Role of the Heterotrimeric G_o Protein α-Subunit on the Cardiac Secretory Phenotype

Cassandra Roeske

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the M.Sc. degree in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

ABSTRACT

Atrial natriuretic factor (ANF) is a polypeptide hormone produced in heart atria, stored in atrial secretory granules and released into the circulation in response to various stimuli. Proper sorting of ANF at the level of the *trans*-Golgi network (TGN) is required for the storage of ANF in these specific granules, and this sorting of hormones has been found to be associated with G-proteins. Specifically, the G_o protein alpha-subunit (Gαo) was established to participate in the stretch-secretion coupling of ANF, but may also be involved in the transporting of ANF from the TGN into atrial granules for storage and maturation. Based on knowledge of Gαo involvement in hormone production in other endocrine tissues, protein-protein interactions of Gαo and proANF and their immunochemical co-localization in granules, the direct involvement of these two proteins in atrial granule biogenesis is probable. In this study, mice were created using the Cre/lox recombination system with a conditional Gαo knockout in cardiocytes to study and characterize ANF production, secretion and granule formation. Deletion of this gene was successful following standard breeding protocols. Characterization and validation of cellular and molecular content of the knockout mice through mRNA levels, protein expression, peptide content, electron microscopy, and electrocardiography determined that a significant phenotypic difference was observed in the abundance of atrial granules. However, Gαo knockout mice did not significantly alter the production and secretion of ANF and only partially prevented granule biogenesis, likely due to incomplete Gαo knockout. These studies demonstrate an involvement of Gαo in specific atrial granule formation.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC	adenylyl-cyclase
ACN	acetonitrile
ANF	atrial natriuretic factor
AngII	angiotensin II
AP-1	adaptor protein-1
BNP	B-type natriuretic peptide
bp	basepair
cGMP	cyclic guanosine monophosphate
ChgA	chromogranin A
ChgB	chromogranin B
CNP	C-type natriuretic peptide
DNA	deoxyribonucleic acid
ECG	electrocardiography
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ET-1	endothelin-1
G6PD	glucose-6-phosphate dehydrogenase
Gαo	G_o protein alpha-subunit
GC	guanylyl-cyclase
GDP	guanosine diphosphate
GIRK1,4	adenosine triphosphate-sensitive K^+ channels
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
IL-1β	interleukin-1 beta
ISG	immature secretory granule
MAS-7	mastoparan-7
mRNA	messenger RNA

MSG	mature secretory granule
NEP	neutral endopeptidase 24.11
NPR-A	natriuretic peptide receptor type-A
NPR-B	natriuretic peptide receptor type-B
NPR-C	natriuretic peptide receptor type-C
PCR	polymerase chain reaction
PTX	pertussis toxin
RAAS	renin-angiotensin-aldosterone system
RGS	regulator of G-protein signaling
RIA	radioimmunoassay
RP-HPLC	reverse-phase high performance liquid chromatography
RT-PCR	real time-polymerase chain reaction
SEM	standard error of the mean
SK4	Ca^{2+}-activated intermediate conductance K^+ channel
SNARE	soluble *N*-ethylmaleimide-sensitive factor activating protein receptors
TBST	tris-buffered saline and 0.1% Tween-20
TCR-α	t-cell receptor alpha
TFA	trifluoroacetic acid
TGN	*trans*-Golgi network
TNFα	tumor necrosis factor-alpha
TREK-1	mechano-gated K^+ channel

ACKNOWLEDGEMENTS

I would like to thank first and foremost my supervisor and mentor, Dr. Adolfo J. de Bold, for without his support, expertise and confidence in me I would not have been honoured with a project under such prestigious leadership. I am very grateful for such an opportunity and I will always hold my experiences at the University of Ottawa Heart Institute as one of my most important accomplishments. I would like to thank Dr. Mercedes Kuroski de Bold, as I was fortunate to have her guidance during my time as a student. I would also like to thank and acknowledge past and present members of the Cardiovascular Endocrinology Lab: Asna Choudhry, Dr. Monica Forero McGrath, and Amy Martinuk for helping me with my project. I would like to express my gratitude to Dr. Tom Lagace and Dr. Simon Lemaire for being a part of my advisory committee. All of the above mentioned individuals have taught me necessary laboratory skills and knowledge and have guided me to become a well-rounded scientist.

I am tremendously thankful to my parents, Carey and Stephen, as they have always been supportive, encouraging and loving. I would also like to acknowledge my brother, Andrew, for believing in me as a sister and a friend. I am very appreciative for their attempts at understanding my project over my two years of graduate work. I am the person I am today because of my family; they have guided and directed me with their words of wisdom and inspiration, and their ultimate faith in me.

The valuable skills, knowledge and relationships I have gained from my experiences and time as a Master's student will forever be remembered. I look forward to the new journeys and adventures to come.

INTRODUCTION

Cardiac natriuretic peptides

In 1964, atrial cardiocytes were examined using electron microscopy and a very distinctive phenotype similar to that of polypeptide hormone-secreting cells was revealed (Jamieson and Palade, 1964). These characteristics of secretory cells were only found in the atria of the heart, and not in the ventricles (Kisch, 1963). Following extensive analysis on the function of these granules in the atria (de Bold et al., 1978), it was found that the atrial extracts induce a very powerful natriuresis, diuresis and lowering of blood pressure upon injection into animals (de Bold et al., 1981). This natriuretic factor was named "Atrial Natriuretic Factor" (de Bold et al., 1981) and was soon after proved to be a polypeptide hormone stored in dense-core atrial secretory granules (Flynn et al., 1983). Almost twenty years after the specific atrial granules were identified, the heart was proven to also possess an endocrine role in addition to its role as a mechanical pump.

Subsequently, two more cardiac natriuretic polypeptide hormones containing similar amino acid sequences to ANF were identified in porcine brain. These other peptides were named B-type natriuretic peptide (BNP) (Sudoh et al., 1988), and C-type natriuretic peptide (CNP) (Sudoh et al., 1990). BNP was first identified in porcine brain, but later found to be co-stored and co-secreted with ANF in atrial secretory granules (Iida et al., 1990). CNP is produced in the endothelium (Suga et al., 1992b); however, it is mainly distributed throughout the central nervous system (Ueda et al., 1991). Together, these three natriuretic peptides maintain cardiovascular homeostasis.

Natriuretic peptide transgenic animal models of ANF, BNP and CNP allowed for insights into their important biological functions. Blood pressure of homozygous and heterozygous ANF knockout mice was markedly increased when challenged with a high-salt diet; however, on a standard salt-diet, ANF knockout mice still displayed significantly elevated blood pressure (John et al., 1995). These knockout mice lacked the presence of specific atrial granules (John et al., 1995); therefore inferring that ANF also plays an important role in atrial secretory granule formation.

Ventricular dysfunction due to cardiac fibrosis was seen in BNP knockout mice. These mice did not differ in their blood pressure compared to wild type mice; however, the concentration of ANF in the ventricles and its expression levels were increased in BNP -/- mice. Specific atrial granules containing ANF were still present in these mice (Tamura et al., 2000).

CNP knockout mice display a very different phenotype than the other natriuretic peptides because of its presence in central and peripheral tissues (Chusho et al., 2001). These mice have severe impairment of endochondral ossification in the long bones, resulting in dwarfism and death in over half the mice by the age of 21 days (Chusho et al., 2001). Transgenic local expression of CNP (Chusho et al., 2001) or infusion of circulating CNP (Fujii et al., 2010) rescues the long bone impairment and early death of these mice.

Atrial natriuretic factor

Biochemistry of ANF

In humans, the ANF gene (*Nppa*) is found on chromosome 1p36 (Ruskoaho, 1992). ANF is synthesized as a prepro-hormone of 151 amino acids, is processed as a pro-hormone with residues 1-126, and is cleaved a third time to form the N-terminus 1-98 (de Bold, 1985) and the C-terminus of residues 99-126 (Flynn et al., 1983). The circulating form of ANF is a 28 amino acid peptide of residues 99-126 (ANF_{99-126}) (de Bold and Flynn, 1983;de Bold, 1985). The structure of ANF contains a disulfide-bonded loop between cysteine residues at amino acids 129 and 145, forming a 17 amino acid ring (de Bold, 1985), which is crucial for its biological activity (Schiller et al., 1986). This disulfide bond is also found in both BNP and CNP's structure as well (Schiller et al., 1986;McGrath et al., 2005) and has been identified in some hormones to have an important function in the trafficking from the Golgi apparatus to the cell membrane for secretion (Gorr et al., 1999).

Natriuretic peptides are highly conserved and as a result the aforementioned applies to other mammalian species (McGrath et al., 2005). Secretory granules of the atria containing ANF store the precursor form, ANF_{1-126} (Oikawa et al., 1984), which is co-stored with BNP in its processed mature form of 32 amino acids, BNP_{77-108} (Yokota et al., 1995). Upon stimulation of these granules, proANF is cleaved into the biologically active form by a transmembrane spanning serine protease termed corin (Yan et al., 2000). Corin was found to regulate the cleavage of proANF to processed ANF using a transgenic mouse model that

lacked functional corin, resulting in hypertension due to significantly low levels of biologically active processed ANF (Yan et al., 2000).

Physiological effects of ANF

ANF exerts potent vasorelaxant properties that can significantly decrease blood pressure (de Bold et al., 1981). This was clearly evident in ANF knockout mice which displayed much higher blood pressure than control mice by up to 40mm mercury (John et al., 1995) and in ANF overexpressing mice whose blood pressure was significantly lower than control mice (Steinhelper et al., 1990). ANF also exerts profound effects on renal function by counteracting the renin-angiotensin-aldosterone system (RAAS) (Cargill and Lipworth, 1995). ANF causes a decrease in sodium reabsorption in the collecting ducts, while increasing glomerular filtration rate, resulting in an increase in diuresis and natriuresis (Melo et al., 2000). ANF also inhibits aldosterone and renin secretion, decreases aldosterone synthesis, and therefore also inhibits angiotensin II (AngII) formation (Zeidel, 1990). This combined inhibition of the RAAS contributes importantly to the maintenance of cardiovascular homeostasis.

ANF has been implicated in the electrophysiological function of the heart because of its ability to regulate multiple ion channels and heart rate by both stimulating and inhibiting the sympathetic activity of the autonomic nervous system (Perrin and Gollob, 2012). The same study also found that mutations in the ANF gene of humans can cause atrial fibrillation (Perrin and Gollob, 2012).

Receptors of ANF

There are three natriuretic peptide receptors: natriuretic peptide receptor-A (NPR-A), natriuretic peptide receptor-B (NPR-B), and natriuretic peptide receptor-C (NPR-C). All three receptors contain an N-terminus extracellular binding domain for natriuretic peptide ligand binding, a membrane spanning domain, and an intracellular domain (Kumar et al., 2001). NPR-A and NPR-B both contain a kinase-homology intracellular domain and a C-terminus guanylyl-cyclase (GC) domain which promotes the production of cyclic guanosine 3',5'-monophosphate (cGMP) (Potter et al., 2006). NPR-C has a much smaller intracellular domain and does not possess any GC activity (Potter et al., 2006).

ANF effects are mediated by binding to its membrane-bound GC receptor, NPR-A (Chinkers et al., 1989). NPR-A is coded by the *Npr1* gene (Tremblay et al., 2002) and is the receptor for both ANF and BNP peptides (Chinkers et al., 1989). The net effect of binding is an increase in intracellular cGMP (Chinkers et al., 1989). Intracellular cGMP affects signaling pathways through interactions with protein kinases, ion channels and phosphodiesterases (Lincoln and Cornwell, 1993). NPR-A knockout mice have shown chronic high blood pressure when put on minimal, normal and high salt diets which greatly resembles essential hypertension (Oliver et al., 1997). These mice do not show a difference in ANF concentrations compared to wild type mice, indicating that production of ANF is not affected by the lack of its receptor (Oliver et al., 1997).

NPR-A preferentially binds ANF and BNP with much stronger affinity than that of CNP (Suga et al., 1992a). Phosphorylation of the NPR-A kinase homology domain is essential for natriuretic peptide activation and effects (Potter and Garbers, 1992;Potter et

al., 2006). NPR-A knockout mice display severe cardiovascular events including hypertension, cardiac hypertrophy and congestive heart failure (Lopez et al., 1995;Oliver et al., 1997;Kuhn et al., 2002). NPR-B is activated primarily by CNP, however will bind ANF and BNP with much lesser affinity (Suga et al., 1992a). NPR-C binds ANF with high affinity, followed by CNP and BNP with lower affinities (Suga et al., 1992a).

ANF concentration in circulation decays drastically with a half-life of approximately 2 minutes (Yandle et al., 1986); however, the half-life has been reported to be anywhere from 1.7-3.1 minutes (Nakao et al., 1986). The extremely low half-life of ANF is due in part to both the activity of neutral endopeptidase 24.11 (NEP) (Stephenson and Kenny, 1987), which cleaves ANF by proteolysis (Erdos and Skidgel, 1989), and its clearance from circulation via NPR-C (Matsukawa et al., 1999). NEP is a transmembrane metallopeptidase which cleaves hydrophobic amino acids of peptides in various tissues distributed throughout the body such as the kidney, epithelial and endothelial cells, and the intestines (Erdos and Skidgel, 1989). NPR-C regulates natriuretic peptide concentrations in the blood through receptor-ligand internalization and further degradation of ANF, BNP and CNP (Nussenzveig et al., 1990). In NPR-C knockout mice, the half-life of ANF is increased and a hypotensive phenotype is observed (Matsukawa et al., 1999).

Healthy individuals have a relatively low concentration of ANF in plasma (25-100 pg/mL) (de Bold, 1985); however, in patients with congestive heart failure and heart disease, ANF concentrations have been seen to be elevated by up to 30-fold (Burnett, Jr. et al., 1986;Cody et al., 1986) and BNP levels by up to 200-fold (Yandle et al., 1993;Langenickel et al., 2000;Potter et al., 2006). BNP plasma concentrations are currently

being used in clinical practice as a marker for heart disease (Potter et al., 2006). Both ANF and BNP expression are increased in the atria in heart disease, but ventricular expression of these peptides in late stages and chronic diseased states are observed as well (de Bold et al., 2001).

Mechanisms of ANF secretion

There are three routes through which ANF progresses throughout the secretory pathway within atrial cardiocytes: constitutive, constitutive-like and regulated.

Constitutive secretion

In the constitutive pathway, newly synthesized proteins are continuously released from the cell in a stimuli-independent manner (Burgess and Kelly, 1987;Arvan and Halban, 2004). Once the proteins are synthesized in the endoplasmic reticulum (ER) and packaged in the Golgi apparatus, vesicles that are not associated with a clathrin coat are immediately trafficked containing these hormones to the plasma membrane for rapid secretion (Burgess and Kelly, 1987). These proteins are not sorted into maturing granules and therefore are separated within the TGN from those destined for regulated secretion (Burgess and Kelly, 1987;Arvan and Halban, 2004). This pathway utilizes the continuous turnover of newly synthesized hormone and its subsequent secretion independent of storage or stimuli (Burgess and Kelly, 1987). In the case of ANF, inhibition of granule formation or targeting

of proteins to the granules within the TGN does not affect basal ANF secretion (Ogawa et al., 1999).

Regulated secretion

In the regulated pathway, hormones are sorted from the Golgi complex into condensing vacuoles and stored in granules until the presence of a stimulus which signals the granules to transport to the plasma membrane and release their contents (Burgess and Kelly, 1987). These secretory granules contain ANF in its pro-hormone form and are retained in the cell until maturation (de Bold et al., 1996). Secretion of ANF from these vesicles is mediated by neuroendocrine and mechanical stimulation (de Bold et al., 1996;McGrath et al., 2005).

The neuroendocrine-stimulated pathway involves agonists such as AngII, endothelin-I (ET-1), vasopressin, glucocorticoids, and α- and β-adrenergic agonists such as phenylephrine (de Bold et al., 1996;de Bold and Bruneau, 2000;McGrath and de Bold, 2005). The most potent neuroendocrine stimulator of regulated ANF secretion is the vasoconstrictor ET-1 (Schiebinger and Gomez-Sanchez, 1990;de Bold et al., 1996), which activates ANF release through the G protein-coupled receptor Gαq (Bensimon et al., 2004;McGrath et al., 2005) and also stimulates the secretion of BNP (Bruneau and de Bold, 1994). Synthesis of ANF is increased in hearts that have been subjected to volume overload and ET-1 stimulation (de Bold and Bruneau, 2000).

Mechanical stimulation of ANF signals through mechanosensitive proteins (de Bold et al., 1996), such as G protein-coupled receptors (GPCRs), which are associated and

localized with the cytoskeleton and organelle membranes (Cote et al., 1997), and have been implicated in various mechanical events within the cell, including stimulus-secretion coupling (Gomperts et al., 1986;Barrowman et al., 1986). Mechanosensation is the ability of a cell to sense a mechanical, physical change in its cytoskeleton and respond by producing a biochemical signal (Storch et al., 2012). Mechanical stimulation of ANF release is due to the stretch-secretion coupling phenomenon documented in atrial cardiocytes (Kuroski de Bold and de Bold, 1991). This pathway involves atrial stretch (Edwards et al., 1988) which rapidly secretes newly synthesized ANF stored in granules (Mangat and de Bold, 1993). Atrial stretch causes an immediate release of ANF from the cell; however, even in the continuous presence of stretch, the amount of ANF release returns to basal levels within 180 minutes even though its stores are not completely depleted (Mangat and de Bold, 1993). This form of regulated ANF secretion may be enhanced by neuroendocrine factors such as ET-1 and AngII (de Bold and Bruneau, 2000).

Constitutive-like secretion

ANF is also secreted from the atria by exocytosis in a constitutive-like manner (Ogawa et al., 1999), which refers to a pathway in which proteins are secreted by exocytosis of vesicles that have budded from immature secretory granules (ISGs) (Arvan et al., 1991;Kuliawat and Arvan, 1992). Constitutive-like secretion has characteristics of both constitutive and regulated secretion because release of secretory products can be either stimulated or unstimulated (Arvan et al., 1991). In the atria, it may be stimulated by mechanical or neuroendocrine factors (de Bold et al., 1996).

Constitutive-like secretion is defined as the release of proteins at the plasma membrane from clathrin-coated vesicles that have budded from ISGs (Kuliawat and Arvan, 1992;Arvan and Castle, 1992;Dannies, 1999). The first destination of these vesicles after budding from ISGs is at the endosome, where the contents of these vesicles have two pathways which they can follow: trafficking to the plasma membrane for stimulated and unstimulated secretion, or trafficking to the lysosome for degradation and recycling (Arvan and Castle, 1998). This indirect route from the TGN to the plasma membrane, requiring an intermediate compartment at the endosome, is distinguished as a separate pathway than that of constitutive secretion, which follows a direct pathway from the TGN to the membrane for release (Kuliawat and Arvan, 1994).

Delivery of proteins via the constitutive-like pathway from ISGs to the cell surface retains a slow half-life of approximately 1-2 hours (Arvan and Castle, 1992). Constitutive-like secretion has been observed in other endocrine organs such as the β-cells of the pancreas (Arvan et al., 1991;Kuliawat and Arvan, 1992).

Secretory granule biogenesis

Properties of secretory granule biogenesis

Formation of secretory granules begins in the rough ER with the synthesis of large precursors of peptides, followed by their transportation through the *cis*-, *medial*- and *trans*-cisternae of the Golgi complex (Kim et al., 2006). It is at the *trans*-most cisterna of the Golgi that peptide hormones are packaged into condensing vacuoles (Arvan and Castle,

1992) that either become enveloped in a lipid bilayer and further budding from the TGN for regulated secretion (Rindler, 1992) or are transported to the plasma membrane for immediate exocytosis via the constitutive pathway (Arvan and Castle, 1998). The TGN and ISGs have many similar features, such as the presence of acid phosphatases, clathrin, luminal acidification, prohormone convertases, and lysosomal hydrolases (Arvan and Castle, 1992;Arvan and Castle, 1998).

One of the distinguishing properties of an ISG compared to a mature secretory granule (MSG) is the presence of a clathrin coat (Schmid, 1997). Clathrin is a triskelion coat protein that assembles in the presence of adaptor protein-1 (AP-1) at the TGN to form a secure proteinaceous membrane for ISGs (Schmid, 1997;Burgess et al., 2011). It plays a critical role in intracellular trafficking of proteins and hormones (Arvan and Castle, 1998) and also mediates the transport of lysosomal hydrolases to the lysosome (Klumperman et al., 1998). Newly synthesized lysosomal hydrolases, packaged in the TGN, are recognized by the mannose-6-phosphate receptor and AP-1 complexes recruited in the TGN to form the clathrin coat on ISGs (Klumperman et al., 1998). Once the clathrin-coated vesicle has budded from the ISG, their route in the cell is determined by the presence of missorted proteins, which are sorted at the endosome, and the secretion of hormones by fusion with the plasma membrane (Kim et al., 2006). Loss of clathrin or its adaptor protein AP-1 in *Drosophila melanogaster* resulted in the loss of formation of secretory granules (Burgess et al., 2011), indicating an important role of these proteins in other secretory pathways.

Once ISGs have formed, several events must take place for maturation of ISGs to MSGs. These include the acidification of the granule milieu to allow for the activation of

carboxypeptidases, removal of missorted proteins and enzymes, loss of clathrin coat, and finally condensation of contents to form electron-dense cores (Arvan and Castle, 1992;Kim et al., 2006). The removal of lysosomal hydrolases and missorted proteins is a crucial step in the maturation of secretory granules (Kim et al., 2006). The last step in the maturation of granules is the priming of the soluble *N*-ethylmaleimide-sensitive factor activating protein receptors (SNARE) complex (Kim et al., 2006), which is comprised of three proteins: syntaxin 1, SNAP-25 and synaptobrevin (Duman and Forte, 2003). SNAREs mediate vesicular trafficking, docking and fusion and are located on both the granule membrane as well as the plasma membrane to establish a tight membrane interaction (Duman and Forte, 2003). The pathway which atrial secretory granules take within the cytoplasm to be transported from the TGN to the plasma membrane for release is through their interaction with microtubules (Iida et al., 1988).

Specific membrane proteins common to granules in all secretory cells have not yet been identified; however, roles for lipids as well as granins have been demonstrated (Kim et al., 2006). Cholesterol was found to be an essential part of membrane formation in granules because of its high concentration in the TGN and its ability to tightly package lipids and proteins destined for the regulated secretory pathway (Kim et al., 2006). Granins, such as chromogranins A and B (ChgA and ChgB), have been identified to drive the budding of granules at the TGN following deletion, knockout (Kim et al., 2001;Huh et al., 2003) and overexpression studies (Mahapatra et al., 2005). Inconsistent results regarding the role of ChgB in granule formation have been observed, whereby one study did not discover significant changes in granule biogenesis in ChgB-downregulated neuroendocrine and

endocrine cells (Kim et al., 2001); however in ChgB-downregulated non-neuroendocrine cells there was a significant decrease in number of secretory granules present (Huh et al., 2003). Unlike ChgB, ChgA is a crucial leader of secretory granule biogenesis (Elias et al., 2010). In the same study that did not find any significance in granule formation due to the loss of ChgB, ChgA-downregulated cells were found to produce significantly fewer granules, which in turn affected the production of regulatory hormones (Kim et al., 2001). Mice with global deletion of ChgA (ChgA-/-) revealed a similar phenotype with a markedly reduced number and size of secretory granules in chromaffin cells which was directly associated with a decrease in catecholamine levels (Kim et al., 2005). This regulated secretory phenotype was restored in ChgA-/- mice with the reintroduction of human ChgA gene expression (Mahapatra et al., 2005). In a different strain of mice produced with global deletion of ChgA, no detectable differences in secretory granule formation or size were discovered; however, overexpression of other granin proteins, such as ChgB, were observed in these animals which may be able to compensate for the loss of ChgA (Hendy et al., 2006).

Sorting of secretory granule proteins

Two hypotheses have been proposed to describe the sorting of proteins for the regulated secretory pathway: the “sorting for entry” hypothesis and the “sorting by retention” hypothesis (Arvan and Castle, 1998). The main difference between these two models is the point at which sorting of secretory proteins occurs within the cell (Arvan and

Castle, 1998). It has been found that these two sorting hypotheses are not mutually exclusive and can occur within the same cell, and also that the same protein in different cell types may use these two different sorting pathways (Gorr et al., 2001).

The most accepted hypothesis is "sorting for entry" and states that sorting of granular proteins occurs within the TGN before the formation of ISGs. This hypothesis requires receptors present in the TGN to be able to "usher" proteins destined for regulated secretion into ISGs. Only selective secretory proteins are bound and retained for entry into the ISG, therefore eliminating the possibility of missorted proteins within this route (Arvan and Castle, 1998).

The hypothesis "sorting by retention" describes the TGN of having less of a direct role in sorting than in the "sorting for entry" model. In this model, sorting occurs within the granules as they mature, removing missorted proteins along the way. There does not exist an "usher", as proposed in the opposing model, because all proteins within the TGN are enter into ISGs and are further sorted here. Lysosomal hydrolases and other non-secretory proteins are removed via receptor-mediated export from the ISGs. "Sorting by retention" is the mechanism that occurs within the β-cells of the pancreas for protein sorting of insulin and is the extensively studied model for this hypothesis (Kuliawat and Arvan, 1994;Arvan and Castle, 1998).

The targeting of proANF to the secretory granules appears to fit the hypothesis of "sorting by retention". Following formation of secretory vesicles from the TGN, sorting of proteins into these vesicles has been found to involve heterotrimeric G-proteins (Konrad et al., 1995) and directional mechanisms (Arvan and Castle, 1998). This hypothesis ensures

proper sorting of proteins for the regulatory route to mature granules through an anchoring mechanism which tethers the regulated proteins to the nascent secretory granules (Dikeakos and Reudelhuber, 2007).

G-Proteins

Heterotrimeric GTP-binding proteins

Heterotrimeric G-proteins are membrane-associated guanine nucleotide-binding proteins that form a tightly bound complex of three subunits in decreasing order of molecular mass: α (39-46 kDa), β (37 kDa) and γ (8 kDa) (Gilman, 1987). The α-subunits possess a guanine nucleotide binding pocket which allows for the exchange of bound guanosine diphosphate (GDP) to guanosine triphosphate (GTP) and can exist on its own after dissociation from the βγ-complex; however, on the other hand the β- and γ-subunits function together as one unit and are referred to as the βγ-dimer (Hepler and Gilman, 1992). Both units are able to stimulate effector pathways once dissociated from the heterotrimeric complex (Hepler and Gilman, 1992;Birnbaumer, 2007). There are 16 mammalian Gα genes, five Gβ genes and 12 Gγ genes to date (Cabrera-Vera et al., 2003;Krumins and Gilman, 2006).

Heterotrimeric G-proteins are involved in two main intracellular processes: signal transduction and targeting of secretory products. Signal transduction occurs when extracellular signals activate a cell surface receptor, i.e. a G-protein coupled receptor (GPCR), and transmits the signal to a target. A GPCR is a seven-transmembrane spanning

helic domain that is linked to a receptor, heterotrimeric G-proteins, and an effector molecule. Ligand binding occurs on the membrane surface, which sends a signal to the intracellular GPCR complex that contains α-, β- and γ-subunits bound to one another. The α-subunits are specifically coupled to different effectors, such as guanylyl-cyclases and protein kinases, and the βγ complex dissociates and activates downstream effectors. Hydrolysis of GTP to GDP and P_i deactivates the receptor and subsequently cannot activate effectors (Gilman, 1987;Hepler and Gilman, 1992).

The formation, trafficking, and fusion of granules to the plasma membrane for release requires the presence and association of one or more G-proteins with the granule cytoskeleton (Burgoyne, 1990;Barr et al., 1991;Gasman et al., 1998). From production of the secretory vesicles in the TGN to the trafficking and subsequent exocytosis, specific proteins are involved to mediate the cytoskeletal fusion, vesicle transport along microtubules in the case of ANF, and vesicle fusion to the plasma membrane (Burgoyne, 1990;Barr et al., 1991). It was found specifically the pertussis toxin-sensitive α-subunits of heterotrimeric G-proteins are involved at the TGN in the formation of secretory vesicles (Barr et al., 1991). G-proteins have been detected in the granule membrane of rat atrial secretory granules (Wildey and Matyas, 1993), and specifically Gαo was found localized on these granules, suggesting an involvement in the regulation of ANF secretion and exocytosis (Wolf et al., 1998;Bensimon et al., 2004).

Gα subfamily

The α-subunit is the most extensively studied of the three, and numerous α-subunits have been identified. In total, there are 16 known mammalian Gα genes (Krumins and Gilman, 2006), which are grouped into four subfamilies based on functional and structural homologies: Gαs (stimulatory), Gαi/o (inhibitory/ other), Gαq/11 and Gα12/13 subunits (Hepler and Gilman, 1992;Offermanns, 2001). Each is unique in its receptor effector, but share a common structure and mechanism of activation (Hepler and Gilman, 1992).

In each family there are two or more subtypes which are classified according to their receptor effector (Cabrera-Vera et al., 2003). The Gαi/o family consists of 9 subtypes, representing the largest subfamily of Gα proteins: α_{i1}, α_{i2}, α_{i3}, α_{oA}, α_{oB}, α_{z}, α_{gust}, α_{t1}, and α_{t2} (Cabrera-Vera et al., 2003). This family is involved in the regulation of adenylyl-cyclases (ACs) and cGMP production (Fields and Casey, 1997). Gαi is represented by its inhibitory actions on the activation of AC and Gαo is represented by its "other" actions, which involve both the inhibition and stimulation of ACs (Fields and Casey, 1997).

One of the characteristics of the Gαi/o family of proteins that allows for the studying of their functions is the use of a toxin from *Clostridium botulinum* that causes the specific inhibition of these subunits (Wettschureck and Offermanns, 2005). This inhibitor of Gαi/o proteins is called Pertussis Toxin (PTX) and acts on the fourth cysteine residue of the COOH-terminal to ADP-ribosylate the protein (Strathmann et al., 1990), preventing it from interacting with its receptor (Wettschureck and Offermanns, 2005). Mastoparan-7

(MAS-7) is the opposite of PTX and can selectively activate the pathway of Gαi/o proteins (Higashijima et al., 1990).

Even though each individual protein is unique, the similarities present between all subunits, subtypes and isoforms brings forth the relevance of redundancy as has been seen in individual gene silencing of α-, β- and γ-subunits and subsequent compensation of other genes (Krumins and Gilman, 2006). The three Gαi subunits, $G\alpha i_{1\text{-}3}$, and two Gαo subunits, $G\alpha o_{A\&B}$, may have overlapping functions and the ability to compensate for the loss of one another because of their highly similar structures and localization within cells and tissues (Wettschureck and Offermanns, 2005).

Gαo subunit

Gαo is one of the lesser studied G-proteins because of its ubiquitous nature and still unknown functions and receptor effectors (Gasman et al., 1998). The specific G-protein subunit Gαo is encoded by the *Gnao1* gene and consists of two splice variants, or isoforms, through alternative splicing: *Gnao1* transcript variant A and *Gnao1* transcript variant B (Strathmann et al., 1990). Gαo is found most abundantly in the brain and central nervous system (Strathmann et al., 1990) (specifically stimulating ionic channels in neurons) (Birnbaumer, 1990); however, lower levels are also found in the heart, pancreas, and other endocrine organs (Asano et al., 1988;Offermanns, 2001). More importantly, greater amounts of Gαo are found within the atrium of the heart as compared to the ventricles (Eschenhagen et al., 1995) by 2.3 fold (McGrath and de Bold, 2009). Furthermore, Gαo was

reported as a granule membrane-associated protein in atrial secretory granules through localization by immunochemistry (Wolf et al., 1998;Bensimon et al., 2004) and proteomic analyses (Muth et al., 2004) in atrial cardiocytes.

The role of Gαo in the central and peripheral nervous systems has been studied by generating Gαo-deficient knockout mice, which show severe malfunctions of their motor and sensory neurons (Wettschureck and Offermanns, 2005). Gαo has been known to modulate cardiac inhibitory muscarinic regulation of L-type Ca^{2+} channels (Valenzuela et al., 1997); however, in the knockout mice this was completely abolished (Wettschureck and Offermanns, 2005). This phenotype was similarly seen in $G\alpha i_2$-deficient mice (Wettschureck and Offermanns, 2005). Full body Gαo knockout mice do not survive long enough to study, therefore tissue-specific Gαo knockout mice have been (Zhao et al., 2010) and are currently being generated. In a pancreas-specific Gαo knockout model, insulin secretion and granule docking in the β-cells was greatly enhanced (Zhao et al., 2010). As well, pancreas-specific $G\alpha o_A$ and $G\alpha o_B$ variant knockout models were created, and found that only $G\alpha o_B$ knockout mice had increased insulin secretion when challenged with glucose (Wang et al., 2011). These results show that $G\alpha o_B$ may have an important role in inhibiting insulin secretion (Wang et al., 2011) and that the two transcript variants of Gαo are mutually exclusive from one another.

Based on earlier studies of G-proteins and their role in the secretory processes of hormones (Barr et al., 1991), such as insulin and the β-cells of the pancreas (Zhao et al., 2010), Gαo was found to have a mechanistic role in the exocytosis of ANF (Bensimon et al., 2004). This study involved the use of both the Gαi/o inhibitor and stimulator, PTX and

MAS-7, respectively, as a pretreatment on isolated rat atria (Bensimon et al., 2004). By stimulating the stretch-secretion coupling regulated pathway of ANF secretion by increasing intraatrial pressure, ANF release was decreased and abolished in the presence of low concentration PTX and high concentration PTX, respectively, and increased in the presence of MAS-7 (Bensimon et al., 2004).

Animal models in research

Knockout mice

Since the complete mapping of the mouse genome 10 years ago (Waterston et al., 2002), genetically modified mice have allowed for significant advancements in research and medicine by modeling human diseased states. Overexpression and knockout strategies of genes within a mouse allows for the understanding of their physiological roles (Davey and MacLean, 2006). Knockout mice for a particular gene involved in embryonic development may die at a very young age and therefore may not provide insight into the gene's function otherwise (Schipani, 2002). A conditional knockout model titled the "Cre/loxP recombination system" has gained popularity as it allows for the deletion of a gene in a tissue-specific or time-specific manner (Sauer, 1998).

Knockout mice for Gαo have been previously generated through homologous recombination (Jiang et al., 1997). These mice displayed severe pathological defects at birth, including a significant decrease in body weight, temperature, and life span (Jiang et al., 1997;Jiang et al., 1998). These mice also suffered from seizures and tremors and

displayed abnormal motor behavior (Jiang et al., 1997;Jiang et al., 1998). Less than half of the mutant mice live to adulthood and less than half of these live past 3 months of age (Jiang et al., 1997;Jiang et al., 1998). For this reason in this thesis, the role of Gαo in atrial cardiocytes is studied using the Cre/lox recombination system.

Cre/lox recombination system

The Cre/lox recombination system utilizes the Cre gene of the life cycle of the P1 bacteriophage to recombine deoxyribonucleic acid (DNA) between two loxP sites at site-specific intersections (Sauer, 1998;Nagy, 2000). Cre/lox recombination utilizes Cre recombinase, a site-specific enzyme whose expression is driven by a promotor and is used to recombine two loxP sites (Sauer, 1998). Identified by their 34 basepair (bp) target sequences, loxP sites are arranged to flank either side of the DNA of interest (Sauer, 1998). The order of the loxP sites determines if the DNA site of interest will be deleted, inserted, translocated or inverted by Cre (Sauer, 1998).

Tissue-specific models require complex breeding strategies of two genetically modified transgenic strains of mice to target recombination of site-specific sequences using a tissue-specific promoter as a guide. By introducing a site-specific Cre recombinase promoter to one mouse and two loxP sites, oriented directly, flanking the gene to another mouse, initial breeding of these two mice produces 50% conditional heterozygous knockout mice with one allele of the knockout after one generation (Sauer, 1998). The second generation, breeding the conditional heterozygous knockout mouse with a

homozygous floxed non-Cre mouse, produces a 25% conditional homozygous knockout mouse of both alleles deleted and only delete the gene in the specific tissue of interest (Sauer, 1998).

In the case of ANF and Gαo, the Cre/lox recombination system for a conditional heart-specific knockout mouse will allow for the understanding of their interactions *in vivo* without severe physiological abnormalities in the central nervous system. Mice expressing two loxP sites directly flanking the fifth and sixth exons of the *Gnao1* gene, Gαo (Figure 1), common to both variants $G\alpha o_A$ and $G\alpha o_B$, will be used (Zhao et al., 2010). To promote loxP recombination to heart muscle, the α-myosin heavy chain (α-MHC) Cre promoter mice will direct this. These mice have been extensively used by other research laboratories and proven to redirect recombination only in heart muscle (Chien, 2001) and no other tissue or cell types (Niu et al., 2005).

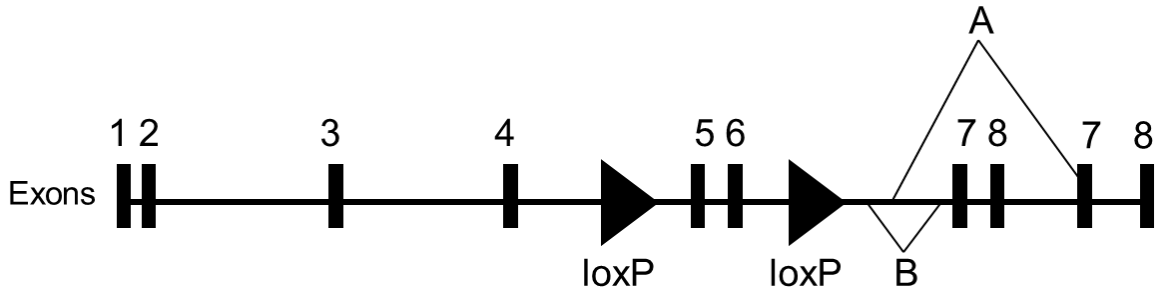


Figure 1: *Mus musculus Gnao1* gene.

Alternative splicing of Gαo produces two transcript variants, $G\alpha o_A$ and $G\alpha o_B$. Two loxP sites are flanking exons 5 and 6 to delete both variants of Gαo by recombination with Cre.

AIM OF STUDY

Immunocytochemical and electron microscopic analyses of atrial secretory granules showed co-localization of Gαo and ANF in atrial cardiocytes (Wolf et al., 1998;Bensimon et al., 2004). Immunochemical and oligonucleotide microarray analysis demonstrated the presence of $G\alpha o_A$ in the atria and its abundance by 2.3 fold compared to the ventricles (McGrath and de Bold, 2009). Further analysis using a yeast-two-hybrid approach with $G\alpha o_A$ as bait revealed protein-protein interactions between proANF and $G\alpha o_A$ (Ogawa et al., 2009). Therefore, it is possible that the specific atrial granule membrane-associated Gαo may play a mechanistic role in the sorting by retention hypothesis that serves to direct proANF to the mature atrial granules.

The aim of this study is to determine the role of Gαo in ANF gene expression and atrial granule biogenesis.

HYPOTHESIS

Gαo cardiac knockout will disrupt ANF granule storage in atrial cardiocytes.

METHODS

Mice

All experiments involving animals were previously approved by the Animal Care Committee of the University of Ottawa and carried out according to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

Gαo-floxed mice with loxP sites flanking exons 5 and 6 (Figure 1), which are common to both isoforms $G\alpha o_A$ and $G\alpha o_B$, were a gift from Dr. Birnbaumer, NIEHS, strain 129SvEv. Disruption of this gene was previously described (Chamero et al., 2011).

α-MHC-Cre mice were purchased from The Jackson Laboratory, strain name B6.FVB-Tg(Myh6-cre)2182Mds/J, strain number 011038. Expression of Cre, driven by the alpha-myosin heavy chain (cardiac-specific) promoter, induces more than 90% recombination in cardiac muscle cells in the presence of two loxP sites.

All experiments were carried out using atrial appendages of both female and male mice aged 8-12 weeks old. Mice were decapitated and hearts immediately excised and rinsed in ice cold 0.9% NaCl.

Cre/lox generation

Standard Cre/lox tissue-specific breeding protocol was followed according to The Jackson Laboratory.

Breeding a Gαo-floxed mouse and an α-MHC-Cre mouse produces a conditional heterozygous knockout mouse (cGαo +/-) that contains α-MHC-Cre and has one deleted Gαo-floxed allele in the heart and one wild type, non-floxed allele in the heart (Figure 2). The first Gαo-floxed allele has recombined with Cre and is deleted from cardiac muscle cells. This mouse also has a heterozygous floxed genotype throughout its genome; however, none of these floxed alleles are recombined. Breeding a Gαo-floxed mouse (that does not contain Cre) and a cGαo+/- mouse produces a conditional homozygous knockout mouse (cGαo-/-) that contains α-MHC-Cre and has both deleted Gαo-floxed alleles in the heart (Figure 3). The second Gαo-floxed allele has recombined with Cre and is deleted from cardiac muscle cells. This mouse is homozygous floxed throughout its genome; however, still none of these floxed alleles are recombined in tissues other than the heart.

All genotypes were produced in Mendelian frequencies. Table 1 represents all the genotypes obtained from the Cre/lox mating scheme and their respective transgenic titles and genotypes. Only the first two mice of the six genotypes were used in experiments: control and cGαo-/-. The homozygous floxed mouse $G\alpha o^{flx/flx}$ was used as the control (Davey and MacLean, 2006;Zhao et al., 2010).

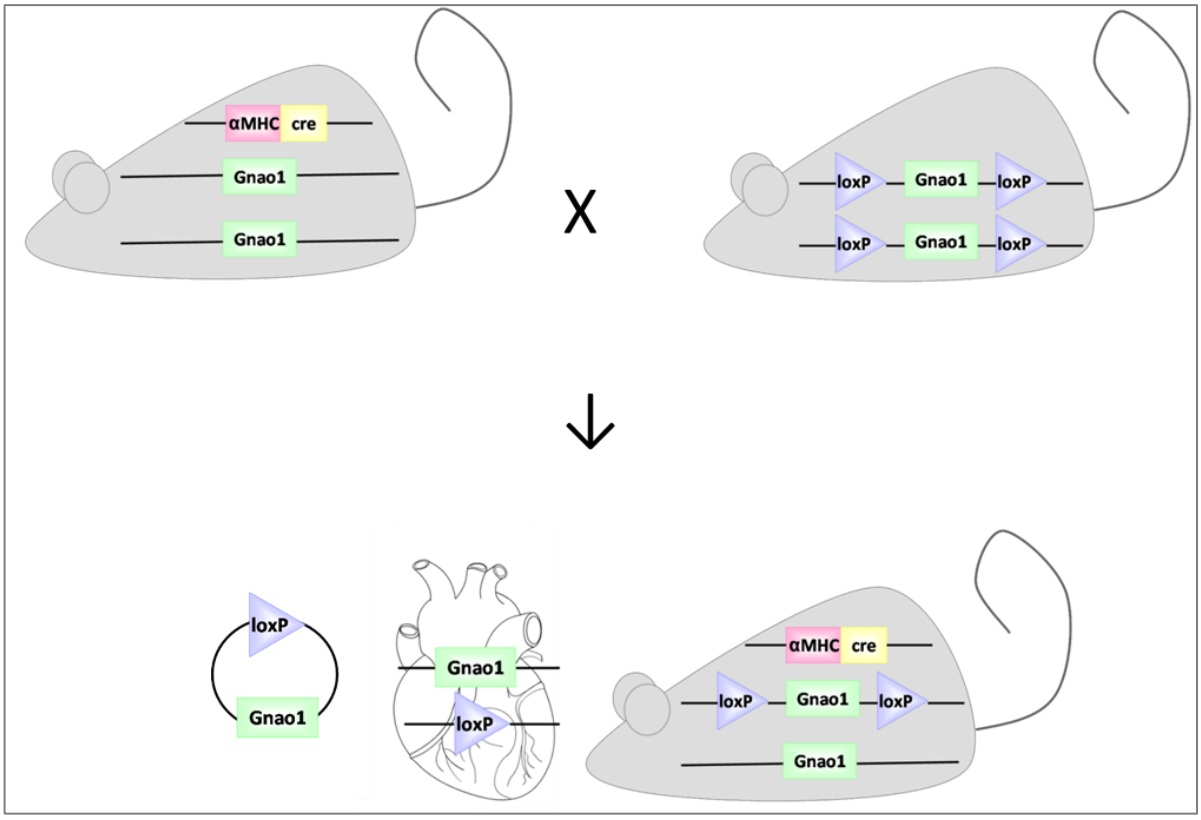


Figure 2: Breeding scheme for F1 generation.

Breeding a Gαo-floxed mouse and an α-MHC-Cre mouse produces a first generation conditional mouse that contains α-MHC-Cre and has one deleted Gαo-floxed allele and one wild type, non-floxed allele. The first Gαo-floxed allele has recombined with Cre and is deleted from cardiac muscle cells.

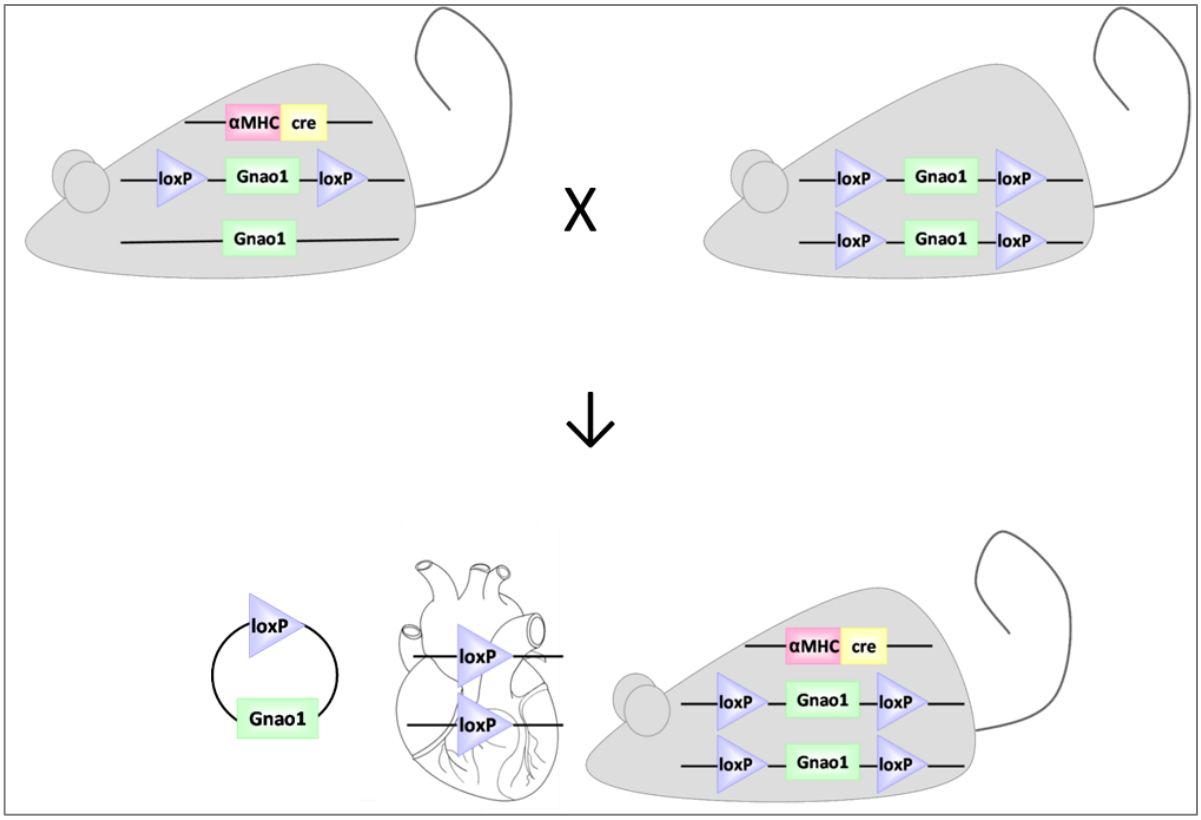


Figure 3: Breeding scheme for F2 generation.

Breeding a Gαo-floxed mouse and a first generation conditional Gαo heterozygous knockout (+/-) mouse produces a second generation mouse that contains α-MHC-Cre and has both deleted Gαo-floxed alleles. The second Gαo-floxed allele has recombined with Cre and is deleted from cardiac muscle cells.

Table 1: Genotypes obtained from F1 and F2 Cre/lox generation breeding scheme.

Only the first two genotypes were used in experiments. "c" represents conditional.

Gαo GENOTYPE	TRANSGENIC TITLE	FULL GENOTYPE
Control	Homozygous floxed control	Wt; $G\alpha o^{Flx/Flx}$
cGαo -/-	Homozygous conditional knockout	αMHC^{Cre}; $G\alpha o^{Flx/Flx}$
cGαo +/-	Heterozygous conditional knockout	αMHC^{Cre}; $G\alpha o^{Flx/Wt}$
	Heterozygous floxed	Wt; $G\alpha o^{Flx/Wt}$
	Hemizygous Cre	αMHC^{Cre}; $G\alpha o^{Wt/Wt}$
	Wild type	Wt; $G\alpha o^{Wt/Wt}$

Genotyping of mice

At 21 days of age, a 0.1 mm tail clipping was taken from each mouse. DNA extraction was performed using the REDExtract-N-Amp™ Tissue PCR Kit (XNAT, Sigma Aldrich) and subsequent polymerase chain reaction (PCR) amplification using REDExtract-N-Amp PCR Reaction Mix (XNAT, Sigma Aldrich) in an Eppendorf Mastercycler Thermal Cycler (Eppendorf Canada). PCR parameters for genotyping the Gαo-floxed alleles, Cre alleles, and control alleles are listed in tables 2 and 3. Oligonucleotide sequences for genotyping are listed in Table 4.

Gαo products were run on a 1% DNA agarose gel using a 1 kilobase ladder (G571A, Promega). Cre products were run on a 1.5% DNA agarose gel using a 50 bp ladder (10488, Invitrogen). Primers were commercially synthesized by Operon.

Table 2: PCR cycling parameters for Gαo-floxed mice.

STEP	TEMPERATURE	TIME	CYCLES
Initial denaturation	94°C	2 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	58°C	1 minute	
Extension	72°C	2 minutes	
Final extension	72°C	7 minutes	1
Hold	4°C	Indefinitely	

Table 3: PCR cycling parameters for α-MHC-Cre mice.

STEP	TEMPERATURE	TIME	CYCLES
Initial denaturation	94°C	1 minute	1
Denaturation	94°C	30 seconds	35
Annealing	58°C	1 minute	
Extension	72°C	2 minutes	
Final extension	72°C	7 minutes	1
Hold	4°C	Indefinitely	

Table 4: Oligonucleotide primer sequences for PCR genotyping.

NAME	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
Gαo-floxed	AAGAATAGAACCTAGGACTGGAGG	GCAGACAAGTGAACAAGTGAAACCC
α-MHC-Cre	ATGACAGACAGATCCCTCCTATCTCC	CTCATCACTCGTTGCATCATCGAC
TCR-α control	CAAATGTTGCTTGTCTGGTG	GTCAGTCGAGTGCACAGTTT

RNA extraction and reverse transcription

Tissues were immediately immersed in TRIzol© Reagent (Invitrogen), stored at -80°C, and homogenized using a Polytron (Kinematica, Inc.) at 70% power for 15-20 sec.

Total ribonucleic acid (RNA) was extracted using the TRIzol© (Invitrogen) method according to the manufacturer's instructions. The quality of the RNA extracted was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 Nano Kit (Agilent Technologies).

Reverse-transcription of total RNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR (RT-PCR) was performed using LightCycler 480 SYBR Green I Master Mix and analyzed on the LightCycler 480 SW 1.5 relative quantification software (Roche Applied Science) according to manufacturer's instructions.

Primer nucleotide sequences were obtained from literature and validated using Primer3 Input Version 0.4.0 (http://frodo.wi.mit.edu). Primer nucleotide sequences are listed in Table 5. Glucose-6-phosphate dehydrogenase (G6PD) was used as the reference gene and concentration ratios were normalized to the calibrator and corrected using primer efficiency. Each gene of interest was done in triplicate. Each primer pair was

validated by conducting a standard curve in triplicate. Primers were commercially synthesized by Operon.

Table 5: Oligonucleotide primer sequences for RT-PCR.

NAME	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
G6PD	CCAGCCTCCTACAAGCACCTCA	AATAGCCCCACGACCCTCAGTA
Gαo	TGCACGAGTCTCTCATGCTCT	AGATGGTCAAGGGTGACTTCT
ANF	GCCGGTAGAAGATGAGGTCA	GGGCTCCAATCCTGTCAATC
BNP	CAGCTCTTGAAGGACCAAGG	AGACCCAGGCAGAGTCAGAA

Western blot

Tissues were immediately placed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate) with 1:100 dilution of protease inhibitor cocktail (P8340, Sigma), stored at -80°C and homogenized using a Polytron at 70% power for 15-20 sec. Homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was collected and protein quantification determined using the Pierce® BCA Protein Assay Kit (Pierce, Thermo Scientific).

Samples were prepared by adding the appropriate amount of protein (7 µg of atrial protein) to 3X Blue loading dye (7722, Cell Signaling Technology) and 10% DTT and boiled for 2 min at 95°C. 30 µL of protein was loaded onto a 4-20% Mini-PROTEAN® TGX™ precast gel (Bio-Rad Laboratories, Inc.) and run at 200 V for 45 min, along with a biotinylated Precision-Plus Protein™ WesternC ladder (161-0399, Bio-Rad Laboratories, Inc.) and mouse brain extract (sc-2253, Santa Cruz Biotechnology, Inc.). The gel was transferred to a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Millipore-Waters Corp) at 100 V for 1 h and blocked for 1 h (blocking buffer: 5% fat-free milk in tris-buffered saline 0.1% Tween-20 (TBST)). Membranes were rinsed 3 times with TBST and incubated overnight at 4°C with antibodies diluted in blocking buffer (Gαo 1:2000 (551, MBL Int.), α-tubulin 1:400 (sc-8035, Santa Cruz Biotechnology, Inc.)). Membranes were rinsed 3 times with TBST and incubated for 1 h at room temperature with secondary antibodies conjugated to horseradish peroxidase diluted in blocking buffer (goat anti-rabbit 1:5000 (sc-2004, Santa Cruz Biotechnolgy, Inc.), goat anti-mouse 1:5000 (1858413, Pierce Thermo Scientific), and

Precision Protein StrepTactin 1:5000 (161-0380, Bio-Rad Laboratories, Inc.)). Membranes were rinsed 3 times with TBST and visualized using the FluroChem Alpha Ease ECL imaging system (Alpha Innotech Corp) following enhancement with Luminata™ Forte chemiluminescence (Millipore-Waters Corp).

Plasma collection

Blood was collected in 1.5 mL tubes containing 15% EDTA and centrifuged at 2,000 g for 20 min at 4°C to isolate plasma. Plasma was transferred to a new tube and stored at -80°C. To each sample, 1 mL of 0.1% trifluoroacetic acid (TFA) was added and passed three times through a pre-wetted Sep-Pak C_{18} cartridge (Millipore-Waters Corp.) with 5 mL 80% acetonitrile (CAN) + 0.1% TFA and 20 mL TFA. The cartridges with the adsorbed peptides were washed with 20 mL TFA and then eluted with 3 mL 80% ACN + 0.1% TFA. Samples were then freeze-dried and reconstituted in 110 µL radioimmunoassay (RIA) buffer and a RIA was performed.

Natriuretic peptide extraction

Tissues were extracted in extractant solution (0.1 N HCl, 1.0 M acetic acid, and 1% NaCl) and homogenized using a Polytron (PT 10-35, Kinematica, Inc.) at 70% power for 15-20 s. For protein calculations, 100 µL of homogenate was removed and stored at -20°C. Samples were centrifuged at 10,000 g for 30 min at 4°C. Supernatants were removed and extracted using pre-wet (5 mL 80% ACN + 0.1% TFA and then 20 mL 0.1% TFA) Sep-Pak C_{18}

cartridges (Millipore-Waters Corp.). Atrial samples were separated into two portions of 1.5 mL: one portion was reconstituted for reverse-phase high-performance liquid chromatography (RP-HPLC) with 0.1% TFA, and the other was reconstituted for RIA in RIA buffer.

Radioimmunoassay

ANF RIA uses the double antibody method and was performed as previously described (Sarda et al., 1989). Anti-rat ANF99-126 and anti-rat BNP64-95 serum (Peninsula Laboratories) showed less than 0.01% cross-reactivity with BNP and ANF peptides, respectively.

ANF RIA was performed on all freeze-dried samples, including RP-HPLC fractions and plasma samples. Each atrial and pancreas sample was reconstituted in 500 µL of RIA buffer (0.1 M sodium phosphate, 0.05 M NaCl, 0.01% sodium azide, 0.1% Triton X-100, and 0.1% heat-treated BSA). Plasma samples were reconstituted in 220 µL of RIA buffer. Dilutions of samples for ANF RIA were 1:1000 for atrial tissue, and 1:10 for RP-HPLC fractions.

Standard curves were generated in-run using rat ANF_{99-126} peptides (PX8895, Advanced ChemTech) with concentrations 1.5625, 3.125, 6.250, 12.50, 25.0, 50.0 and 100.0 pg/0.1 mL diluted in RIA buffer. To 100 µL of sample or standard (in duplicate), 100 µL of ANF antiserum diluted 1:20,000 (RAB 005-24, Phoenix Pharmaceuticals) was added and incubated at 4°C for 4 h out of direct sunlight. At the appropriate counts per minute

(9000-10,000), 100 µL of iodinated ANF_{99-126} was added to each tube and incubated at 4°C for 18 h out of direct sunlight. 100 µL each of goat anti-rabbit IgG serum (GAR-500, Phoenix Pharmaceuticals) and 5% normal rabbit serum was added to each tube and incubated at room temperature for 2 h. Following addition of 1.5 mL of 6.25% polyethylene glycol (Sigma Aldrich), tubes were centrifuged at 2700 rpm using a Beckman J-6 centrifuge at 4°C for 45 min. Supernatants were discarded and radioactive pellets were counted for 3 min in a gamma counter (1272 CliniGamma Counter, LKB Wallac). Low and medium quality controls (12.5 pg/0.1 mL and 25.0 pg/0.1 mL) were used.

Reverse-phase high-performance liquid chromatography

Extracted atrial freeze-dried tissues were reconstituted in 1 mL 0.1% TFA. RP-HPLC was conducted in a C_{18} column using a linear gradient elution profile from 15% to 85% ACN in 0.1% TFA at 1.5 mL/min over a total period of 80 min. 3 mL fractions were collected every 3 min and monitored at 275 nm. 100 µL of 1 mg/mL BSA was added to each fraction prior to freeze-drying. Each fraction underwent ANF quantitation by RIA.

Electron microscopy

Tissues were dissected and fixed in Karnovsky's glutaraldehyde (4% paraformaldehyde, 5% glutaraldehyde, 1 M sodium hydroxide, 0.2 M phosphate buffer) overnight and washed three times in washing solution (0.1 M Sorensen's phosphate buffer pH 7.4, sucrose, 1% calcium chloride). Tissues were post-fixed in 1% osmium tetroxide.

Embedding of tissue consisted of propylene oxide and epon. Tissues were sectioned, stained with lead and uranyl and observed at the electron microscope. A Transmission Electron Microscope (JEM-1230, JEOL Ltd.) was used to visualize the sections and AMT Image Capture Engine Software (Advanced Microscopy Techniques, Corp.) to capture section images.

Atrial specific granulation assessment was conducted by counting granules in each image (field) and normalizing this number to the magnification of the field. Microscopic sampling followed a previously published protocol (de Bold, 1978).

Electrocardiographic telemetry

Electrocardiography (ECG) transmitters (ETA-F20, Data Science International) were implanted in mice pretreated with subcutaneous buprenorphine (0.05 mg/kg) and anesthetized with isoflurane (1.5-2%). A 2-3 cm midline abdominal incision was made and an ETA-F20 (Data Sciences International) ECG transmitter unit placed within the abdominal cavity. Telemetry data gathering began on post-surgery day 3 using the PhysioTel Receiver Model Carrier (RPC-1, Data Sciences International). Quasi-lead II ECG recordings were continuously acquired at a 1 kHz sampling rate with commercially available software (Data Sciences International) for three days. Analysis was conducted using Ponemah Physiology Platform software (Data Sciences International).

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Student t-tests were performed to determine statistical significance between groups. $P \leq 0.05$ was considered significant.

RESULTS

Generation of Gαo Cre/lox mice

Breeding scheme

To examine the role of Gαo in atrial cardiocyte phenotype, a conditional Gαo-floxed mouse in which two LoxP sites exist in both alleles flanking the fifth and sixth exons common to the two variants ($G\alpha o_A$ and $G\alpha o_B$) (Figure 1) was used. By the introduction of the Cre enzyme, the LoxP sites recombine to delete the fifth and sixth exons of Gαo, producing a condensed mRNA sequence, deleting the C-terminal functional amino acids. The N-terminus of Gαo lacks the functional binding domains to its signaling pathways with adenylyl-cyclase and phospholipase C, as well as its binding to the Gβγ complex (Birnbaumer, 2007;Zhao et al., 2010).

Through standard breeding of the Gαo-floxed mouse and the α-MHC-Cre mouse, conditional Gαo heart-specific knockout mice were derived after two generations of mating (Figures 2 & 3). Continuous breeding of an α-MHC-Cre mouse to a Gαo-floxed mouse produced six genotypes of mice (Table 1). No Cre toxicity was observed in these mice (Baba et al., 2005) because no two animals expressing α-MHC-Cre were mated. This maintains a constant level of Cre in a mouse since high expression levels of these transgenes have been shown to lead to gene dysfunctions and further toxic effects, causing nonspecific effects to obscure primary effects of the gene deletion (Chien, 2001). These mice will always be Cre hemizygous and limit any confusion in genotyping which can only

distinguish the presence of Cre and not the number of alleles present. Also, $G\alpha o^{flx/flx}$ mice were used as the control mice to correct for any possible effects of targeted floxed alleles (MacLean et al., 2008).

Standard breeding of a Gαo-floxed mouse and an αMHC-Cre mouse for the F1 knockout generation can be seen in Figure 2, and the F2 generation can be seen in Figure 3. Breeding of these mice induced greater than 90% recombination in cardiac muscle cells by deletion of the flanked exons of Gαo. cGαo-/- mice (conditional Gαo heart-specific knockout mice) were no different in body weight and did not display any motor or behavioural defects compared to control mice.

Genotyping of mice

Genotyping tail tissue using Gαo-floxed primers revealed only one band at the floxed allele size in samples L510-L516, which indicates these animals are homozygous for floxed Gαo alleles (Figure 4A). Samples L517-L523 show two bands, one at the floxed allele size and one at the wild type allele size, which indicates these animals are heterozygous and contain one of each allele. Genotyping of the same mouse with ventricular DNA results in a band at 442 bp if they have at least one floxed allele and contain Cre. This can be depicted in Figure 4B of ventricular DNA as well as in Figure 4C of atrial DNA. Samples L520-L523 do not contain Cre as seen in Figure 5A, and therefore do not reveal a band at the 442 bp size because the gene cannot be deleted without activation by Cre. A faint band is visible at the floxed and/or wild type allele size (dependent on if the animal is homozygous or heterozygous for floxed) even with a band at 442 bp because this Cre

induces at least 90% recombination and therefore it is possible few alleles are not recombined.

Figure 5A represents the presence of Cre from tail tissue of the same 14 mice in Figure 4. Samples L510-L519 show one band at the 300 bp size and this indicates the presence of Cre in the genome. Since all these samples also have at least one floxed allele, Cre activates these alleles and produces a deleted Gαo gene in these 10 samples. The presence of a 200 bp size band represents the internal control for this multiplex PCR and indicates the absence of Cre from the genome. This means that even if there is a floxed Gαo allele, it is not recombined and no knockout occurs. Figures 5B and 5C are DNA isolated from ventricles and atria, respectively. These two gels confirm the presence or absence of Cre from tail tissue DNA in the preliminary stages of genotyping mice.

Genotyping ventricular DNA confirm that samples L510-L516 are cGαo-/- (Cre/ flx flx), L517-L519 are cGαo+/- (Cre/ flx wt), and L520-L523 are control cGαo+/+ (Wt/ flx wt). The following genotypes were disregarded in all further analyses: heterozygous conditional knockout (cGαo+/-), heterozygous floxed, hemizygous Cre, and wild type mice (Table 1).

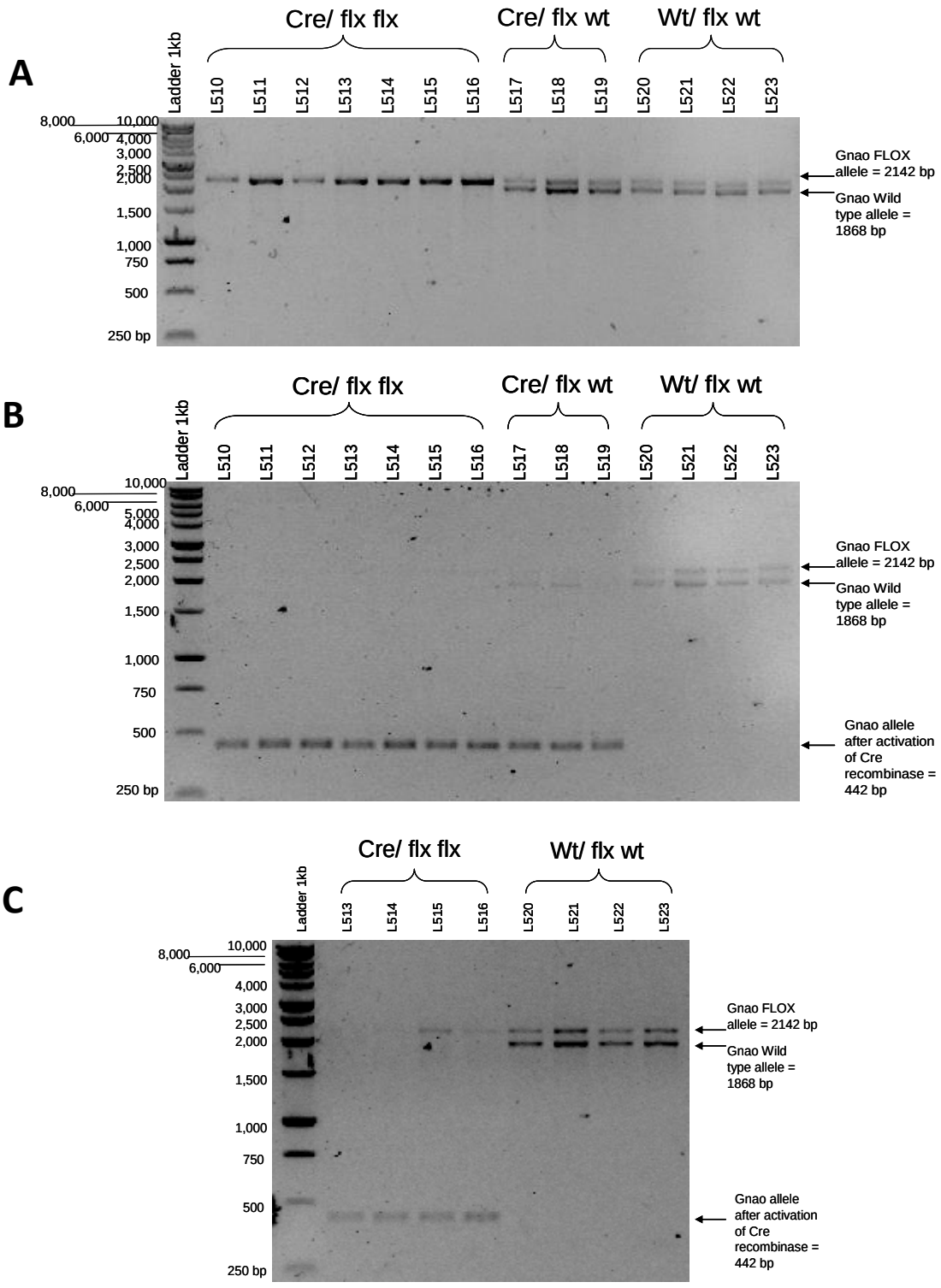


Figure 4: Analysis of genomic DNA isolated from tissue using Gαo-floxed primers.

Genotyping of A) tail tissue, B) ventricular tissue, and C) atrial tissue using Gαo-floxed primers to distinguish between the wild type, heterozygous or homozygous Gαo-floxed

mice. A 2142 bp gene product represents a Gαo allele containing two loxP sites in the Gαo gene; an 1868 bp gene product represents a Gαo wild type allele; and a 442 bp gene product represents a Gαo allele after activation by Cre recombinase thereby producing a deleted allele and truncated band. Samples L513-L516 are homozygous knockout mice (they are homozygous for Gαo-floxed and express Cre). Samples L520-L523 do not express Cre and will therefore not produce a band at 442 bp.

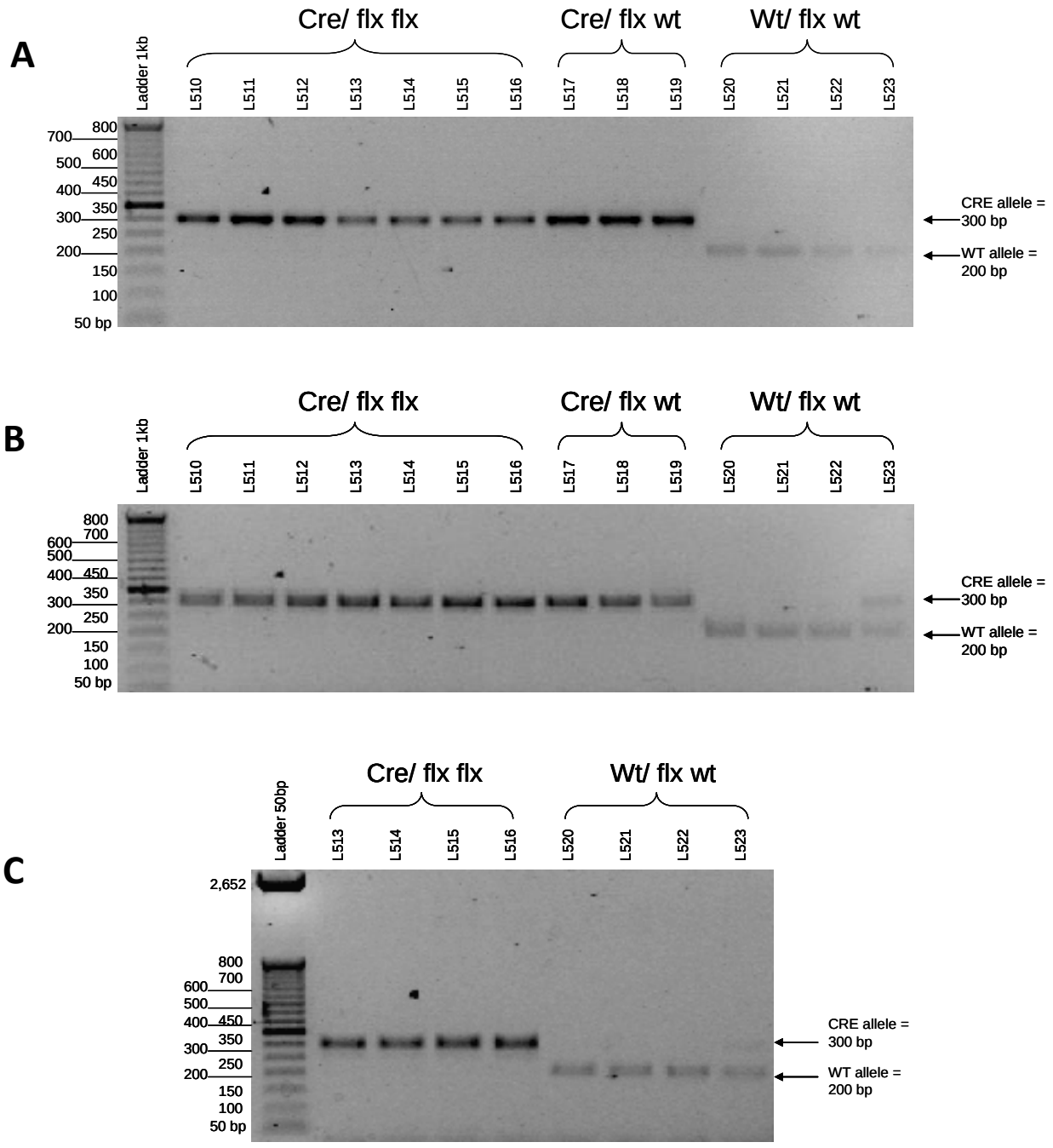


Figure 5: Analysis of genomic DNA isolated from tissue using α-MHC-Cre primers.

Genotyping of A) tail tissue, B) ventricular tissue, and C) atrial tissue using α-MHC-Cre primers to distinguish between the mice carrying the Cre enzyme or those without. A 300 bp gene product represents the presence of Cre in the genome (one Cre allele) and a 200 bp gene product represents the internal control in a sample without the presence of Cre.

Characterization of Gαo Cre/lox mice

Gene expression in atrial tissue

The expression of Gαo, ANF and BNP were analyzed in atrial tissue extracts using RT-PCR. Significantly differentially expressed genes in homozygous knockout mice include Gαo, ANF and BNP.

In order to determine the extent of the deletion of Gαo, expression of Gαo transcripts were analyzed. In cGαo-/- mice, Gαo mRNA was significantly reduced by 90% ($P < 0.001$) that of control mice (Figure 6). These results correspond to the almost complete inactivation (90%) of both alleles of the Gαo gene in cGαo-/- mice. These results confirm the conditional gene knockout has occurred in the heart.

ANF and BNP gene expression were analyzed to observe the effects of Gαo inactivation in the hormones co-stored in the granules that co-localize Gαo. Surprisingly, 23% of ANF was knocked down in cGαo-/- mice compared to control (Figure 7). Statistical significance ($P < 0.05$) of conditional animals was observed in the cGαo-/- mice (Figure 7). Interestingly, BNP had a more significant expression change than ANF (Figure 8). In cGαo-/- mice, BNP gene expression was downregulated significantly by 63% ($P < 0.01$).

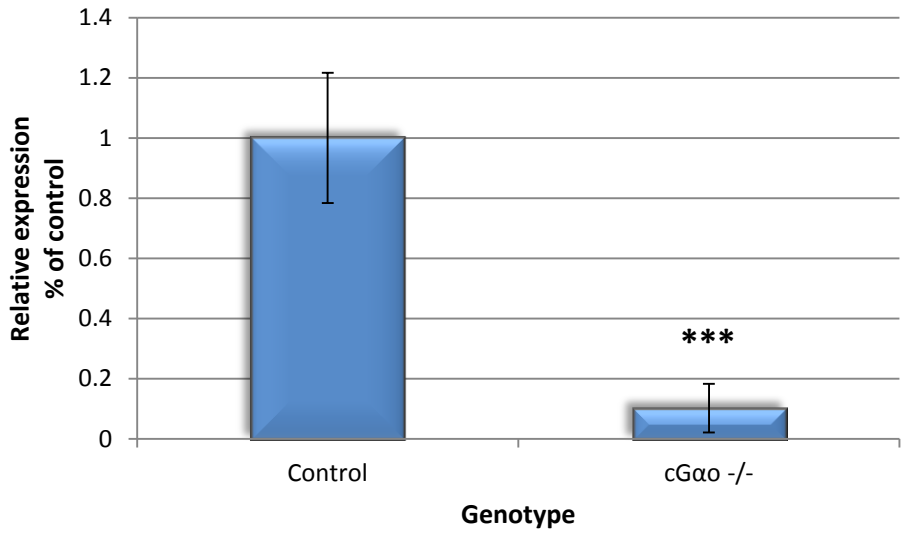


Figure 6: Gαo relative expression levels in atrial tissue.

Expression levels of Gαo in control and cGαo-/- genotypes through RT-PCR. Values are mean ± SEM; n = 6; *** $P < 0.001$.

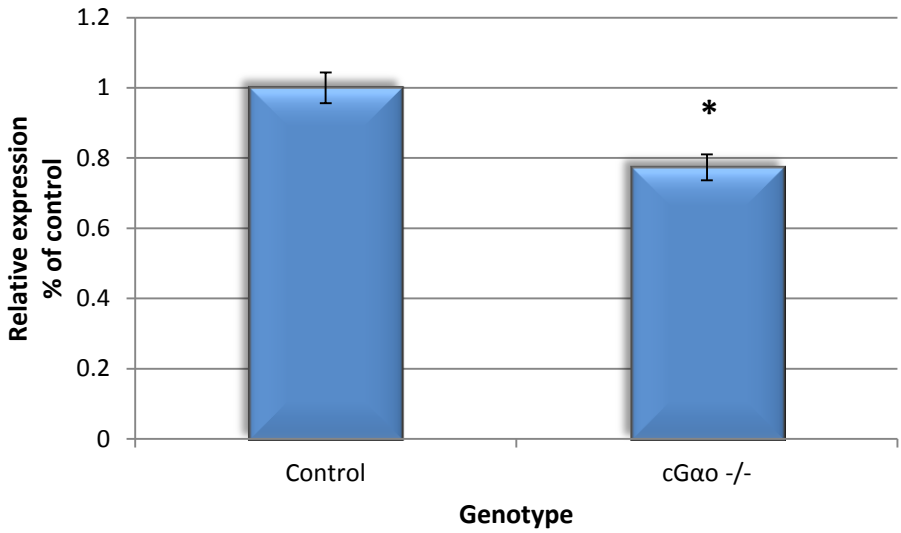


Figure 7: ANF relative expression levels in atrial tissue.

Expression levels of ANF in control and cGαo-/- genotypes through RT-PCR. Values are mean ± SEM; n = 6; * $P < 0.05$.

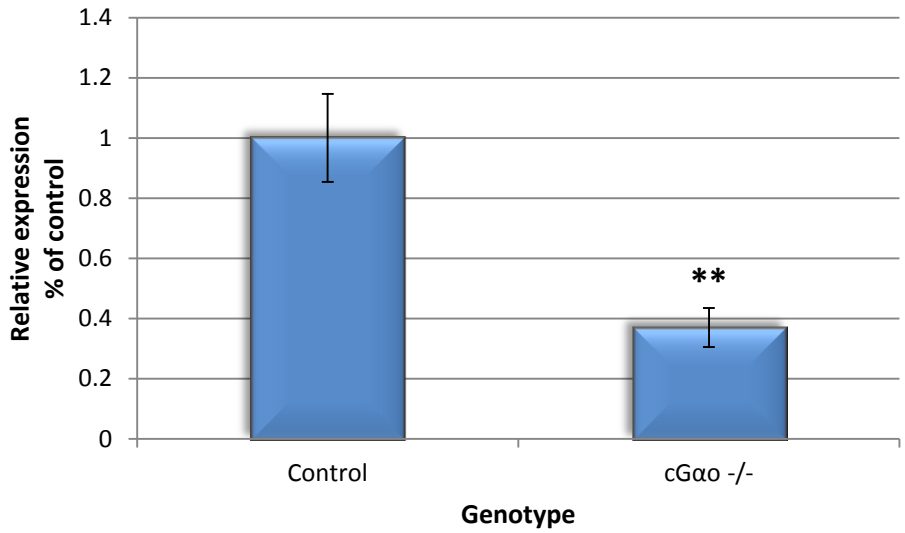


Figure 8: BNP relative expression levels in atrial tissue.

Expression levels of BNP in control and cGαo-/- genotypes through RT-PCR. Values are mean ± SEM; n = 6; ** $P < 0.01$.

Protein expression in atrial tissue

Protein expression analysis of the atria in control and cGαo-/- mice using a specific Gαo polyclonal antibody by western blotting allowed for the determination of the presence or absence of Gαo protein production in conditional knockout animals. Gαo protein expression was much less abundant in cGαo-/- mice compared to control mice, which was very robust (Figure 9). These results correspond to the RT-PCR data of a significant downregulation of Gαo gene expression.

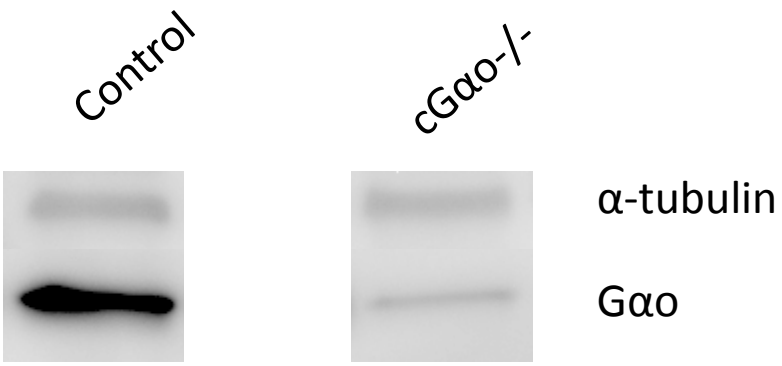


Figure 9: Protein expression of Gαo in atrial tissue.

Western blot using specific Gαo polyclonal antibody in control and cGαo-/- mice.

Representative band of n = 4.

Peptide content and molecular forms of ANF

ANF and BNP peptide content in atrial tissue

Quantification of peptide content in atrial tissue extracts of control and cGαo-/- mouse genotypes using specific ANF and BNP antibodies in a double antibody RIA allowed for the determination of intracellular concentrations of ANF and BNP, respectively. The atrial ANF content in the cGαo-/- mice did not show a statistically significant difference (16489 pg/mg of tissue) compared to the atrial concentration of ANF in control mice (18546 pg/mg of tissue) (Figure 10).

Intracellular BNP content was found to be statistically significant for cGαo-/- mice ($P \leq 0.05$) (Figure 11). Control mice had an average BNP concentration of 118 pg/mg of tissue; while the cGαo-/- mice were considerably lower at an average concentration of 84 pg/mg of BNP in tissue.

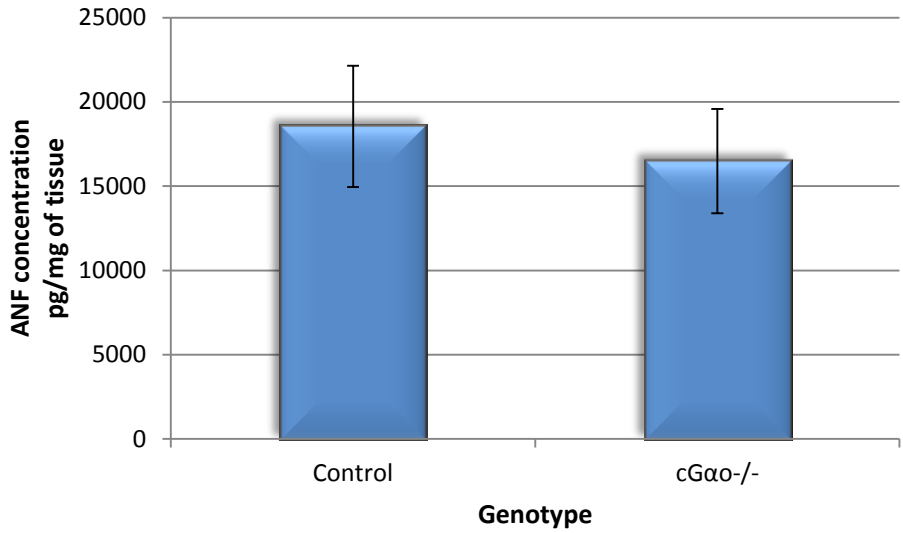


Figure 10: Concentration of ANF peptide in atrial tissue.

Intracellular content of ANF in control and cGαo-/- genotypes through ANF RIA. Values are mean ± SEM; n = 6.

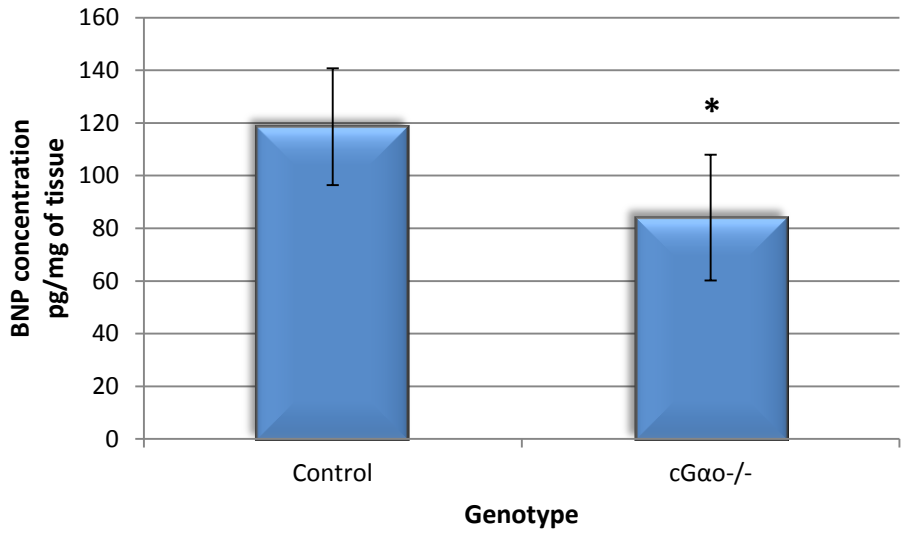


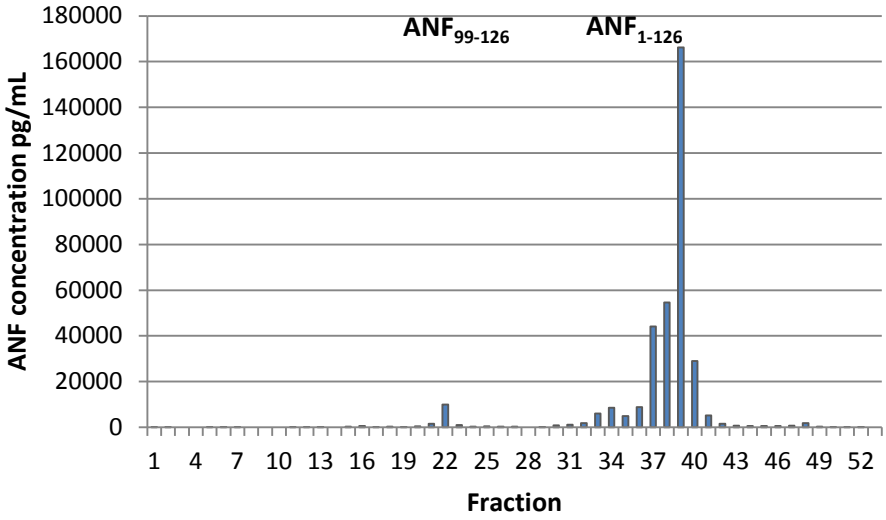
Figure 11: Concentration of BNP peptide in atrial tissue.

Intracellular content of BNP in control and cGαo-/- genotypes through BNP RIA. Values are mean ± SEM; n = 6; * P ≤ 0.05.

Molecular forms of intracellular ANF in atrial tissue

Molecular forms of intracellular ANF in atrial tissue extracts were examined to determine the ratio content of low molecular weight processed ANF (ANF_{99-126}) and high molecular weight proANF (ANF_{1-126}) by RP-HPLC analysis. Each fraction underwent a subsequent ANF RIA to determine ANF content. RP-HPLC profiles showed the presence of both forms of ANF in each genotype, with an approximate ratio of 90% proANF to 10% processed ANF. The predominant intracellular form of ANF is proANF, and can be seen in the control (Figure 12A) and cGαo-/- (Figure 12B) mice in fractions 38-40. The processed form of ANF can be found in fractions 21-22 in both genotypes (Figure 12). Similar profiles of proANF and ANF are seen between both genotypes, with the cGαo-/- mice not revealing a significant change in stoichiometry of proANF to ANF.

A



B

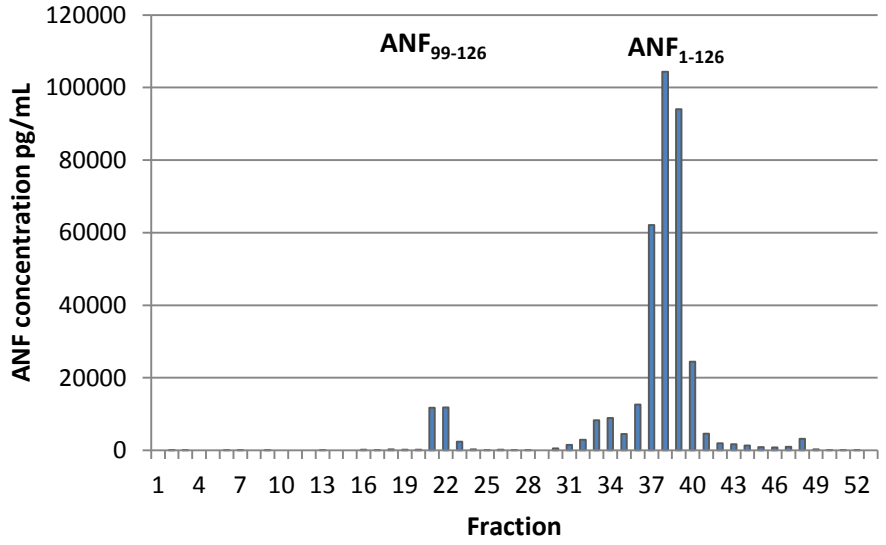


Figure 12: Molecular forms of intracellular ANF in atrial tissue.

RP-HPLC profiles of intracellular ANF through ANF RIA in (A) Control mice and (B) cGαo -/-.

N = 1.

ANF peptide content in plasma

Quantification of circulating peptide content in both genotypes was performed using a specific ANF antibody in a double antibody RIA. A total of four and five animals were used in control and cGαo-/- mice, respectively. No significant differences were observed between cGαo-/- mice (34.64 pg/mL) compared to control mice (50.612 pg/mL) (Figure 13).

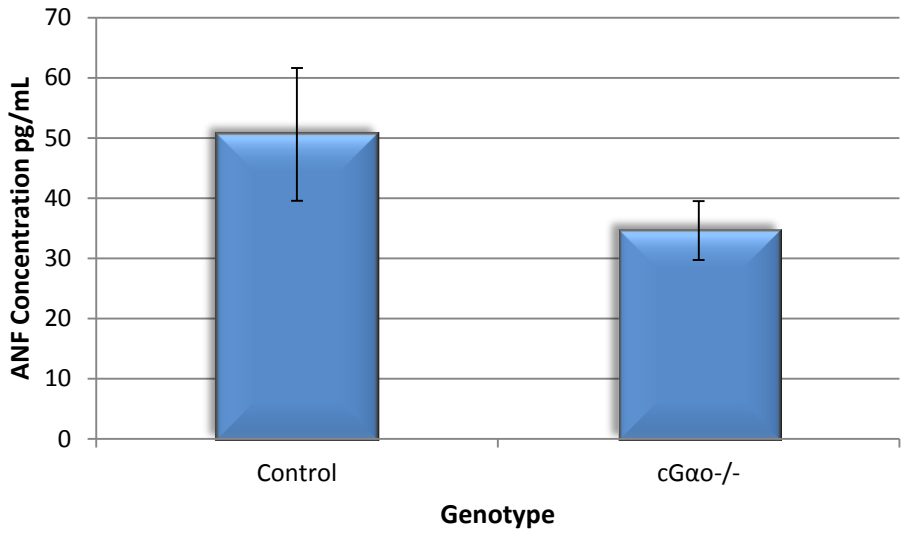


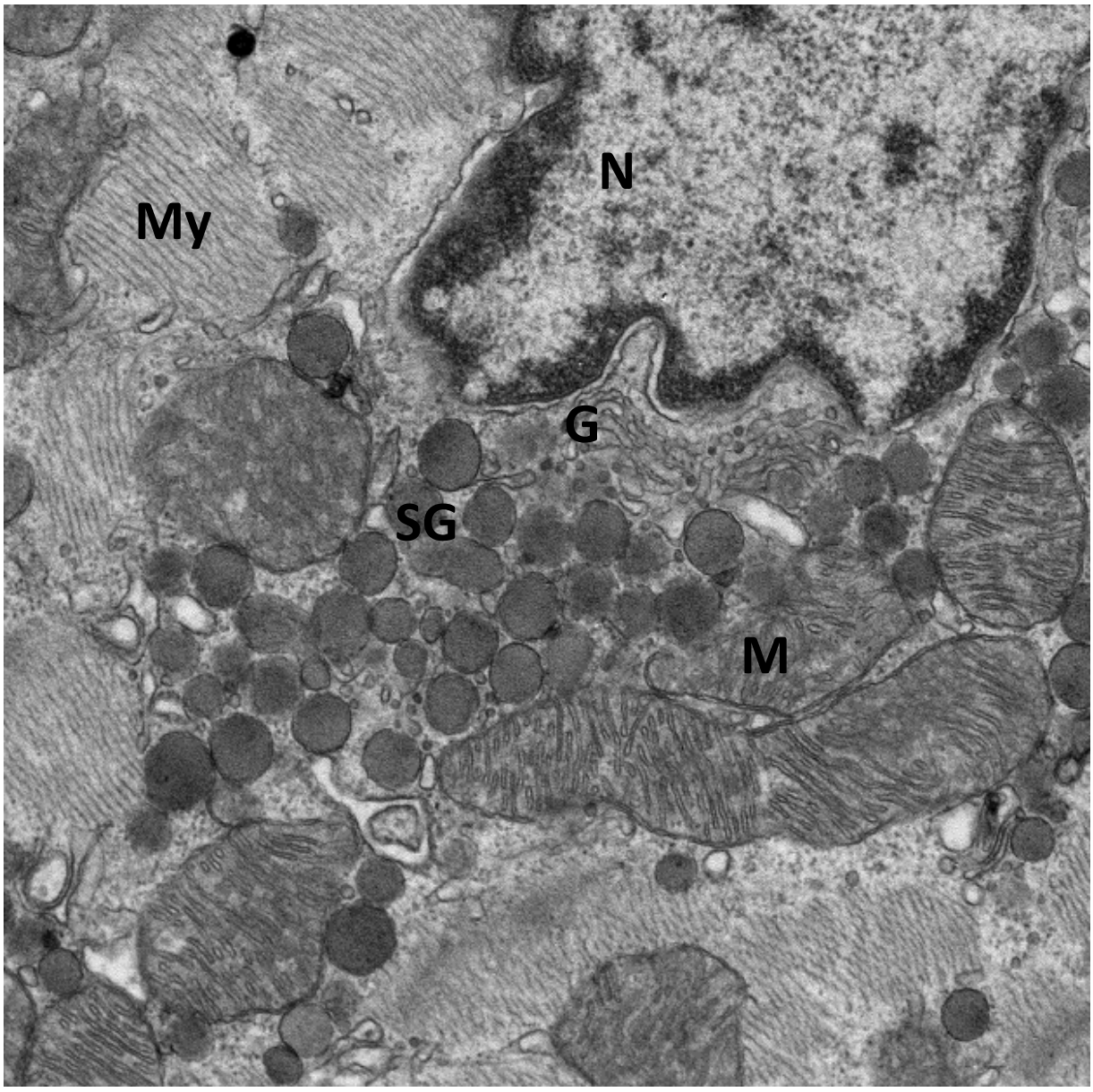
Figure 13: Concentration of ANF peptide in plasma.

Circulating ANF peptide content in control and cGαo-/- genotypes through ANF RIA. Values are mean ± SEM; n = 4 and 5 for control and cGαo-/-, respectively.

Granule density in atrial tissue

Morphological analysis of the atria by electron microscopy and granule counting in each field allowed for the determination of granule density in control and cGαo-/- mice. Initial morphological analysis of atrial cardiocytes was conducted by observing tissues through the electron microscope and it appeared that there were less granules in the cGαo-/- mice than in control. Further analysis of granule counting was conducted on ninety-five fields and 98 randomly chosen fields from control and cGαo-/- mice, respectively. A total of three animals per genotype were used, with two blocks per animal and one section per block. A highly significant ($P < 0.001$) difference of volume occupied by granulated regions was detected in cGαo-/- mice compared to the control (Figure 15). An average of 7.3 granules/μm^2 were present in control mice; whereas an average of 3.6 granules/μm^2 were seen in cGαo-/- mice.

A



B

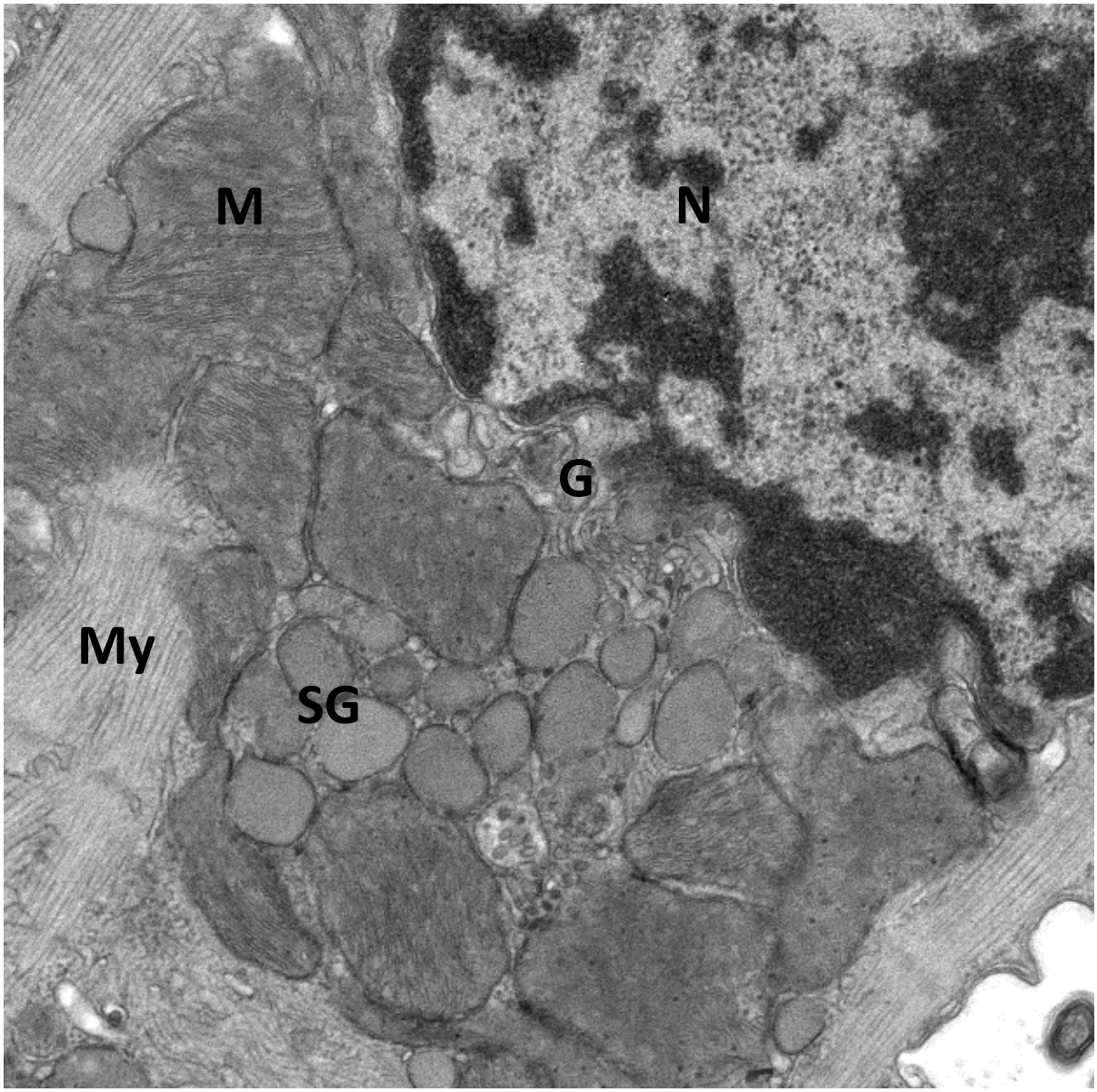


Figure 14: Electron microscopy images of atrial tissue.

Longitudinally sectioned atrial muscle cell in (A) Control mice and (B) cGαo -/-, illustrating general features present: atrial secretory granules (SG), Golgi complex (G), nucleus (N), mitochondria (M), and myofibrils (My). Magnification 5000 X.

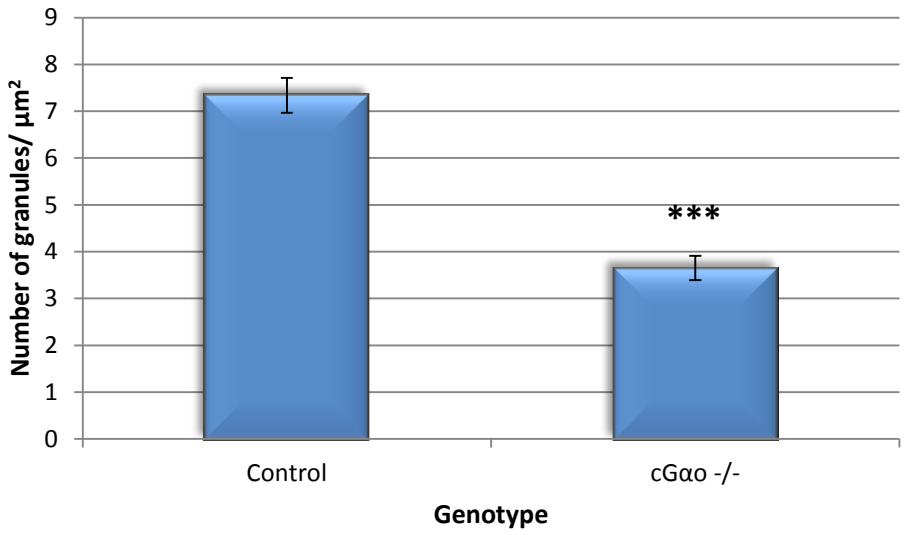


Figure 15: Specific granule density in atrial tissue.

N per group = 3 animals/genotype x 2 blocks/animal x 1 section/block x 95, 98 fields/section for control and cGαo-/- mice, respectively. Values are mean ± SEM; *** P < 0.001.

Electrocardiography

In order to determine differences in electrical conductance of cGαo-/-mice, telemetry recordings via an implantable telemetry unit were used. A three-day continuous recording system was used in three mice per genotype. Parameters analyzed in these mice include: RR-intervals, heart rate, R-height, P-height, QRS-interval, PR-interval, and QR-interval. These are common interval and height measurements when determining electrocardiography of mice. Each parameter assessed were representative recordings of a mouse during daytime hours (at rest), and during nighttime hours (while awake). Both genotypes have similar recordings and none of the aforementioned parameters were significantly different in the cGαo-/- mice compared to the control mice (Table 6). An ECG tracing representing four cardiac cycles is shown (Figure 16), depicting no significant differences in intervals and heights observed between genotypes. Background noise from implantable telemetry units in mice are common, and can be seen in the second cycle in Figure 16A and in the third cycle in Figure 16B. These are artifacts and do not represent parameters assessed in these mice.

Table 6: ECG parameters in mice.

Values are averages of the same time point (4-7pm or 4-7am) over three days. N = 3; RR-I: interval between R waves in milliseconds; HR: heart rate in beats per minute; R-H: height of the R wave in milliVolts; P-H: height of the P wave in milliVolts; QRS-I: interval of the QRS complex in milliseconds; PR-I: interval between the P wave and R wave in milliseconds; and QR-I: interval between the Q wave and the R wave in milliseconds.

Phase/ time	Genotype	RR-I (ms)	HR (bpm)	R-H (mV)	P-H (mV)	QRS duration (ms)	PR-I (ms)	QR-I (ms)
Rest 4-7pm	Control	107.4864	569.1028	0.725167	0.087531	15.43949	34.4616	6.712252
Rest 4-7pm	cGαo-/-	106.8187	572.3695	0.852373	0.076315	14.30988	33.22616	7.217987
Awake 4-7am	Control	107.071	576.5367	0.735165	0.083329	15.08417	33.66375	6.611529
Awake 4-7am	cGαo-/-	106.3489	576.0036	0.84694	0.079072	14.34907	33.42748	7.252358

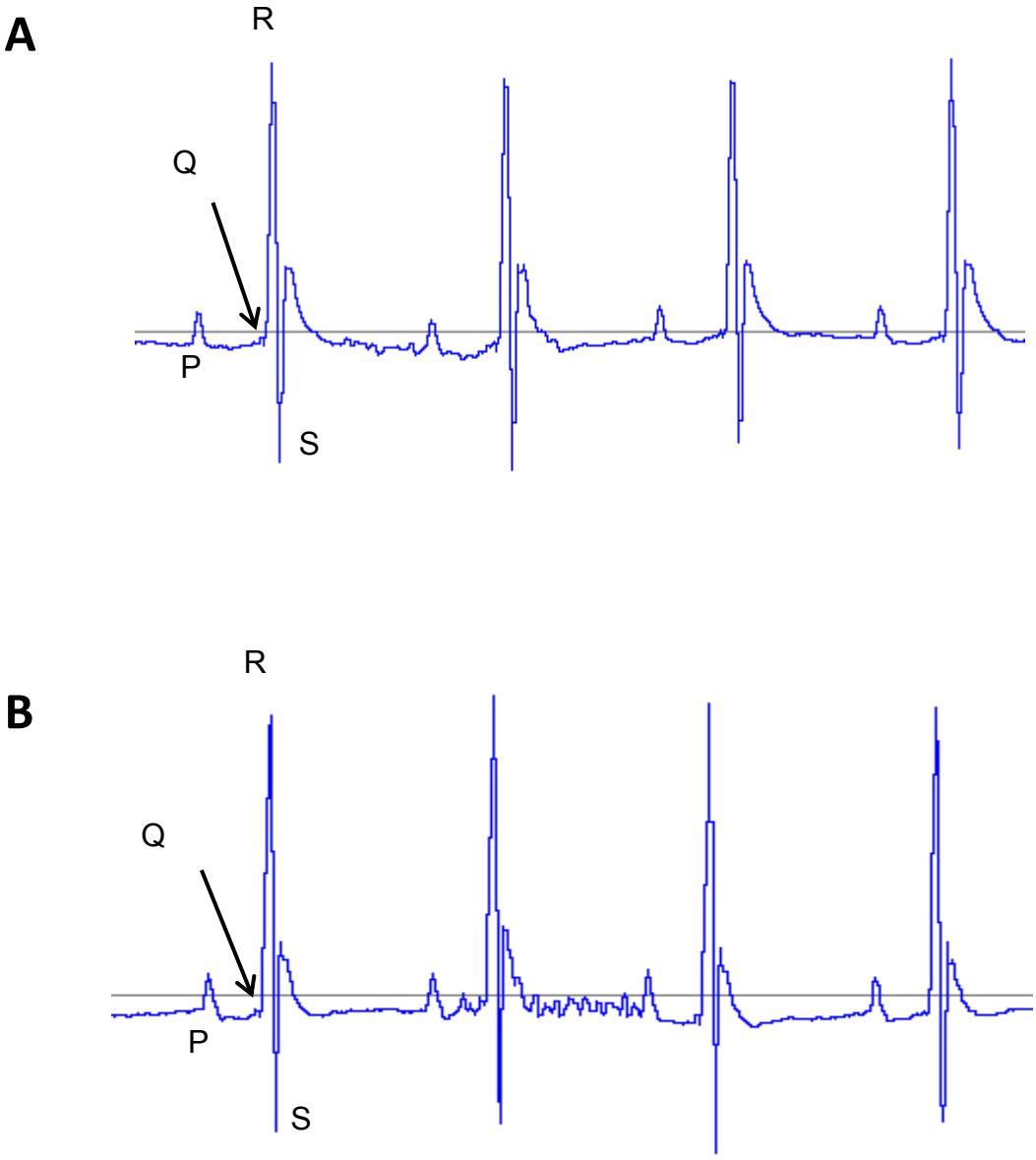


Figure 16: ECG tracings of four cardiac cycles in mice.

Four cardiac cycles are shown in A) control mice, and B) cGαo-/- mice. P: P wave; Q: Q wave; R: R wave; S: S wave. N = 3.

DISCUSSION

Generation of Gαo Cre/lox mice

Previously, the homologous recombinant knockout mouse for the *Gnao1* gene demonstrated severe pathologies and very early lethality (Jiang et al., 1997). These mice displayed austere motor and neurological symptoms and experienced frequent seizures, and therefore were not a candidate for studying gene deletions (Jiang et al., 1997). Cre/lox recombination of Gαo in a pancreas-specific tissue knockout was previously performed, and these mice do not display the complications that are present in the full body knockout mice for Gαo (Zhao et al., 2010).

Therefore, the first step of this project was to successfully generate a conditional, heart-specific knockout of Gαo in mice using an α-MHC-Cre mouse line and Gαo-floxed mouse line. This α-MHC-Cre line have been found to effectively delete the targeted gene in heart muscle without recombining the gene in other tissues or organs (Frantz et al., 2013). The homozygous floxed mouse $G\alpha o^{flx/flx}$ was used as the control (Davey and MacLean, 2006;Zhao et al., 2010) as opposed to the wild-type littermates to eliminate unforeseen effects of the targeted floxed allele due to the presence of the neomycin resistance gene cassette, which has been previously found to cause hypomorphic alleles (MacLean et al., 2008). Following published breeding protocols for Cre/lox mice (Niu et al., 2005), a heart-specific Gαo knockout mouse was produced in Mendelian frequencies through two generations of mating. These mice appeared normal and did not display

abnormal motor or neurological symptoms. There were no gross morphological features in the heart associated with this deletion. Therefore, the heart-specific deletion of Gαo was successful after two generations of mating.

Characterization of Gαo Cre/lox mice

The second part of this project was to characterize Gαo mutant mice using gene expression analyses, peptide content and peptide profiles, secretory granule content, and electrocardiography.

Gαo gene and protein expression in atrial cardiocytes

The precise extent of the Gαo knockout was analyzed using mRNA analyses in each genotype. Mice with the conditional Gαo deletion after two generations (cGαo-/-) demonstrated a significant loss of Gαo mRNA by recombining 90% of cardiac cells (Figure 5). Therefore, 10% of the *Gnao1* gene is still remaining in atrial tissue. Protein expression analysis using a western blot revealed the remaining 10% presence of Gαo in the cGαo-/- mice was able to still produce small amounts of protein in tissue (Figure 9). The amount of protein in the cGαo-/- mice is much lower than that of the control mice. Therefore, there is a significant downregulation of Gαo gene in atrial tissue of cGαo-/- mice (90%) through the Cre/lox recombination system, which was shown through protein expression as well. These results reveal an almost complete knockout of Gαo has occurred in the heart and that the deletion of the gene transcripts resulted in a loss of protein production also.

Although the Cre/lox recombination system is leading edge technology to drive *in vivo* studies of genes, there are potential problems that may arise. The most important is the efficiency of the recombination; high efficiency of recombination of the floxed alleles by Cre is required in a sufficient number of cells in order to exert the appropriate

physiological outcome (Chien, 2001). This causes an event called mosaicism, which occurs when gene inactivation is only induced in a subset of cells, leaving a population of wild-type cells where the gene has not been inactivated (Kwan, 2002). The subpopulation of wild-type cells can obscure the phenotype of the inactivated cells and have also been found to be able to fully rescue the inactivation phenotype (Kwan, 2002), which is exceedingly likely what has occurred with these conditional Gαo knockout mice. An approach identified to deal with this problem of mosaicism is to label the populations of cells at a cellular level by introducing a reporter gene to the targeted gene (Kwan, 2002). Inactivation of the targeted gene and reporter gene allows the labeling of these cells and can be further visualized to distinguish the mutant and wild-type cells (Kwan, 2002). A less direct approach to identifying these cells is to use *in situ* hybridization or immunostaining (Kwan, 2002).

Additionally, the location of the loxP sites inserted into the gene of interest is crucial and recombined alleles can cause partial protein products due to a small in-frame deletion instead of a frameshift deletion when the loxP sites are activated by Cre (Turlo et al., 2010). The ideal strategy for the placement of the loxP sites is to flank the extremities of the gene to cause a substantial frameshift deletion (Turlo et al., 2010). The mice used in this Gαo study had loxP sites flanking exons 5 and 6 and recombined alleles would only produce a small in-frame deletion. This deletion may create a partial protein product that could still be partially functional (Turlo et al., 2010).

Gαo regulation of ANF expression, production and secretion

ANF gene transcripts can be controlled by various stimuli and mechanisms to both downregulate and upregulate its expression. It was found that on a sodium-free diet and in water deprivation states, ANF transcripts were drastically reduced (Takayanagi et al., 1985); while hemodynamic stress (Day et al., 1987) and rats treated with ET-1 (Fukuda et al., 1989) and DOCA-salt (Takayanagi et al., 1985) increased ANF gene expression. Thus, regulatory elements on the ANF promoter allow for the downregulation or upregulation of ANF transcripts.

It was previously reported that ANF and Gαo directly interact at the protein level (Ogawa et al., 2009) and are co-localized in atrial secretory granules (Bensimon et al., 2004). Based on these findings, it was postulated that Gαo may be involved in the transporting of ANF into granules at the level of the TGN. In order to determine the level at which Gαo may affect ANF production, investigations on gene expression, intracellular ANF content and intracellular molecular profiles of ANF were conducted. It was found that the 90% loss of Gαo did not significantly affect the overall ANF endocrine activity of the heart based on only marginal changes in ANF gene expression and peptide content, as well as no changes in circulating ANF peptide content or intracellular processing.

ANF mRNA transcripts were differentially expressed in cGαo-/- mice through a downregulation of 23% (Figure 7); however, this did not affect the production of ANF within atrial cardiocytes as shown with only a minor reduction of ANF peptide by RIA (Figure 10). Although this decrease in ANF content in the cGαo-/- mice is noticeably less (16489 pg/mg compared to 18546 pg/mg in control mice), this value does not carry any

statistical significance. Since it is known that proANF enters ISGs, it was essential to pursue the possibility that Gαo may be involved in the interaction of targeting proANF to granules and in effect cause a disturbance in intracellular forms of ANF in cGαo-/- mice. In terms of the molecular forms of intracellular proANF and processed ANF, in normal mice this ratio is approximately 90% proANF and 10% processed ANF. This ratio was consistent in both genotypes of mice (control and cGαo-/-) (Figure 12), indicating that Gαo does not affect the intracellular peptide profiles and molecular forms of ANF in atrial cardiocytes.

Circulating levels of ANF peptide, as quantified by performing a RIA on plasma of mice in both genotypes, were unchanged between cGαo-/- and control mice (Figure 13). Overall, a noticeably lower abundance of ANF peptide content was found in this line of mice as compared to all previous studies that published ANF values in various strains of mice. The significantly low abundance of atrial ANF and circulating ANF in these mice might be a result of the genetic background and strain of mouse. Various parameters in the pancreas, such as pancreas mass, β-cell mass, α-cell mass, islet mass, and total islet number, were assessed in seven common strains of inbred mice, including C57BL/6 and 129SvEv (Bock et al., 2005). All of the aforementioned parameters excluding pancreas mass demonstrated a distinct variation between strains of mice (Bock et al., 2005). Of particular interest are the 129SvEv agouti coloured mice because this is the main background of the cGαo-/- mice produced in this study. The 129SvEv mice in comparison to all other six strains demonstrated significantly fewer number of islets and smallest β-cell to body mass ratio, and ranked as the second smallest islet mass to body mass ratio overall (Bock et al., 2005). In this study, the most important predictor of the number of islets and

total islet volume in the pancreas was genetic background (Bock et al., 2005). The results from the 129SvEv mice in the pancreas study may be transferrable to the 129SvEv background of the Gαo-floxed mice in terms of low quantity of endocrine activity present in the endocrine tissues tested in comparison to the other strains of mice. Furthermore, in a study that examined ANF levels in five strains of mice, significant interstrain variation was demonstrated between number of auricular ANF granules and auricular ANF concentration (Mifune et al., 2012). The number of auricular granules varied from 34.9 granules to 126.7 granules in an area of 382.7 μm^2, and the concentration of ANF in the atria varied from 44.1 to 101.5 μg/g of tissue between strains (Mifune et al., 2012). Even though there are significant differences in the production and storage of ANF in the mice studied, there were no significant differences observed in the circulating levels of ANF in the plasma (177.8 to 194.0 pg/mL) or the systolic blood pressure (108.6 to 116.9 mmHg) (Mifune et al., 2012). Although the control mice used in this study for Gαo cannot be compared to the wild type mice studied above due to improper genetic comparison properties (transgenic vs. non transgenic), it is noteworthy that both the auricular ANF concentration (average concentration 18.546 μg/g tissue) and circulating ANF concentration in the control and conditional knockout mice of Gαo (average concentration 42.626 pg/mL) is suggestively less than any of the five strains of mice studied above in the heart (average concentration 61.46 μg/g tissue and 185.12 pg/mL, respectively) (Mifune et al., 2012). These results above infer that the genetic variation demonstrated in the pancreas and atria may be due to underlying differences in the mechanisms of hormone synthesis and secretion between strains of mice (Bock et al., 2005;Mifune et al., 2012).

A further possibility of the unexpectedly low levels of ANF in the atria and plasma may be due to the transgenic properties and presence of the floxed alleles in the Gαo mice. Since the loxP sites are inserted into introns and are excised during transcription, mice expressing loxP sites also express the normal gene product (Chien, 2001); however, it has been found that floxed genes can cause hypomorphic alleles, whereby a partial loss of gene function has occurred (MacLean et al., 2008). Hyperandrogenization was seen in control mice expressing the floxed allele of the *androgen receptor*, without the presence of Cre (MacLean et al., 2008).

Overall, the profound amount of endocrine activity in the atria of the heart in terms of ANF production and secretion may conceal small changes in this peptide due to the 90% loss of Gαo. However, it is evident there is a feedback mechanism occurring in this system to alter the transcript levels of ANF to a great extent in the cGαo-/- mice.

Gαo regulation of BNP expression and production

Based on the fact that atrial granules co-store both ANF and BNP, BNP transcripts and peptide content were analyzed in atrial cardiocytes. Interestingly, the inactivation of Gαo in these mice did affect BNP gene expression (Figure 8) and peptide content (Figure 11), and these results were more significantly differentiated than those of ANF. As shown, BNP mRNA content was downregulated by 63% in cGαo-/- mice (Figure 11). These findings suggest a likely interaction of Gαo and BNP, which was not predicted. The cGαo-/- mice

peptide content showed a correlation with their mRNA levels, both having decreased significantly ($P < 0.001$).

The majority of the literature on BNP is focused on its role in cardiac pathologies and its use as a biomarker or therapeutic agent (Synetos et al., 2008). BNP is not as extensively characterized as ANF in terms of its role in the secretory pathway (Synetos et al., 2008), and therefore little information has been published regarding the importance of its cellular and molecular machinery with respect to hormone production and storage in atrial secretory granules. It was previously believed that there were distinct pools of secretory granules in atrial cardiocytes containing ANF and BNP, ANF only, or BNP only following immuno-labeled microscopy images of heart tissue (Hasegawa et al., 1991). It was later determined that that these granules contained ANF (McGrath et al., 2005); however, the results presented in this study may suggest otherwise. The relationship between the 63% downregulation of BNP gene expression, 29% downregulation of intracellular BNP peptide, and 51% fewer granules in cGαo-/- mice compared to control mice suggest there may be specific atrial granules that contain only BNP, not ANF, and since BNP's mRNA levels are affected, its production and storage in granules may be as well. Furthermore, studies have found a disassociation of the cardiac hormones ANF and BNP in terms of production and secretion (Yokota et al., 1995), and therefore it is possible that the relationship between Gαo and ANF in granules is different than that of Gαo and BNP.

The amount of BNP peptide in comparison to ANF both in intracellular content and plasma content is significantly less (Thibault et al., 1992;Goetze et al., 2006); owing to the

possibility that if BNP content is altered even slightly, there would be a more visible change in its expression and peptide content than ANF, which is produced and released in comparatively vast quantities. If there are different pools of granules (ANF and BNP, ANF only, or BNP only) then it may be suggested that Gαo is the leader of granule formation in at least one of the pools containing BNP. Based on the knowledge that ANF release is different for neurohumoral and mechanical stimuli by receptor-mediated stimulation and stretch-secretion coupling, respectively, it is also likely that there are separate pools of granules produced for these pathways (Mangat and de Bold, 1993).

In cardiac dysfunction, ANF and BNP gene expression and secretion are both increased; whereas, in inflammation, only BNP gene expression and secretion were upregulated (de Bold, 2009). This finding that BNP, but not ANF, gene expression and peptide content in the heart was changed was demonstrated recently through studies involving pro-inflammatory cytokines (de Bold, 2009). Furthermore, it was found that there is a relationship between G-proteins and pro-inflammatory cytokines; as well, the receptors for cytokines are GPCRs (Schaub et al., 2006). Pro-inflammatory cytokines direct functions within immune cells (Schaub et al., 2006), and specific cytokines such as tumor necrosis factor-alpha (TNFα) and interleukin-1 beta (IL-1β) were found to selectively up-regulate BNP both at the gene expression level and protein level following a study on cardiac allograft rejection (de Bold, 2009). Both TNFα and IL-1β are well studied cytokines and are markers in heart failure due to their raised plasma concentrations (Aukrust et al., 2005). These results may suggest that Gαo is involved in the immunomodulatory role of BNP and could be the Gα-subunit in the receptor for cardiac cytokines that regulate their

activity. In line with the view that cytokines and hormones regulate cellular functions (Schaub et al., 2006), the same may be true for cytokines to regulate hormones and vice versa. Chronic congestive heart failure was found associated with various inflammatory processes and cytokines as well, which mediate both production and secretion of BNP from the heart; however, this mediation by cytokines can be independent of heart failure (de Bold, 2009). Moreover, the recent discovery that the upregulation of Gαo is a critical feature and predictor of heart failure in mice (Zhu et al., 2008) implicates an involvement for Gαo in the immunomodulatory role of BNP in heart failure.

Overall, the results of BNP presented in this study indicate the potential for multiple storage pools of cardiac hormones with the possibility of different interactions of Gαo with each pool, and as well Gαo may mediate the inflammatory processes of the atria resulting in a substantial change in BNP peptide levels.

Gαo modulates the production of atrial secretory granules

The production of ANF knockout mice proved to be a very intriguing find in terms of its involvement in granule biogenesis. Heterozygous full body ANF knockout mice produced half the amount of secretory granules than wild type mice, and ANF homozygous knockout mice did not produce any granules at all (John et al., 1995), establishing ANF as a prominent leader in atrial granule biogenesis. The hypothesis of this study was that Gαo is involved in atrial secretory granule biogenesis and it was demonstrated here that indeed, Gαo is involved in granule production.

Upon initial morphological examination of atrial cardiocytes under the electron microscope, it was noted that although atrial secretory granules were present in both genotypes, there appeared to be fewer in number in the cGαo-/- mice (Figure 14). Further analysis consisted of granule counting in each image and the numbers of granules were normalized to the magnification of the image. It was found that while atrial secretory granule formation and the recruitment of ANF to granules do not require the presence of Gαo, there was a highly significant ($P < 0.001$) difference of volume occupied by granulated regions in the cGαo-/- mice than in the control mice (Figure 15). Therefore, unlike ANF in atrial cardiocytes and ChgA in chromaffin cells, Gαo is not a key leader in atrial secretory granule biogenesis, but may still serve a role in their production as only half the number of granules were produced in cGαo-/- mice. This suggests that other proteins and mechanisms are able to still produce granules in the cGαo-/- mice, albeit not to the same extent than in control mice. Based on the functional and structural redundancy of G-proteins, it may be possible that multiple G-proteins are required to produce atrial secretory granules by leading proANF to its storage.

Secretory granule biogenesis is a complex process, which is still not fully understood, whose molecular mechanisms and activations differ based on cell and tissue type (Kim et al., 2006). There are many steps and pathways a protein or hormone must take in order to be packaged and stored in these granules, and these processes involve numerous molecular partners such as proteins and enzymes (Kim et al., 2006). The involvement of G-proteins in granule biogenesis became evident following studies using inhibitors of GTP hydrolysis or using non-hydrolysable GTP analogues, and finding that the

formation of vesicles was absent (Barr et al., 1991). Many G-proteins have been found to regulate the production in granules in different endocrine systems, and specifically Gαo regulates granules within the β-cells of the pancreas by preventing the ability of vesicles to dock properly to the plasma membrane (Zhao et al., 2010). As well, both Gαo and Gαi proteins regulate insulin secretion in the pancreas, indicating another role for G-proteins in this secretory pathway (Konrad et al., 1995;Wang et al., 2011).

As mentioned in the previous section involving regulation of BNP, the significant difference in granule density between the cGαo-/- mice and the control mice may be in part due to the potential for multiple pools of stored cardiac hormones and that Gαo may be involved in the production of one or more of these storage pools. If this is the case and Gαo is involved in the granules storing BNP, and to a lesser extent those storing ANF, these results suggest the heart as an endocrine organ is even more complex than previously believed. On the other hand, the vast quantities of ANF present in the atria, as mentioned in the section involving regulation of ANF, may obscure the 51% decrease in granules in cGαo-/- mice and this would agree with previous results of their direct interaction and co-localization.

Gαo is not involved in the electrical conductance of the heart

Evidence has suggested G-protein α-subunits of GPCRs directly couple to ion channels, regulating both contraction and conductance of the heart (Eschenhagen et al., 1995). In a study involving mice with the global deletion of $G\alpha i_2$, the electrical conductance

of the heart was affected and ventricular cardiac arrhythmias were pronounced (Zuberi et al., 2010). These mice revealed a reduced ventricular refractory period and ventricular tachycardia, confirming the coupling of the inhibitory $G\alpha i_2$ to cardiac ion channels (Zuberi et al., 2010).

It was previously found that Gαo plays a direct role in the muscarinic regulation of Ca^{2+} channels in the central nervous system (Valenzuela et al., 1997), and upon further investigation in the heart this inhibitory effect was completely abolished in Gαo-/- mice (Offermanns, 2001). The full body Gαo-/- mice display an increase in heart rate, which is described as a defect in short-term heart rate dynamics (Zuberi et al., 2008). This same study stated that conditional gene targeting approaches are required to define the precise involvement of Gαo in cardiocytes and cardiac function because the full body Gαo-/- mice are problematic to study (Zuberi et al., 2008). These Gαo-/- mice were subsequently observed for atrial contractility because the inotropic and chronotropic regulation of the heart can be abolished by PTX (Boknik et al., 2009), which inhibits the Gαi/o subfamily of G-proteins (Zuberi et al., 2010); however, it was found that there were no differences between knockout and control mice (Boknik et al., 2009;Zuberi et al., 2010). Furthermore, a constitutively active Gαo (Gαo*) mouse was produced that overexpressed Gαo by 3- to 15-fold that of control mice (Zhu et al., 2008). Gαo* mice displayed increased systolic blood pressure, rate of contraction due to myocyte shortening, and enhanced Ca^{2+} channel handling and density (Zhu et al., 2008).

The discovery that Gαo affects the stretch-secretion coupling of ANF suggested that stretch-activated potassium channels may be involved in this regulation and interaction

(Bensimon et al., 2004;Ogawa et al., 2009). Microarray analyses of rat heart tissue revealed multiple potassium channels particularly abundant in the atria of the heart as compared to the ventricles, specifically Ca^{2+}-activated intermediate conductance K^+ channels (SK4), mechano-gated K^+ channels (TREK-1), and adenosine triphosphate-sensitive K^+ channels (GIRK1 and GIRK4) (Ogawa et al., 2009). These channels are involved with secretory granules, stretch-stimulated activity, and are known to be coupled with the Gαi/o subunit (Ogawa et al., 2009). Accordingly, Gαo protein in the atria directly interacts with SK4, as determined by a yeast-two-hybrid (Ogawa et al., 2009).

Based on these above findings, electrocardiographic parameters of cGαo-/- mice were analyzed. In order to optimally assess the extent to which Gαo plays a role in the conductance of the heart, implantable telemetry units were used and resting and awake parameters were assessed. In both resting and awake phases of the mouse, the loss of Gαo did not affect the conductance of the heart. Even though the electrical parameters measured (Table 6) and the cardiac rhythmic cycles (Figure 16) in these mice were not significantly altered, these results do not confirm nor deny the involvement of Gαo in the regulation of SK4, TREK-1, GIRK1 or GIRK4 channels based on the inability of the Cre/lox system to completely delete Gαo in the heart.

Summary of Gαo regulation in atrial cardiocytes

Since Gαo greatly altered transcript levels of both ANF and BNP, it can be inferred that a feedback mechanism exists in the endocrine heart that is affected by the loss of Gαo

in cGαo-/- mice. Although these exact mechanisms were not identified in this study, it is essential that this interaction be studied further to gain insight into the regulation of cardiac natriuretic peptides for purposes such as therapeutic targets for cardiovascular diseases.

In the present view of literature, proANF and BNP are co-stored in secretory granules within atrial cardiocytes (McGrath et al., 2005). Previously, small populations of granules containing only proANF or only BNP were identified by immuno-labeling techniques (Hasegawa et al., 1991). Therefore, it is possible that the significant decrease in granule density seen in cGαo-/- mice in conjunction with the reduction in BNP peptide content and BNP gene expression in the atria may indicate the presence of multiple populations of granules whereby those that are regulated by Gαo contain a larger abundance of BNP than ANF. Although these interpretations may be far-reaching for Gαo to regulate BNP-containing granules, the results presented in this study suggest this possibility. This relationship is extremely important in defining the role of Gαo in the production of granules since there were demonstrably fewer granules in the cGαo-/- mice, establishing the involvement of Gαo in the targeting, storage and transporting of ANF and BNP in atrial secretory granule biogenesis.

The relationship between the differential regulation of BNP in the heart and inflammation is well established (Vesely and de Bold, 2009). The fact that BNP was significantly more affected by the Gαo knockout than ANF may provide further insights into the relationship of Gαo and heart failure by implicating a further relationship with inflammatory cytokines. Since Gαo was identified to be an important factor in cardiac

disease, these results (a decrease in Gαo leads to a decrease in ANF and BNP peptide) corroborate with the study that overexpressing Gαo produces increased heart function in mice (Zhu et al., 2008). Therefore, Gαo can be implicated as a key factor in regulating natriuretic peptides and cardiac diseased states in murine models.

Gαo regulates insulin granule dynamics by acting as a repressor and inhibitor of vesicular docking in pancreatic β-cells during normal physiological functions (Zhao et al., 2010). In pancreatic-specific conditional Gαo-/- mice, more insulin vesicles are docked at the plasma membrane by 35-100% that of control β-cells (Zhao et al., 2010). Therefore, in the pancreatic β-cell model, Gαo acts as a key regulator of granule docking by interacting at either the docking or priming station of cells to prevent the oversecretion of insulin (Zhao et al., 2010). In chromaffin cells, Gαo inhibits granule fusion to the plasma membrane by controlling the priming of granules, therefore regulating catecholamine secretion (Gasman et al., 1997). Since it was found that trimeric G-proteins are closely associated with the actin cytoskeleton, further analysis of the role of Gαo in neuroendocrine and endocrine tissues have arisen. The most profound result from this study is the 51% decrease in granule density in cGαo-/- mice compared to control mice. This finding may prove Gαo to be one of the key regulators in atrial secretory granules, as it is a key regulator in both β-cells and chromaffin cells.

Limitations of this study were mainly pertaining to the conditional knockout of Gαo using the Cre/lox recombination system and the background strain of mice used. The fact that only a 90% gene expression reduction was observed in these mice raises the possibility that the remaining 10% of functional gene may be able to mask the inactivated phenotype

and rescue the normal phenotype in this tissue. Even though significant differences were observed in most analyses conducted on cGαo-/- mice, the endocrine activity of the heart is so vast that it is possible the 90% loss cannot impact its function as much as if there were a full 100% gene knockout. Also noteworthy is the placement of the loxP sites in the Gαo-floxed mice. Since the floxed sequences were inserted before exon 5 and after exon 6, a partial protein product can still be produced; although, it is said to be nonfunctional (Zhao et al., 2010). The more optimal strategy would have been to place these sites at the extremities of the gene exons to produce a more substantial frameshift and reduce the production of any protein product (Turlo et al., 2010). Profound interstrain variation of mice in secretory cells has been demonstrated through the number of islets and β-cell mass in the pancreas and number of ANF granules and auricular ANF concentration in the heart (Bock et al., 2005;Mifune et al., 2012). Furthermore, a study found that five transgenic lines (produced from implantation of the transgene for constitutively active Gαo into pseudopregnant females) of the overexpression of Gαo that differed only in the founder mice displayed significantly different mRNA and protein levels of Gαo (Zhu et al., 2008), indicating that even within one transgenic colony there can be substantial variation of the transgene. These discrepancies in the efficiency of Cre in conditional knockout lines can cause interpretation of the results to be very challenging (Bao et al., 2013). Lastly, the structural and functional redundancy of subfamilies of Gα-proteins allows for the possibility of compensation and redundancy (Birnbaumer, 2007) occurring in the heart of cGαo-/- mice.

Although it is clear from the present study that Gαo is involved in atrial secretory granule biogenesis and cardiac hormone storage, the precise relationship was not identified. The abundance of endocrine activity in the atria suggests there may be an array of mechanisms, proteins and factors working to maintain cardiovascular homeostasis and the loss of one of these components may be compensated by another. The overall mechanisms and pathways involved in cardiac peptide secretion are still largely unknown (Goetze et al., 2006). Further studies are required to understand the production and secretion of cardiac hormones.

CONCLUSION

The present study demonstrates the importance of the G-protein, Gαo, in the regulation of atrial cardiocytes through three mechanisms – ANF and BNP synthesis, BNP production, and atrial secretory granule production. These may be either indirectly or directly related, the relationship of these to be determined through future research. All of these strongly implicate the role of Gαo in the production of granules and BNP hormone with the possibility that BNP may be regulated independently of ANF and stored in granules other than those which co-store ANF and BNP.

Furthermore, the complexity of the secretory pathway in atrial cardiocytes suggests many molecular mechanisms are at work to maintain the endocrine activity of the atria and that the loss of one protein may be sustained by other proteins, especially the redundancy present in Gα-proteins. Implications of the present results in cardiac endocrinology include the direct positive interaction of Gαo and BNP (and to a lesser extent the relationship between Gαo and ANF), based on the results of the significant downregulation of both BNP gene expression and peptide production as well as demonstrably fewer atrial granules.

G-proteins and GPCRs are common drug targets which can be either inhibited or activated by various drugs (Cabrera-Vera et al., 2003). The structural and functional similarities in both the proteins themselves and their extracellular binding domain (Cabrera-Vera et al., 2003) make them the most pursued therapeutic targets, however delivery of the drugs may present challenges (Fredriksson et al., 2003). Future studies may suggest a potential role for Gαo as a therapeutic target in cardiovascular disease.

The data presented here suggest the involvement of Gαo in atrial secretory function. There is evidence supporting a relationship between Gαo and cardiac hormones; however, the relationship is still not clear. This involvement was established in the characterization of secretory granular density and peptide content in atrial cardiocytes.

FUTURE DIRECTIONS

To further understand the secretory function of atrial cardiocytes and the role of Gαo in its regulation, a number of subsequent experiments could be conducted both on mice from this colony and other genetically modified mice. Proposed initial future studies regarding characterization of cGαo-/- mice include analyzing gene expression of granule proteins such as ChgA and ChgB through RT-PCR to further our understanding of the involvement of granular components throughout the secretory pathway. Furthermore, if there is a relationship between Gαo and cytokines in the heart, a cytokine ELISA array would allow for the quantitative comparison of cytokines in atrial and ventricular tissues of cGαo-/- and control mice.

In order to determine the relationship between Gαo and BNP within atrial secretory granules, immuno-gold labeled atrial sections of cGαo-/- mice can be analyzed under the electron microscope labeled with specific antibodies for both Gαo and BNP. Also of interest in this experiment would be the labeling of ANF in atrial tissues to distinguish between pools of granules containing ANF, BNP, or both, and to validate the presence or absence of Gαo in these granules.

The remaining 10% wild-type Gαo population of cells in cardiocytes can be distinguished from the population of cells that Gαo has been conditionally inactivated by introducing a reporter gene to the Gαo-floxed alleles, as suggested in other studies (Kwan, 2002). This would provide a detailed tracing of the efficiency of the conditional knockout, while in turn visualizing the pockets of cells that still contain the normal alleles which might be obscuring the inactivated phenotype of Gαo. Moreover, sequencing of the conditional

Gαo transcripts to determine if a frameshift deletion has occurred to ensure a protein with functional properties is not produced (Yang et al., 2009) since it is possible with the location of the loxP sites in these Gαo-floxed mice to produce a partial protein product.

Additionally, challenging the cGαo-/- mice involving a volume expansion experiment would further define the role of Gαo in the stretch-secretion coupling of ANF, which was previously identified to be a participant in this phenomenon (Bensimon et al., 2004). Blood pressure monitoring would be performed on these animals to determine if Gαo is involved in the stretch-stimulated secretion of ANF.

To determine if other Gα proteins are mediating the specific loss of Gαo, double knockout animals would have to be produced. Double knockout mice are becoming increasingly more popular to provide a true genetic analysis of genes that have overlapping functions and structures (Chien, 2001). By crossing the respective single knockout animals (cGαo-/- with a $G\alpha i^{flxflx}$), double knockout mice of all subunits of the Gαi/o protein subfamily would be achieved, correcting for any compensation that might occur between these two proteins since all three isoforms of Gαi ($G\alpha i_{1,2,3}$) are also present in the heart (Zuberi et al., 2008). Mating this colony of mice (cGαo-/-) to those with floxed sequences for $G\alpha i_{1,2,3}$ would ensure the knockout is again directed to heart to avoid any central nervous defects, and a heart-specific knockout would be sufficient to achieve the results with respect to defining involvement of G-proteins in the cardiovascular endocrine activity.

Lastly, a new cGαo-/- colony could be produced by creating new Gαo-floxed mice with loxP sites inserted into different regions than the current Gαo-floxed mouse. These loxP sites would be inserted into regions flanking opposite ends of the gene, with sites

around exons 1 and 6 to further reduce the possibility that a partial protein product is produced since the location and distance between loxP sites has been found to be crucial in determining the success of the conditional deletion (Turlo et al., 2010).

These investigations would provide a significant framework that may be accomplished to further define the role of Gαo in the atrial secretory pathway. The results of the present investigation and subsequent future experiments may suggest a potential for Gαo as a therapeutic target in cardiovascular diseases. Since it is widely known that GPCRs are a common therapeutic target in diseased states (Cabrera-Vera et al., 2003), and that their diverse structures and functions are highly sought after for drug therapy (Fredriksson et al., 2003), it seems only logical to further pursue the role of Gαo in the production, storage and secretion of cardiac hormones, namely ANF and BNP.

REFERENCES

Arvan, P., and D.Castle. 1992. Protein sorting and secretion granule formation in regulated secretory cells. *Trends Cell Biol.* 2:327-331.

Arvan, P., and D.Castle. 1998. Sorting and storage during secretory granule biogenesis: looking backward and looking forward. *Biochem. J.* 332 (Pt 3):593-610.

Arvan, P., and P.A.Halban. 2004. Sorting ourselves out: seeking consensus on trafficking in the beta-cell. *Traffic.* 5:53-61.

Arvan, P., R.Kuliawat, D.Prabakaran, A.M.Zavacki, D.Elahi, S.Wang, and D.Pilkey. 1991. Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* 266:14171-14174.

Asano, T., R.Semba, N.Kamiya, N.Ogasawara, and K.Kato. 1988. Go, a GTP-binding protein: immunochemical and immunohistochemical localization in the rat. *J. Neurochem.* 50:1164-1169.

Aukrust, P., L.Gullestad, T.Ueland, J.K.Damas, and A.Yndestad. 2005. Inflammatory and anti-inflammatory cytokines in chronic heart failure: potential therapeutic implications. *Ann. Med.* 37:74-85.

Baba, Y., M.Nakano, Y.Yamada, I.Saito, and Y.Kanegae. 2005. Practical range of effective dose for Cre recombinase-expressing recombinant adenovirus without cell toxicity in mammalian cells. *Microbiol. Immunol.* 49:559-570.

Bao, J., H.Y.Ma, A.Schuster, Y.M.Lin, and W.Yan. 2013. Incomplete cre-mediated excision leads to phenotypic differences between Stra8-iCre; Mov10l1 and Stra8-iCre; Mov10l1 mice. *Genesis.*1-10.

Barr, F.A., A.Leyte, S.Mollner, T.Pfeuffer, S.A.Tooze, and W.B.Huttner. 1991. Trimeric G-proteins of the trans-Golgi network are involved in the formation of constitutive secretory vesicles and immature secretory granules. *Febs Lett.* 294:239-243.

Barrowman, M.M., S.Cockcroft, and B.D.Gomperts. 1986. Two roles for guanine nucleotides in the stimulus-secretion sequence of neutrophils. *Nature* 319:504-507.

Bensimon, M., A.Chang, M.L.Kuroski-de Bold, A.Ponce, D.Carreras, and A.J.de Bold. 2004. Participation of G Proteins in Natriuretic Peptide Hormone Secretion from Heart Atria. *Endocrinology* 145:5313-5321.

Birnbaumer, L. 1990. G proteins in signal transduction. *Annu. Rev. Pharmacol. Toxicol.* 30:675-705.

Birnbaumer, L. 2007. Expansion of signal transduction by G proteins. The second 15 years or so: from 3 to 16 alpha subunits plus betagamma dimers. *Biochim. Biophys. Acta* 1768:772-793.

Bock, T., B.Pakkenberg, and K.Buschard. 2005. Genetic background determines the size and structure of the endocrine pancreas. *Diabetes* 54:133-137.

Boknik, P., S.Grote-Wessels, G.Barteska, M.Jiang, F.U.Muller, W.Schmitz, J.Neumann, and L.Birnbaumer. 2009. Genetic disruption of G proteins, G(i2)alpha or G(o)alpha, does not abolish inotropic and chronotropic effects of stimulating muscarinic cholinoceptors in atrium. *Br. J. Pharmacol.* 158:1557-1564.

Bruneau, B.G., and A.J.de Bold. 1994. Selective changes in natriuretic peptide and early response gene expression in isolated rat atria following stimulation by stretch or endothelin-1. *Cardiovasc. Res.* 28:1519-1525.

Burgess, J., M.Jauregui, J.Tan, J.Rollins, S.Lallet, P.A.Leventis, G.L.Boulianne, H.C.Chang, B.R.Le, H.Kramer, and J.A.Brill. 2011. AP-1 and clathrin are essential for secretory granule biogenesis in Drosophila. *Mol. Biol. Cell* 22:2094-2105.

Burgess, T.L., and R.B.Kelly. 1987. Constitutive and regulated secretion. *Annu. Rev. Cell. Biol.* 3:243-293.

Burgoyne, R.D. 1990. Secretory vesicle-associated proteins and their role in exocytosis. *Annu. Rev. Physiol* 52:647-659.

Burnett, J.C., Jr., P.C.Kao, D.C.Hu, D.W.Heser, D.Heublein, J.P.Granger, T.J.Opgenorth, and G.S.Reeder. 1986. Atrial natriuretic peptide elevation in congestive heart failure in the human. *Science* 231:1145-1147.

Cabrera-Vera, T.M., J.Vanhauwe, T.O.Thomas, M.Medkova, A.Preininger, M.R.Mazzoni, and H.E.Hamm. 2003. Insights into G protein structure, function, and regulation. *Endocr. Rev.* 24:765-781.

Cargill, R.I., and B.J.Lipworth. 1995. The role of the renin-angiotensin and natriuretic peptide systems in the pulmonary vasculature. [Review]. *Br. Clin. Pharmacol.* 40:11-18.

Chamero, P., V.Katsoulidou, P.Hendrix, B.Bufe, R.Roberts, H.Matsunami, J.Abramowitz, L.Birnbaumer, F.Zufall, and T.Leinders-Zufall. 2011. G protein G(alpha)o is essential for vomeronasal function and aggressive behavior in mice. *Proc. Natl. Acad. Sci. U. S. A* 108:12898-12903.

Chien, K.R. 2001. To Cre or not to Cre: the next generation of mouse models of human cardiac diseases. *Circ. Res.* 88:546-549.

Chinkers, M., D.L.Garbers, M.S.Chang, D.G.Lowe, H.M.Chin, D.V.Goeddel, and S.Schulz. 1989. A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* 338:78-83.

Chusho, H., N.Tamura, Y.Ogawa, A.Yasoda, M.Suda, T.Miyazawa, K.Nakamura, K.Nakao, T.Kurihara, Y.Komatsu, H.Itoh, K.Tanaka, Y.Saito, M.Katsuki, and K.Nakao. 2001. Dwarfism and early death in mice lacking C-type natriuretic peptide. *Proc. Natl. Acad. Sci. U. S. A* 98:4016-4021.

Cody, R.J., S.A.Atlas, J.H.Laragh, S.H.Kubo, A.B.Covit, K.S.Ryman, A.Shaknovich, K.Pondolfino, M.Clark, M.J.Camargo, R.M.Scarborough, and J.A.Lewicki. 1986. Atrial natriuretic factor in normal subjects and heart failure patients. Plasma levels and renal, hormonal, and hemodynamic responses to peptide infusion. *J. Clin. Invest.* 78:1362-1374.

Cote, M., M.D.Payet, M.N.Dufour, G.Guillon, and N.Gallo-Payet. 1997. Association of the G protein alpha(q)/alpha11-subunit with cytoskeleton in adrenal glomerulosa cells: role in receptor-effector coupling. *Endocrinology* 138:3299-3307.

Dannies, P.S. 1999. Protein hormone storage in secretory granules: mechanisms for concentration and sorting. *Endocr. Rev.* 20:3-21.

Davey, R.A., and H.E.MacLean. 2006. Current and future approaches using genetically modified mice in endocrine research. *Am. J. Physiol Endocrinol. Metab* 291:E429-E438.

Day, M.L., D.Schwartz, R.C.Wiegand, P.T.Stockman, S.R.Brunnert, H.E.Tolunay, M.G.Currie, D.G.Standaert, and P.Needleman. 1987. Ventricular atriopeptin. Unmasking of messenger RNA and peptide synthesis by hypertrophy or dexamethasone. *Hypertension* 9:485-491.

de Bold, A.J. 1978. Morphometric assessment of granulation in rat atrial cardiocytes: effect of age. *J. Mol. Cell. Cardiol.* 10:717-724.

de Bold, A.J. 1985. Atrial natriuretic factor: a hormone produced by the heart. *Science* 230:767-770.

de Bold, A.J. 2009. Cardiac natriuretic peptides gene expression and secretion in inflammation. *J. Investig. Med.* 57:29-32.

de Bold, A.J., H.B.Borenstein, A.T.Veress, and H.Sonnenberg. 1981. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extracts in rats. *Life Sci.* 28:89-94.

de Bold, A.J., and B.G.Bruneau. 2000. Natriuretic Peptides. *In* Handbook of Physiology, Section 7: The Endocrine System, Volume III: Endocrine Regulation of Water and Electrolyte Balance. J.C.S. Fray, and M.H. Goodman, editors. American Physiological Society by Oxford University Press, 2000, 377-409.

de Bold, A.J., B.G.Bruneau, and M.L.de Bold. 1996. Mechanical and neuroendocrine regulation of the endocrine heart. *Cardiovasc. Res.* 31:7-18.

de Bold, A.J., and T.G.Flynn. 1983. Cardionatrin I - a novel heart peptide with potent diuretic and natriuretic properties. *Life Sci.* 33:297-302.

de Bold, A.J., K.K.Ma, Y.Zhang, M.L.de Bold, M.Bensimon, and A.Khoshbaten. 2001. The physiological and pathophysiological modulation of the endocrine function of the heart. *Can. J. Physiol Pharmacol.* 79:705-714.

de Bold, A.J., J.J.Raymond, and S.A.Bencosme. 1978. Atrial specific granules of the rat heart: light microscopic staining and histochemical reactions. *Journal of Histochemistry & Cytochemistry* 26:1094-1102.

Dikeakos, J.D., and T.L.Reudelhuber. 2007. Sending proteins to dense core secretory granules: still a lot to sort out. *J. Cell Biol.* 177:191-196.

Duman, J.G., and J.G.Forte. 2003. What is the role of SNARE proteins in membrane fusion? *Am. J. Physiol Cell Physiol* 285:C237-C249.

Edwards, B.S., R.S.Zimmerman, T.R.Schwab, D.M.Heublein, and J.C.Burnett, Jr. 1988. Atrial stretch, not pressure, is the principal determinant controlling the acute release of atrial natriuretic factor. *Circ. Res.* 62:191-195.

Elias, S., C.Delestre, M.Courel, Y.Anouar, and M.Montero-Hadjadje. 2010. Chromogranin A as a crucial factor in the sorting of peptide hormones to secretory granules. *Cell Mol. Neurobiol.* 30:1189-1195.

Erdos, E.G., and R.A.Skidgel. 1989. Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. *FASEB J.* 3:145-151.

Eschenhagen, T., U.Laufs, W.Schmitz, H.Scholz, A.Warnholtz, J.Weil, and H.-J.Schafer. 1995. Enrichment of G protein α-subunit mRNAs in the AV-conducting system of the mammalian heart. *J. Mol. Cell. Cardiol.* 27:2249-2263.

Fields, T.A., and P.J.Casey. 1997. Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins. *Biochem. J.* 321 (Pt 3):561-571.

Flynn, T.G., M.L.de Bold, and A.J.de Bold. 1983. The amino acid sequence of an atrial peptide with potent diuretic and natriuretic properties. *Biochem. Biophys. Res. Commun.* 117:859-865.

Frantz, S., M.Klaiber, H.A.Baba, H.Oberwinkler, K.Volker, B.Gabetaner, B.Bayer, M.Abebetaer, K.Schuh, R.Feil, F.Hofmann, and M.Kuhn. 2013. Stress-dependent dilated cardiomyopathy in mice with cardiomyocyte-restricted inactivation of cyclic GMP-dependent protein kinase I. *Eur. Heart J.* 34:1233-1244.

Fredriksson, R., M.C.Lagerstrom, L.G.Lundin, and H.B.Schioth. 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol. Pharmacol.* 63:1256-1272.

Fujii, T., Y.Komatsu, A.Yasoda, E.Kondo, T.Yoshioka, T.Nambu, N.Kanamoto, M.Miura, N.Tamura, H.Arai, M.Mukoyama, and K.Nakao. 2010. Circulating C-type natriuretic peptide (CNP) rescues chondrodysplastic CNP knockout mice from their impaired skeletal growth and early death. *Endocrinology* 151:4381-4388.

Fukuda, Y., Y.Hirata, S.Taketani, T.Kojima, S.Oikawa, H.Nakazato, and Y.Kobayashi. 1989. Endothelin stimulates accumulations of cellular atrial natriuretic peptide and its messenger RNA in rat cardiocytes. *Biochem. Biophys. Res. Commun.* 164:1431-1436.

Gasman, S., S.Chasserot-Golaz, P.Hubert, D.Aunis, and M.F.Bader. 1998. Identification of a potential effector pathway for the trimeric Go protein associated with secretory granules. Go stimulates a granule-bound phosphatidylinositol 4-kinase by activating RhoA in chromaffin cells. *J. Biol. Chem.* 273:16913-16920.

Gasman, S., S.Chasserot-Golaz, M.R.Popoff, D.Aunis, and M.F.Bader. 1997. Trimeric G proteins control exocytosis in chromaffin cells. Go regulates the peripheral actin network and catecholamine secretion by a mechanism involving the small GTP-binding protein Rho. *J. Biol. Chem.* 272:20564-20571.

Gilman, A.G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615-649.

Goetze, J.P., L.Friis-Hansen, J.F.Rehfeld, B.Nilsson, and J.H.Svendsen. 2006. Atrial secretion of B-type natriuretic peptide. *Eur. Heart J.* 27:1648-1650.

Gomperts, B.D., M.M.Barrowman, and S.Cockcroft. 1986. Dual role for guanine nucleotides in stimulus-secretion coupling. *Fed. Proc.* 45:2156-2161.

Gorr, S.U., X.F.Huang, D.J.Cowley, R.Kuliawat, and P.Arvan. 1999. Disruption of disulfide bonds exhibits differential effects on trafficking of regulated secretory proteins. *Am. J. Physiol* 277:C121-C131.

Gorr, S.U., R.K.Jain, U.Kuehn, P.B.Joyce, and D.J.Cowley. 2001. Comparative sorting of neuroendocrine secretory proteins: a search for common ground in a mosaic of sorting models and mechanisms. *Mol. Cell Endocrinol.* 172:1-6.

Hasegawa, K., H.Fujiwara, H.Itoh, K.Nakao, T.Fujiwara, H.Imura, and C.Kawai. 1991. Light and electron microscopic localization of brain natriuretic peptide in relation to atrial natriuretic peptide in porcine atrium. Immunohistocytochemical study using specific monoclonal antibodies. *Circulation* 84:1203-1209.

Hendy, G.N., T.Li, M.Girard, R.C.Feldstein, S.Mulay, R.Desjardins, R.Day, A.C.Karaplis, M.L.Tremblay, and L.Canaff. 2006. Targeted ablation of the chromogranin a (Chga) gene: normal neuroendocrine dense-core secretory granules and increased expression of other granins. *Mol. Endocrinol.* 20:1935-1947.

Hepler, J.R., and A.G.Gilman. 1992. G proteins. *Trends Biochem. Sci.* 17:383-387.

Higashijima, T., J.Burnier, and E.M.Ross. 1990. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. *J Biol. Chem.* 265:14176-14186.

Huh, Y.H., S.H.Jeon, and S.H.Yoo. 2003. Chromogranin B-induced secretory granule biogenesis: comparison with the similar role of chromogranin A. *J. Biol. Chem.* 278:40581-40589.

Iida, H., W.M.Barron, and E.Page. 1988. Monensin turns on microtubule-associated translocation of secretory granules in cultured rat atrial myocytes. *Circ. Res.* 62:1159-1170.

Iida, T., Y.Hirata, N.Takemura, K.Togashi, S.Nakagawa, and F.Marumo. 1990. Brain natriuretic peptide is cosecreted with atrial natriuretic peptide from porcine cardiocytes. *Febs Lett.* 260:98-100.

Jamieson, J.D., and G.E.Palade. 1964. Specific granules in atrial muscle cells. *J. Cell Biol.* 23:151-172.

Jiang, M., G.Boulay, K.Spicher, M.J.Peyton, P.Brabet, L.Birnbaumer, and U.Rudolph. 1997. Inactivation of the G alpha i2 and G alpha o genes by homologous recombination. *Receptors. Channels* 5:187-192.

Jiang, M., M.S.Gold, G.Boulay, K.Spicher, M.Peyton, P.Brabet, Y.Srinivasan, U.Rudolph, G.Ellison, and L.Birnbaumer. 1998. Multiple neurological abnormalities in mice deficient in the G protein Go. *Proc. Natl. Acad. Sci. U. S. A* 95:3269-3274.

John, S.W., J.H.Krege, P.M.Oliver, J.R.Hagaman, J.B.Hodgin, S.C.Pang, T.G.Flynn, and O.Smithies. 1995. Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science* 267:679-681.

Kim, T., M.C.Gondre-Lewis, I.Arnaoutova, and Y.P.Loh. 2006. Dense-core secretory granule biogenesis. *Physiology. (Bethesda.)* 21:124-133.

Kim, T., J.H.Tao-Cheng, L.E.Eiden, and Y.P.Loh. 2001. Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. *Cell* 106:499-509.

Kim, T., C.F.Zhang, Z.Sun, H.Wu, and Y.P.Loh. 2005. Chromogranin A deficiency in transgenic mice leads to aberrant chromaffin granule biogenesis. *J. Neurosci.* 25:6958-6961.

Kisch, B. 1963. A significant electron microscopic difference between the atria and the ventricles of the mammalian heart. *Exp. Med. Surg.* 21:193-221.

Klumperman, J., R.Kuliawat, J.M.Griffith, H.J.Geuze, and P.Arvan. 1998. Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein AP-1, clathrin, and syntaxin 6-positive vesicles. *J. Cell Biol.* 141:359-371.

Konrad, R.J., R.A.Young, R.D.Record, R.M.Smith, P.Butkerait, D.Manning, L.Jarett, and B.A.Wolf. 1995. The heterotrimeric G-protein Gi is localized to the insulin secretory granules of beta-cells and is involved in insulin exocytosis. *J Biol. Chem.* 270:12869-12876.

Krumins, A.M., and A.G.Gilman. 2006. Targeted knockdown of G protein subunits selectively prevents receptor-mediated modulation of effectors and reveals complex changes in non-targeted signaling proteins. *J. Biol. Chem.* 281:10250-10262.

Kuhn, M., R.Holtwick, H.A.Baba, J.C.Perriard, W.Schmitz, and E.Ehler. 2002. Progressive cardiac hypertrophy and dysfunction in atrial natriuretic peptide receptor (GC-A) deficient mice. *Heart* 87:368-374.

Kuliawat, R., and P.Arvan. 1992. Protein targeting via the "constitutive-like" secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment. *J. Cell Biol.* 118:521-529.

Kuliawat, R., and P.Arvan. 1994. Distinct molecular mechanisms for protein sorting within immature secretory granules of pancreatic β-cells. *J. Cell Biol.* 126(1):77-86.

Kumar, R., N.Grammatikakis, and M.Chinkers. 2001. Regulation of the atrial natriuretic peptide receptor by heat shock protein 90 complexes. *J. Biol. Chem.* 276:11371-11375.

Kuroski de Bold, M.L., and A.J.de Bold. 1991. Stretch-secretion coupling in atrial cardiocytes. Dissociation between atrial natriuretic factor release and mechanical activity. *Hypertension* 18:III-169-III-178.

Kwan, K.M. 2002. Conditional alleles in mice: practical considerations for tissue-specific knockouts. *Genesis.* 32:49-62.

Langenickel, T., I.Pagel, K.Hohnel, R.Dietz, and R.Willenbrock. 2000. Differential regulation of cardiac ANP and BNP mRNA in different stages of experimental heart failure. *Am. J. Physiol.* 278:H1500-H1506.

Lincoln, T.M., and T.L.Cornwell. 1993. Intracellular cyclic GMP receptor proteins. *FASEB J.* 7:328-338.

Lopez, M.J., S.K.F.Wong, I.Kishimoto, S.DuBois, V.Mach, J.Friesen, D.L.Garbers, and A.Beuve. 1995. Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for natriuretic peptide. *Nature* 378:65-68.

MacLean, H.E., W.S.Chiu, C.Ma, J.F.McManus, R.A.Davey, R.Cameron, A.J.Notini, and J.D.Zajac. 2008. A floxed allele of the androgen receptor gene causes hyperandrogenization in male mice. *Physiol Genomics* 33:133-137.

Mahapatra, N.R., D.T.O'Connor, S.M.Vaingankar, A.P.Hikim, M.Mahata, S.Ray, E.Staite, H.Wu, Y.Gu, N.Dalton, B.P.Kennedy, M.G.Ziegler, J.Ross, and S.K.Mahata. 2005. Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *J. Clin. Invest* 115:1942-1952.

Mangat, H., and A.J.de Bold. 1993. Stretch-induced atrial natriuretic factor release utilizes a rapidly depleting pool of newly synthesized hormone. *Endocrinology* 133:1398-1403.

Matsukawa, N., W.J.Grzesik, N.Takahashi, K.N.Pandey, S.Pang, M.Yamauchi, and O.Smithies. 1999. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. *Proc. Natl. Acad. Sci. USA* 96:7403-7408.

McGrath, M.F., and A.J.de Bold. 2005. Determinants of natriuretic peptide gene expression. *Peptides* 26:933-943.

McGrath, M.F., and A.J.de Bold. 2009. Transcriptional analysis of the mammalian heart with special reference to its endocrine function. *BMC. Genomics* 10:254.

McGrath, M.F., M.L.de Bold, and A.J.de Bold. 2005. The endocrine function of the heart. *Trends Endocrinol. Metab* 16:469-477.

Melo, L.G., M.E.Steinhelper, S.C.Pang, Y.Tse, and U.Ackermann. 2000. ANP in regulation of arterial pressure and fluid-electrolyte balance: lessons from genetic mouse models. *Physiol Genomics* 3:45-58.

Mifune, H., Y.Nishi, Y.Tajiri, and A.Yabuki. 2012. Different A-type natriuretic peptide level in five strains of mice. *J. Vet. Med. Sci.* 74:499-502.

Muth, E., W.J.Driscoll, A.Smalstig, G.Goping, and G.P.Mueller. 2004. Proteomic analysis of rat atrial secretory granules: a platform for testable hypotheses. *Biochim. Biophys. Acta* 1699:263-275.

Nagy, A. 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis.* 26:99-109.

Nakao, K., A.Sugawara, N.Morii, M.Sakamoto, T.Yamada, H.Itoh, S.Shiono, Y.Saito, K.Nishimura, T.Ban, and a.l.et. 1986. The pharmacokinetics of alpha-human atrial natriuretic polypeptide in healthy subjects. *European Journal of Clinical Pharmacology* 31:101-103.

Niu, Z., W.Yu, S.X.Zhang, M.Barron, N.S.Belaguli, M.D.Schneider, M.Parmacek, A.Nordheim, and R.J.Schwartz. 2005. Conditional mutagenesis of the murine serum response factor gene blocks cardiogenesis and the transcription of downstream gene targets. *J. Biol. Chem.* 280:32531-32538.

Nussenzveig, D.R., J.A.Lewicki, and T.Maack. 1990. Cellular mechanisms of the clearance function of type C receptors of atrial natriuretic factor. *J. Biol. Chem.* 265:20952-20958.

Offermanns, S. 2001. In vivo functions of heterotrimeric G-proteins: studies in Galpha-deficient mice. *Oncogene* 20:1635-1642.

Ogawa, T., M.Forero McGrath, P.G.Burgon, M.L.Kuroski de Bold, T.Georgalis, and A.J.de Bold. 2009. Role of potassium channels in stretch-promoted atrial natriuretic factor secretion. *Journal of the American Society of Hypertension* 3:9-18.

Ogawa, T., M.Vatta, B.G.Bruneau, and A.J.de Bold. 1999. Characterization of natriuretic peptide production by adult heart atria. *Am. J. Physiol. Heart Circ. Physiol.* 276:H1977-H1986.

Oikawa, S., M.Imai, A.Ueno, S.Tanaka, T.Noguchi, H.Nakazato, K.Kangawa, A.Fukuda, and H.Matsuo. 1984. Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. *Nature* 309:724-726.

Oliver, P.M., J.E.Fox, R.Kim, H.A.Rockman, H.S.Kim, R.L.Reddick, K.N.Pandey, S.L.Milgram, O.Smithies, and N.Maeda. 1997. Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc. Natl. Acad. Sci. USA* 94(26):14730-14735.

Perrin, M.J., and M.H.Gollob. 2012. The role of atrial natriuretic peptide in modulating cardiac electrophysiology. *Heart Rhythm.* 9:610-615.

Potter, L.R., S.Abbey-Hosch, and D.M.Dickey. 2006. Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr. Rev.* 27:47-72.

Potter, L.R., and D.L.Garbers. 1992. Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization. *J. Biol. Chem.* 267:14531-14534.

Rindler, M.J. 1992. Biogenesis of storage granules and vesicles. *Curr. Opin. Cell Biol.* 4:616-622.

Ruskoaho, H. 1992. Atrial natriuretic peptide: synthesis, release, and metabolism. *Pharmacological Reviews* 44:479-602.

Sarda, I.R., M.L.de Bold, and A.J.de Bold. 1989. Optimization of atrial natriuretic factor radioimmunoassay. *Clin. Biochem.* 22:11-15.

Sauer, B. 1998. Inducible gene targeting in mice using the Cre/lox system. *Methods* 14:381-392.

Schaub, M.C., M.A.Hefti, and M.Zaugg. 2006. Integration of calcium with the signaling network in cardiac myocytes. *J. Mol. Cell Cardiol.* 41:183-214.

Schiebinger, R.J., and C.E.Gomez-Sanchez. 1990. Endothelin: a potent stimulus of atrial natriuretic peptide secretion by superfused rat atria and its dependency on calcium. *Endocrinology* 127:119-125.

Schiller, P.W., F.Bellini, G.Dionne, L.A.Maziak, R.Garcia, A.De Lean, and M.Cantin. 1986. Synthesis and activity profiles of atrial natriuretic peptide (ANP) analogs with reduced ring size. *Biochem. Biophys. Res. Commun.* 138:880-886.

Schipani, E. 2002. Conditional gene inactivation using cre recombinase. *Calcif. Tissue Int.* 71:100-102.

Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66:511-548.

Steinhelper, M.E., K.L.Cochrane, and L.J.Field. 1990. Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes. *Hypertension* 16:301-307.

Stephenson, S.L., and A.J.Kenny. 1987. The hydrolysis of alpha-human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase-24.11. *Biochem. J.* 243:183-187.

Storch, U., M.Schnitzler, and T.Gudermann. 2012. G protein-mediated stretch reception. *Am. J. Physiol Heart Circ. Physiol* 302:H1241-H1249.

Strathmann, M., T.M.Wilkie, and M.I.Simon. 1990. Alternative splicing produces transcripts encoding two forms of the alpha subunit of GTP-binding protein Go. *Proc. Natl. Acad. Sci. U. S. A* 87:6477-6481.

Sudoh, T., K.Kangawa, N.Minamino, and H.Matsuo. 1988. A new natriuretic peptide in porcine brain. *Nature* 332:78-81.

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

Suga, S., K.Nakao, K.Hosoda, M.Mukoyama, Y.Ogawa, G.Shirakami, H.Arai, Y.Saito, Y.Kambayashi, K.Inouye, and H.Imura. 1992a. Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* 130:229-239.

Suga, S., K.Nakao, H.Itoh, Y.Komatsu, Y.Ogawa, N.Hama, and H.Imura. 1992b. 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* 90:1145-1149.

Synetos, A., K.Aznaouridis, and S.Lerakis. 2008. Brain natriuretic peptide in cardiovascular diseases. *Am. J. Med. Sci.* 335:477-483.

Takayanagi, R., I.Tanaka, M.Maki, and T.Inagami. 1985. Effects of changes in water-sodium balance on levels of atrial natriuretic factor messenger RNA and peptide in rats. *Life Sci.* 36:1843-1848.

Tamura, N., Y.Ogawa, H.Chusho, K.Nakamura, K.Nakao, M.Suda, M.Kasahara, R.Hashimoto, G.Katsuura, M.Mukoyama, H.Itoh, Y.Saito, I.Tanaka, H.Otani, and M.Katsuki. 2000. Cardiac fibrosis in mice lacking brain natriuretic peptide. *Proc. Natl. Acad. Sci. U. S. A. 2000. Apr. 11. ;97. (8.):4239. -44.* 97:4239-4244.

Thibault, G., C.Charbonneau, J.Bilodeau, E.L.Schiffrin, and R.Garcia. 1992. Rat brain natriuretic peptide is localized in atrial granules and released into the circulation. *Am. J. Physiol* 263:R301-R309.

Tremblay, J., R.Desjardins, D.Hum, J.Gutkowska, and P.Hamet. 2002. Biochemistry and physiology of the natriuretic peptide receptor guanylyl cyclases. *Mol. Cell Biochem.* 230:31-47.

Turlo, K.A., S.D.Gallaher, R.Vora, F.A.Laski, and M.L.Iruela-Arispe. 2010. When Cre-mediated recombination in mice does not result in protein loss. *Genetics* 186:959-967.

Ueda, S., N.Minamino, M.Aburaya, K.Kangawa, S.Matsukura, and H.Matsuo. 1991. Distribution and characterization of immunoreactive porcine C- type natriuretic peptide. *Biochem. Biophys. Res. Commun.* 175:759-767.

Valenzuela, D., X.Han, U.Mende, C.Fankhauser, H.Mashimo, P.Huang, J.Pfeffer, E.J.Neer, and M.C.Fishman. 1997. G alpha(o) is necessary for muscarinic regulation of Ca2+ channels in mouse heart. *Proc. Natl. Acad. Sci. U. S. A* 94:1727-1732.

Vesely, D.L., and A.J.de Bold. 2009. Cardiac natriuretic peptides gene expression and secretion in inflammation. *J. Investig. Med.* 57:29-32.

Wang, Y., S.Park, N.S.Bajpayee, Y.Nagaoka, G.Boulay, L.Birnbaumer, and M.Jiang. 2011. Augmented glucose-induced insulin release in mice lacking G(o2), but not G(o1) or G(i) proteins. *Proc. Natl. Acad. Sci. U. S. A* 108:1693-1698.

Waterston, R.H., K.Lindblad-Toh, E.Birney, J.Rogers, J.F.Abril, P.Agarwal, R.Agarwala, R.Ainscough, M.Alexandersson, P.An, S.E.Antonarakis, J.Attwood, R.Baertsch, J.Bailey, K.Barlow, S.Beck, E.Berry, B.Birren, T.Bloom, P.Bork, M.Botcherby, N.Bray, M.R.Brent, D.G.Brown, S.D.Brown, C.Bult, J.Burton, J.Butler, R.D.Campbell, P.Carninci, S.Cawley, F.Chiaromonte, A.T.Chinwalla, D.M.Church, M.Clamp, C.Clee, F.S.Collins, L.L.Cook, R.R.Copley, A.Coulson, O.Couronne, J.Cuff, V.Curwen, T.Cutts, M.Daly, R.David, J.Davies, K.D.Delehaunty, J.Deri, E.T.Dermitzakis, C.Dewey, N.J.Dickens, M.Diekhans, S.Dodge, I.Dubchak, D.M.Dunn, S.R.Eddy, L.Elnitski, R.D.Emes, P.Eswara, E.Eyras, A.Felsenfeld, G.A.Fewell, P.Flicek, K.Foley, W.N.Frankel, L.A.Fulton, R.S.Fulton, T.S.Furey, D.Gage, R.A.Gibbs, G.Glusman, S.Gnerre, N.Goldman, L.Goodstadt, D.Grafham, T.A.Graves, E.D.Green, S.Gregory, R.Guigo, M.Guyer, R.C.Hardison, D.Haussler, Y.Hayashizaki, L.W.Hillier, A.Hinrichs, W.Hlavina, T.Holzer, F.Hsu, A.Hua, T.Hubbard, A.Hunt, I.Jackson, D.B.Jaffe, L.S.Johnson, M.Jones, T.A.Jones, A.Joy, M.Kamal, E.K.Karlsson, D.Karolchik,

A.Kasprzyk, J.Kawai, E.Keibler, C.Kells, W.J.Kent, A.Kirby, D.L.Kolbe, I.Korf, R.S.Kucherlapati, E.J.Kulbokas, D.Kulp, T.Landers, J.P.Leger, S.Leonard, I.Letunic, R.Levine, J.Li, M.Li, C.Lloyd, S.Lucas, B.Ma, D.R.Maglott, E.R.Mardis, L.Matthews, E.Mauceli, J.H.Mayer, M.McCarthy, W.R.McCombie, S.McLaren, K.McLay, J.D.McPherson, J.Meldrim, B.Meredith, J.P.Mesirov, W.Miller, T.L.Miner, E.Mongin, K.T.Montgomery, M.Morgan, R.Mott, J.C.Mullikin, D.M.Muzny, W.E.Nash, J.O.Nelson, M.N.Nhan, R.Nicol, Z.Ning, C.Nusbaum, M.J.O'Connor, Y.Okazaki, K.Oliver, E.Overton-Larty, L.Pachter, G.Parra, K.H.Pepin, J.Peterson, P.Pevzner, R.Plumb, C.S.Pohl, A.Poliakov, T.C.Ponce, C.P.Ponting, S.Potter, M.Quail, A.Reymond, B.A.Roe, K.M.Roskin, E.M.Rubin, A.G.Rust, R.Santos, V.Sapojnikov, B.Schultz, J.Schultz, M.S.Schwartz, S.Schwartz, C.Scott, S.Seaman, S.Searle, T.Sharpe, A.Sheridan, R.Shownkeen, S.Sims, J.B.Singer, G.Slater, A.Smit, D.R.Smith, B.Spencer, A.Stabenau, N.Stange-Thomann, C.Sugnet, M.Suyama, G.Tesler, J.Thompson, D.Torrents, E.Trevaskis, J.Tromp, C.Ucla, A.Ureta-Vidal, J.P.Vinson, A.C.Von Niederhausern, C.M.Wade, M.Wall, R.J.Weber, R.B.Weiss, M.C.Wendl, A.P.West, K.Wetterstrand, R.Wheeler, S.Whelan, J.Wierzbowski, D.Willey, S.Williams, R.K.Wilson, E.Winter, K.C.Worley, D.Wyman, S.Yang, S.P.Yang, E.M.Zdobnov, M.C.Zody, and E.S.Lander. 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420:520-562.

Wettschureck, N., and S.Offermanns. 2005. Mammalian G proteins and their cell type specific functions. *Physiol Rev.* 85:1159-1204.

Wildey, G.M., and A.E.Matyas. 1993. Detection of low molecular weight GTP-binding proteins associated with rat atrial secretory granules. *J. Mol. Cell. Cardiol.* 25:459-468.

Wolf, W.P., K.Spicher, H.Haase, and W.Schulze. 1998. Immunocytochemical localization of the G-protein sub-unit, G(o) alpha, in rat heart. Implications for a role of G(o) alpha in secretion of cardiac hormones. *J. Mol. Cell Cardiol.* 30:1149-1162.

Yan, W., F.Wu, J.Morser, and Q.Wu. 2000. Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. *Proc. Natl. Acad. Sci. U. S. A* 97:8525-8529.

Yandle, T.G., A.M.Richards, A.Gilbert, S.Fisher, S.Holmes, and E.A.Espiner. 1993. Assay of brain natriuretic peptide (BNP) in human plasma: evidence for high molecular weight BNP as a major plasma component in heart failure. *J. Clin. Endocrinol. Metab.* 76:832-838.

Yandle, T.G., A.M.Richards, M.G.Nicholls, R.Cuneo, E.A.Espiner, and J.H.Livesey. 1986. Metabolic clearance rate and plasma half life of alpha-human atrial natriuretic peptide in man. *Life Sci.* 38:1827-1833.

Yang, S.H., M.O.Bergo, E.Farber, X.Qiao, L.G.Fong, and S.G.Young. 2009. Caution! Analyze transcripts from conditional knockout alleles. *Transgenic Res.* 18:483-489.

Yokota, N., B.G.Bruneau, B.E.Fernandez, M.L.Kuroski de Bold, L.A.Piazza, H.Eid, and A.J.de Bold. 1995. Dissociation of cardiac hypertrophy, myosin heavy chain isoform expression, and natriuretic peptide production in DOCA-salt rats. *Am. J. Hypertens.* 8:301-310.

Zeidel, M.L. 1990. Renal actions of atrial natriuretic peptide: regulation of collecting duct sodium and water transport. *Annu. Rev. Physiol* 52:747-759.

Zhao, A., M.Ohara-Imaizumi, M.Brissova, R.K.Benniger, Y.Xu, Y.Hao, J.Abramowitz, G.Boulay, A.C.Powers, D.Piston, M.Jiang, S.Nagamatsu, L.Birnbaumer, and G.Gu. 2010. G{alpha}o represses insulin secretion by reducing vesicular docking in pancreatic {beta} cells. *Diabetes*.

Zhu, M., A.A.Gach, G.Liu, X.Xu, C.C.Lim, J.X.Zhang, L.Mao, K.Chuprun, W.J.Koch, R.Liao, G.Koren, B.C.Blaxall, and U.Mende. 2008. Enhanced calcium cycling and contractile function in transgenic hearts expressing constitutively active G alpha o* protein. *Am. J. Physiol Heart Circ. Physiol* 294:H1335-H1347.

Zuberi, Z., L.Birnbaumer, and A.Tinker. 2008. The role of inhibitory heterotrimeric G proteins in the control of in vivo heart rate dynamics. *Am. J. Physiol Regul. Integr. Comp Physiol* 295:R1822-R1830.

Zuberi, Z., M.Nobles, S.Sebastian, A.Dyson, S.Y.Lim, R.Breckenridge, L.Birnbaumer, and A.Tinker. 2010. Absence of the inhibitory G-protein Galphai2 predisposes to ventricular cardiac arrhythmia. *Circ. Arrhythm. Electrophysiol.* 3:391-400.