrane assisting with phagocytosis (Wesolowski *et al.* 2012). In neurons, SNAP29 localizes at the synapse and regulates the disassembly of the SNARE core complex (Pan *et al.* 2005). Other studies implicate SNAP29 to be localized to intracellular membrane structures within the cell during endocytic recycling of membrane receptors (Rapaport *et al.* 2010). A recent study done by Kang and colleagues in 2011 studied the function of SNAP29 in *C. elegans* and found that the protein is most likely involved in the fusion of Golgi vesicles to the recycling endosome. This same group also suggests that SNAP29 plays a role in secretion from intestinal cells. Despite all this, the role of SNAP29 within the cardiac myocyte has not previously been characterized. As a result, its function can only be inferred from previous studies. If SNAP29 is solely involved in the endosomal recycling pathway within the cardiac myocyte, it is not surprising that the trends observed to do not correlate with pre-pro-ANP or pre-pro-BNP levels within the cell. Instead, this post-Golgi SNARE may hold multiple trafficking functions within the cardiac myocyte which may or may not include the constitutive secretion of ANP and BNP. Further experiments would have to be conducted to test its functional importance.

Finally, syntaxin 5A and pre-pro-ANP were found to be absent in fibroblasts, in both control and after drug treatment (Figure 11). This suggests that syntaxin 5A may not play a role in the constitutive secretion of ANP and BNP in fibroblasts. However, the absence of pre-pro-ANP conflicts with previous studies done by Cameron and colleagues (2000), in which ANP was expressed in sheep cardiac fibroblasts after treatment with TGF-β. My findings could suggest that ANP is not secreted from rat myofibroblasts, or it could be the case that the dosage of TGF-β was not high enough to stimulate transformation of fibroblasts to myofibroblasts. The study conducted by Cameron and colleagues (2000) was also conducted on adult animals, while my study was conducted on neonates. A possible explanation for this discrepancy may be that there are changes to natriuretic peptide secretion from fibroblasts with age. In contrast to pre-pro-ANP and syntaxin 5A, pre-pro BNP, syntaxin 18 and SNAP29 levels were expressed in fibroblasts (Figure 11). Expression levels for all three proteins remained the same post-treatment. It may be possible that syntaxin 18 and SNAP29 assist in the trafficking of pre-pro-BNP, but

because their expression levels do not change with myofibroblast transformation, it is likely that syntaxin 18 and SNAP29 play other roles in vesicular trafficking within the cell.

There were no significant changes in syntaxin 5A, syntaxin 18 and SNAP29 expression between atria and ventricle. Furthermore, the expression of these three SNARE proteins post-treatment with fetal bovine serum or phenylephrine was opposite to the trends I expected. Taken together, my observations provide evidence towards the conclusion that these three SNARE proteins do not play a role in the constitutive secretion of ANP and BNP, and may be important in another protein trafficking pathway within the cardiac myocyte.

4.2 Role of syntaxin 1A and SNAP25 in regulated ANP secretion

The purpose of studying the transcriptional activity and expression profiles of syntaxin 1A and SNAP25 was to gain some insight on the chamber-specific role these two SNARE proteins play in the exocytosis of ANP. Having previously been suggested to form a complex important in ANP secretion (Peters *et al.* 2006), defining the age-dependent expression profiles of syntaxin 1A and SNAP25, and furthermore, the transcription of their corresponding genes could assist in suggesting a functional role for these SNARE proteins. Looking at chamber-specific differences may also provide insight into the localization of these SNARE proteins within the heart.

4.2.1 Chamber-specific trends in transcriptional activity and protein expression

The findings from my experiments confirm the discovery of Peters and colleagues (2006) that syntaxin 1A and SNAP25 are expressed at higher levels, if not solely in the adult atria compared to neonates. This increase corresponds with an increase in pre-pro-ANP levels in adult atrial tissue (Figure 13). Although there is little to no protein expression in neonates, promoter activity for both syntaxin 1A and SNAP25 is observed in both 1 day and 7 day atrial myocytes (Figure 14a).

Compared to MIN6 cells, our positive control which has been shown in previous studies to express both syntaxin 1A and SNAP25 (Ohara-Imaizumi *et al.* 2004), less promoter activity is observed in

primary neonatal myocytes. With this in mind, it is conceivable that the downstream peptide synthesis of syntaxin 1A and SNAP25 may be too low to significantly be detected via immunoblotting. If protein synthesis is indeed relative to transcriptional activity, then the absence of SNAP25 expression in the neonatal atria can also be explained relative to the expression of syntaxin 1A (Figure 13d), as syntaxin 1A transcriptional activity is approximately twofold greater than SNAP25 (Figure 14a).

In the ventricle, syntaxin 1A levels increase in 14 day and adult tissue relative to 1 day and 7 day neonatal myocytes. Its correlation with pre-pro-ANP expression was not established as pre-pro-ANP levels were not high enough to be detected via immunoblotting (Figure 13c). This is presumably due to the ventricle's little contribution to natriuretic peptide secretion during normal physiological conditions. Most natriuretic peptide secretion occurs in atrial myocytes via a readily releasable pool of granules. Ventricular myocytes also secrete ANP at low levels (McGrath *et al.* 2005). However, its contribution to natriuretic peptide secretion is predominately observed during chronic overload (Rushkoaho 2003).

Based on my findings, it is feasible to imagine that syntaxin 1A may additionally be involved in the exocytosis and/or vesicle trafficking of other peptides in the cardiac myocyte. The high levels of syntaxin 1A expression in the absence of pre-pro-ANP in the ventricle (Figure 13d), is suggestive that the synthesis of this SNARE protein may be required for other functions besides natriuretic peptide secretion. This would also explain why in the atria, such high amounts of protein are detected in comparison to SNAP25. In fact, previous studies have outlined the stoichiometric ratio of the syntaxin 1A-SNAP25 complex to be 2:1 in neuronal cells (Lerman *et al.* 2000). However, the contrast in syntaxin 1A to SNAP25 expression is so large that is likely that syntaxin 1A is required at a significant amount for other functions. In the neuron alone, syntaxin 1A not only functions in calcium-mediated exocytosis (Li *et al.* 1995), but also in the disassembly of the SNARE protein complex (Kee and Scheller 1996) and interacts with several SNARE-associated proteins besides SNAP25 including Munc proteins (Shen *et al.* 2007) and synaptotagmin (Kee and Scheller 1996). It has also been implicated to interact with potassium channels in pancreatic beta cells to induce glucose-stimulated insulin secretion and potassium channels on cardiac myocytes to regulate the excitability of the heart (Chao *et al.* 2011). Aside from its regulation of potassium channels (Chao *et al.* 2011, Kang *et al.* 2004), the function of syntaxin 1A in the cardiac myocyte has not

been characterized as of yet. However, its multi-faceted role in other tissues suggests that the high levels of syntaxin 1A expression seen in atrial and ventricular myocytes are probably required for a secondary function. Additional experiments would be required to demonstrate the functional role of syntaxin 1A within the cardiac myocyte and its stoichiometric expression when interacting with SNAP25 at the cardiac myocyte membrane.

SNAP25, like pre-pro-ANP, was also undetectable in the ventricle (Figure 13b). Similar to the atria, SNAP25 promoter activity was not as high as syntaxin 1A in neonatal ventricular myocytes (Figure 14a) and may have been too low to be detected with Western blot. It is worth noting, however, that SNAP25 expression was also absent in adult ventricular tissue (Figure 13b). These findings suggest that SNAP25 plays a more pivotal role in the adult atria relative to the ventricle.

HL-1 and H9c2 cell lines were also used to study the transcriptional activity of atrial and ventricular cardiac myocytes. HL-1 cells, a tumor cell line derived from AT-1 atrial myocytes, are commonly used in the study of atrial myocytes due to its ability to maintain cardiac myocyte phenotype and contractility throughout serial passage. Similar to primary atrial myocytes, HL-1 cells contain ANP-containing granules. They also display similar gene expression profiles to adult cardiac myocytes, similar electrophysiological properties, and receptor expression (White, Constantin and Claycomb 2004). H9c2 cells are derived from the lower half of a 13 day embryonic rat heart mostly consisting of ventricle tissue. This cell line replicates but does not contract *in vitro.* It holds similar phenotypic and cellular properties to ventricular primary myocytes, but also skeletal muscle cells (Parameswaran *et al.* 2013). Kimes and Brandt (1976), who originally established the H9c2 cell line, indicate that although the cell line displays properties shared by both heart and skeletal muscle cells, morphologically, it is more similar to a skeletal muscle cell. However, studies have shown that H9c2 cells do share electrophysiological characteristics and respond to G-protein signalling similar to adult cardiac myocytes (Hescheler *et al.* 1991). Furthermore, when treated with hypertrophic stimuli such as ET-1 and ANGII, H9c2 cells display similar cell growth and up-regulation of hypertrophic genes to primary cardiac myocytes (Watkins *et al.* 2011). This makes H9c2 cells a primarily useful cell model to study cardiac myocytes in response to heart disease (Parameswaran *et al.* 2013). Due to their origins, HL-1 was used to study the transcriptional

activity of syntaxin 1A and SNAP25 in atrial cardiac myocytes, while H9c2 was used as a model for studying ventricular cardiac myocytes.

Syntaxin 1A and SNAP25 transcriptional activity did not change across the two cell lines. Similar to the trends seen in primary cells, syntaxin 1A again displayed greater promoter activity (Figure 14b). As both HL-1 and H9c2 cells have previously been found to display characteristics of adult cardiac myocytes, it may be possible that the adult and not solely neonatal cells display similar levels of syntaxin 1A and SNAP25 promoter activity in the atria and the ventricle. This phenotype would have to be supported by studies done on primary adult myocytes.

All in all, I propose that syntaxin 1A and SNAP25 form a secondary SNARE complex in neonatal and adult cardiac myocytes which assists with the regulated secretion of ANP. As the ventricle is the main site of constitutive secretion, SNARE protein levels are not as high in this chamber. I propose the following: Low levels of both SNARE proteins are transcribed and translated in neonatal atrial cells to assist with regulated ANP secretion. This explains why syntaxin 1A and SNAP25 transcriptional activity is observed in atrial myocytes but protein levels are low or absent. Since regulated ANP secretion occurs at much lower levels in the ventricle (as noted in previous studies by a lack of secretory granules (Bloch *et al.* 1986)), it is expected that the transcriptional activity and protein synthesis in this chamber would be significantly lower. With age, however, natriuretic peptide secretion increases from the cardiac myocyte (Clerico *et al.* 2006), increasing the demand for the SNARE proteins required in its exocytosis. The primary SNARE complex, consisting of syntaxin 4 and SNAP23, identified in both neonates and adults (Peters *et al.* 2006), is not sufficient enough to meet the demands of the cell. As a result, an increase in protein synthesis and transcription of syntaxin 1A and SNAP25 occurs to assist with the increased exocytosis of ANP.

It is possible, however, that transcriptional activity does not correspond with protein synthesis. Though transcription may be occurring, post-transcriptional and post-translational regulation may keep the protein from being synthesized until required at a future time or during a particular state. Thus, it may be that at the neonatal stage, transcription of both syntaxin 1A and SNAP25 occurs, but the peptide

synthesis of syntaxin 1A solely takes place. In this case, I still propose that the secondary complex assists with regulated natriuretic peptide secretion, but instead of an increase in transcription as a response to increased ANP secretion, a post-transcriptional or post-translational mechanism occurs in which the previously transcribed mRNA transcript is processed into its peptide product.

4.2.2 Functional significance of SNAP25 and syntaxin 1A: Use of botulinum neurotoxins

In addition to characterizing the expression profiles and transcriptional activity of SNAP25 and syntaxin 1A, I also sought to investigate their functional role through the use of botulinum neurotoxins (BoNT). 7 day myocytes were transfected with BoNT/A and BoNT/C to proteolytically cleave SNAP25 and syntaxin 1A respectively. ANP secretion was then measured using an ANP-targeted ELISA to examine the effects of syntaxin 1A and SNAP25 proteolysis. Pre-pro-ANP levels within the cell were also examined using Western blot.

My findings display that the cleavage of SNAP25 with BoNT/A (with the exception of the atrial control group) consistently decreases ANP secretion (Figure 15a), a finding that is slightly more exaggerated in both atrial and ventricular myocytes stimulated with endothelin-1. Replicates of this experiment would have to be conducted to make any significant conclusions, but from my observations, the resulting decrease in ANP secretion displays the importance of SNAP25 in ANP exocytosis. Unfortunately, SNAP25 protein levels were not detectable with Western blot making the efficacy of BoNT/A difficult to determine. Similar to the results discussed in *section 4.2.1*, SNAP25 expression may have been too low to be detected via immunoblotting.

Although the exact localization and function of SNAP25 in cardiac myocytes remains to be characterized, its interaction with syntaxin 1A does not seem integral to the exocytosis of ANP, as the cleavage of syntaxin 1A with BoNT/C does not decrease ANP secretion (Figure 15a). Immunoblotting analysis, however, displays that syntaxin 1A protein levels decrease in both atrial and ventricular cells treated with BoNT/C relative to their GFP control groups, confirming the efficacy of the neurotoxin (Figure 15b).

Cleavage of both SNAP25 and syntaxin 1A resulted in a decrease in pre-pro-ANP levels in the atria (Figure 15c). Pre-pro-ANP levels were too low to be detected in the ventricle. The contrasting result in pre-pro-ANP levels and ANP secretion in atrial myocytes treated with BoNT/C suggest a difference in the functional role of syntaxin 1A and SNAP25 in ANP release.

In agreement with my proposal in *section 4.2.1*, the syntaxin 1A-SNAP25 complex likely assists with the regulated exocytosis of ANP. Evidence that syntaxin 1A and SNAP25 are not involved in constitutive secretion, but regulated secretion has previously been shown in constitutively secreting cell lines, where stimulation by these SNARE proteins resulted in a release of peptides from stored vesicles (Bittner *et al.* 1996). The reason why secretion of ANP is impaired in cells treated with BoNT/A, and not BoNT/C, may be because the little SNAP25 expressed in these myocytes is crucial to the SNARE complex involved in regulated exocytosis of ANP. This is why an even larger decrease is observed in ANP secretion levels for atrial and ventricular myocytes treated with both BoNT/A and endothelin-1, as oppose to those treated with BoNT/C. Although levels of SNAP25 are indetectable in the ventricle (Figure 15d), I propose that the little regulated secretion occurring in the ventricle does involve SNAP25, and cleavage of this protein is enough to elicit an observable decrease in ventricular ANP secretion.

Syntaxin 1A may also contribute to this complex, albeit at lower levels in neonatal cells relative to adult cells. Indeed, cleaving this SNARE with BoNT/C results in a decrease in pre-pro-ANP levels within the atrial cardiac myocyte (Figure 15c). However, this result is not extrapolated onto secreted ANP levels (Figure 15a). Thus, it is possible that syntaxin 1A plays a small role in the regulated release of ANP, although not significant enough to decrease ANP secretion. Similar to what was proposed in *section 4.2.1,* it may be that syntaxin 1A plays a secondary role in vesicle trafficking within the cardiac myocyte, which is why its cleavage did not significantly impair the exocytosis of ANP. It is possible that the complex Peters and colleagues (2006) also found in neonatal myocytes consisting of syntaxin 4 and SNAP23 may be assisting with ANP secretion in compensation for the lack of syntaxin 1A. The functional role of syntaxin 1A in the cardiac myocyte remains to be completely characterized. It may be that this SNARE plays additional functions in vesicle trafficking, perhaps interfering with the processing of ANP pro-

peptides in the rough endoplasmic reticulum. This would support the decrease observed in pre-pro-ANP levels with BoNT/C transfection (Figure 15c).

4.3 Importance of CRE in SNAP25 and syntaxin 1A promoter activity

David Boyce, a former member of the Tsushima lab, previously suggested that the CRE site and TATA box on the SNAP25 promoter may be essential to the transcriptional activity of this SNARE. Using a firefly luciferase/renilla assay, he showed that the minimum promoter length for SNAP25 transcription in HL-1 and H9c2 cell lines contains the CRE and TATA box elements (Figure 5). As a follow-up to this study, I further investigated the role of CRE in SNAP25 and syntaxin 1A transcriptional activity by stimulating 7 day atrial and ventricular myocytes, transfected with syntaxin 1A and SNAP25 promoters, with FOR/PMA for 24 hours. Forskolin and PMA are stimulators of cAMP and PKC, respectively, causing increased binding of downstream transcription factor CREB (cAMP-response element binding protein) to CRE. Treatment of cells should result in increased promoter activity of STX1A and SNAP25 (Figure 17).

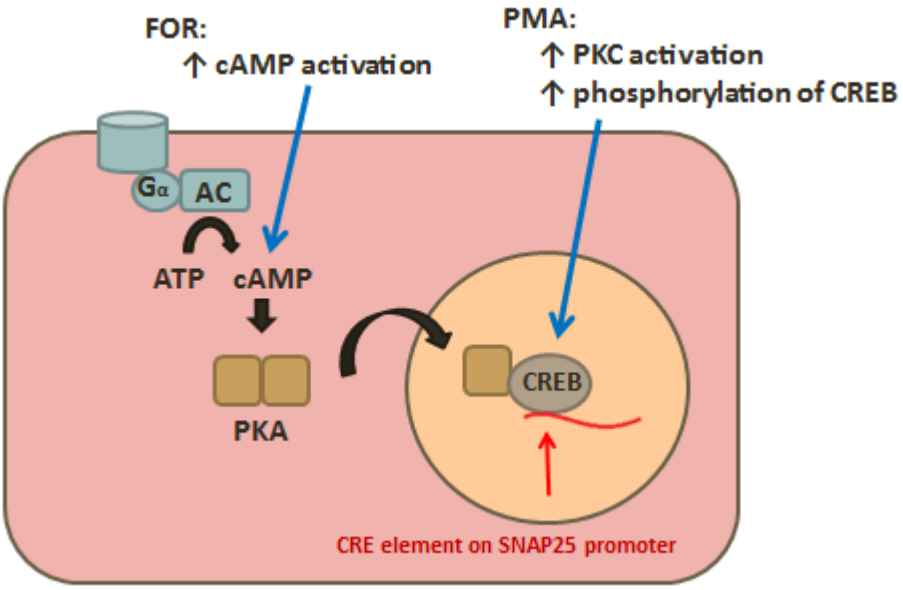


Figure 17. Activation of CRE by forskolin/PMA through synthesis of cAMP and PKC, respectively. Forskolin increases cAMP within the cell, which then binds to PKA. PKA dissociates and a component of the protein moves into the nucleus where it phosphorylates CREB. CREB can also be phosphorylated downstream of PKC signalling which can be activated by phorbol 12-myristate 13-acetate (PMA). CREB binding stimulates transcription of target genes by binding to CRE promoter sites.

Contrary to what I expected, no significant changes in syntaxin 1A and SNAP25 transcriptional activity were observed post-treatment in both atrial and ventricular myocytes. Taking this into consideration, it is conceivable that the CRE element is not essential to the transcription of syntaxin 1A and SNAP25. The resulting element, the TATA box, may thus play an important role in the transcription of these genes. In mammalian cells, the TATA box is known to recruit RNA polymerase II (RNAPII) when TATA binding protein (TBP) is bound. TBP binds to the TATA box when in complex with transcriptional factors such as TFIID. Once RNAPII is recruited, the transcription of the gene can take place (Yang *et al.* 2007). It has previously been shown that TATA-containing genes are highly regulated by nucleosomes, chromatin regulators and factors that interact with the TATA box binding protein (TBP). These genes are characterized as being highly responsive to environmental stressors or pressures, which explain why they are so meticulously regulated (Basehoar *et al.* 2004). Regulation of transcription factors that interact with the TATA box on the promoter region of syntaxin 1A and SNAP25 may then be the driving factor for their transcription in the cell. Perhaps increases in ANP secretion, an environmental stressor, are sensed by regulatory transcription elements within the nucleus of cardiac myocytes. These elements may then interact with TBP, regulating its binding to the TATA box, and recruitment of RNAPII in transcription of syntaxin 1A and SNAP25.

It is surprising that in both atrial and ventricular myocytes, transcriptional activity post-treatment mirrors that of control cells. I would have predicted that atrial cells would have a higher sensitivity to FOR/PMA as the atrium is the predominant site of calcium-mediated exocytosis. It has been shown in both hippocampal neurons and pancreatic endocrine cells that elevated intracellular calcium levels increase binding to CRE via phosphorylation of the Ser133 site on CREB (Bading *et al.* 1993; Vallejo 1994). It is postulated that Ser133 may be phosphorylated by PKA, as seen when stimulated by cAMP (Figure 19) or CaMKs (Ca^{2+}/calmodulin-dependent protein kinases) which are auto-phosphorylated in the presence of calcium (Vallejo 1994). It is unclear, however, whether CRE is only activated in response to increases in nuclear calcium concentrations, or cytoplasmic calcium, as an additional promoter site in hippocampal neurons, SRE, is suggested to increase transcription in response to cytoplasmic calcium (Hardingham *et al.*1997). In summary, CREB-CRE binding is found to regulate the transcription of genes in both neuronal and pancreatic endocrine cells. It is apt that these cells are also characteristic models of

calcium-mediated exocytosis (e.g. neurotransmitters and insulin). It is therefore conceivable that CRE may be an essential component of SNARE protein transcription in cardiac myocytes, during the calcium-mediated exocytosis of ANP.

Despite the results seen in my experiments, the possibility that CRE is an essential element is still promising. It may be possible that the concentration of FOR/PMA used to treat the cells was not high enough to elicit a response. However, due to time constraints, a concentration assay could not be conducted. Treatment protocol was instead replicated from Shimada and colleagues (2007), who used 10uM forskolin and 20 nM PMA to stimulate SNAP25 promoter activity in granulosa cells transfected with truncated SNAP25 promoter-luciferase constructs. Their treatment was conducted over a 4-hour span. To optimize treatment time for my study, a preliminary assay was conducted to test the efficacy of FOR/PMA at the indicated concentration for 4 hours versus 24 hours (Appendix B). A 24 hour treatment elicited greater transcriptional activity in both atrial and ventricular myocytes, suggesting the continuous stimulation of promoter activity with time. In both time-sensitive treatments, transcriptional activity did not greatly change with FOR/PMA treatment. To test if this was a product of limited sample size, the experiment was replicated three times using the 24 hour treatment protocol. My findings, again, displayed no significant changes.

Conducting a concentration assay before replicating this experiment and further looking into the importance of the TATA box on the SNAP25 and syntaxin 1A promoter site may prove useful in corroborating David's initial findings that the CRE site and TATA box are essential elements in the promoter activity of these SNARE proteins.

Conclusions

5.1. Syntaxin 5A, syntaxin 18, and SNAP29 are not responsible for the constitutive secretion of ANP and BNP, but may be constituents of the fetal gene program during hypertrophy.

With age, the ER to Golgi SNARE protein syntaxin 5A increases in expression, the Golgi to ER syntaxin 18 decreases in expression and the expression of the post-Golgi SNAP29 does not change. My initial hypothesis predicted that these SNARE proteins may be important in the constitutive secretion of ANP and BNP in cardiac myocytes and fibroblasts. This finding would have been realized if the expression profiles of these SNARE proteins supported a significant increase in anterograde transport and decrease in retrograde transport in the ventricle relative to the atria, parallel to an increase in natriuretic peptide secretion seen with age. However, there were no differences in atrial and ventricular protein expression. With fetal bovine serum or phenylephrine treatment, syntaxin 5A, syntaxin 18 and SNAP29 displayed opposite trends in SNARE protein expression to what I would expect when constitutive secretion increases. I propose that the genes transcribing syntaxin 5A, syntaxin 18 and SNAP29 may be up-regulated during post-natal development, and down-regulated during fetal bovine serum or phenylephrine-induced hypertrophy, in conjunction with the theory of fetal gene reprogramming. These findings suggest that syntaxin 5A, syntaxin 18 and SNAP29 are not involved in the constitutive secretion of ANP and may be important in another peptide trafficking pathway.

5.2. Syntaxin 1A and SNAP25 may assist with regulated ANP secretion in atrial and ventricular neonatal myocytes, then up-regulated during adulthood.

As many of these experiments were based on a limited sample size of one, no definitive conclusions can be made. However, from my data I infer that syntaxin 1A and SNAP25 are transcribed in both atria and ventricle. Both proteins are expressed, albeit low levels, in neonatal atrial and ventricular myocytes to assist with the regulated exocytosis of ANP. Treatment with botulinum neurotoxins indicates that even small amounts of SNAP25 present in 7 day neonatal myocytes are important to the exocytosis of ANP. Syntaxin 1A may also interact with SNAP25 in the exocytosis of this peptide, but not enough to elicit a decrease in ANP secretion when the SNARE protein is cleaved. I suggest that syntaxin 1A has

other protein-trafficking functions, which is why its protein levels are conceivably higher than SNAP25. With age, the requirement of additional SNARE proteins is realized as ANP secretion levels go up. Thus, in adults, up-regulation of this SNARE complex is observed, mostly in the atria as this is the main site of regulated ANP secretion.

5.3. The CRE site on the SNAP25 promoter may not be essential to the transcriptional activity the SNAP25 gene.

FOR/PMA treatment of atrial and ventricular cardiac myocytes does not result in an increase in transcriptional activity suggesting that the CRE site on the SNAP25 or syntaxin 1A promoter is not a main regulatory element of these genes' transcription. Further studies would need to be conducted to make any significant conclusions. I propose that the TATA box may play a more significant role in the transcription of these SNARE proteins in response to environmental stressors eliciting ANP release, such as an increase in pre-load in the heart.

Future Experiments

6.1 Replicate experiments in adult cardiac myocytes.

The discovery of syntaxin 1A and SNAP25 in the atria was originally observed in adult cardiac myocytes (Peters *et al.* 2010). In order to speculate about any age-dependent changes at the cellular level or draw any conclusions about the up-regulation of syntaxin 1A and SNAP25 transcription in adults, these experiments would have to be replicated in adult cardiac myocytes. Transfection of these cells with the promoters used in this study, stimulating with endothelin-1, then comparing adult promoter activity to neonatal cells may provide further insight into the importance of each SNARE at an adult age. Furthermore, cleaving these SNARE proteins with botulinum neurotoxins could provide evidence towards whether the up-regulation of syntaxin 1A protein levels, seen with age, indeed corresponds with an increase in regulated ANP secretion. If a decrease in ANP levels is observed with BoNT/C treatment (contrary to the lack of change observed in 7 day neonates), this would provide more tangible evidence that syntaxin 1A is important in regulated ANP secretion when levels increase in adulthood.

I would also aim to replicate the experiments conducted in this current study to increase my sample size and determine any significant changes.

6.2 RT-PCR

In this study, promoter activity, protein level expression, and correlating ANP secretion levels were studied for both syntaxin 1A and SNAP25. Although transcriptional activity was investigated using the secreted-luciferase assay, I would also want to characterize the mRNA profiles of these two SNARE proteins in atrial and ventricular myocytes and tissue. Evaluating these two SNARE proteins at different sections of the protein synthesis pathway may paint a clearer picture as to where the proteins are regulated – post-transcriptionally, post-translationally, or at the peptide level – and whether these trends correlate with each other. It would also be interesting to look at the mRNA expression of syntaxin 1A and SNAP25 in HL-1 and H9c2 cells as gene expression in these cells are suggested to mirror that of adult cardiac myocytes (White, Constantin and Claycomb 2004) (Hescheler *et al.* 1991).

6.3 Transient knockdown, Conditional knockout

The functional role syntaxin 1A and SNAP25 play in ANP secretion was analyzed through the transfection of neonatal myocytes with botulinum neurotoxins, conducting an endothelin-1 stimulated secretion assay, and measuring ANP levels in the media with an ELISA. Although BoNT cleavage is site-directed, and results in a decrease in the corresponding SNARE protein levels, conducting a transient knock down study with shRNA or siRNA constructs, would be a more effective method in observing the changes in ANP secretion. Stimulating these myocytes with endothelin-1 and hypertrophy-inducing compounds such as fetal bovine serum and phenylephrine would prove useful in studying the function of these SNARE proteins during heart disease. Alternatively, but more difficult, ANP secretion levels could be studied in transgenic mice with a conditional knockout using targeted loxP sites. Arash Bouramandi, an undergraduate student in the Tsushima lab, is currently characterizing the heart of syntaxin 1A-knockout mice with the Langendorff perfusion apparatus and measuring corresponding ANP secretion levels during heart disease simulations (e.g.hypoxia, ischemia).

6.4 Syntaxin 1A-SNAP25 SNARE complex: co-immunoprecipitation (coIP)

Syntaxin 1A and SNAP25 are mostly characterized to interact with each other in a complex. This complex consists of other SNARE proteins such as synaptobrevin/VAMP and SNARE-associated proteins such as synaptotagmin. This current study characterizes each SNARE's promoter activity and protein expression individually rather than looking at the complex as a whole. Using co-immunoprecipitation, the amount of bound syntaxin 1A and SNAP25 can be characterized across ages, and the importance of each SNARE protein to the complex, substantiated in exocytosis.

6.5 Confocal Imaging/TIRF

Syntaxin 1A, SNAP25 and pro-ANP can be tagged using fluorescent markers and viewed under a confocal microscope to define their localization in non-treated cells and cells treated with stimulators of ANP secretion such as endothelin-1. Using this method, we would also be able to confirm the co-localization of these SNARE proteins with intracellular granules containing pro-ANP.

Using total internal reflection fluorescence (TIRF) microscopy, live-cell imaging and videos can also be observed. TIRF is particularly useful in observing secretion events. Contrasting the interaction of syntaxin 1A and SNAP25 with pro-ANP containing vesicles versus the interaction of SNAP23 and syntaxin 4 during exocytosis may provide insight into the rationale behind a secondary complex in adult myocytes. Perhaps one complex predominantly mediates exocytosis during regulated ANP release. Alternatively, one or both SNARE complexes may be constitutively expressed. Observing the differences in SNARE protein composition in atrial and ventricular myocytes could prove useful in understanding their function.

6.6 Optimize concentration conditions: FOR/PMA treatment

Conducting a preliminary concentration assay for FOR/PMA on cells transfected with syntaxin 1A and SNAP25 promoters may be crucial to uncovering the importance of CRE in syntaxin 1A and SNAP25 promoter activity. The concentration used was replicated from a study done on ovarian cells, while my study was done on cardiac myocytes. Replicating this study with an optimal concentration may make a difference in the stimulation of SNAP25 and syntaxin 1A promoter activity.

References

Bading H, Ginty DD, and Greenberg ME. 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science*. 260(5105):181-186.

Bark C, Hahn KM, Ryabinin AE, Wilson MC. 1995. Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neuiral development. *Proc. Natl. Acad. Sci. USA*. 92(5):1510-1514.

Basehoar AD, Zanton SJ, and Pugh BF. 2004. Identification and distinct regulation of yeast TATA box-containing genes. *Cell*. 116(5): 699-709.

Baum J and Duffy HS. 2011. Fibroblasts and myofibroblasts: what are we talking about? *J Cardiovasc Pharmacol.* 57(4): 376–379.

Bennet MK, Garcia-Arraras JE, Elferink LA, Peterson K, Fleming AM, Hazuka CD, Scheller RH. 1993. The syntaxin family of vesicular transport receptors. *Cell.* 74(5):863-873.

Bernardo BC, Weeks KL, Pretorius L, and McMullena JR. 2010. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacol Ther.* 128(1): 191–227.

Bloch KD, Seidman JG, Naftilan JD, Fallon JT, Seldman CE. 1986. Neonatal atria and ventricles secrete atrial natriuretic factor via tissue-specific secretory pathways. *Cell*. 47(5):695-702.

Block MR, Glick BS, Wilcox CA, Wieland FT, Rothman, JE. 1988. Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *PNAS*. 85(21): 7852-7856.

Boyce, D. 2013. Regulation of SNAP-25 promoter activity in cardiac myocytes. Biology Honours Thesis. York University: Toronto, Ontario.

Brose, N. 2014. All roads lead to neuroscience: the 2013 nobel prize in physiology or medicine. *Neuron.* 81(4): 723-727.

Cameron V and Ellmers LJ. 2003. Minireview: Natriuretic peptides during development of the fetal heart and circulation. *Endocrinology*. 144(6):2191-2194.

Cameron VA, Rademaker MT, Ellmers LJ, Espiner EA, Nicholls MG, Richards AM. 2000. Atrial (ANP) and brain natriuretic peptide (BNP) expression after myocardial infarctin in sheep: ANP is synthesized by fibroblasts infiltrating the infarct. 141(12):4690-4697.

Carr CM and Rizo J. 2010. At the junction of SNARE and SM protein function. *Curr Opin Cell Biol.* 22(4):488-495.

Chao C, Liang T, Kang Y, Lin X, Xie H, Feng ZP, Gaisano HY. 2011. Syntaxin-1A inhibits K_{ATP} channels by interacting with specific conserved motifs within sulfonylurea receptor 2A. *J Mol Cell Cardiol.* 51(5):790-802.

Chao CC, Mihic A, Tsushima RG, Gaisano HY. 2011. SNARE protein regulation of cardiac potassium channels and atrial natriuretic factor secretion. *J Mol Cell Cardiol.* 50(3):401-407.

Chapman ER. 2002. Synaptotagmin: a Ca2+ sensor that triggers exocytosis? *Nat Rev Mol Cell Bio.* 3(7):498-508.

Chen YA and Scheller RH. 2001. SNARE-mediated membrane fusion. *Nat Rev Mol Cell BIol.* 2(2):98-106.

Clary DO, Griff IC, and Rothman JE. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell*. *61*(4):709-721.

Clerico A, Emdin M, Panteghini M, Passino C, Recchia F, and Vittorini S. 2006. Natriuretic Peptides. A. Clerico, M. Emdin (Ed.). Milan, Italy: Springer.

Dascher C, Matteson J, and Balch WE. 1994. Syntaxin 5 regulates endoplasmic reticulum to Golgi transport. *J Biol Chem*. 269(47): 29363-29366.

de Bold AJ, Borenstein HB, Veress AT, and Sonnenberg H. 1981. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extracts in rats. *Life Sci.* 28(1): 89–94.

Dietz J. 2005. Mechanisms of atrial natriuretic peptide secretion from the atrium. *Cardiovasc. Res.* 68(1):8-17.

D'Souza SP, Davis M, and Baxter GF. 2004. Autocrine and paracrine actions of natriuretic peptides in the heart. *Pharmacol Therapeut*.101(2): 113-129.

Fasshauer D, Sutton RB, Brunger AT, and Jahn R. 1998. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q-and R-SNAREs. *Proc. Natl. Acad. Sci. USA.* 95(26):15781-15786.

Ferlito M, Fulton WB, Zauher MA, Marbán E, Steenbergen C, Lowenstein CJ. 2010. VAMP-1, VAMP-2, and syntaxin-4 regulate ANP release from cardiac myocytes. *J Mol Cell Cardiol.* 49(5): 791-800.

Fernandez I, Ubach J, Dulubova I, Zhang X, Sudhof TC, Rizo J. 1998. Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. Cell. 94(6):841-849.

Flynn TG, de Bold ML, and de Bold AJ. 1983. The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. *Biochem Bioph Res Co.* 117(3): 859–865.

Frey N, Katus HA, Olson EN, Hill JA. 2004. Hypertrophy of the heart: a new therapeutic target? *Circulation.* 109(13):1580-1589.

Giraudo CG, Eng WS, Melia TJ, Rothman JE. 2006. A clamping mechanism involved in SNARE-dependent exocytosis. *Science*. 313(5787):676-680.

Gordon DE, Bond LM, Sahlender DA, Peden AA. 2010. A targeted siRNA screen to identify SNAREs responsible for constitutive secretion in mammalian cells. *Traffic*.11(9):1191-1204.

Hardingham GE, Chawla S, Johnson CM, Bading H. 1997. Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature*. 385:260-265.

Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. 1991. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res*. 69(6):1476-1486.

Iinuma T, Aoki T, Arasaki K, Hirose H, Yamamamoto A, Samata R, Hauri H, Arimitsu N, Tagaya M, Tani K. 2009. Role of syntaxin 18 in the organization of endoplasmic reticulum subdomains. *J Cell Sci.* 122(10): 1680-1690.

Jahn R and Scheller RH. 2006. SNAREs - engines for membrane fusion. *Nat Rev Mol Cell Biol.* 7(9): 631-643.

Jamieson JD and Palade GE. 1964. Specific granules in atrial muscle cells. *J Cell Biol.* 23(1):151–172.

Kang Y, Leung YM, Manning-Fox JE, Xia F, Xie H, Sheu L,Gaisano HY. 2004.Syntaxin-1A inhibits cardiac KATP channels by its actions on nucleotide binding folds 1 and 2 of sulfonylurea receptor 2A. *J Biol Chem. 279*(45):47125-47131.

Kee Y and Scheller RH. 1996. Localization of synaptotagmin-binding domains on syntaxin. *J Neurosci*.16(6):1975-1981.

Kimes BW and Brandt BL.1976. Properties of a clonal muscle cell line from rat heart. *Exp Cell Biol.* 98(2), 367-381.

Kimura K, Mizoguchi A, Ide C. 2003. Regulation of growth cone extension by SNARE proteins. *J. HIstochem. Cytochem.* 51(4):429-433.

Konhilas JP and Leinwand LA. 2006. Partnering up for cardiac hypertrophy. *Circ Res.* 98(8):985-987.

Lam PL, Leung Y, Sheu L, Ellis J, Tsushima RG, Osborne LR, Gaisano HY. 2005. Transgenic Mouse overexpressing syntaxin-1A as a diabetes model. *Diabetes*. 54(9):2744-2754.

Lerman JC, Robblee J, Fairman R, Hughson FM. 2000. Structural analysis of the neuronal SNARE protein syntaxin-1A. *Biochemistry.* 39(29): 8470-8479.

Levin ER, Gardner DG, and Samson WK. 1998. Natriuretic peptides. *New Engl J Med*. 339(5): 321-328.

Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Sudhof TC. 1995. Ca^{2+}-dependent and independent activities of neural and non-neural synaptotagmins. *Nature*. 275:594-599.

Mantymaa P, Vuolteenaho O, Marttila M, and Ruskoaho H. 1993. Atrial stretch induces rapid increase in brain natriuretic peptide but not in atrial natriuretic peptide gene expressionin vitro. *Endocrinology.* 133(3): 1470-1473

.

Mao LM, Tang Q, and Wang JQ. 2007. Protein-kinase C-regulated cAMP response element-binding protein phosphorylation in cultured rat striatal neurons. *Brain Res Bull.* 72(4): 302-308.

McGrath MF and de Bold AJ. 2005. Determinants of natriuretic peptide gene expression. *Peptides.* 26(6):933-943.

McGrath MF, Kuroski and de Bold ML, de Bold AJ. 2005. The endocrine function of the heart. *Trends Endocrin Met*. 16(10): 469-477.

Montecucco C and Schiavo G. 1994. Mechanism of action of tetanus and botulinum neurotoxins. *Mol Microbiol.*13(1):1-8.

Montecucco C and Schiavo G. 1995. Structure and function of tetanus and botulinum neurotoxins. *Q Rev Biophys.* 28(4):423-472.

Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, Shirakami G, Jougasaki M, Obata K, Yasue H. 1991. Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. *J Clin Invest*. 87(4):1402-1412.

Nakagawa O, Ogawa Y, Itoh H, Suga S, Komatsu Y, Kishimoto I, Nishino K, Yoshimasa T, Nakao K. 1995. Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. *J Clin Invest.* 96(3):1280-1287.

Nakamura K, Anitha A, Yamada K, Tsuji M, Iwayama Y, Hattori E, Toyota T, Suda S, Takei N, Iwata Y, et al. 2008. Genetic and expression analyses reveal elevated expression of syntaxin 1A (STX1A) in high functioning autism. *Int J Neuropsychop*. 11(8)1073-1084.

Novick P and Schekman R. 1979. Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. 76(4):1858–1862.

Novick P, Field C, and Schekman R. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell*. 21(1):205-215.

Ohara-Imaizumi M, Nishiwaki C, Nakamichi Y, Kikuta T, Nagai S, Nagamatsu S. 2004. Correlation of syntaxin-1 and SNAP-25 clusters with docking and fusion of insulin granules analysed by total internal reflection fluorescence microscopy. *Diabetologia*. 47(12), 2200-2207.

Oyler GA, Polli JW, Wilson MC, Billingsley ML. 1991. Developmental expression of the 25-kDA synaptosomal-associated (SNAP-25) in rat brain. *Proc. Natl. Acad. Sci. USA*. 88(12):5247-5251.

Pan PY, Cai Q, Lin L, Lu PH, Duan S, Sheng ZH. 2005. SNAP-29-mediated modulation of synaptic transmission in cultured hippocampal neurons. *J Biol Chem.* 280(27):25769-25779.

Pandey KN. 2005. Biology of natriuretic peptides and their receptors. *Peptides*. 26(6):901-932.

Parameswaran S, Kumar S, Verma RS, Sharma RK. 2013. Cardiomyocyte culture—an update on the in vitro cardiovascular model and future challenges. *Can J Physiol Pharm*. 91(12), 985-998.

Peters CG, Miller DF and Giovannucci DR. 2006. Identification, localization and interaction of SNARE proteins in atrial cardiac myocytes. *J Mol Cell Cardiol*. 40(3): 361-374.

Raizaida V, Thakore K, Luo W, McGuire PG. 2001.Cardiac chamber-specific alterations of ANP and BNP expression with advancing age and with systemic hypertension. *Mol Cell Biochem*. 216(1-2):137-140.

Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. 2007. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail Rev.*12(3-4):331-343.

Rapaport D, Lugassy Y, Sprecher E, Horowitz M. 2010. Loss of SNAP29 impairs endocytic recycling and cell motility. *PloS one.* 5(3):e9759.

Ryabinin AE, Sato TN, Morris PJ, Latchman DS, Wilson MC. 1996. Immediate upstream promoter regions required for neurospecific expression of SNAP-25. *J Mol Neurosci.* 95(6):201-210.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N, Rosenmund C. 2001. Complexins regulate a late step in Ca^{2+}-Dependent neurotransmitter release. *Cell*. 104(1):71-81.

Ruskoaho H. 2003. Cardiac hormones as diagnostic tools in heart failure. *Endocr Rev*. 24(3): 341-356.

Sadoul K, Lang J, Montecucco C, Weller U, Regazzi R, Catsicas S, Wollheim CB, Halban PA. 1995. SNAP-25 is expressed in islets of langerhans and is involved in insulin release. *J Cell Biol.* 128(6):1019-1028.

Schulz-Knappe P, Forssmann K, Herbst F, Hock D, Pipkorn R, and Forssmann WG. 1988. Isolation and structural analysis of “urodilatin”, a new peptide of the cardiodilatin-(ANP)-family, extracted from human urine. *Klinische Wochenschrift*. 66(17): 752-759.

Schweitz H, Vigne P, Moinier D, Frelin C, Lazdunski M. 1992. A new member of the natriuretic peptide family is present in the venom of the green mamba (Dendroaspis angusticeps). *J Biol Chem*. *267*(20): 13928-13932.

Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ. 2007. Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell*. 128(1):183-195.

Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ. 2007. Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell*. 128(1):183-195.

Shimada M, Yanai Y, Okazak T, Yamashita Y, Sriraman V, Wilson MC, RIchards JS. 2007. Synaptosomal-associated protein 25 gene expression is hormonally regulated during ovulation and

is involved in cytokine/chemokine exocytosis from granulosa cells. *Mol Endocrinol.* 21(1):2487-2502.

Simpson P, McGrath A, and Savion S. 1982. Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ Res*. 51(6):787-801.

Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE.1993. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell.* 75(3):409-418.

Su Q, Mochida S, Tian JH. Mehta R, Sheng ZH. 2001. SNAP-29: a general SNARE protein that inhibits SNARE disassembly and is implicated in synaptic transmission. *PNAS*. 98(24):14038-14043.

Sudhof TC and Rothman JE. 2009. Membrane fusion: grappling with SNARE and SM proteins. *Science*. 323(5913):474–477.

Sudoh T, Minamino N, Kangawa K, Matsuo H. 1990. C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Bioph Res Co*. 168(2): 863-870.

Sudoh TK, Kangawa N, Minamino N, Matsuo H. 1988. A new natriuretic peptide in porcine brain. *Nature(Lond.).* 332:78-81.

Suga SI, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H. 1992. Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-beta. Possible existence of "vascular natriuretic peptide system". *J Clin Invest*. 90(3): 1145-1149.

Sutton R, Fasshauer D, Jahn R, Brunger A. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 angstrom resolution. *Nature.* 395(6700):347-353.

Thibault G, Amiri F, and Garcia R. 1999. Regulation of natriuretic peptide secretion by the heart. *Annu Rev Physiol.* 61(1):193-217.

Tsuruda T, Boerrigter G, Huntley BK, Noser JA, Cataliotti A, Costello-Boerrigter LC, Chen HH, Burnett Jr JC. 2002. Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases. *Circ Res*. 91(12):1127-1134.

Turton K, Chaddock JA, Acharya KR. 2002. Botulinum and tetanus neurotoxins: structure, function and therapeutic utility. *Trends Biochem Sci.* 27(11):552-558.

Vallejo M. 1994. Transcriptional control of gene expression by cAMP-response element binding proteins. *J Neuroendocrinol.* 6(6): 587-596.

van den Bogaart G,Holt MG, Bunt G, Riedel D, Wouters FS, Jahn R. 2010. One SNARE complex is sufficient for membrane fusion. *Nat Struct Mol Biol.* 17(3): 358-364.

Vo T and Hardy DB. 2012. Molecular mechanisms underlying the fetal programming of adult disease. *J Cell Commun Signal.* 6(3): 139-153.

Wang TJ, Larson MG, Levy D, Leip EP, Benjamin EJ, Wilson PWF, Sutherland P, Omland T, Vasan RS. 2002. Impact of age and sex on plasma natriuretic peptide levels in healthy adults. *Am J Cardiol*. 90(3):254-258.

Watkins SJ, Borthwick GM, and Arthur HM. 2011.The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. *In Vitro Cell Dev-An.7*(2):125-131.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell*. 92(6):759-772.

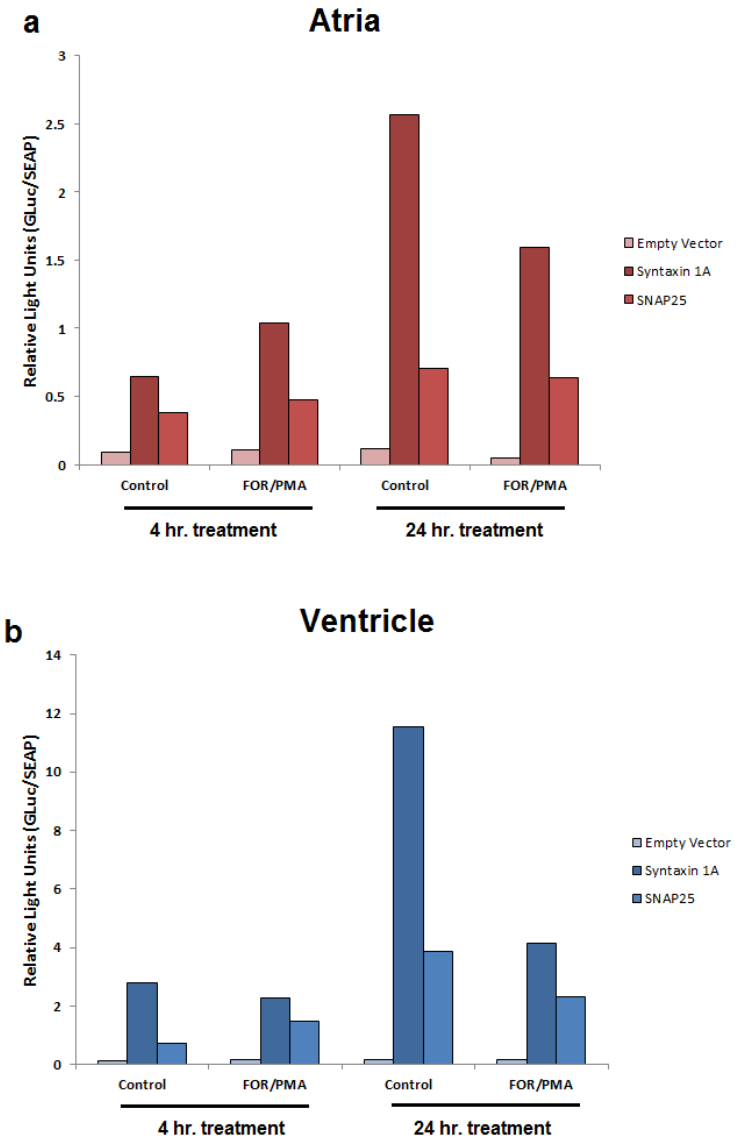
Wesolowski J, Caldwell V, Paumet F. A novel function for SNAP29 (Synaptosomal-Associated Protein of 29 kDa) in mast cell phagocytosis. *PloS one*. *7*(11): e49886.

White SM, Constantin PE, and Claycomb WC. 2004.Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. *Am J Physiol-Heart C.* 286(3), H823-H829.

Appendix A: Primary and Secondary Antibodies

Antibody	Company	Catalog Number	Anti-Mouse or Anti-Rabbit Antibody (Ab)	Dilution Factor
Primary Antibodies				
Anti-syntaxin 5A	Sigma	WH0006811M1	Monoclonal Mouse Ab	1:1000
Anti-syntaxin 18	SYSY	110 183	Polyclonal Rabbit Ab	1:1000
Anti-SNAP29	Abcam	ab138500	Monoclonal Rabbit Ab	1:1000
Anti-syntaxin 19	Abcam	ab94538	Polyclonal Rabbit Ab	1:1000
Anti-syntaxin 1A	Sigma	S0664	Monoclonal Mouse Ab	1:1000
Anti-SNAP25	Sigma	S5187	Monoclonal Mouse Ab	1:1000
Anti-syntaxin 4	SYSY	110 042	Polyclonal Rabbit Ab	1:1000
Anti-SNAP23	SYSY	111 202	Polyclonal Rabbit Ab	1:1000
Anti-ANP	Millipore	AB5490	Polyclonal Rabbit Ab	1:2000
Anti-GAPDH	Abcam	ab8245	Monoclonal Mouse Ab	1:1000
Secondary Antibodies				
anti-mouse				1:40000
anti-rabbit				1:40000
IRDye 800CW Goat anti-Mouse IgG	LI-COR	926-32210		1:10000
IRDye 680CW Goat anti-Rabbit IgG	LI-COR	926-68071		1:10000

Appendix B: Optimizing FOR/PMA Treatment Time



Appendix B. Transcriptional activity of syntaxin 1A and SNAP25 in 7 day (a) atrial and (b) ventricular rat myocytes treated with 10 μM forskolin and 20 nM phorbol 12-myristate 13-acetate (FOR/PMA) for 4 hours or 24 hours. Exposure time to FOR/PMA was manipulated to determine if the effects of these compounds were time-dependent. There were no significant changes in FOR/PMA treated cells relative to non-treated cells with the exception of the atrial and ventricular control group at 24 hours (n=1). 24 hour treated cells displayed slightly higher activity. Thus, 24 hours was chosen as the treatment time in the above experiments.

Appendix C: Copyright License Agreements

I. Figure 1. Function of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in target organs

About NEJM Permissions

Reuse of Content within a Thesis or Dissertation

Content (full-text or portions thereof) may be used in print and electronic versions of your dissertation or thesis without formal permission from the Massachusetts Medical Society, Publisher of the New England Journal of Medicine.

The following credit line must be printed along with the copyrighted material:

"Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

II. Figure 2. Role of SNARE proteins, SNARE-associated proteins, and SM proteins in neuronal exocytosis

ELSEVIER LICENSE
TERMS AND CONDITIONS

Aug 02, 2014

This is a License Agreement between Nikki Natividad ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Nikki Natividad
License number	3440940049536
License date	Aug 02, 2014
Licensed content publisher	Elsevier
Licensed content publication	Neuron
Licensed content title	All Roads Lead to Neuroscience: The 2013 Nobel Prize in Physiology or Medicine

Licensed content author	Nils Brose
Licensed content date	19 February 2014
Licensed content volume number	81
Licensed content issue number	4
Number of pages	5
Start Page	723
End Page	727
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	Role of SNARE Proteins in Natriuretic Peptide Secretion by the Heart
Expected completion date	Aug 2014
Estimated size (number of pages)	92
Elsevier VAT number	GB 494 6272 12

III. Figure 6. Localization of SNARE and SNARE-associated proteins implicated to play a role in the constitutive secretion pathway

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Aug 24, 2014

This is a License Agreement between Nikki Natividad ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

License Number	3441630940717
License date	Aug 03, 2014

Order Content Publisher	John Wiley and Sons
Order Content Publication	Traffic
Order Content Title	A Targeted siRNA Screen to Identify SNAREs Required for Constitutive Secretion in Mammalian Cells
Licensed copyright line	© 2010 John Wiley & Sons A/S
Order Content Author	David E. Gordon,Lisa M. Bond,Daniela A. Sahlender,Andrew A. Peden
Order Content Date	Jun 10, 2010
Start page	1191
End page	1204
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 8
Will you be translating?	No
Title of your thesis / dissertation	Role of SNARE Proteins in Natriuretic Peptide Secretion by the Heart
Expected completion date	Aug 2014
Expected size (number of pages)	92