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ROLE OF APELIN AND ENDOTHELIN SYSTEMS IN THE PAIN ASSOCIATED WITH SICKLE CELL DISEASE

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DEDICATION

"We all grow up with the weight of history on us. Our ancestors dwell in the attics of our brains as they do in the spiraling chains of knowledge hidden in every cell of our bodies."

~Shirley Abbott

"Trust in the Lord with all your heart, and do not lean on your own understanding. In all your ways acknowledge him, and he will make straight your paths."

~Proverbs 3:5-6

I would like to dedicate this dissertation to my dad and mom, Gene and Virginia Smith, who have been so encouraging and supportive throughout this whole process. I would also like to dedicate this to my grandmothers whose love and encouragement were guiding lights for me. I would also like to dedicate this work to Larry White, Jr. who served as a reminder for why this work is important.

ACKNOWLEDGEMENTS

I would first like to thank God who has made all things possible. I would like to especially thank my lab mate and former womb mate Sherika Smith for sticking it out with me through this whole process and making lab work go a little bit easier (most of the time). I would also like to especially thank my mentor, Dr. Sarah Sweitzer for also sticking it out with me and making incredible sacrifices to see me come to the completion of this journey. I would like to extend a special thank-you to my co-mentor, Dr. Claudia Grillo for always being willing to help with anything and giving great advice. I would also like to thank my committee for guiding me through this journey with tremendous help and expertise.

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ABSTRACT

Sickle cell disease (SCD) is a common genetic blood disorders that is characterized by painful vaso-occlusive episodes (VOEs), which are the major cause of hospitalizations for these patients. One of the mechanisms that may contribute to the development of painful VOEs is the imbalance between vasoconstrictors and vasodilators. This thesis focuses on the vasoconstrictors endothelin-1 (ET-1), which is elevated during VOEs and is pro-nociceptive, and angiotensin II (Ang II) and the vasodilator apelin, which has anti-nociceptive properties. This thesis tested two hypotheses: 1) an imbalance between the vasoconstrictive and pro-nociceptive systems and vasodilatory and anti-nociceptive systems contributes to pain in children with SCD and 2) the pain associated with VOEs in SCD involves the contralateral sensitizing effects of ET-1 through central mechanisms. The first aim of this thesis explored the impact of an imbalance between apelin and either endothelin or Ang II on pain measures in children with SCD. The second aim determined the pain neuroaxis location of the sensitizing/desensitizing effect of ET-1 and the effect of endothelin system activation on the apelin system. The last aim explored associations between genetic variability in the apelin receptor gene and pain measures. An imbalance between apelin and endothelin was correlated with underlying baseline pain and the frequency of VOEs while Ang II was correlated with acute procedural pain. In our models of acute VOEs, the mechanism responsible for the contralateral

sensitizing effect of ET-1 is, at least in part, mediated centrally in the spinal cord. In contrast, at the level of the primary afferent neuron, repeated exposure to ET-1 has a desensitizing effect. Furthermore in a sex-dependent manner, activation of the endothelin system decreased the peripheral apelin system with a concomitant increase in the central apelin system. Single nucleotide polymorphisms in the apelin receptor gene in children with SCD had strong trends for associations with indirect pain measures, namely increases in health care utilization. Overall, these results support the postulates that contralateral sensitizing effects of ET-1 occurs through central mechanisms in the spinal cord and that an imbalance between the vasoconstrictive and pronociceptive systems and vasodilatory and antinociceptive systems may contribute to pain associated with SCD.

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List of Abbreviations

ACS ..... Acute chest syndrome

CGRP ..... Calcitonin gene-related peptide

DRG ..... Dorsal root ganglion

ET-1 ..... Endothelin-1

HbF ..... Fetal hemoglobin

HbS ..... Hemoglobin S

HbSS ..... Homozygous sickle hemoglobin

HU ..... Hydroxyurea

NO ..... Nitric oxide

NOS ..... Nitric oxide synthase

SCA ..... Sickle cell anemia

SCD ..... Sickle cell disease

SNP ..... Single nucleotide polymorphism

SS-RBC ..... Sickle shaped red blood cells

VOE ..... Vaso-occlusive episode

CHAPTER 1:

Introduction

1.1 Sickle Cell Disease

Sickle cell disease (SCD) is one of the most common genetic blood disorders. SCD results from a single nucleotide polymorphism (A to T) in the β-globin gene that leads to the polymerization of hemoglobin S molecules (HbS) and eventually the formation of "sickle" shaped red blood cells upon deoxygenation (Figure 1.1). It is estimated that approximately 70,000 to 100,000 individuals in the United States have SCD (Hassell, 2010). The healthcare costs associated with this chronic illness are estimated to be close to $1.1 billion annually in the U.S. and these costs do not factor in the loss of productivity and the diminished quality of life that accompany this disease (Kauf et al., 2009).

1.1.1 Hemoglobinopathies associated with SCD

There are several forms of SCD that differ in severity and pathophysiology. The most common, and one of the most severe forms, is the HbSS (homozygous sickled hemoglobin) genotype or sickle cell anemia (SCA). It is estimated that in Africa, approximately 280,000 children are born with SCA each year (Makani et al., 2010). The life expectancy for these patients is almost 28 years below race-matched asymptomatic populations (Edwards et al., 2005). Other common forms include the HbSC and HbSβ^{+}-thalassemia genotypes, which typically exhibit milder clinical symptoms, and the HbSβ^{0}-thalassemia genotype, which is similar to HbSS in severity (Serjeant, 2013).

Fetal hemoglobin (HbF) is usually produced in the first two years of life and is the predominant form of hemoglobin for the first two months, and it is associated with protection from the adverse effects of the HbS allele (Schnog et

al., 2004; Steinberg, 2005; Akinsheye et al., 2011). Patients with a higher percentage of HbF typically have lower rates of mortality and a lower frequency of painful VOEs compared to patients with a lower percentage of HbF, and patients with persistent HbF (>20%) usually show no complications related to SCD (Schnog et al., 2004). Although HbF is produced during the first two years of life, the levels dramatically decrease over the first six months after birth (Edoh et al., 2006). The decrease in HbF corresponds with the appearance of clinical symptoms in children with SCD, which can occur as early as 6-12 months of age (Steinberg, 1999).

1.1.2 Clinical Symptoms

SCD is a multifaceted disease that has many clinical complications. These complications can include anemia, stroke, multi-organ damage, acute chest syndrome, and recurrent painful vaso-occlusive crises (VOEs). Acute chest syndrome (ACS) is one of the leading causes of hospitalization and death in SCD patients (Schnog et al., 2004). It is characterized by fever, shortness of breath, and chest pain and is thought to be caused by fat or thrombotic embolism and occlusion in the pulmonary vasculature (Schnog et al., 2004). The risk factors associated with ACS include having the HbSS genotype, lower levels of fetal hemoglobin, and previous episodes of ACS (Schnog et al., 2004; Stuart and Nagel, 2004).

Vaso-occlusive episodes (VOEs) are the hallmark features of SCD. Vaso-occlusion is caused by the polymerization of HbS (sickled hemoglobin) after deoxygenation, which leads to rigid sickled shaped red blood cells (SS-RBCs)

that become occluded in the microvasculature (Figure 1.2). Occlusion of the blood vessels leads to hypoxia, ischemia, tissue damage, inflammation of the vasculature, and extreme pain (Ballas et al., 2012). Occlusion in small and large blood vessels in the brain can lead to brain injury, such as stroke, in some patients, especially young children (Moser et al., 1996). On average, SCD patients have about 2 episodes per year that require health care utilization and approximately 17% of patients have more than 3 crises per year (Smith and Scherer, 2010).

1.2 Vaso-occlusion in SCD

1.2.1 Vascular component

Vaso-occlusion in SCD is a complicated process that depends on many factors such as physical obstruction of blood vessels by SS-RBCs, adhesion of blood cells to the endothelium, as well as imbalances in vasomotor tone that lead to reduced blood flow (Ergul et al., 2004; Chiang and Frenette, 2005). Occlusion of the blood vessels leads to localized ischemia, which results in tissue damage, which in turn leads to pain through the release of certain molecular mediators (Stinson and Naser, 2003). The interactions between the SS-RBCs and the endothelium are thought to contribute to vaso-occlusion through several different mechanisms including disturbing the balance between vasoconstrictors and vasodilators in the vessels (Ergul et al., 2004). The endothelium produces many vasoactive compunds involved in constriction and dilation, such as endothelins and nitric oxide (NO) (Graido-Gonzalez et al., 1998). In SCD, both vasoconstrictors and vasodilators are upregulated, and this upregulation along

with an impairment in NO-dependent vasodilation leads to vascular instability (Nath et al., 2004).

1.2.2 Pain component

It is believed that the release of chemical mediators, such as inflammatory mediators, from the endothelium lead to activation of nociceptors, which are nerve fibers responsible for transmitting pain signals (Stuart and Nagel, 2004). Acute painful crises occurs in approximately 70% of patients and is responsible for 90% of hospital admissions for adult SCD patients, but the frequency of these crises is highly variable among patients (Steinberg, 1999; Ballas, 2005). The duration of the painful crisis can also vary considerably between patients, but on average, patients report a duration of 4 or 5 days; however, for some patients, it can last for up to 2-3 weeks (Stinson and Naser, 2003). In one study examining children and adolescents with SCD, older adolescents typically reported a longer duration of pain episodes, than younger children (Shapiro et al., 1995). The areas most commonly affected during a painful crisis are the chest, back, abdomen, and the extremities, and typically, multiple areas are involved simultaneously (Steinberg, 1999). The pain is often described as “throbbing”, “sharp”, “dull”, “stabbing”, and “shooting”, and some of these descriptors, such as “stabbing” and “shooting” indicate a neuropathic pain component and suggest that central as well as peripheral mechanisms may be involved (Ballas, 2005; Smith and Scherer, 2010).

Although patients on average have about 2 crises per year, one study reported that adults with SCD experienced SCD pain on 54.5% of the 31,017

days surveyed (Smith et al., 2008). Another study found that children and adolescents reported SCD pain on 8.4% of the 1,515 days surveyed (Dampier et al., 2002). These reports suggest that although acute pain is the hallmark complication of vaso-occlusion, this pain can become more chronic in nature in adults, which may possibly be due to peripheral or central sensitization.

Nociceptive pain involves: 1.) the transduction of signals from mechanical, thermal, or chemical stimuli into nerve impulses through primary afferent nociceptors, 2.) transmission of these signals to second order neurons in the spinal cord then eventually to higher brain centers, 3.) modulation of these signals in the dorsal horn of the spinal cord and input from ascending and descending pathways, and 4.) perception of the nociceptive signal due higher brain centers (Figure 1.3). The source of nociceptive pain in SCD most likely includes chemical stimuli such as substance P, calcitonin gene-related peptide (CGRP), bradykinin, and prostaglandins that are produced after ischemic tissue damage, which in turn activate or sensitize nociceptors in the damaged tissue (Ballas, 2005).

1.3 Vascular regulation in SCD

Another component besides interactions of SS-RBCs with the endothelium that contributes to VOEs may be the dysregulation of vasomotor tone. Patients with SCD show impairment in endothelium-dependent vasodilation, and the amount of NO production under basal conditions in these patients is similar or lower than healthy controls (Belhassen et al., 2001). In a transgenic mouse model of sickle cell disease, vascular responses to vasodilators are altered, and

when vasodilatory components that help produce nitric oxide synthase (NOS), the molecule responsible for producing NO, are inhibited, an overactive vasoconstrictive system is revealed (Nath et al., 2000). These results suggest that there is some impairment in vascular regulation in SCD. It has been speculated that occlusion in one part of the blood vessel leads to the release of certain vasoactive substances from damaged endothelial cells, such as vasoconstrictive peptides that promote vaso-occlusion downstream from the original site of vaso-occlusion (Kaul et al., 1996). It may also be possible that vasoactive humoral factors could be responsible for the extension of pain from the original site of painful crisis (Kaul et al., 1996). We believe that it is the vasoactive and nociceptive actions of endothelin-1, a potent vasoconstrictive and nociceptive agent, that are some of the factors responsible for this phenomenon.

1.4 Role of Vascular Regulators in Pain

1.4.1 Vasoconstrictor: Endothelin-1

Endothelin-1 (ET-1) was identified in 1988 as a potent vasoconstrictor (Yanagisawa et al., 1988; Rubanyi and Polokoff, 1994). ET-1 is a 21 amino acid peptide that is formed from the 39 amino acid precursor peptide Big Endothelin (Big ET) by endothelin converting enzyme (Figure 1.4). Unexpectedly in early human studies, administration of ET-1 produced severe and long lasting pain (Dahlof et al., 1990). This discovery led to the investigation of ET-1 as a potential pain mediator. Further studies, identified elevated plasma ET-1 levels in many disease states in which pain is a major symptom, such as VOEs and ACS in SCD (Hammerman et al., 1997; Graido-Gonzalez et al., 1998).

In VOEs associated with SCD, the time course of elevated ET-1 plasma levels parallels the temporal profile of pain in these patients (Hammerman et al., 1997; Graido-Gonzalez et al., 1998; Rybicki and Benjamin, 1998; Ergul et al., 2004; Tharaux et al., 2005). Furthermore, down-regulation of ET-1 gene expression has been found in SCD patients treated with hydroxyurea (HU), which is a drug that has been shown to decrease the rate and intensity of VOEs (Brun et al., 2003; Odievre et al., 2007). HU has been shown to reduce in half the levels of circulating ET-1 in children with SCD (Lapoumeroulie et al., 2005). The induction of ET-1 mRNA and the release of ET-1 from endothelial cells are stimulated by sickled red blood cells from homozygous sickle cell (HbSS) patients (Phelan et al., 1995; Shiu et al., 2002). This provides further evidence that ET-1 may be an important factor in facilitating vaso-occlusive episodes. Increased production of ET-1 has been shown in endothelial cells exposed to plasma from SCD patients during acute chest syndrome (ACS). The highest levels of ET-1 stimulation came from plasma samples that were taken four days prior to hospital admittance for VOEs (Hammerman et al., 1997).

Many different cells produce ET-1 including endothelial cells, vascular smooth muscle cells (Lerman et al., 1991), cardiac myocytes (Suzuki et al., 1993; Harada et al., 1997), neurons (Hasue et al., 2005), mast cells (Liu et al., 1998), and macrophages (Ehrenreich et al., 1990). Both physiological and pathophysiological mediators modulate the synthesis and release of ET-1. Nitric oxide and prostacyclin are two well-studied mediators which inhibit the production of ET-1 (Kopetz et al., 2002). In contrast, pro-inflammatory cytokines,

angiotensin II, norepinephrine, bradykinin, mechanical stress, peripheral tissue injury, and hypoxia stimulate the production of ET-1 (Moreau et al., 1997; Ahn et al., 1998; Carducci and Jimeno, 2006). Within the vasculature, ET-1 release from endothelial cells is polarized with release being toward the smooth muscle interface. This suggests that plasma ET-1 elevations after vascular injury most likely result from spill-over from the smooth muscle as opposed to it being from the circulation (Wagner et al., 1992).

ET-1 exerts its effects through two G-protein coupled receptors, the ET_A and ET_B receptors. Binding of ET-1 to the ET_A and ET_B receptors located on vascular smooth muscle cells leads to vasoconstriction. In contrast, binding of ET-1 to ET_B receptors located on the endothelium leads to the synthesis of vasodilators such as NO (Bourque et al., 2011). Similarly, in pain modulation the two receptors are thought to variably regulate the pain response depending upon the tissue in which the receptors are expressed.

The endothelin system is has been identified at all levels of the pain signaling pathway. Peripheral endings of nociceptors are located in the epidermis of the skin and have been shown to be modulated by the local endothelin system. ET_B receptors have been identified on endothelial cells (Ghoneim et al., 1993), smooth muscle cells (Shetty et al., 1993), macrophages (Sakurai-Yamashita et al., 1997), and keratinocytes within the dermis and epidermis of the skin. In the DRG, ET_B receptors have been identified on satellite glial cells and Schwann cells that myelinate nociceptors, but not on nociceptors (Pomonis et al., 2001; Berti-Mattera et al., 2006; Chichorro et al., 2010). ET_A

receptors have been identified on the peripheral endings of the nociceptors, on nerve axons, and the nociceptors cell bodies located in the dorsal root ganglion (DRG) (Pomonis et al., 2001). In the DRG, ET_A receptors are expressed in small, medium, and large-sized nociceptors (Chichorro et al., 2010). Within the brain, ET_A receptors have been identified in the hypothalamus, reticular formation, pontine tegmentum, locus coeruleus, and substantia nigra (Kurokawa et al., 1997; Yamada and Kurokawa, 1998). ET-1 is expressed in the epidermis and in laminae I-V of the spinal cord (Yoshizawa et al., 1989). Similar to the opposite effects observed for the vasculature, the endothelin receptors also show opposite effects on pain processing depending on the location and the pain model. Activation of ET_A receptors typically induces nociception while activation of the ET_B receptor typically leads to anti-nociception (Gokin et al., 2001; Khodorova et al., 2002; Khodorova et al., 2003). In summary, the endothelin system is functional across the pain neuroaxis and likely participates in signaling cascades which can modulate acute and chronic pain.

Following injection into the human forearm, ET-1 was found to cause acute pain and sensitization to mechanical and other noxious stimulation (Ferreira et al., 1989; Dahlof et al., 1990; Hans et al., 2007). Similarly in rodents, injection of ET-1 into the hindpaw or directly onto the sciatic nerve elicits spontaneous nociceptive behaviors (Davar et al., 1998; Gokin et al., 2001). These spontaneous nociceptive behaviors are age and sex dependent (McKelvy et al., 2007). Significantly greater nociceptive behaviors have been reported in younger animals and decrease with age. In addition, clear sex differences have

been observed at early ages, with increased nociception in males compared to females. These sex differences appear to decrease with maturation. Similarly, nociceptive priming effects of ET-1 demonstrate both age- and sex-dependence. The priming effect of ET-1 produces altered nociceptive behaviors in response to a second exposure to ET-1 when compared to a first exposure. In neonatal rats, priming with ET-1 increased subsequent nociceptive behaviors in males but decreased behaviors in females. (McKelvy and Sweitzer, 2009). This is likely important in certain disease states, such as sickle cell disease, in which there is repeated exposures to ET-1.

1.4.2 Vasoconstrictor: Angiotensin II

Similar to ET-1, angiotensin II (Ang II) is a potent vasoconstrictor and plays an important role in the regulation of vasomotor tone (Nguyen Dinh Cat and Touyz, 2011). The actions of Ang II are mediated by two G protein coupled receptors: AT1 and AT2. The vasoconstrictive properties of Ang II are mediated by binding to the AT1 receptor. In contrast, lower affinity binding to the AT2 receptors functions as a negative feedback to induce vasodilation (Nguyen Dinh Cat and Touyz, 2011). In addition to its roles in the cardiovascular system, Ang II may also play a role in pain processing or modulation. It has been documented that Ang II and its receptors are produced in parts of the pain neuroaxis and may contribute to the development of pain hypersensitivity (Chakrabarty et al., 2008; Imboden et al., 2009; Chakrabarty et al., 2013). Depending on whether Ang II is injected peripherally or centrally, the particular pain model, or the species it can

be either anti-nociceptive or nociceptive (Irvine and White, 1997; Georgieva and Georgiev, 1999; Marques-Lopes et al., 2009).

1.4.3 Vasodilator: Apelin

Apelin is a recently discovered peptide that has been many different physiological effects throughout the body. It was first isolated from bovine stomach tissue in 1998 by Tatemoto et al. and was found to be the endogenous ligand for the orphan G-protein coupled receptor, APJ. Apelin is formed from a 77 amino acid preproapelin, which is cleaved by angiotensin-converting enzyme 2 to form apelin-36 and other small biologically active fragments such as apelin-13 and apelin-12 (Cheng et al., 2012). The APJ receptor is a G-protein coupled receptor that couples to $G_{\alpha i/o}$ to inhibit the activity of adenylyl cyclase and cause the phosphorylation of ERK1/2 when activated (O'Carroll et al., 2013). Apelin and its receptor are found throughout the body and they are involved in several functions including, but not limited to, the regulation of cardiovascular and body fluid homeostasis (Barnes et al., 2010). In the vasculature, apelin, through its interaction with the APJ receptor, causes NO-dependent vasodilation when acting on endothelial cells and vasoconstriction when acting directly on smooth muscle cells, suggesting that it may play an important role in the maintenance of vascular tone (Kleinz et al., 2005; Japp et al., 2008; Maguire et al., 2009).

In addition to its location in the vasculature, apelin has been found in the nerve fibers of neurons in several key areas of the central nervous system involved in the nociceptive pathway such as the periaqueductal grey and dorsal

raphe nucleus (Reaux et al., 2002). In the rat, APJ receptors have been found in both neuronal and glial cells in the central nervous system (Medhurst et al., 2003). Recently, apelin has been shown to have a modulatory role in nociception. When injected centrally, apelin-13 has anti-nociceptive effects in both a visceral and acute pain model, which is thought to occur through both the APJ and μ-opioid receptors (Xu et al., 2009; Lv et al., 2012). Mice lacking the gene for apelin have lower thresholds for thermal nociception compared to wild-type mice, which further suggests that apelin may play a role in pain processing or modulation (Kasai et al., 2011). Currently, the involvement of apelin in the pain associated with SCD is unknown.

1.5 Previous Results

Previously, we have shown that ET-1 in the plasma of children with SCD is related to baseline pain, suggesting a role for ET-1 in existing vaso-occlusive pain (Schlenz et al., 2012). Since ET-1 appears to play a role in VOEs and also elicits nociception in both humans and animals, our lab uses it to model an acute VOE in the rat hindpaw. We have previously shown that repeated administrations of ET-1 into the same hindpaw elicit a sex dependent response, with males exhibiting sensitization and females exhibiting desensitization; however, when the second administration is given in the opposite hindpaw as the first administration, both sexes exhibit sensitization. What is unknown is whether or not this contralateral sensitization is specific for ET-1 and the location of this sensitizing effect.

Building upon these previous results, this dissertation seeks to explore 1) if ET-1 produces a systemic pain sensitization to other algogens and 2) the balance between pronociceptive ET-1 and potentially antinociceptive and vasodilatory apelin peptide in the pain associated with SCD.

1.6 Hypothesis

This thesis will test two hypotheses: 1) an imbalance between the vasoconstrictive and pro-nociceptive systems and vasodilatory and anti-nociceptive systems contributes to pain in children with SCD and 2) the pain associated with VOEs in SCD involves the contralateral sensitizing effects of ET-1 through central mechanisms.

1.7 Specific Aims

Specific Aim 1: To determine the impact of the balance between vasoconstrictive and pronociceptive regulators (endothelin and angiotensin II) and vasodilatory and antinociceptive apelin on pain in pediatric SCD patients.

Specific Aim 2: To determine whether the sensitizing/desensitizing effect of ET-1 is specific for ET-1 and is mediated centrally (spinal cord) or at the level of the primary afferent neuron and to determine the effect of endothelin system activation on the apelin system.

Specific Aim 3: To examine the apelin system separately in pediatric SCD patients focusing on the genetic variability within the apelin receptor gene.

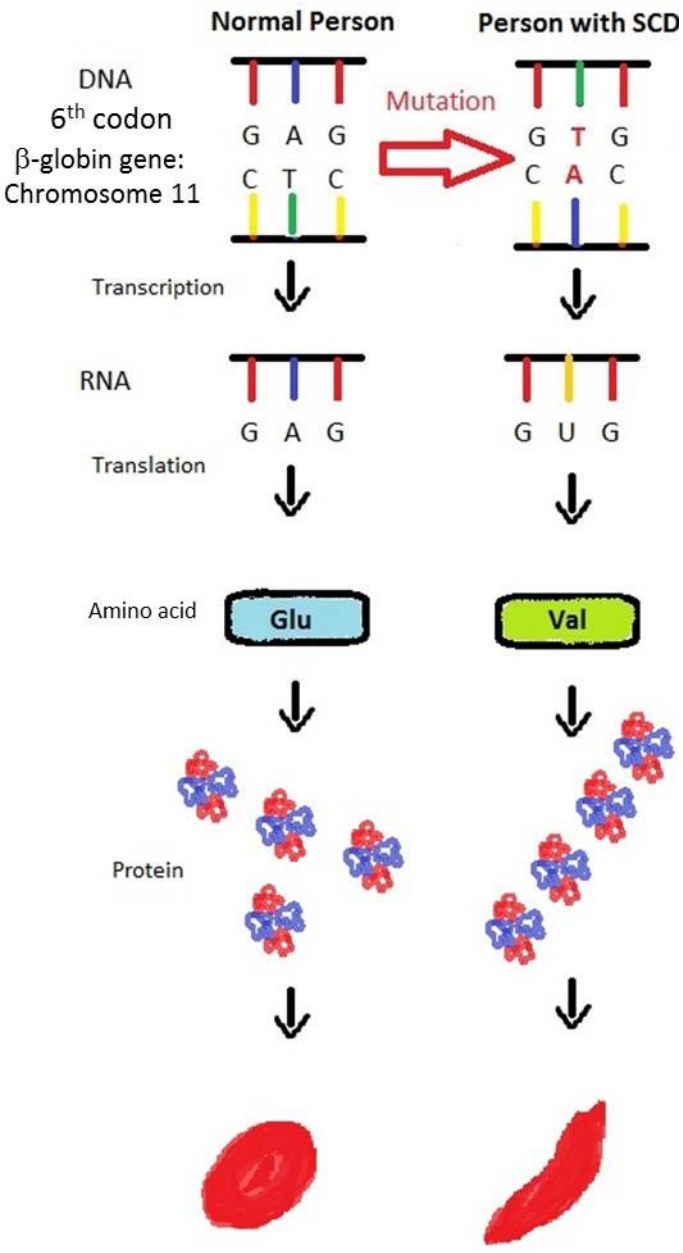

Figure 1.1 Sickle cell disease: From the genetic to the tissue level. Figure adapted from "A case study of the effects of mutation: Sickle cell anemia" Understanding Evolution. University of California Museum of Paleontology. 02 May 2014 <http://evolution.berkeley.edu/evolibrary/article/mutations_06>.

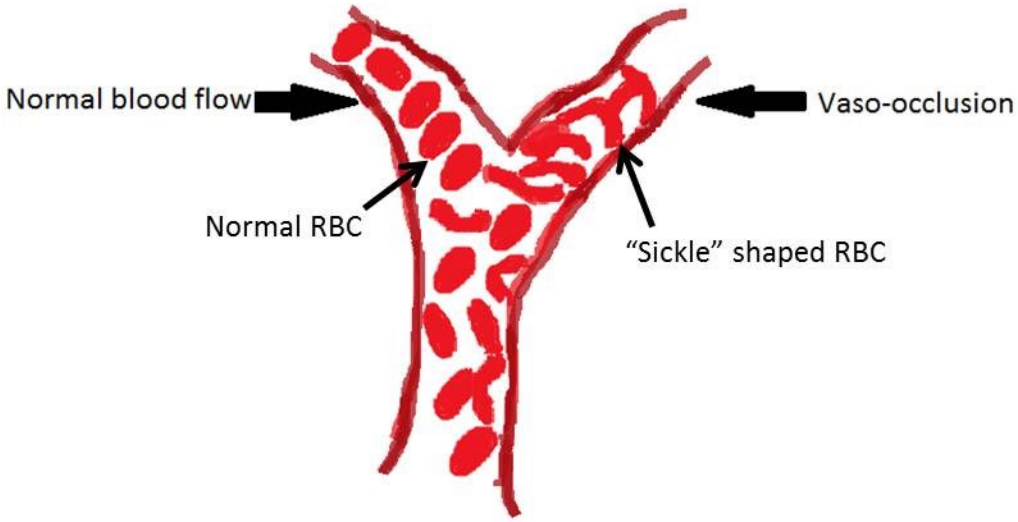

Figure 1.2 Vaso-occlusion due to sickle-shaped red blood cells. In small blood vessels, normal red blood cells can flow easily through the vessel. However, sickle-shaped red blood cells (RBC) are more rigid and more readily adhere to the endothelium, thereby causing occlusion of the vessel.

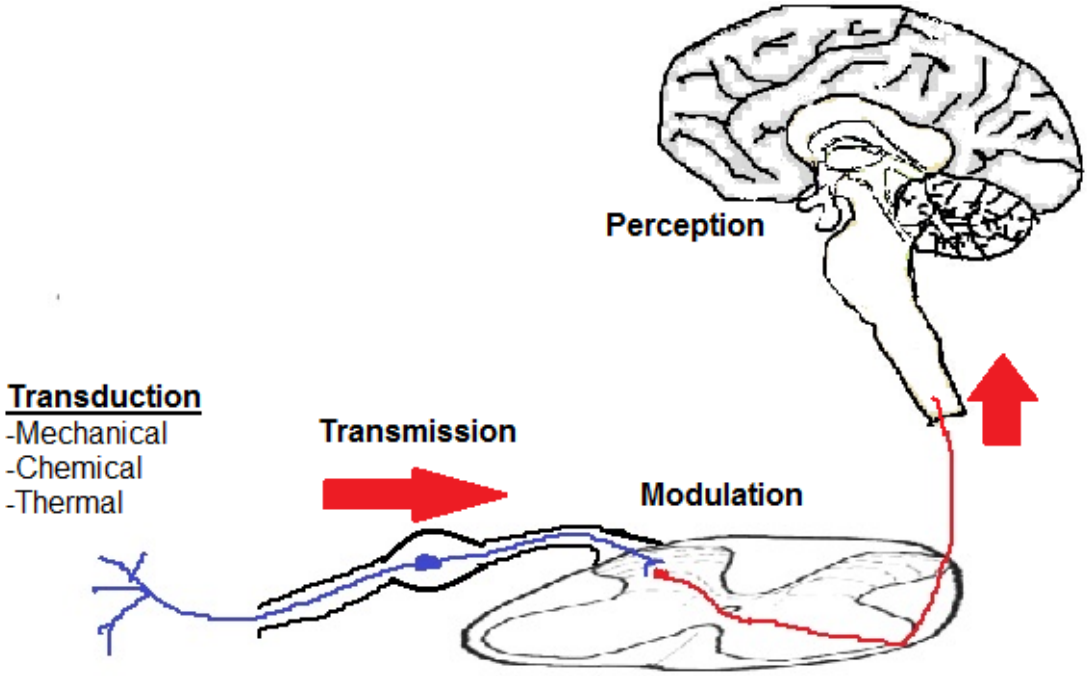

Figure 1.3 Physiology of pain Outlines the four stages of pain processing: 1) Transduction: translation of a mechanical, chemical, or thermal signal into an electrical impulse, 2) Transmission: process of by which the impulse is sent to the spinal cord and to the brain, 3) Modulation: dampening or amplification of the impulse due to ascending or descending inputs usually into the spinal cord, and 4) Perception: the conscious awareness of pain due to the integration of the other three stages of pain processing

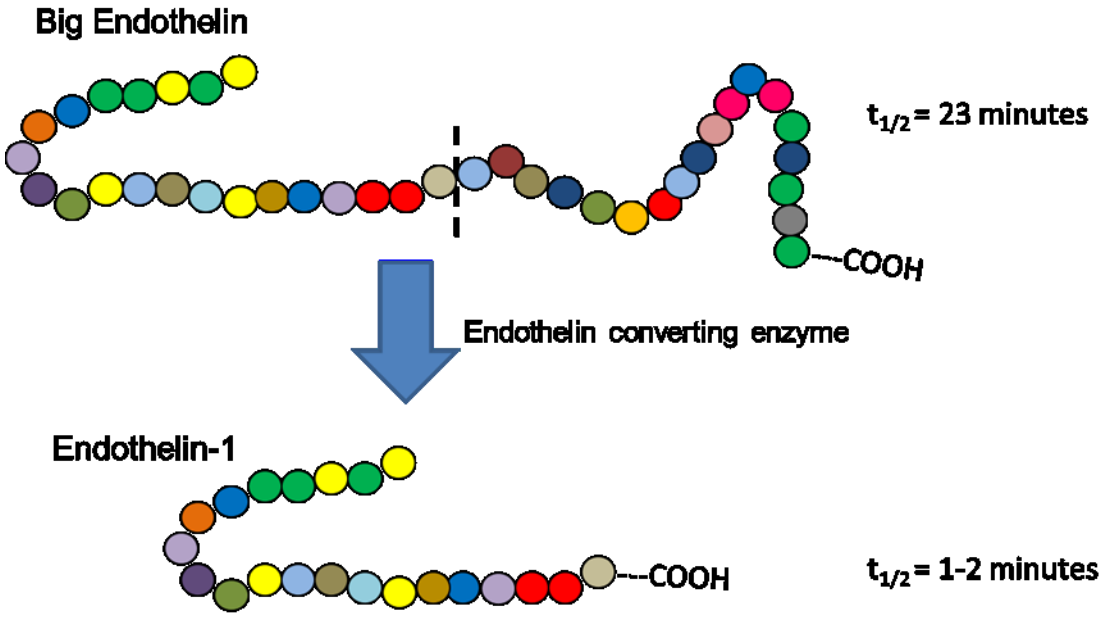

Figure 1.4 Conversion of Big ET to ET-1. The 39 amino acid peptide Big ET is converted to the 21 amino acid peptide ET-1 by endothelin converting enzyme. The plasma half-life of Big ET is estimated to be around 23 minutes while the half-life of ET-1 is estimated to be between 1 and 2 minutes.

CHAPTER 2:

Modulation of Pain in Pediatric Sickle Cell Disease: Understanding the Balance Between ET Mediated Vasoconstriction and Apelin Mediated Vasodilation[1]

[1]Smith T, Schlenz A, Schatz J, Sweitzer SM. Modulation of Pain in Pediatric Sickle Cell Disease: Understanding the Balance between ET mediated vasoconstriction and apelin mediated vasodilation. To be submitted to *Blood Cells, Molecules and Diseases*

2.1 Introduction

Studies have shown that ET-1 levels are increased in SCD patients compared to healthy controls (Graido-Gonzalez et al., 1998; Werdehoff et al., 1998) and these increased levels are even more pronounced during an acute VOE (Ergul et al., 2004). Interestingly, the time course of ET-1 plasma elevations parallels VOE-associated pain symptoms with a peak in plasma levels and pain at the height of the VOE and then slow return to baseline that requires several weeks (Graido-Gonzalez et al., 1998). In addition to its vasoactive properties, ET-1 also causes nociception when injected into both humans and animals (Ferreira et al., 1989; Dahlof et al., 1990; Davar et al., 1998; Gokin et al., 2001; Hans et al., 2007; McKelvy et al., 2007; Smith et al., 2014).

Previously, we have shown that endothelin variables in the plasma of pediatric SCD patients is related to baseline pain such that higher plasma levels of ET-1 were found in children with higher baseline pain (Schlenz et al., 2012). In contrast, lower plasma levels of Big ET, the precursor to ET-1, were found in children with a higher frequency of recent VOEs possibly a result of recent conversion to vasoactive ET-1. From these findings of excessive endothelin mediated vasoconstriction in children with SCD, we were interested in the role of vasodilatory systems in SCD. We have recently begun to explore the role of apelin, a novel vasoactive peptide with vasodilatory properties, in the pain associated with SCD.

Apelin (Tatemoto et al., 1998) is formed from the 77 amino acid preproapelin, which is cleaved by angiotensin-converting enzyme 2 to form

apelin-36 and other biologically active fragments such as apelin-17 and apelin-13 (Cheng et al., 2012). Apelin is the endogenous ligand for the orphan G-protein coupled receptor, APJ. In addition to its role in the vasculature, the apelin-APJ system has also been recently implicated in having a modulatory role in nociception since apelin and its receptor are found in several brain regions associated with pain (Xu et al., 2009; Kasai et al., 2011; Lv et al., 2012). In the vasculature, apelin's interaction with its receptor causes nitric-oxide (NO) dependent vasodilation when acting on endothelial cells (Japp et al., 2008; Maguire et al., 2009). Recently, it has been proposed that there is a shift in favor of ET-1-mediated vasoconstriction and away from NO-mediated vasodilation during an acute VOE in SCD patients (Ergul et al., 2004).

To date, there have been no studies that explore the potential relationship between apelin and ET-1 in the pain associated with SCD. The general hypothesis of this work is that an imbalance between the vasoconstrictive and pronociceptive systems and vasodilatory and antinociceptive systems modulate SCD-associated pain. The goal of this study was to examine the balance between ET-1 or its precursor, Big ET, and apelin in a cohort of pediatric SCD patients and how this balance correlates with acute chest syndrome (ACS), procedural pain, and pain history.

2.2 Methods

2.2.1 Participants

This study used a subsample of 47 children with SCD ages 2 to 18 (m = 9.98, SD = 4.78; 22 male, 25 female) that were participating in a larger study of

procedural pain in SCD. Due to the goals of the larger study, children between the ages of 10 and 12 (the transition from pre- to post-pubescence) were excluded. Forty-seven children had adequate plasma available for apelin analysis by ELISA. For the ratios, 46 children had both ET-1 and apelin data and 43 children had both Big ET and apelin data available. The ET-1 and Big ET ELISAs were conducted as part of the previous study and details about those results can be found in Schlenz, *et al.* (2012). For the child ratings reported below, only children over the age of 5 completed ratings, resulting in a sample of 37 children for the apelin/ET-1 analysis and 36 children for the apelin/Big ET analysis.

2.2.2 Procedures

Children and their caregivers were approached at routine hematologist visits for participation. Children commonly receive routine venipuncture at these visits. Venipuncture was chosen to represent a standardized painful stimulus. After consent and assent procedures were completed, children and caregivers completed baseline (pre-venipuncture) ratings of pain and caregivers completed a background questionnaire. Once the venipuncture was completed, children and caregivers completed ratings of the child's pain during the procedure. Children were also video recorded from the time they entered the exam room to the end of the venipuncture, in order to obtain observational ratings of pain. Medical record reviews were conducted after the child's visit using a structured coding method.

2.2.3 Background and medical information

Caregivers completed a background information questionnaire to obtain demographic information. Children rated their pain using the Wong Baker Faces Scale. Caregivers rated their child's pain using a visual analog scale (VAS). Observational ratings of pain were also taken at baseline and during the procedure using the modified version of the Observational Scale of Behavioral Distress. Reviews of children's medical charts were used to establish history of ACS, VOEs, and SCD genotype. Of the 47 children in this sample, 12 had a history of acute chest syndrome and 35 did not. For recent VOE history, we measured the number of hospitalizations, emergency department visits, and outpatient contacts for pain in the previous 24 months. The SCD genotypes were divided into the more severe phenotype (HbSS and HbSβ°) and more mild-moderate phenotype (HbSC and HbSβ^{+}). Of the 47 children, 34 were categorized as having the more severe phenotype and 13 were categorized as having the more mild-moderate phenotype for the apelin levels and apelin to ET-1 ratio and 33 severe and 11 mild to moderate for the apelin to Big ET ratio.

2.2.4 ELISA

Blood collection and plasma separation for this sample of participants has been described previously (Schlenz et al., 2012). Briefly, blood was collected into EDTA vacutainer tubes and placed on ice for plasma isolation within 30 minutes of blood collection. After isolation, plasma was stored at -80°C until further analysis by ELISA. ELISA kits for ET (1-21) (Cat no. BI-20052) and Big ET (Cat no. BI-20082) were purchased from ALPCO Immunoassays and apelin-

36 (EK-057-15) was purchased from Phoenix Pharmaceuticals. ELISAs for ET-1 and Big ET-1 were performed in triplicate and ELISAs for apelin-36 were performed in duplicate according to the respective assay protocols. A standard curve was plotted from the standards of each kit using Prism software (GraphPad Software Inc, San Diego, CA), which was then used to extrapolate the sample concentrations from each plasma sample.

2.2.5 Data analysis

This study used an exploratory analysis to examine relationships between apelin and ratios of apelin to ET-1 and Big ET to the primary outcomes: baseline pain, procedural pain, acute chest syndrome and recent VOE history. For VOE history, there was one outlier (a child with 58 documented pain episodes) who was removed from analysis. The relationships between apelin, the two ratios, baseline pain, procedural pain, and VOE history were assessed using correlations. The relationship between apelin, the two ratios, ACS, and disease severity were assessed via t-test. Descriptive information on age and gender differences for apelin is also provided using correlation and t-test analysis, respectively. Information on age and gender for ET-1 and Big ET can be found in a prior publication (Schlenz et al., 2012).

The ratio variables demonstrated a positive skew that was corrected with log-transformation. Additionally, to ensure that the ratios were equally associated with both apelin and the endothelin variables, we examined correlations between the ratios, apelin, ET-1, and Big ET. The log-transformed ratio for apelin/ET-1 was highly associated with ET-1 (r = -.84) whereas the

untransformed ratio was more equally associated with both apelin (r = .41) and ET-1 (r = -.47). Both the log-transformed and untransformed results can be found in Table 1. For outcome variables, the three baseline and procedural pain ratings all demonstrated a positively skewed distribution and were log-transformed. In addition, because baseline ratings of pain were associated with significantly greater procedural pain, regression was used to remove variance in procedural pain that could be explained by baseline ratings. This approach allowed for the procedural pain ratings to solely reflect pain from the procedure. Finally, due to the exploratory nature of this study, we were careful to evaluate the impact of outliers (defined as values exceeding three standard deviations from the mean of the variable).

2.3 Results

Table 2.1 provides correlation results and these findings are explained descriptively below. Apelin plasma levels had a range of 0.87 to 10.74 ng/mL (m = 2.74; SD = 1.73). The ranges for plasma ET-1 and Big ET were 0.21 to 4.25 pg/mL (excluding the children whose levels exceeded 10 pg/mL) and 0.0 to 1.57 pg/mL (m = 0.93, m = 0.39; SD = 0.96, SD = 0.30), respectively.

2.3.1 Demographics

Apelin was significantly related to age, with older age children displaying lower plasma apelin levels. There was no statistically significant difference in mean apelin levels between male (m = 2.53, SD = 1.42) and female (m = 2.61, SD = 1.12) children. There were also no significant differences in the ratio data between male and female children.

2.3.2 Correlation with pain measures

Apelin alone was not significantly related to any of the baseline pain ratings (caregiver, child, or observational), procedural pain ratings, or recent VOE history. The apelin/ET-1 ratio was not significantly related to procedural pain or recent VOE history. However, the apelin/ET-1 ratio was negatively correlated to observational baseline pain ratings (r = -0.32, p = 0.032) (Figure 2.1). The apelin/Big ET ratio was significantly correlated with caregiver ratings of baseline pain (r = -.30, p = .049) and approached significance in relation to child ratings of baseline pain (r = -.29, p = .088). The apelin/Big ET ratio was not significantly related to the remaining baseline and procedural pain variables or VOE history. However, when the same outlier noted above for VOE history was removed, a statistically significant correlation was found between the apelin/Big ET ratio and VOE history, r = .32, p = .041 (Figure 2.2).

2.3.3 Relationship to ACS

There was no statistically significant difference in apelin levels between children with (m = 2.20, SD = .91) and without (m = 2.71, SD = 1.37) a history of acute chest syndrome, p = .221. There was also no statistically significant difference in apelin/ET-1 ratio values for children with (m = 5.87, SD = 5.58) and without (m = 4.22, SD = 4.11) a history of acute chest syndrome, p = .282. There was also no significant difference in apelin/Big ET ratio values for children with (m = .93, SD = .35) and without (m = .79, SD = .36) a history of acute chest syndrome, p = .257.

2.3.4 Relationship to disease severity

There were no significant differences found between the more severe disease phenotypes and the more mild-moderate phenotypes for either the plasma apelin levels (p = 0.5302) or the apelin/ET-1 ratio (p = 0.5961). There was a trend for a significant difference between the severe and mild-moderate phenotype for the apelin/Big ET ratio (p = 0.079) (Figure 2.3).

2.3.5 Relationship of apelin to ET-1 and Big ET

There was a significant positive correlation between plasma apelin levels and ET-1 levels (p = 0.0478, r = 0.290). No significant correlation was found between apelin and Big ET.

2.4 Discussion

In this study we sought to examine vasodilatory and antinociceptive plasma apelin expression in children with SCD. In addition, this study sought to determine how the balance between vasodilatory apelin and vasoconstrictive endothelin plasma levels impacts pain reports in children with SCD. Plasma apelin expression was related to age, with decreased levels as a child ages, but no relationship was found between apelin and baseline pain, procedural pain, or history of recent VOEs. Similarly, the ratio between apelin and ET-1 was not related to procedural pain or recent VOEs, but it was negatively correlated to observational baseline pain. In contrast, the ratio between apelin and Big ET was found to positively correlate to history of VOEs such that increased ratios of the two peptides was associated with an increased number of VOEs. The results from this study suggest that an imbalance in the apelin and endothelin

systems may contribute to an increasing number of VOEs and baseline pain in children with SCD. This is the first study that has demonstrated a potential relationship between the apelin and endothelin systems in VOEs, which are one of the hallmark complications associated with SCD.

Vascular tone is maintained by a delicate balance of vasoconstrictors and vasodilators, and in diseases like SCD, it is believed that there is an imbalance in these systems that may further contribute to vaso-occlusion and the pain associated with vaso-occlusion (Nath et al., 2000; Ergul et al., 2004). Our previous work has shown that increased plasma endothelin levels were more closely associated with greater baseline pain in children with SCD (Schlenz et al., 2012). In humans, intravenous infusion of apelin into arteries causes endothelial NO-mediated vasodilation (Japp et al., 2008). In the vasculature, binding of apelin to the APJ receptor produces NO dependent vasodilation (Japp et al., 2008; Maguire et al., 2009). Impairment in endothelium-dependent vasodilation with decreased NO production under basal conditions and during times of wall shear stress such as experienced during a VOE has been reported in patients with SCD (Belhassen et al., 2001). These current findings are the first to suggest that impairment in endothelium-dependent vasodilation in SCD may be the result of an imbalance between ET-1-mediated vasoconstriction and apelin-mediated vasodilation.

In human endothelial and vascular smooth muscle cells exposed to hypoxic conditions, apelin expression is significantly up-regulated, which may be a compensatory response to low oxygen levels in the tissue (Eyries et al., 2008).

Although the current study did not show a relationship between apelin levels alone and pain history or ratings, there are several limitations to the study design which might contribute to this negative finding. The first being that apelin was sampled at a single time point from patients during disease steady state. Children who had a recent VOE or were taking current pain medications had been excluded from participating in the study. It is possible that changes in apelin expression may be more pronounced during periods of vascular crisis when vasodilation has been shown to be significantly impaired in SCD patients (Belhassen et al., 2001). Similarly, the imbalance between the apelin and endothelin systems found in this study may be more pronounced during a crisis. In addition, contributions of the apelin system in the current study may be underestimated based on our single sampling method since apelin has a short plasma half-life of less than 8 minutes (Japp et al., 2008).

In the current study, the ratio of apelin to Big ET was positively correlated with a higher frequency of VOEs. An increase in the ratio can result from increased apelin levels or decreased Big ET levels. We have previously shown that lower plasma levels of Big ET were correlated with more recent VOEs (Schlenz et al., 2012). The lower levels of Big ET may also result from an increased conversion of Big ET into the more active peptide, ET-1, by endothelin converting enzyme-1.

In the current study, apelin was found to decrease with age in children with SCD. This decrease in plasma apelin levels with age parallels the decrease in circulating ET-1 with age in children with SCD (Lapoumeroulie et al.,

2005).This suggests that there is developmental regulation of the vaso-regulatory systems in SCD. These results are also consistent with animal studies showing a decrease in plasma apelin levels in aged rats (22 months) compared to young rats (3 months), which may suggest a developmental role for these mediators in the vascular system (Sauvant et al., 2014). It should also be noted that the range of plasma level of apelin found in our study (0.87-10.74 ng/mL) is much higher than levels (0.0493 to 0.273 ng/mL) found in healthy adult volunteers in another study, which may be due to the decrease in apelin levels with age (Zhen et al., 2013). In addition, ET-1-induced spontaneous nociceptive behaviors in rodents are age dependent (McKelvy et al., 2007). Significantly greater ET-1-induced nociceptive behaviors have been reported in younger animals compared to older animals. Similarly, a priming effect with ET-1 has been characterized in young rodents in which increased ET-1 induced nociceptive behaviors are produced by a second exposure to ET-1 (McKelvy and Sweitzer, 2009). This is important in certain disease states, such as sickle cell disease, in which there are repeated exposures to ET-1 with each subsequent VOE.

Extrapolation of the current findings to larger patient populations is limited by the small sample size. Although we attempted to separate the children by SCD genotype, we did not find any significant differences between the phenotypes for apelin or the two ratios. A larger sample size may reveal significant differences in plasma apelin levels and the ratios between the more severe genotypes and the more mild-moderate genotypes in regards to painful VOEs, since the different genotypes have been shown to influence the frequency

of VOEs and pain factors (Platt et al., 1991). Another limitation of this study is the short half-lives of plasma apelin and ET-1 and Big ET, which are less than 8 minutes, 1-2 minutes, and 23 minutes, respectively (Hemsen et al., 1995; Galie et al., 2004; Japp et al., 2008).

This is the first study to explore the potential relationship between apelin and ET-1 in the pain associated with SCD. The current results support the general postulate that children with SCD with higher numbers of VOEs may have a greater imbalance between the vasoconstrictive and pro-nociceptive systems and vasodilatory and anti-nociceptive systems. Further studies in this area are needed to define and characterize the relationships between the apelin and endothelin systems in pain-associated with SCD. If larger studies confirm that the balance between apelin and endothelin are important in SCD then modulating this target may be a unique therapeutic approach to treat and prevent VOEs.

Table 2.1. Correlations for age, apelin, ratios, baseline and procedural pain, and recent VOEs.
Note. The upper diagonal displays correlations with log-transformed ratio values whereas the lower diagonal displays correlations with untransformed ratio values. † $p<0.10$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Variables	1	2	3	4	5	6	7	8	9	10	11
1. Age	-	-.29*	-.02	.07	-.29†	-.07	-.42**	-.35	-.30*	-.49**	.36*
2. Apelin	-.29*	-	.29*	.51**	-.14	-.03	.03	.03	-.01	.21	-.19
3. Apelin/ET-1 Ratio	.05	.41**	-	.61**	-.24	-.12	-.32*	.05	-.11	.03	.16
4. Apelin/Big ET Ratio	.25†	.43**	.74**	-	-.29†	-.30*	-.23	-.07	-.22	.04	.23
5. Child Report (Baseline Pain)	-.29†	-.14	-.25	-.22	-	.53**	.21	-.08	.09	-.06	-.14
6. Caregiver Report (Baseline Pain)	-.07	-.03	-.20	-.20	.53**	-	-.01	.28	.00	.19	-.01
7. Obs. Distress (Baseline Distress)	-.42**	.03	-.24	-.20	.21	-.01	-	.24	.24	.08	-.19
8. Child Report (Procedural Pain)	-.35*	.03	-.10	-.23	-.08	.28	.24	-	.27	.37*	-.13
9. Caregiver Report (Procedural Pain)	-.30*	-.01	-.14	-.35*	.09	.00	.24	.27	-	.18	-.21
10. Obs. Distress (Procedural Distress)	-.49***	.21	.03	-.10	-.06	.19	.08	.37*	.18	-	.00
11. RecentVOEs	.36*	-.19	.18	.27†	-.14	-.01	-.19	-.13	-.21	.00	-

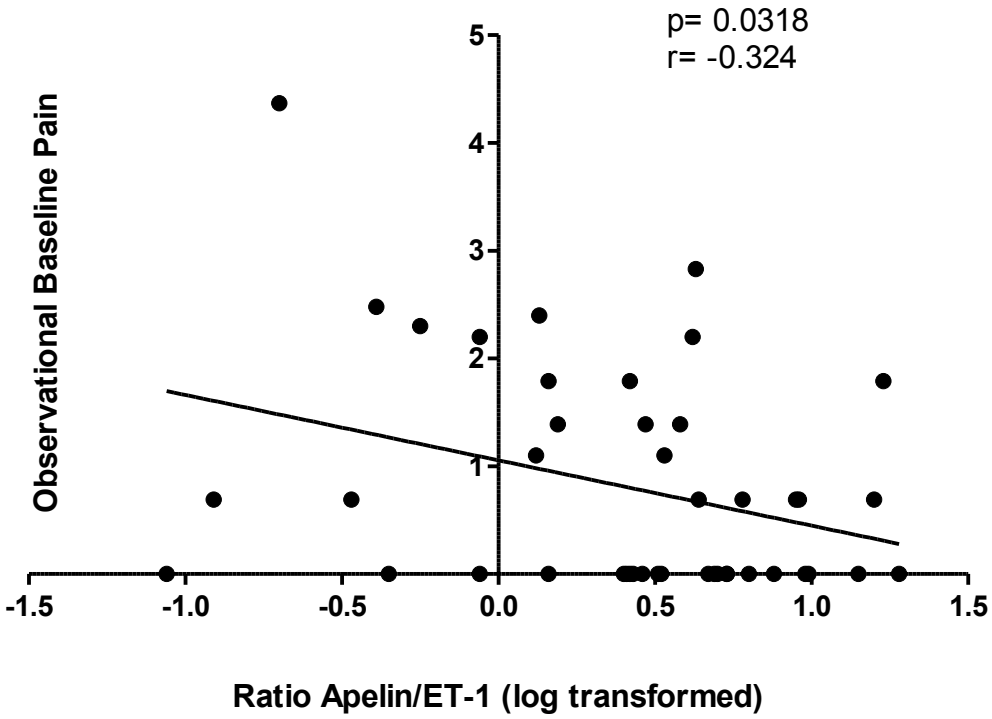

Figure 2.1 Negative correlation for the ratio between apelin and ET-1 and observational baseline pain ratings. Higher ratings of observational baseline are significantly associated with lower ratios of apelin to ET-1 (*N* = 42).

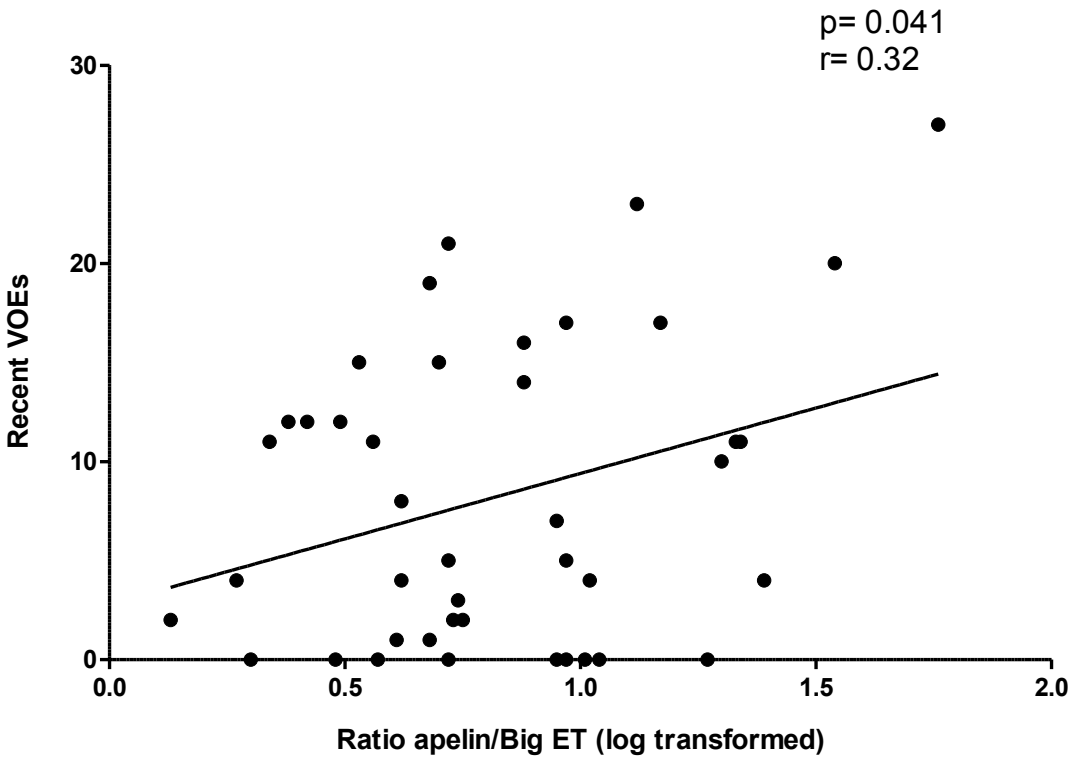

Figure 2.2 Positive correlation for the ratio between apelin and Big ET and recent VOEs. Higher ratio of apelin to Big ET are significantly associated with a higher number of recent VOEs (*N* = 42).

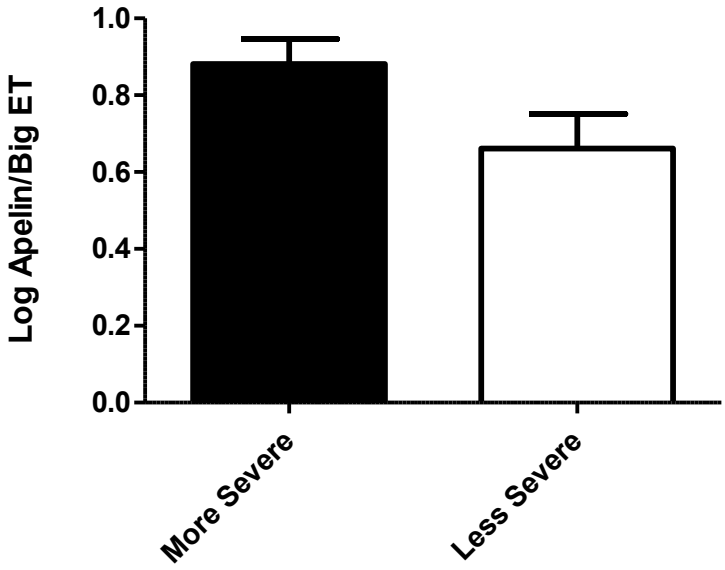

Figure 2.3 Relationship between disease severity phenotype and ratio between apelin and Big ET. Trend for significantly higher ratio of apelin to Big ET in children with the more severe disease phenotype (*N* = 32) compared to children with the less severe phenotype (*N* = 11).

CHAPTER 3:

Understanding the balance between the vasoconstrictor angiotensin II and the vasodilator apelin in the pain associated with sickle cell disease

3.1 Introduction

This study sought to examine the involvement of another potent vasoconstrictor, Ang II in the complications associated with SCD. This study also sought to understand the potential balance between Ang II and the vasodilator apelin in these complications since apelin has been shown to reverse Ang II induced vasoconstriction in isolated arteries (Zhong et al., 2007). Angiotensin II is similar to ET-1 in that it is a potent vasoconstrictor with an important role in regulating vasomotor tone (Nguyen Dinh Cat and Touyz, 2011) and also has expression and receptors at multiple levels of the pain neuroaxis and may be involved in modulating pain hypersensitivity (Chakrabarty et al., 2008; Imboden et al., 2009; Chakrabarty et al., 2013). Interestingly the role of angiotensin II as a pain modulator appears to be complex. Both pronociceptive and antinociceptive effects of angiotensin II have been reported. Anatomical site of administration, pain model, and species have all been shown to modify the functions of angiotensin II in the pain neuroaxis (Irvine and White, 1997; Georgieva and Georgiev, 1999; Marques-Lopes et al., 2009).

The goal of this study was to determine if the imbalance between ET-1-induced vasoconstriction and apelin-induced vasodilation in children with SCD is specific for these two vasoactive peptides or more global and includes other vasoactive and potentially pain modulating endogenous peptides such as angiotensin II. Both ET-1 and apelin are synthesized locally in the endothelial cells of the vasculature and released in a polarized fashion from endothelial to smooth muscle cells. Hence, plasma levels of ET and apelin represent only a

small fraction of tissue levels at any given time. In contrast, Ang II is a circulating peptide that is cleaved from angiotensinogen via multiple steps with a terminal cleavage to Ang II by angiotensin converting enzyme in the lungs. Thus, plasma levels of Ang II are believed to be representative of levels acting on the vasculature. In the current study, we postulate that plasma Ang II levels will display similar relationships to pain and symptoms as ET-1 because of the similar vasoactive and pronociceptive properties exhibited by these two endogenous peptides.

3.2 Methods

The same plasma samples used for the previous chapter were used for this study. The methods for the sample collection and more details on the primary outcome measures can be found in methods from Schlenz et al. (2012).

The enzyme immunoassay kit for Ang II was purchased from RayBiotech, Inc. (Cat#: EIA-ANGII-1, Norcross, GA). The assay was performed in duplicate according to the manufacturer's instructions. A standard curve was plotted from the standards in the kit using Prism software (GraphPad Software, La Jolla, CA), which was used to extrapolate the Ang II concentration in each plasma sample. The percentage of absorbance in relation to the blank optical density was used in the determination of Ang II concentration in the samples. Log-transformed values for Ang II concentrations were used since the untransformed values were highly skewed and variable. The values for the ratio between Ang II and apelin were also log transformed for the same reason. Five samples could not be analyzed; four samples contained Ang II concentrations that were below the level

of detection and one sample contained a concentration that was above the level of detection.

The primary outcomes explored for this study were baseline pain, procedural pain, ACS, recent VOE history, and SCD phenotype (disease severity). The relationships between Ang II or the ratio between Ang II to apelin and baseline or procedural pain ratings or recent VOEs were analyzed using correlations. A t-test was used to assess the relationship between Ang II or the ratio and ACS or SCD phenotype. The relationship between Ang II or the ratio and age or gender was also explored using either correlation or a t-test. The different procedural and baseline pain ratings are presented as log-transformed values because the original values exhibited a positive skew. For the procedural pain ratings, since baseline pain ratings were associated with significantly greater procedural pain ratings, regression analysis was used to remove variance in procedural pain that could be attributed to baseline pain.

3.3 Results

3.3.1 Demographics

The untransformed Ang II levels ranged from 9.0-6,244 pg/mL (m = 408.9, SD = 1053.3). There were no significant differences in Ang II plasma levels between males and females. However, a significant correlation was found between Ang II and age (p = 0.0096, r = -0.438) with older children displaying lower levels of Ang II (Figure 3.1). The ratio between Ang II and apelin did not reveal a difference for either gender or age.

3.3.2 Correlation with pain measures

No relationship was found between Ang II plasma concentration and the number of recent VOEs or observational baseline pain ratings (p = 0.582, r = -0.097; p = 0.966, r = 0.008, respectively). A positive correlation was found between observational procedural pain ratings and Ang II where higher pain ratings are significantly associated with a higher level of plasma Ang II (p = 0.0038, r = 0.497) (Figure 3.2). No relationships were found between the ratio of Ang II to apelin and the number of recent VOEs or observational baseline pain ratings. Higher observational procedural pain ratings were significantly correlated with a higher ratio of Ang II to apelin (p = 0.026, r = 0.387) (Figure 3.3).

3.3.3 Relationship to ACS

There was a trend for a difference between children with a history of ACS in the past 12 months and children without a history of ACS in relation to plasma Ang II levels. Children who did not have a history of ACS tended to have higher levels of plasma Ang II levels (p = 0.074) (Figure 3.4). No differences were found between children with or without a history of ACS in relation to the ratio between Ang II and apelin.

3.3.4 Relationship to disease severity

There was also a strong trend for a difference between severe (HbSS and HbSβ°) and mild-moderate (HbSC and HbSβ^{+}) phenotypes with children who have the more mild-moderate phenotype having higher levels of Ang II (p = 0.054) (Figure 3.5). This difference was significant when the untransformed

values of Ang II were used in the analysis (data not shown). No differences were found between the two disease severity phenotypes and the ratio between Ang II and apelin.

3.3.5 Relationship of Ang II to apelin

Higher plasma Ang II levels were also significantly associated with higher levels of apelin ($p = 0.012$, $r = 0.426$) (Figure 3.6).

3.4 Discussion

This study examined plasma levels of angiotensin II in children with SCD with the goal of determining the potential involvement of the angiotensin system in pain and symptoms associated with SCD. In addition, this study sought to determine how the balance between vasodilatory apelin and vasoconstrictive angiotensin II plasma levels impacts pain reports in children with SCD. Similar to the endothelin and apelin systems, Ang II plasma levels also decrease with age, suggesting global developmental changes in vasoregulatory systems. Future studies comparing vasoregulatory peptide plasma levels in children with and without SCD are needed to determine whether the developmental changes found in this study are specific for SCD. Interestingly, a significant positive correlation between Ang II and procedural pain ratings was found, suggesting that Ang II may be involved in acute pain in children with SCD.

The goal of this study was to determine if the imbalance between vasoconstriction and vasodilation is specific for the endothelin system or whether the imbalance is global and might involve other vasoactive and pain modulating peptides such as angiotensin II. These results suggest that the imbalance seen

between apelin and ET-1 in the previous chapter is specific for ET-1 rather than a general imbalance between apelin and vasoconstrictors. Previous studies examining ET-1 in SCD have found increased plasma levels during VOEs (Graido-Gonzalez et al., 1998; Rybicki and Benjamin, 1998; Lapoumeroulie et al., 2005). Furthermore, elevated ET-1 plasma levels slowly decrease over the course of several weeks which temporally parallels the resolution of pain in children with SCD (Graido-Gonzalez et al., 1998). Previous findings from our laboratory have found that plasma ET-1 levels are correlated with baseline pain in children with SCD. The precursor Big ET levels are negatively correlated with higher frequency of recent VOEs in children with SCD, suggesting that decreased Big ET plasma levels may result from increased conversion to the active ET-1. Similarly, ratios of apelin to ET-1 or Big ET have also been found to correlate with baseline pain and VOEs (Chapter 2). In contrast to previous findings from our laboratory, no significant relationships were found for the ratio between Ang II and apelin and any of the SCD specific pain measures. These findings suggest that the relationships identified with apelin and ET-1 or Big ET and SCD related pain measures are specific for the endothelin system. This is the first study that has demonstrated a potential relationship between angiotensin II and procedural pain in children with SCD, but not a relationship with SCD-associated pain.

There is a growing preclinical literature suggesting that while Ang II plays an important role in the regulation of vasomotor tone by inducing vasoconstriction, it may also have a role in pain hypersensitivity through Ang II

receptor type 1 and 2 receptors (Chakrabarty et al., 2008; Imboden et al., 2009; Chakrabarty et al., 2013). Depending on whether Ang II is injected peripherally or centrally, the particular pain model, or the species it can be either anti-nociceptive or pro-nociceptive (Irvine and White, 1997; Georgieva and Georgiev, 1999; Marques-Lopes et al., 2009). It has been recently suggested that Ang II may act as a neuropeptide with multiple effects in the nervous system including modulation of pain transmission in the spinal cord (Nemoto et al., 2013). For instance, intrathecal administration of Ang II produces nociceptive behaviors in rodents which can be blocked by both Ang II receptor inhibitors and opioids (Nemoto et al., 2013). Furthermore, inhibition of Ang II in the rodent brain is anti-nociceptive in acute pain models and this is thought to be mediated through the endogenous opioid system (Takai et al., 1996). This suggests that the central actions of Ang II, possibly in the caudal ventrolateral medulla (Marques-Lopes et al., 2009), may have a role in enhancing acute pain (Takai et al., 1996). In contrast, in spontaneously hypertensive rats, decreased pain sensitivity is reported, and this sensitivity can be attenuated with angiotensin I receptor antagonists (Irvine et al., 1995). This suggests that peripheral, as opposed to central, angiotensin II may be anti-nociceptive in pathological states. Furthermore, peripheral angiotensin II has been postulated to increase the release of endogenous opioids to facilitate analgesia (Irvine and White, 1997). In the current study, increased peripheral plasma levels were associated with increased acute procedural pain in children with SCD which is opposite of what would be predicted from preclinical studies. One potential reason is that

measurement of plasma Ang II does not rule out a potential central site of action of the peripherally circulating angiotensin II or centrally produced and released angiotensin II. In fact, tissue levels of Ang II may be more important than circulating plasma levels in modulating both vascular reactivity and pain sensitivity (Schuijt et al., 2002).

Interestingly, especially in light of the vascular pathology in SCD, the vascular disease status may impact whether Ang II exerts anti-nociceptive or pro-nociceptive activity. Ang II has been postulated to play a role in pathological endothelial cell dysfunction and vascular hyper-reactivity, both of which may be involved in SCD (Schujt et al., 2002). Two sub-phenotypes that have been identified in the complications associated with SCD: viscosity-vaso-occlusive and vasculopathy sub-phenotypes (Kato et al., 2009). The viscosity-vaso-occlusive sub-phenotype is associated with acute painful VOEs and ACS while the vasculopathy or endothelial dysfunction sub-phenotype is associated with stroke, pulmonary hypertension, and chronic leg ulcers, which also has a pain component (van der Land et al., 2013). So even though in this study Ang II was not found to be associated with the frequency of recent VOEs and baseline pain, which is thought to be related to vaso-occlusive pain, it may be possible that it is instead involved in the vasculopathy/endothelial dysfunction sub-phenotype.

Compared to an earlier study that found that Ang II levels in normal children range from 5-103 pg/mL, the children in this study display much higher levels of Ang II and a much larger variability (9-6,244 pg/mL) (Broughton Pipkin et al., 1981). This difference could be attributed to the different ethnicities

studied or it could be due to the particular pathologies associated with SCD. It has been noted that in transgenic sickle cell mice, removal of the vasodilatory component unmasks an upregulation of vasoconstrictive systems, and the high levels of Ang II found in this study may lend to the possibility that Ang II could be one of these systems (Nath et al., 2000). A consequence of the imbalance in vasoconstrictors and vasodilators is vascular instability, which may contribute to a state of vasculopathy and endothelial dysfunction (Hebbel, 2014). One possible candidate for contributing to the state of vasculopathy and endothelial dysfunction is Ang II since it has been implicated in contributing to endothelial dysfunction in other pathologies (Taguchi et al., 2011). This study also confirms our finding of a decrease in Ang II levels with age.

As with any clinical study, there are several limitations in the current study. The first limitation is the small sample size, which makes extrapolation of the current findings to larger patient populations limited. An attempt to separate the children by SCD genotype resulting in even small sample numbers did not show any significant differences between the phenotypes for Ang II. Another limitation of this study is the measurement of Ang II in blood plasma, which is only capturing circulating levels as opposed to actual tissue levels. This is further confounded by the short half-life of Ang II. In the rat, the half-life of Ang II is estimated to be around 16 seconds in the plasma (Al-Merani et al., 1978), and according to the manufacturer of the Ang II enzyme immunoassay kit, the half-life of Ang II in human plasma is approximately 30 seconds. The time between

blood collection and the placement on ice varied for each participant, which may explain the large variation in the Ang II levels.

One of the most interesting findings is that while we have previously shown a correlation between the endothelin (and apelin) systems and SCD-related pain but not procedural pain, the current study suggests that Ang II is not involved in SCD-related pain but is involved in acute pain. This suggests that future clinical studies should explore the utility of endothelin antagonists in SCD-related pain conditions and Ang II inhibitors on acute pain in children with SCD. This study also highlights the importance of discriminating the causes of pain in children with SCD as the different forms of pain may be responsive to varied pharmacotherapy approaches.

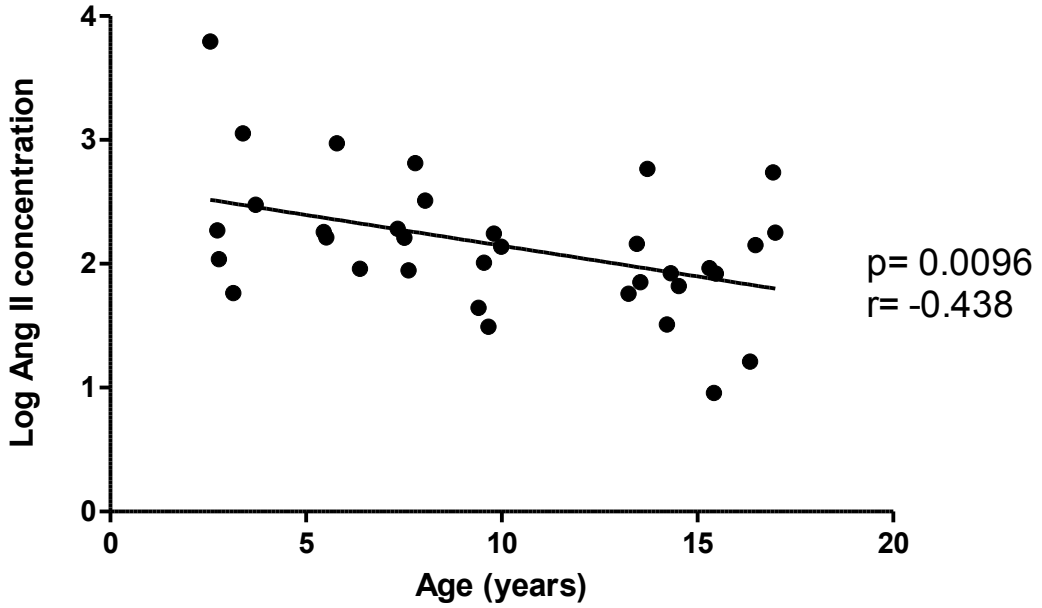

Figure 3.1 Negative correlation between plasma Ang II levels and age. Lower plasma levels of Ang II are significantly correlated with older children (N = 34).

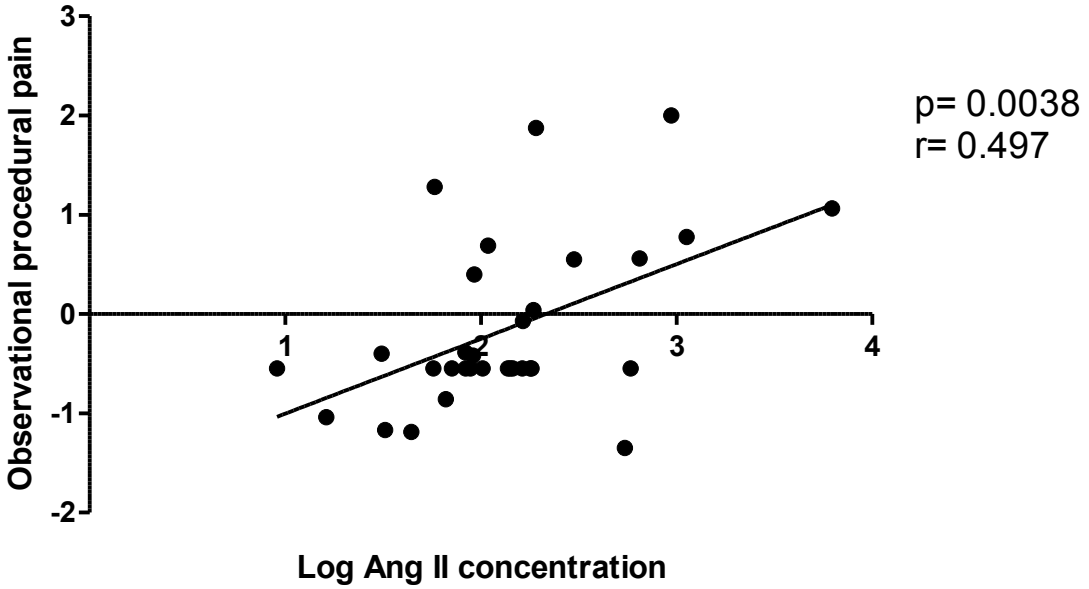

Figure 3.2 Positive correlation between Ang II plasma levels and observational procedural pain ratings Higher plasma levels of Ang II were significantly correlated to higher ratings of procedural pain in children with SCD (N = 32)

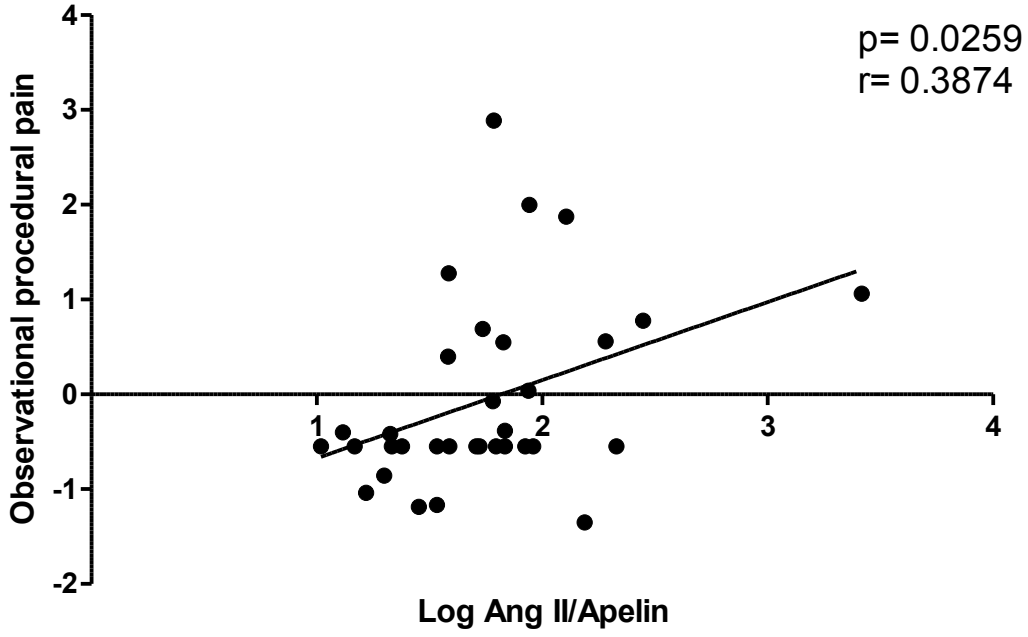

Figure 3.3 Positive correlation between ratio of Ang II to apelin and observational procedural pain ratings. A higher ratio of Ang II to apelin in the plasma was significantly correlated with higher ratings of observational procedural pain (N = 33).

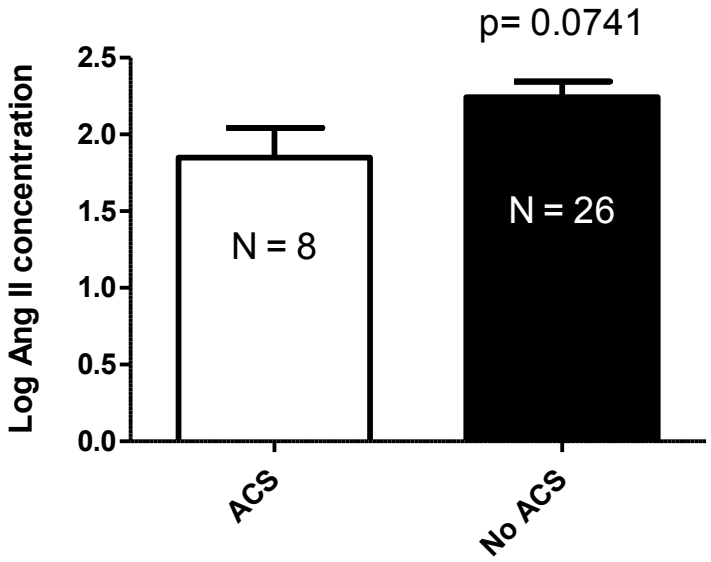

Figure 3.4 Plasma levels of Ang II and history of ACS. Trend for higher levels of plasma Ang II in children without a history of ACS compared to children with a history of ACS in the past 12 months.

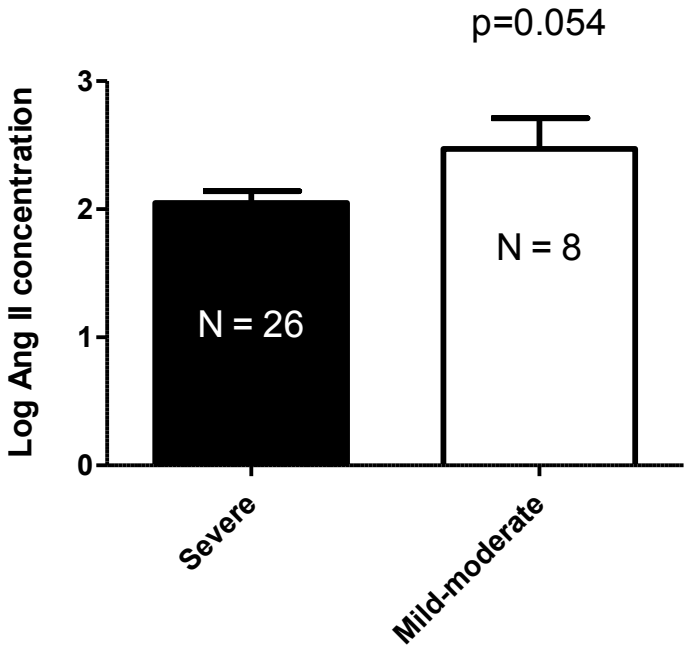

Figure 3.5 Plasma levels of Ang II and disease severity. Trend for higher levels of plasma Ang II levels in children with a less severe phenotype of SCD (children with the HbSC and HbSβ^+ genotypes) compared to children with the more severe phenotype (HbSS and Hbβ°).

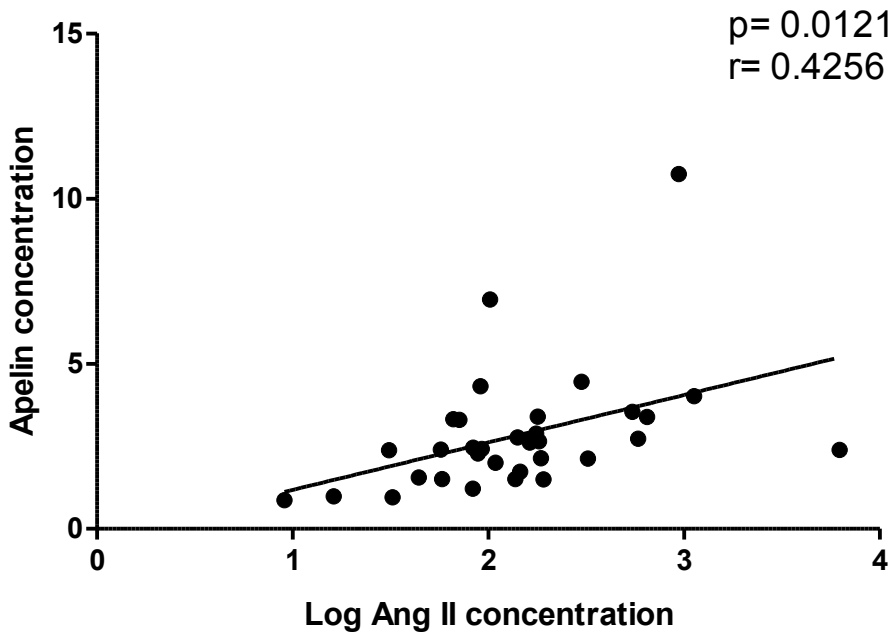

Figure 3.6 Positive correlation between Ang II and apelin. Higher plasma levels of Ang II were significantly correlated with higher plasma levels of apelin (N = 34).

CHAPTER 4:

Endothelin-1-induced priming to capsaicin in young animals[1]

[1] Smith T, Beasley S, Smith S, Mark I, Sweitzer SM (2014) Endothelin-1-induced priming to capsaicin in young animals. Neurosci Lett. Reprinted here with permission of publisher

4.1 Introduction

Vaso-occlusive episodes (VOEs) accompanied by extreme pain is a hallmark feature of sickle cell disease (SCD). The vasoactive peptide endothelin-1 (ET-1) has been found to be elevated in SCD patients during an acute VOE (Graido-Gonzalez et al., 1998). Endothelin-1 is a 21 amino acid peptide known to be a potent vasoconstrictor which causes acute pain and sensitization to mechanical and other noxious stimulation when injected into the human forearm (Ferreira et al., 1989; Dahlof et al., 1990; Hans et al., 2007). When ET-1 is injected into the rat hindpaw (Gokin et al., 2001) or directly onto the sciatic nerve (Davar et al., 1998), it induces spontaneous nociceptive behaviors. Repeated administration of ET-1 onto the sciatic nerve of adult male rats causes desensitization as evident by a reduction in nociceptive behaviors (Fareed et al., 2000). Previous results from our lab have shown that prior exposure to ET-1 alters behavioral responses to subsequent exposure to ET-1 at the same location in a sex dependent manner with sensitization in males and desensitization in females (McKelvy and Sweitzer, 2009). However, when the second administration of ET-1 was in the contralateral hindpaw a sensitization was observed in both males and females.

To determine whether the contralateral increase in ET-1-induced nociception is a result of central sensitization, this study applied capsaicin to the contralateral hindpaw and examined secondary mechanical hyperalgesia, which is when a noxious mechanical stimulus becomes even more noxious, and neuronal activation in the dorsal horn of the spinal cord using c-fos. This study

tested the hypothesis that a priming dose of ET-1 produces central sensitization, which will cause an increase capsaicin-induced secondary mechanical hyperalgesia in the contralateral hindpaw and c-Fos expression in the spinal cord.

4.2 Methods

4.2.1 Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Efforts were made to limit the amount of distress and the number of animals used. Male and female Sprague-Dawley (Charles River Laboratories, MA) pups were housed with dams on a 12 hour light/dark cycle with food and water available *ad libitum*. Each litter was culled at 10-12 pups.

4.2.2 ET-1 and capsaicin administration and behavioral analysis

Sterile saline or ET-1 (Enzo Life Sciences, Farmingdale, NY, USA) dissolved in deionized water was administered (5.25 pmol) in 10 µL (1.31 ng/µL) subcutaneously using an insulin syringe into the left plantar hindpaw on postnatal day 7 (P7). Animals were videotaped, and the number of spontaneous paw flinches was counted. On postnatal day 11 (P11), capsaicin cream (0.1% Capzaisin-HP, Chattem, Inc, Chattanooga, TN, USA) or control lotion (Johnson & Johnson Baby Lotion) was applied topically to the dorsum of the right hindpaw. There were four treatment groups for both sexes (n=5-6 for each group and sex): saline (P7)+control (P11), saline (P7)+capsaicin (P11), ET-1 (P7)+control (P11), and ET-1 (P7)+capsaicin (P11). Before application of capsaicin, the baseline

paw withdrawal threshold was measured in the plantar right hindpaw using sequential von Frey filaments ranging from 0.04 g to 1.4 g. Each filament was applied a total of five times and the first filament that elicited a sustained response was considered the paw withdrawal threshold. This method was used at 20, 40, 60, and 120 minute time points following capsaicin application.

4.2.3 Fos immunohistochemistry

Two hours post-capsaicin on P11, all animals were deeply anesthetized with isoflurane then transcardially perfused with cold phosphate buffered saline (PBS) and 4% paraformaldehyde followed by isolation of the vertebral columns. Spinal cords were isolated and equilibrated in a cyroprotecting solution (30% sucrose in PBS). Spinal cords were mounted in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and sliced into serial 40 µm transverse sections. Sections were stored in anti-freeze solution (ethylene glycol and sucrose in PBS) at -20°C until processed for immunofluorescence or immunoperoxidase. Free-floating sections were blocked in normal horse serum before being incubated with polyclonal rabbit anti-c-Fos (EMD Biosciences, Billerica, MA, USA, 1:5000) overnight at 4°C. Following a wash, the tissue sections were then incubated with donkey anti-rabbit Alexa Flor 594 (Invitrogen, Carlsbad, CA, USA, 1:200) or biotinylated donkey anti-rabbit (Jackson Immunoresearch, West Grove, PA, USA, 1:1000) for 1.5 hours at room temperature then washed, slide mounted, and coverslipped using Vectashield (Vector Laboratories, Burlingame, CA, USA). For immunoperoxidase, sections were incubated in HRP-streptavidin (Jackson Immunoresearch, 1:1600) for 1

hour at room temperature followed by exposure to DAB and mounting on gelatin coated slides. The total number of c-Fos positive cells was counted in the L3-L5 dorsal horns by an experimenter blinded to treatment. Due to the diffuse expression of c-Fos expression across the dorsal horn in spinal cords from relatively immature animals, the expression was counted in both superficial and deeper dorsal horns.

4.2.4 Data Analysis

One-way ANOVA was used for comparing the number of ET-1 induced paw flinching on postnatal day 7 (treatment and sex). Two-way ANOVA was used for comparing time versus treatment between sexes in the capsaicin induce mechanical hyperalgesia study. One-way ANOVA was used for comparing the number of c-Fos positive neurons (treatment and sex). The conservative Bonferroni post-test was used for all analysis and a p-value < 0.05 was considered significant. All statistical analysis was done with GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA).

4.3 Results

4.3.1 Behavioral Analysis

Administration of ET-1on P7 elicited a significantly greater number of paw flinches compared to saline administration in both male and female rats ($p<0.05$; Fig. 4.1 A). No differences between sexes was observed on P7 (ET-1 female vs. ET-1 male, $p>0.05$; saline female vs. saline male, $p>0.05$).

Administration of capsaicin to the contralateral dorsal hind paw on P11, produced secondary mechanical allodynia, which is defined as a non-noxious

stimuli becoming noxious at a site beyond the area of injury, in the plantar hind paw (Fig 4.1 B,C). In saline control animals, the duration of secondary allodynia was sex-dependent (Fig 4.1 B,C). In control males not previously exposed to ET-1, topical capsaicin produced secondary mechanical allodynia at all time points examined including 120 minutes post-capsaicin (Fig. 4.1 B). In control females not previously exposed to ET-1, topical capsaicin produced secondary mechanical allodynia of a short duration (Fig 4.1 C). Secondary mechanical allodynia was only observed at 20 minutes after capsaicin administration. At 40 minutes post-capsaicin administration control saline females had significantly higher paw withdrawal thresholds compared to capsaicin-treated control saline males ($p<0.05$). This suggests sex-dependent capsaicin-induced secondary allodynia in neonatal rats.

Priming with ET-1on P7, did not alter the magnitude or duration of capsaicin-induced secondary mechanical hyperalgesia in males. There were no significant differences found between the primed and unprimed males at any of the time points (Fig. 4.1 B). In contrast, priming with ET-1 on P7, sensitized females to capsaicin induced secondary mechanical hyperalgesia ($p<0.01$ versus control across the time course) demonstrated by a significant reduction in paw withdrawal threshold across the entire 120 minutes (Fig 4.1 C). Interestingly, females primed with ET-1 on P7 had greater capsaicin induced secondary mechanical hyperalgesia as measured by a lower paw withdrawal threshold when compared to males primed with ET-1 on P7 ($p<0.05$ across time).

Administration of vehicle cream did not alter paw withdrawal thresholds across the time course in primed and unprimed males and females (data not shown).

4.3.2 Fos expression

ET-1 priming significantly increased capsaicin-induced c-Fos expression in both males and females ($p<0.001$) compared to the unprimed animals and compared to control (Fig. 4.2 A-C). C-Fos expressing neurons were found in both superficial and deeper dorsal horn lamina (see Fig. 4.2 A). Animals that were primed with ET-1 and received capsaicin on P11 had significant c-Fos expression in the right dorsal horn (Fig. 4.2 A-C) compared to the animals who received saline on P7 and capsaicin on P11. Primed males treated with capsaicin had significantly greater c-Fos compared to primed females treated with capsaicin ($p<0.001$). Interestingly, priming also increased c-Fos expression on the contralateral paw in males but not females (data not shown).

4.4 Discussion

The nociceptive response to capsaicin was sex dependent with males developing a longer lasting capsaicin induced secondary mechanical hyperalgesia compared to females. Priming with ET-1 on P7 sensitized females to capsaicin induced secondary mechanical hyperalgesia. These results also demonstrate that capsaicin causes a greater increase in Fos expression in the dorsal horns of ET-1 primed animals compared to ET-1 naïve animals, with a larger induction in males as compared to females. The results of this study provide more evidence for the hypothesis that pain early in life can alter the response to subsequent noxious stimuli and this alteration may be sex

dependent. The neonatal period has been found to be a critical period for injury-induced changes to nociceptive processing, and these changes affect subsequent responses to noxious stimuli (Fitzgerald, 2005). In children with sickle cell disease, the risk of experiencing a painful crisis occurs in the first year of life and increases thereafter (Gill et al., 1995).

In female rats, capsaicin induced a short duration secondary mechanical hyperalgesia on postnatal day 11. Priming with ET-1 on postnatal day 7 increased the duration of capsaicin-induced secondary mechanical hyperalgesia. In addition, priming with ET-1 led to greater capsaicin induced secondary mechanical hyperalgesia in females as compared to males. These results correspond with research that has reported sexually dimorphic differences in nociceptive thresholds in adulthood after neonatal injury (LaPrairie and Murphy, 2007). They also relate to clinical research that has shown that female sickle cell anemia patients have a higher incidence of painful crisis compared to male sickle cell anemia patients (Gill et al., 1995).

The significantly higher Fos expression seen in the ET-1 pre-treated animals suggests that the priming effect of ET-1 may be centrally mediated at the level of the spinal cord. However, it cannot be ruled out that ET-1 priming may also have supraspinal effects on descending modulation, such as enhancing facilitation of nociceptive inputs or reducing descending inhibition (Porreca et al., 2002; Hohmann et al., 2005). It is possible that similar to inflammatory pain, ET-1-induced nociception may cause a shift in the balance from inhibition to facilitation in the capsaicin treated animals (Ren and Dubner, 2002). In contrast

to prior reports showing no Fos expression in the contralateral dorsal horns of adult rats after treating with capsaicin in the neonatal period, we show Fos expression in the contralateral dorsal horns (Hohmann et al., 2005). However, this difference may be explained by the fact that in the prior report, Fos expression was measured in adulthood as opposed to the neonatal period. At this time it is not exactly clear what the functional significance is of the activation of contralateral neurons. One possible explanation is that the activation of these contralateral neurons could be due to the activation of polysynaptic pathways involving neuronal connections to the brainstem (Kaczmarek, 2002). The observation of Fos expression in the deeper dorsal horns may be explained by an increase in the response of wide dynamic range and low-threshold neurons, which has been shown to happen after neonatal chronic inflammation (Ruda et al., 2000; Peng et al., 2003).

The repetitive nociceptive pain experienced by sickle cell patients early in life may contribute to the development of chronic pain experienced by some patients later in life (Smith and Scherer, 2010). Because SCD patients may experience repeated painful crises throughout the infant and childhood periods, it is important to find better ways to effectively relieve pain during these critical periods.

Together, the results of this study suggest that pain in early life may alter future responses to painful stimuli at both the behavioral and neuronal level and the response at the behavioral level is sexually dimorphic. These results also

suggest that, at least in part, the contralateral sensitizing effect is mediated centrally in the spinal cord.

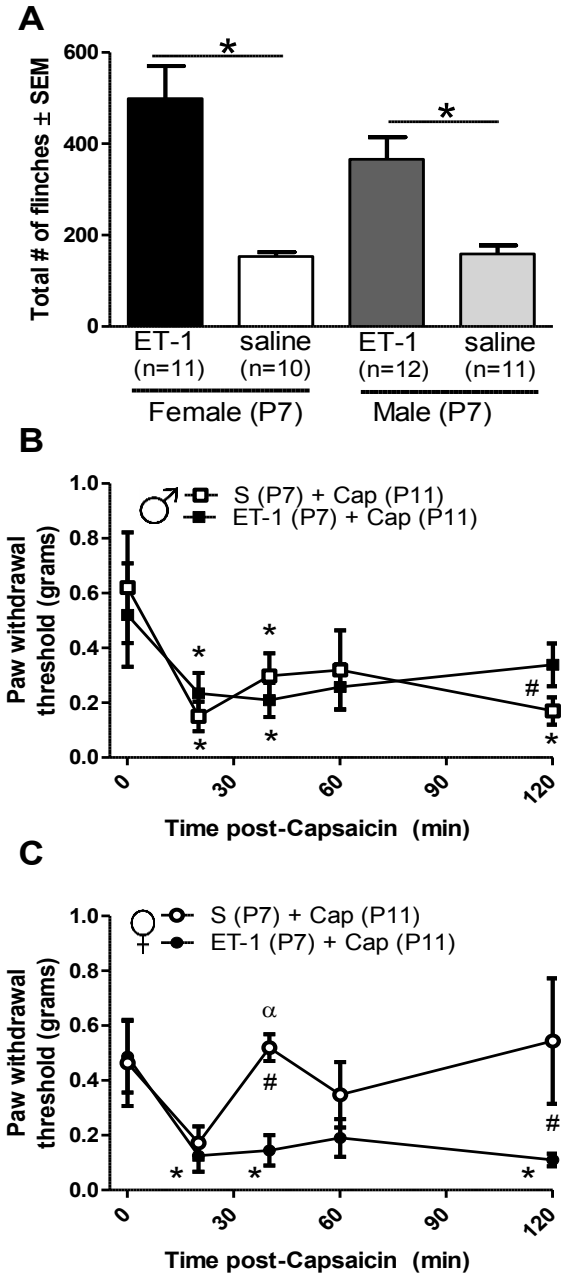

Figure 4.1 ET-1-induced spontaneous behavior and capsaicin-induced secondary allodynia. Sex-dependent ET-1 priming on capsaicin induced secondary allodynia and spinal cord c-fos expression. **(A)** Intraplantar ET-1 administration increased the total number of spontaneous paw flinches on postnatal day 7 (*$p<0.05$ ET-1 vs saline same sex). **(B)** In males, priming had no effect on capsaicin induced secondary allodynia in the contralateral hind paw (*$p<0.05$ vs time 0 baseline, $^{\#}p<0.05$ vs primed animals). **(C)** In females, priming prolongs capsaicin-induced secondary allodynia in the contralateral hind paw (*$p<0.05$ vs baseline, $^{\#}p<0.05$ vs primed animals, $^{\alpha}p<0.05$ vs. males).

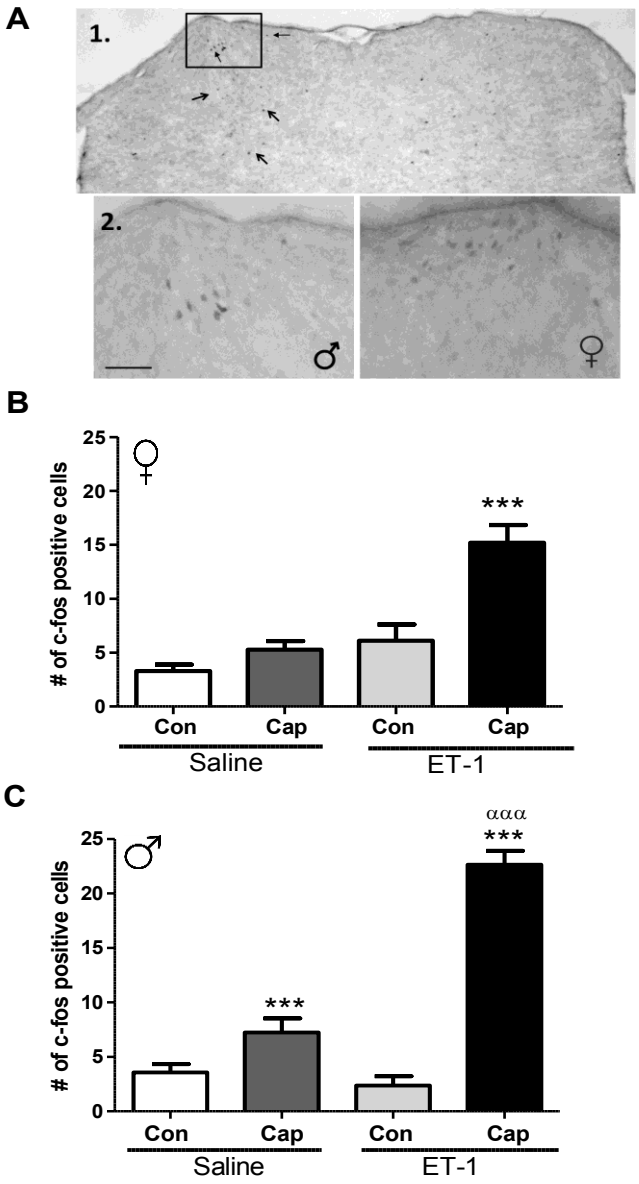

Figure 4.2 ET-1 priming effect on capsaicin-induced c-Fos expression. (A) Representative micrographs of c-Fos staining in the right dorsal horn of the spinal cord. **(A1)** Ipsilateral and contralateral dorsal horn from male ET-1 + Capsaicin treatment. Large arrows point to deep dorsal horn c-Fos positive neurons. Small arrows point to superficial dorsal horn c-Fos positive neurons. Box indicates area that is shown in higher magnification in figure A2. **(A3)** Ipsilateral dorsal horn from female ET-1 + Capsaicin treatment. Scale bar represents 500 μm in A1 and 50 μm in A2 and 3. **(B)** In females, priming increases capsaicin-induced c-Fos in the dorsal horn (***p<0.001 vs. control). **(C)** In males, priming increases capsaicin-induced c-Fos in the dorsal horn (***p<0.001 vs. control; $^{\alpha\alpha\alpha}$p<0.001 vs. females).

CHAPTER 5:

Examining the Endothelin and Apelin Systems in Animal and *in vitro* Model of Acute VOEs[1]

[1]Smith, T and Sweitzer, S.M. Endothelin-1 induced desensitization in primary afferent neurons. Submitted in part to *Neuroscience Letters*. 06/16/2014

5.1 Introduction

Our lab has previously used an acute administration of ET-1 in rats to model an acute VOE associated with a sickle cell crisis. In this model, repeated administration of ET-1 results in a sex-dependent priming effect. In males, ET-1 exposure produces sensitization to a second exposure to ET-1 and this ET-1-induced sensitization is associated with down-regulation of the ET_B receptor (McKelvy and Sweitzer, 2009). The ET_B receptor produces vasodilation in the vasculature and release of endogenous opioids from keratinocytes in the skin, suggesting that ET_B receptor activation has analgesic effects in the skin (Mazzocchi et al., 1998; Khodorova et al., 2003). In contrast, in females, prior exposure to ET-1 produces desensitization to a second exposure to ET-1 and causes an up-regulation of the ET_B receptor (McKelvy and Sweitzer, 2009). Furthermore, in the previous chapter we showed that priming with ET-1 enhances capsaicin-induced secondary hyperalgesia in both males and females and that this is accompanied by enhanced c-Fos activation in dorsal horn neurons.

The first goal of this phase of the project was to determine if ET-1-induced sensitization is also mediated at the primary afferent neuron level. To examine this, primary DRG cell cultures were kept in culture for four days to mimic the timeline (P7-P11) in our *in vivo* model and exposed to ET-1 to simulate an acute VOE. Calcium imaging was used to measure neuronal activity in these DRG cultures after exposure to ET-1. The second step of this phase of the project, builds upon our previous clinical findings that the ratio between apelin and

endothelin correlates with VOEs and baseline pain in children with SCD. This phase sought to examine the role of the apelin system in our rodent and *in vitro* model of acute VOEs. These studies entailed western analysis of APJ receptor expression in the pain pathway, using cultured DRG cells to examine the apelin-ET-1 functional intersection in afferent neurons, and skin release assays to determine the impact of ET-1 on apelin release.

5.2 Methods

5.2.1 Cell Culture

Dorsal root ganglia (DRG) from adult male rats were dissected and collected in ice cold Tyrode buffer (132 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM dextrose, 5 mM HEPES). DRGs were digested using a solution of dispase (1 mg/mL, Gibco 171050-041) and collagenase (2 mg/mL, Roche 1088831) in Tyrode buffer and incubation in a shaking water bath at 37°C for 45 minutes. Following digestion, the DRGs were triturated with a fire polished Pasteur pipet followed by separation using an OptiPrep (Sigma D1556) gradient. Cells were centrifuged at 1900 rpm for 20 minutes then the bottom layer was isolated and the cells were counted.

The DRG cells (both neurons and support cells) were seeded in 96 well black clear bottom plates (Costar, Corning Inc.) coated with poly-D-lysine at a density of approximately 60,000-80,000 cells per well (day 0). Cells were maintained in Minimum Essential Medium Eagle media (Sigma Aldrich M0643) (supplemented with 20% glucose, 10% FBS (Invitrogen 16000-036), 1%

penicillin-streptomycin solution (Invitrogen 15070-063), and 10 µg/ml of NGF (Millipore 01-125).

5.2.2 Calcium imaging for ET-1 experiment

Cells were maintained in culture for 4 days and on the 5th day, calcium imaging was performed. On day 0 (the day of culture), cells were either treated with 1.1 nmole of ET-1 (22 µM, Enzo Life Sciences) or vehicle (sterilized deionized water). On day 4, cells were first washed with either Ca^{2+}-containing NaCl-based extracellular (EC) buffer (130 mM NaCl, 2.5 mM KCl, 1mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM HEPES, 5mM glucose) with 2.5 mM probenecid (Molecular Probes P36400) or Ca^{2+}-free NaCl-based EC buffer then incubated with Fluo-4AM solution (1 µM, Molecular Probes, Invitrogen F14201) containing pluronic acid at 37°C for 1 hour. After incubation with the dye solution, cells were washed twice with the appropriate NaCl-based EC buffer, then 190 µl of the appropriate buffer was added to each well. After incubation at 37°C for 1 hour, the plate was loaded into the BioTek Synergy 2 plate reader and the BioTek Gen5™ Data Analysis Software was used to perform reader control. After a 5 minute delay, the cells were baselined every 5 seconds for 30 seconds. Following the baseline measures, either 10 µL of ET-1 (0.55µM) or 10 µL of vehicle (sterile filtered deionized water) was injected into the appropriate wells by hand. The change in fluorescence was then measured every 3 seconds for 3 minutes. Fluorescence was excited at 485 nm and emission was measured at 528 nm. Temperature was maintained at 37°C. A total of 6 wells were measured for each treatment group.

5.2.3 Drug treatment and Ca^{2+} Imaging for ET-1 time course

The cells were treated with either ET-1 (2.2 μM, Alexis Biochemicals) or vehicle for a period of 4 days. On day 0, one set of cells was treated with ET-1 and the others treated with vehicle. On day 1, the culture media was changed and a different set of cells was treated with ET-1 and the others treated with vehicle. This was repeated on days 2 and 3 with a different set of cells being pre-treated once with ET-1 during the treatment period. There were a total of 6 wells for each treatment group.

On day 4, calcium imaging was performed. Cells were loaded with the dye solution as mentioned previously. The plate reader was programmed to have a 5 minute delay, after which, baseline measurements were taken every 5 seconds for 30 seconds for the first set of wells. ET-1 (10 μl, either 5.5 μM or 0.55 μM) or vehicle (10 μl) was added to the appropriate wells, then readings were made continuously every 3 seconds for 3 minutes.

5.2.4 Calcium imaging for capsaicin experiment

The cells were treated with either vehicle (sterile filtered deionized water) or 1.1 nmole of ET-1 (22 μM, American Peptide, Sunnyvale, CA on day 0 (the day of cell culture). On day 4, calcium imaging was performed. Following the baseline measures, either 10 μL of capsaicin (1μM) or 10 μL of vehicle (DMSO in NaCl-based EC buffer) was added to the appropriate wells (5 wells/treatment group) and the change in fluorescence measured every 3 seconds for 5 minutes.

5.2.5 Western blot analysis

On postnatal day 7, rats received either an intraplantar injection of ET-1 (3.3 nmol American Peptide) or saline. Postnatal day 11 rats were given an overdose of isoflurane and the hindpaw skin and spinal cords were collected and frozen at -80°C until ready for analysis. The tissues were homogenized and a bicinchoninic acid assay (BCA assay) was used to determine protein concentration for each sample. For Western blot analysis, 33 µg of protein was loaded for each sample onto a 4-15% Tris HCl Criterion gel (BioRad). The separated proteins were then transferred to a PVDF membrane. The membrane was blocked in 5% milk for one hour at room temperature, then incubated with the rabbit anti-APJ receptor antibody ([1:1000], Santa Cruz Biotechnology) overnight at 4°C. Following a few washes, the membrane was incubated in donkey anti-rabbit secondary antibody ([1:4000], Jackson Immunoresearch Laboratories) for one hour at room temperature. Protein expression was visualized using enhanced chemiluminescence (ECL). Beta-actin was used as a loading control and the ratio of APJ receptor expression to beta-actin expression was used in the analysis.

5.2.6 Apelin-ET-1 calcium imaging

Cells were cultured as previously mentioned from adult male rats. Two paradigms were explored for this experiment. In the first paradigm, the cells were primed with either 1.1 nmole of ET-1 (5.5 µM, American Peptide), a combination of ET-1 and 2 nmole of pyroglutamylated apelin-13 (10.1 µM, American Peptide), apelin alone, or vehicle on the day of culture. On day 4,

calcium imaging was performed and cells were either challenged with 0.55 μM of ET-1 or vehicle. In the second paradigm, the cells were primed with either 1.1 nmole of ET-1 or vehicle on the day of culture. On day 4, calcium imaging was performed and cells were challenged with either 0.55 μM of ET-1, a combination of ET-1 and 5.5 μM of apelin, or apelin alone. Following baseline measures, the peptides were added to the appropriate wells (5 wells/treatment group) and the change in fluorescence was measured every 3 seconds for 3 minutes.

5.2.7 Skin release assay and ELISA

On postnatal day 11, hindpaw skin was collected from both male and female rats and maintained in artificial interstitial fluid (aIF). Skin release fractions were collected before incubation with an ET_B receptor agonist (IRL-1620, American Peptide), during incubation with agonist, and after incubation with agonist. The skin release fractions were then analyzed for apelin (Phoenix Pharmaceuticals) using EIA.

5.2.8 Data analysis

GraphPad Prism Software was used for all statistical analysis. For comparison between treatment groups for all of the Ca^{2+} imaging experiments, one-way ANOVA followed by a Bonferroni post hoc test was employed. One-way ANOVA followed by a Bonferroni post-hoc test was also employed for all of the Western blot results. For the skin release assay, a two-way ANOVA was used to determine the effects of both sex and treatment with the ET_B receptor agonist. $P<0.05$ was considered to be statistically significant.

5.3 Results

5.3.1 Effect of ET-1 on $[Ca^{2+}]_i$ in afferent neurons

To determine whether behavioral desensitization is driven by afferent neuron desensitization, an *in vitro* model using cultured DRG neurons and calcium imaging was developed. The experimental design was to apply ET-1 on day 0 of culture and then measure ET-1 stimulated Ca^{2+} influx four days later. On day 4 in culture, application of 0.55 μM of ET-1 onto naïve cells significantly increased intracellular Ca^{2+} compared to vehicle treated cells ($p<0.001$) (Figure 5.1 A). However, priming with 22 μM of ET-1 on day 0 followed by a challenge with 0.55 μM of ET-1 on day 4 did not produce a significant influx of Ca^{2+} into the cultured afferent neurons (Figure 5.1 A). The ET-1-induced increase in intracellular Ca^{2+} due to ET-1 is dependent on extracellular Ca^{2+}, as removal of Ca^{2+} from the extracellular buffer prevents ET-1-induced Ca^{2+} transients (Figure 5.1 A inset). Similarly, a reduction in area under the curve (AUC) (Fig 5.1 B) and maximum peak height (Fig 5.1 C) is seen in ET-1 primed cells compared to unprimed cells exposed to ET-1 for the first time at the time of imaging (Fig 5.1 B). The time to the calcium peak is similar between treatments with a trend to a more rapid peak in cells exposed to ET-1 compared to vehicle (Fig 5.1 D).

5.3.2 Time course of priming effect of ET-1

To examine the time course of the priming effect, cells were primed with 4, 3, 2, or 1 day before the ET-1 challenge dose. Application of low dose (0.55 μM, Fig 5-2 A) or high dose (5.5 μM, Fig 5.2 B) of ET-1 produced a statistically significant rise in $[Ca^{2+}]_i$. Priming of the cells with 2.2 μM of ET-1 on Day 1, 2, or

3 of culture produced a time-dependent blockade of the ET-1 stimulated rise in $[Ca^{2+}]_i$ when a low challenge dose of ET-1 was used (Fig 5.2 A, C). In contrast, priming on any day suppressed calcium transients when a high challenge dose of ET-1 was used (Fig 5.2 B, C). Measurement of the maximum peak height (Fig 5.2 E) showed similar time dependent priming with low but not high dose ET-1 as the area under the curve measurements (Fig 5.2 C). The time to peak was similar in all treatments using a low dose ET-1 challenge (Fig 5.2 D). In contrast, the time to peak was delayed by priming on days 1 and 3 when a high dose of ET-1 was used to challenge (Fig 5.2 D). This data suggests that both the day of priming and the challenge dose of ET-1 have a significant impact on ET-1 induced desensitization in primary afferent neurons.

5.3.3 Effect of ET-1 priming on capsaicin-induced increase in $[Ca^{2+}]_i$

As we have previously demonstrated (Chapter Four) in this thesis, prior exposure to ET-1 can modify subsequent behavioral nociceptive responses to capsaicin. The purpose of this experiment was to determine whether that effect of ET-1 priming on capsaicin responses might be mediated at the level of the afferent neuron. Treatment of cells with capsaicin on day 4 causes a significant increase in $[Ca^{2+}]_i$ compared to cells treated with vehicle on day 4 ($p<0.001$) (Figure 5.3). Priming with ET-1 on day 0 then challenging with capsaicin causes significantly lower increase in $[Ca^{2+}]_i$ compared to cells treated with vehicle on day 0 then given capsaicin on day 4 ($p<0.001$) (Figure 5.3).

5.3.4 APJ receptor expression in rodent hindpaw skin and spinal cord

No significant differences in APJ receptor expression were found in the hindpaw skin between the normal, saline, or ET-1 treated P11 male rats; however, there was a trend for a decrease in APJ receptor expression in ET-1 treated rats (Figure 5.4 A). However in the spinal cord of male rats given ET-1, there was a significant increase in APJ receptor expression compared to male rats given saline of P7 ($p<0.05$) (Figure 5.4 B). No significant differences in APJ receptor expression in the spinal cord were found between female rats given ET-1 on P7 and female rats given saline on P7. This data suggests that APJ may play a sex dependent role in ET-1 induced priming.

5.3.5 Apelin-ET-1 interactions in afferent neurons

Cultured DRG neurons were used to examine whether apelin can modify ET-1-induced Ca^{2+} transients in afferent neurons. For the first paradigm, there were significantly greater increases in $[Ca^{2+}]_i$ in cells primed with vehicle or apelin alone then challenged with 0.55 μM of ET-1 compared to ET-1 primed cells challenged with ET-1 (Figure 5.5 A, C) and all vehicle challenged groups (Figure 5.5 B, C). There were no significant changes in fluorescence in vehicle challenged groups as expected (Figure 5.5 B, C). The time to peak was significantly greater in apelin primed cells challenged with ET-1 compared to unprimed and apelin primed cells challenged with vehicle (Figure 5.5 D). The maximum peak height was significantly greater in ET-1 challenged unprimed cells compared to all other treatment groups except unprimed vehicle challenged and apelin primed ET-1 challenged cells (Figure 5.5 E).

For the second paradigm, a challenge with 0.55 μM of ET-1or a combination of ET-1 and 2 nmoles of apelin in unprimed cells causes the greatest change in fluorescence compared to cells challenged with vehicle or apelin (Figure 5.6 A, C) and all ET-1 primed cells (Figure 5.6 B, C). There was a significantly greater AUC for ET-1 primed cells challenged with a combination of ET-1 and apelin compared to ET-1 primed cells that were challenged with ET-1 (Figure 5.6 C). Apelin causes a slower time to calcium peak in both primed and unprimed cells compared to cells challenged with vehicle or ET-1 (Figure 5.6 D). Unprimed cells challenged with either ET-1 alone or a combination of ET-1 and apelin had significantly greater maximum peak height compared to all other treatment groups except unprimed cells challenged with vehicle (Figure 5.6 E).

5.3.6 Skin release assay

The hindpaw skin isolated from male rats showed a significant decrease in release of apelin when the ET_B receptor agonist, IRL-1620, was applied compared to the time before and after the agonist was applied ($p<0.05$) (Figure 5.7). This decrease in apelin release was also significantly lower than the apelin levels measured in the hindpaw skin from female rats when the agonist was applied. No differences were found in apelin release between the periods before, during, and after application of the agonist for female rats.

5.4 Discussion

In this study, we examined the endothelin system in an *in vitro* model correlate of our animal model of localized acute VOEs as well as the apelin system in our animal model. We found that in our *in vitro* model in primary

afferent neurons from DRGs, ET-1 causes a large influx of Ca^{2+} in naïve cells, but a “desensitized” response in ET-1 primed cells. The ET-1 priming effect is dependent upon the timing of the priming dose as well as on the dose of the ET-1 challenge. Similarly, ET-1 priming desensitizes primary afferent neurons to subsequent challenge with capsaicin.

To begin to understand whether the apelin system may be involved in ET-1-induced changes in nociception and afferent neuron activation we examined expression of the apelin APJ receptor in our rodent model. We found sex-dependent changes in APJ expression in the spinal cord; however in the skin of male animals exposed to a priming dose of ET-1, no significant changes were seen. At the level of the DRG, apelin did not completely attenuate or potentiate ET-1 induced calcium transients in primed or unprimed cells suggesting that apelin does not have a direct effect on afferent neurons in modulating nociception; however it may have an impact on calcium channel opening as evident by its effect on time to peak for the calcium transients. In contrast, ET-1 induced release of apelin from the skin was sex-dependent with a decrease in apelin release following treatment with an ET_B receptor agonist in males, but not females. Overall, this data suggests that behavioral desensitization following priming with ET-1 may be at the level of the afferent neuron and that if apelin modulates this behavioral desensitization it may occur by modifying the skin microenvironment and the initial activation of the afferent neurons or potentially at the afferent neuron-spinal cord synapse.

The present calcium imaging results demonstrating ET-1 priming induced desensitization is supported by previous studies which showed that ET-1 causes an increase in $[Ca^{2+}]_i$ in neuronal cells, while repeated administration of ET-1 onto mouse neuroblastoma-rat DRG hybrid cells reduces $[Ca^{2+}]_i$ transients (Zhou et al., 2001; Yamamoto et al., 2006). These studies also found that the ET-1-induced increase in $[Ca^{2+}]_i$ is mediated by activation of the ET_A receptor (Zhou et al., 2001). However these previous studies also found that the increase in $[Ca^{2+}]_i$ comes from intracellular stores, while this present study found that this increase was due to the influx from extracellular Ca^{2+}. The difference in the source of $[Ca^{2+}]_i$ increases in our study and the aforementioned studies may be explained by the much larger dose of ET-1 used in our study to prime the cells as well as a potential species difference in DRG neurons.

Depending on the cell type and species investigated, ET-1-induced influx of extracellular Ca^{2+} can be due to direct or indirect regulation of voltage gated Ca^{2+} channels as well as non-selective cation channel and store-operated Ca^{2+} channel activation (Tykocki and Watts, 2010). In mammalian parasympathetic neurons, ET-1 causes an increase in $[Ca^{2+}]_I$ by activating receptor-operated Ca^{2+} channels (Nishimura et al., 1991). In many cell types, ET-1-induced Ca^{2+} mobilization is caused by a mixture of voltage-dependent Ca^{2+} influx, voltage-independent Ca^{2+} influx, and the release of Ca^{2+} from intracellular stores (Tykocki and Watts, 2010). One possible explanation for voltage-dependent Ca^{2+} influx due to ET-1 may be the effect of ET-1 on sodium channels. ET-1, through the ET_A receptor, lowers the threshold for activation of tetrodotoxin-resistant Na^+

channels in DRG neurons thereby increasing the excitability of the neurons (Zhou et al., 2002). In DRG neurons, ET-1 also enhances neuronal excitability by suppressing delayed-rectifier type of K^+ currents (Feng and Strichartz, 2009). An explanation for voltage-independent Ca^{2+} influx due to ET-1 may be its effects on the opening of cation channels. In rat glioma cells, the major source of ET-1-induced increase in $[Ca^{2+}]_I$ was found to be from an influx of extracellular Ca^{2+} possibly through the opening of non-selective cation channels such as TRPV1 channels (Lin et al., 1992).

Priming with ET-1 also desensitized the $[Ca^{2+}]_i$ response to capsaicin, which is the chemical found in chili peppers that produces a burning sensation when applied externally or ingested. Capsaicin activates the TRPV1 ion channel on DRG neurons, more specifically on small diameter neurons, which leads to an influx of Ca^{2+} (Caterina et al., 1997). It has been shown that application of capsaicin onto DRG neurons causes an increase in $[Ca^{2+}]_i$, while repeated applications of capsaicin leads to desensitized response of a reduction in $[Ca^{2+}]_i$ (Vellani et al., 2001; Yamamoto et al., 2006). Our result of a decreased response to capsaicin in ET-1 primed cells is somewhat in conflict with a previous study showing an enhancement of the increase in $[Ca^{2+}]_i$ after application of ET-1 followed by capsaicin (Yamamoto et al., 2006). However, this difference may be explained by the fact that the neurons used in our study were incubated with ET-1 for hours before being removed and challenged with capsaicin days later, while the previous published study applied ET-1 seconds

before adding capsaicin looking at an acute modulator role versus a long-term modulatory role.

In our animal model, priming with ET-1 appears to cause a decrease in APJ receptor expression in hindpaw skin of male rats, while in the spinal cord of male rats, ET-1 priming causes a significant increase in APJ receptor expression. The APJ receptor is a G-protein coupled receptor that couples to $G_{\alpha i/o}$ to inhibit the activity of adenylate cyclase (O'Carroll et al., 2013). When apelin is injected centrally into rodents, either intrathecally or intracerebroventricularly, it induces anti-nociception through APJ and opioid receptor activation in visceral and acute pain models (Lv et al., 2012). It is possible that long lasting changes induced by ET-1 priming cause changes in the expression of APJ receptors in the spinal cord due to compensatory mechanisms, which may influence the central modulatory role of apelin signaling in nociception. In isolated skin tissue, application of an ET_B receptor agonist causes a significant decrease in the release of apelin, which was only seen in male rats. In hindpaw skin of rats, activation of ET_B receptors causes anti-nociception by causing the release of endogenous opioids from keratinocytes, which are then thought to act on nociceptors to cause hyperpolarization (Khodorova et al., 2002; Khodorova et al., 2003).

The results of this study suggest that endothelin-induced sensitization upon repeated administration may not be occurring at the primary afferent level. Furthermore, priming with ET-1 produces a long lasting desensitization of afferent neurons to subsequent ET-1 and the alternative algogen capsaicin. This

suggests that behavioral sensitization to repeat ET-1 exposure may be occurring in the skin, in the spinal cord, or in the brain. Future studies are needed to begin to understand how the different levels of the pain neuroaxis respond to repeated exposure to ET-1. This preliminary analysis of the apelin system in ET-1 induced sensitization/desensitization suggests that in response to endothelin, there is a decrease in the peripheral apelin system and a concomitant increase in the central apelin system that is sex dependent. Further studies are needed to more fully understand whether apelin plays a significant role in acute ET-1-induced nociception or in the ET-1-induced priming effect.

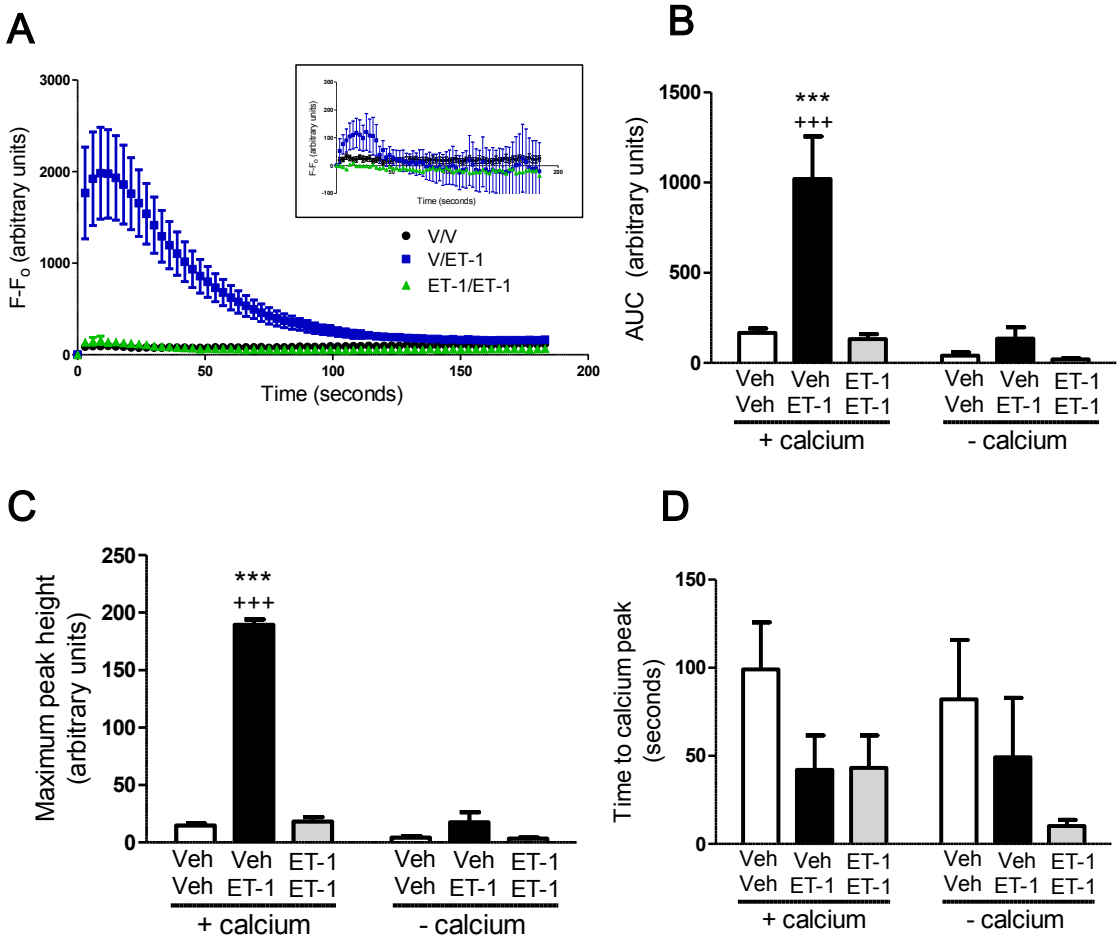

Figure 5.1 ET-1-induced increase in Ca^{2+} due to influx of Ca^{2+} not from release of intracellular stores and priming with ET-1 prevents ET-1-induced Ca^{2+} influx. Cells incubated in buffer containing Ca^{2+} exhibited a significant increase in intracellular Ca^{2+} upon treatment with ET-1 compared to cells treated with vehicle ($p<0.001$) when measuring the change in fluorescence **(A)**, the area under the curve **(B)**, and the maximum peak height **(C)**. Cells primed with ET-1 on day 0 then treated with ET-1 at the time of imaging show a significant decrease in Ca^{2+} influx compared to vehicle primed cells treated with ET-1 at the time of imaging ($p<0.001$). Cells incubated in Ca^{2+} buffer did not show an increase in intracellular Ca^{2+} upon treatment with ET-1 **(A inset)**.

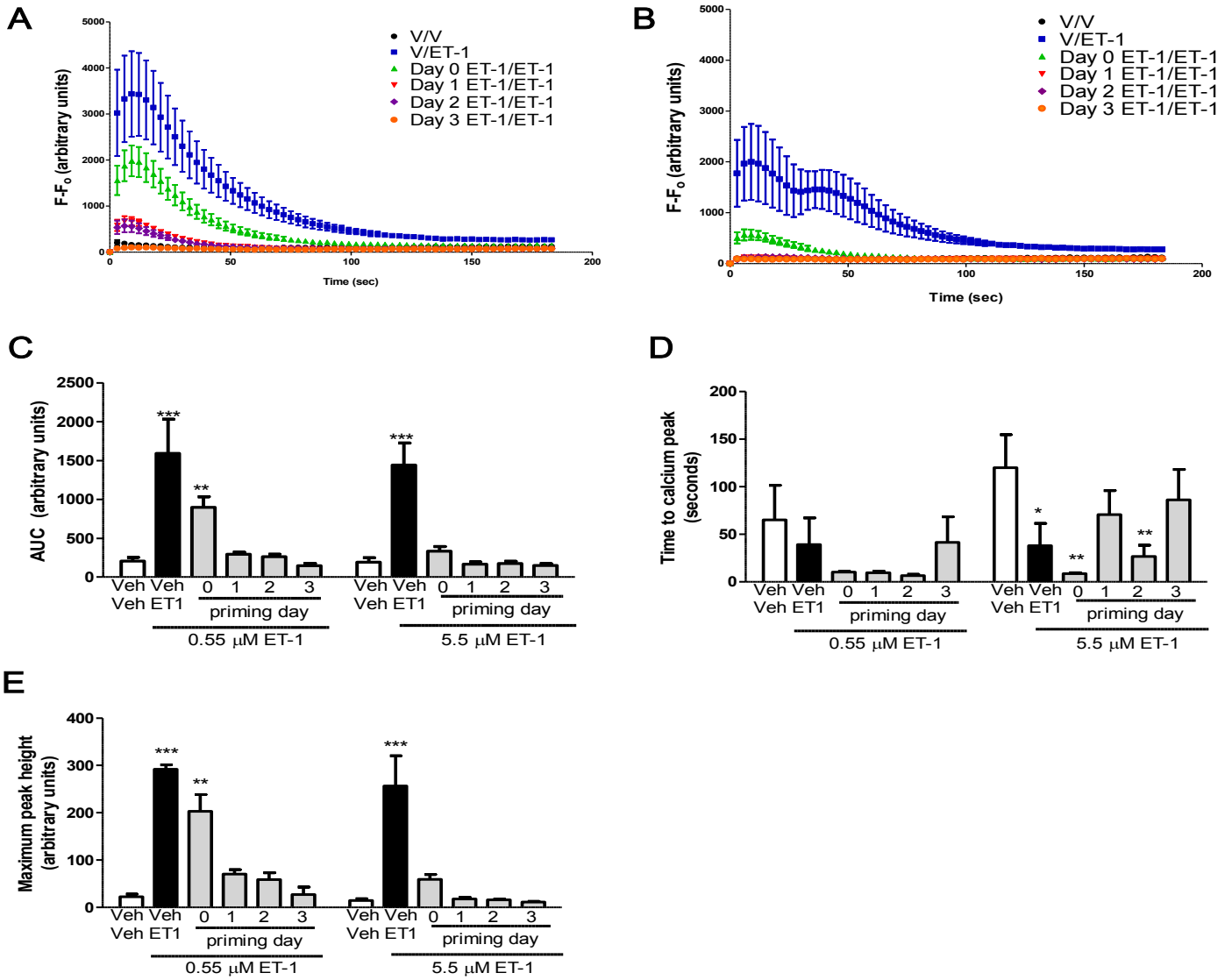
A
B
C
D
E
V/V
V/ET-1
Day 0 ET-1/ET-1
Day 1 ET-1/ET-1
Day 2 ET-1/ET-1
Day 3 ET-1/ET-1
F-F0 (arbitrary units)
Time (sec)
AUC (arbitrary units)
Time to calcium peak (seconds)
Maximum peak height (arbitrary units)
Veh
Veh ET1
0 1 2 3
priming day
0.55 μM ET-1
5.5 μM ET-1

Figure 5.2 Time course for prevention of ET-1-induced Ca^{2+} influx due to priming effect of ET-1. Reducing the priming dose of ET-1 to 2.2 μM reveals a particular time course for the decrease in $[Ca^{2+}]_i$. **(A, C)** Cells primed with 2.2 μM of ET-1 on day 0 and challenged with 0.55 μM of ET-1 on day 4 show a significantly smaller increase in $[Ca^{2+}]_i$ compared to cells treated with ET-1 only at the time of imaging (***$p<0.001$) and this level of increase in $[Ca^{2+}]_i$ is significantly different from vehicle treated cells (**$p<0.01$). **(B, C)** Cells primed with 2.2 μM of ET-1 on day 0 and challenged with 5.5 μM of ET-1 on day 4 show a significantly smaller increase in $[Ca^{2+}]_i$ compared to cells only treated with ET-1 at the time of imaging (***$p<0.001$) and this level of $[Ca^{2+}]_i$ is not significantly different from vehicle treated cells. **(D)** The time to calcium peak appeared to be more rapid for cells treated with ET-1 for the first time on the day of imaging with 5.5 μM ET-1 compared to cells challenged with vehicle (*$p<0.05$). This was also true for cells primed with ET-1 on days 0 and 2 and challenged with 5.5 μM ET-1 (**$p<0.01$). **(E)** Maximum peak height for unprimed ET-1 treated cells and cells primed on day 0 and challenged with 0.55 μM ET-1 had significantly greater maximum peak heights compared to unprimed vehicle treated cells (***$p<0.001$ and **$p<0.01$, respectively).

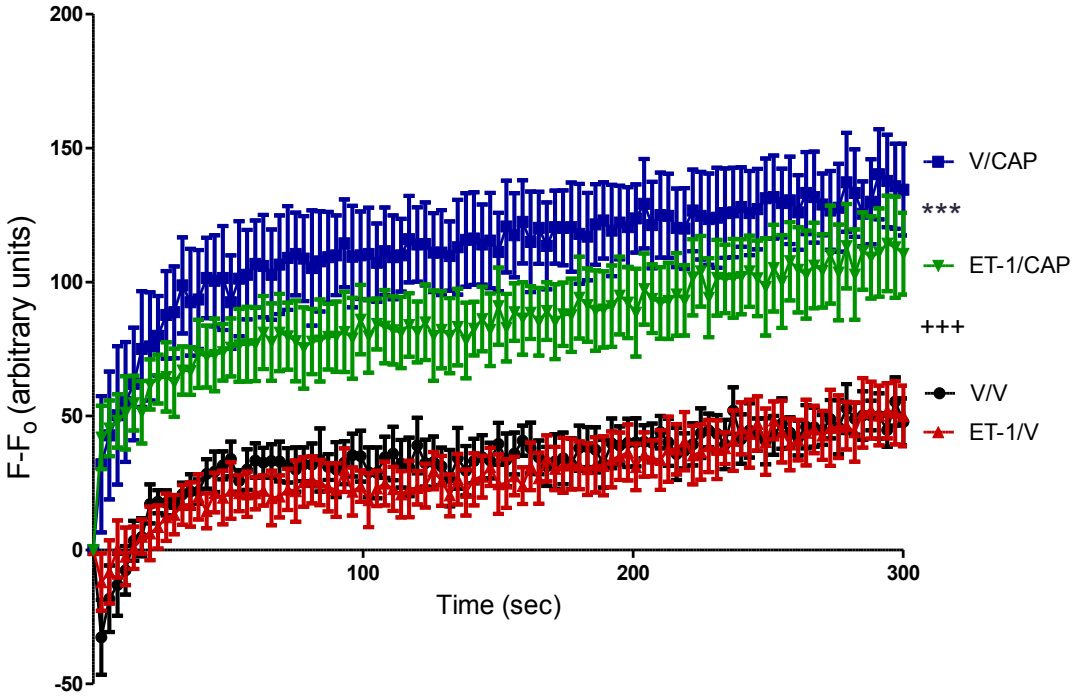

Figure 5.3 Pre-treatment with ET-1 causes decrease in capsaicin-induced influx of Ca^{2+}. Capsaicin caused a significant increase in $[Ca^{2+}]_i$ compared to cells treated with vehicle on day 4 (+++ $p<0.001$) There is a significantly greater difference from baseline in capsaicin treated cells primed with vehicle on day 0 compared to capsaicin treated cells primed with ET-1 on day 4 (***$p<0.001$). There was no significant difference between cells vehicle treated cells primed with vehicle on day 0 and vehicle treated cells primed with ET-1 on day 0.

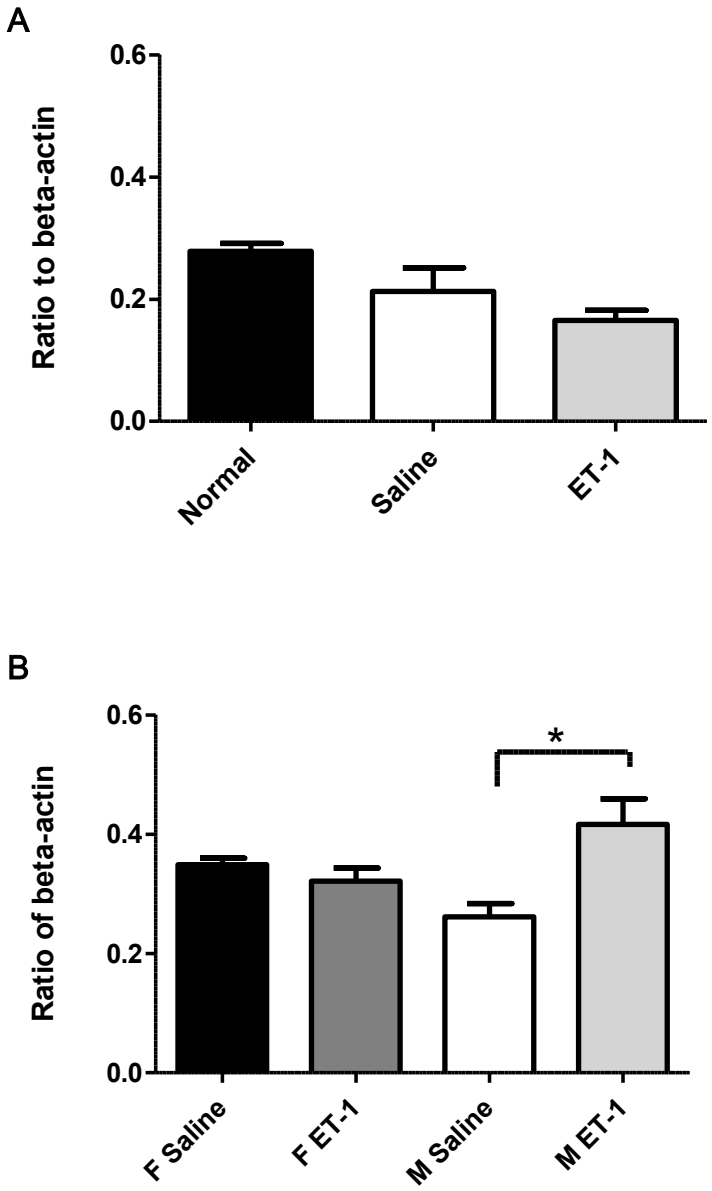

Figure 5.4 APJ receptor expression in hindpaw skin and spinal cord of postnatal day 11 rats. (A) No significant differences in APJ receptor expression in the male hindpaw were found between the normal (n = 2), saline treated (n = 9), and ET-1 treated rats (n = 13), but there appeared to be a slight trend for a decrease in APJ receptor expression in ET-1 treated rats. **(B)** There was a significant increase in APJ receptor expression in the spinal cord of male rats after treatment with ET-1 (n = 3) compared to saline treated (n = 2) rats (*$p<0.05$). There is no significant difference in APJ receptor expression in the spinal cord of saline (n = 4) and ET-1 treated (n = 5) female rats.

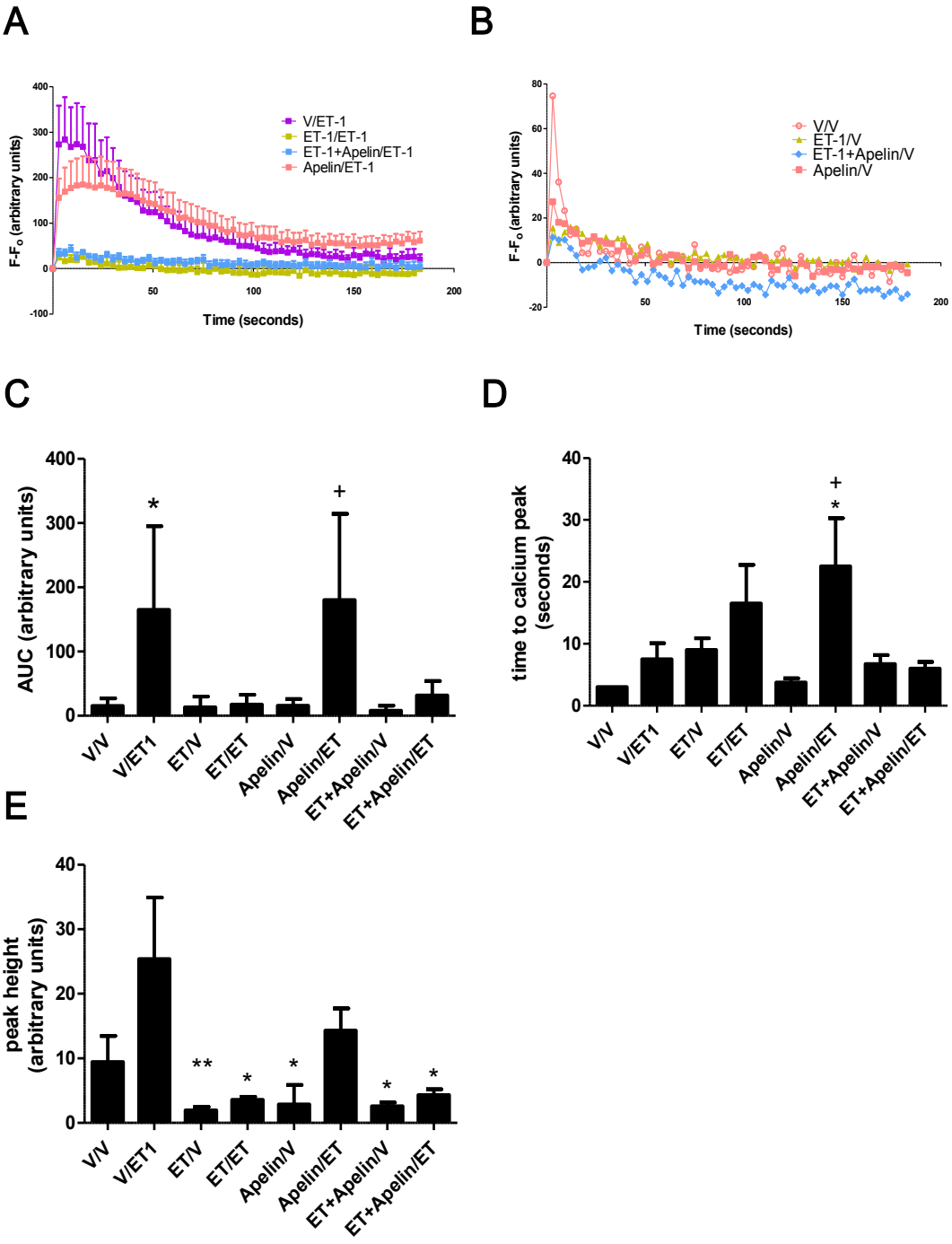
A
B
C
D
E
V/ET-1
ET-1/ET-1
ET-1+Apelin/ET-1
Apelin/ET-1
V/V
ET-1/V
ET-1+Apelin/V
Apelin/V
F-F0 (arbitrary units)
Time (seconds)
AUC (arbitrary units)
time to calcium peak (seconds)
peak height (arbitrary units)
V/V
V/ET1
ET/V
ET/ET
Apelin/V
Apelin/ET
ET+Apelin/V
ET+Apelin/ET

Figure 5.5 Effect of apelin on ET-1-induced changes in $[Ca^{2+}]_i$ (Paradigm 1). **(A)** ET-1 challenged cells. Cells primed with vehicle or apelin alone had the largest changes in fluorescence compared to the ET-1 or ET-1+apelin primed cells. **(B)** Vehicle challenged cells. There were no significant changes in fluorescence in these cells regardless of the priming peptide. **(C)** Area under curve for $F\text{-}F_o$ graphs. Vehicle primed cells challenged with ET-1 had significantly larger AUC compared to all other treatment groups except Apelin/ET and ET+apelin/ET treatment groups (*$p<0.05$ vs. V/ET). Apelin primed cells challenged with ET-1 had significantly larger AUC compared to all other groups except V/ET treated cells (+$p<0.05$ vs. Apelin/ET). **(D)** Time to calcium peak. Apelin primed cells challenged with ET-1 had significantly longer time to calcium peak compared to V/V (*$p<0.05$) and Apelin/V (+$p<0.05$) treatment groups. **(E)** Maximum peak height. ET-1 challenged unprimed cells had a significantly larger peak height compared to all other groups except V/V and Apelin/ET treated cells (**$p<0.01$, *$p<0.05$ vs. V/ET).

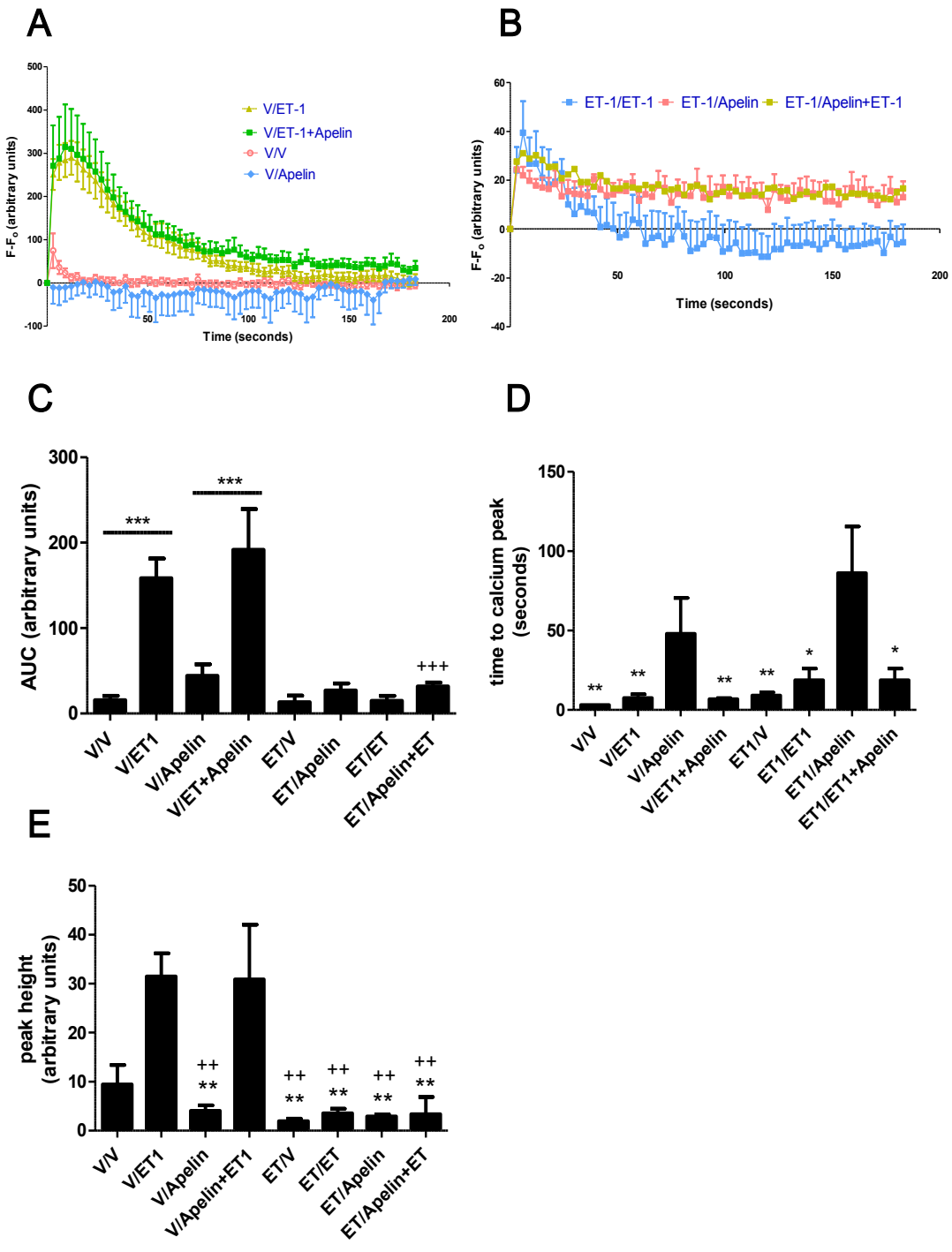
A
V/ET-1
V/ET-1+Apelin
V/V
V/Apelin
F-F0 (arbitrary units)
Time (seconds)
B
ET-1/ET-1
ET-1/Apelin
ET-1/Apelin+ET-1
F-F0 (arbitrary units)
Time (seconds)
C
AUC (arbitrary units)
V/V
V/ET1
V/Apelin
V/ET1+Apelin
ET/V
ET/Apelin
ET/ET
ET/Apelin+ET
D
time to calcium peak (seconds)
V/V
V/ET1
V/Apelin
V/ET1+Apelin
ET1/V
ET1/ET1
ET1/Apelin
ET1/ET1+Apelin
E
peak height (arbitrary units)
V/V
V/ET1
V/Apelin
V/Apelin+ET1
ET/V
ET/ET
ET/Apelin
ET/Apelin+ET

Figure 5.6 Effect of apelin on ET-1-induced changes in calcium imaging (Paradigm 2). (A) Unprimed cells. ET-1 causes the greatest change in fluorescence. Apelin does not cause an increase in $[Ca^{2+}]_i$ and it does not enhance ET-1-induced increases in $[Ca^{2+}]_i$. **(B)** ET-1 primed cells. ET-1 causes a "desensitized" response in ET-1-primed cells. The combination of apelin with ET-1 causes less of a desensitized response in these cells. **(C)** Area under curve for both primed and unprimed cells. ET-1 and ET-1+apelin treated unprimed cells have significantly greater AUC compared to vehicle treated cells (***p<0.001). There was a significantly smaller AUC for the ET-1 treated ET-1 primed cells compared to the apelin+ET-1 treated ET-1 primed cells (+++p<0.001). **(D)** Time to calcium peak for primed and unprimed cells. Apelin only treated primed cells have a significantly slower time to calcium peak compared to cells treated with ET-1 or vehicle (**p<0.01, *p<0.05 vs. ET1/Apelin). **(E)** Maximum peak height for primed and unprimed cells. The unprimed cells treated with ET-1 or a combination of ET-1 and apelin had a significantly greater peak height compared to V/apelin (**p<0.01 vs. V/ET; ++p<0.01 vs. V/Apelin+ET). The peak height for these cells was also significantly greater than all of the ET-1 primed treatment groups.

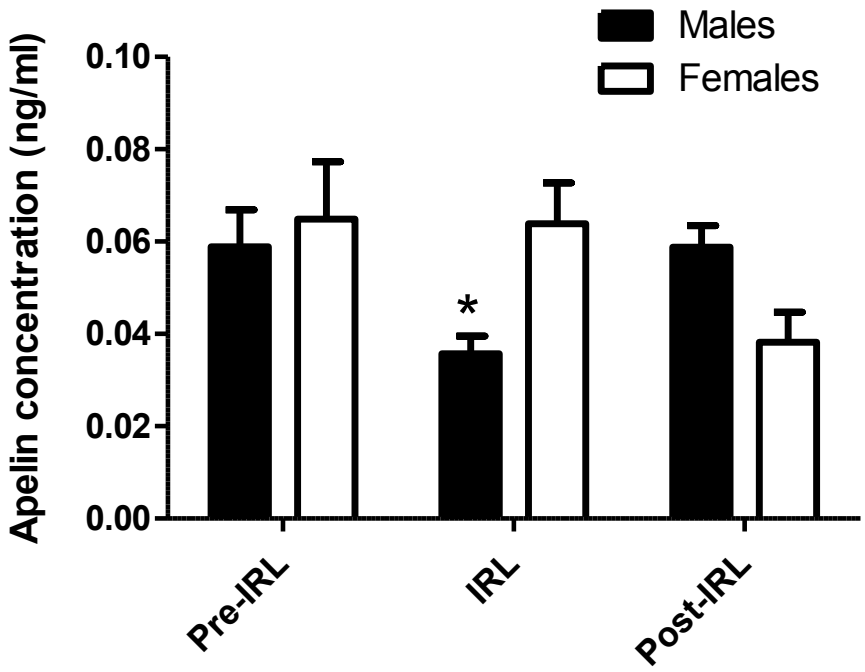

Figure 5.7 Release of apelin after activation of ET_B receptor in hindpaw skin of male and female rats. In the hindpaw skin of male rats, activation of the ET_B receptor using the agonist IRL-1620 (IRL) leads to a significant decrease in the release of apelin compared to the time before and after the application of the agonist (*$p<0.05$). There was also a significant difference between the apelin levels after agonist application in the males and females (*$p<0.05$). There were no significant differences in the release of apelin between the treatment time points for the female rats.

CHAPTER 6:

Exploring Apelin Receptor Single Nucleotide Polymorphisms in Children with SCD

6.1 Introduction

Although the apelin system has not been directly linked to the complications of SCD, it has been examined in other pathologies such as cardiovascular disease, stroke, and pulmonary hypertension (Hata et al., 2007; Sarzani et al., 2007; Falcao-Pires et al., 2009; Jin et al., 2012). Several single nucleotide polymorphisms (SNPs) have been identified for the APJ receptor gene, *APLNR*, and some of these SNPs have been studied for associations with some of the aforementioned pathologies. One study found an association for a SNP (rs9943582) found in the 5'-flanking region and the risk for brain infarction in a Japanese population (Hata et al., 2007). Associations have also been found between two SNPs found in the 3'-untranslated region (3'-UTR) (rs746886 and rs2282623) and blood pressure response to low-sodium intervention in a Chinese population (Zhao et al., 2010a). Using these previous studies, we decided to focus on these particular SNPs of the *APLNR* gene to carry out an association study focusing on pain measures in SCD. We chose these particular SNPs because either they had clinical relevance to SCD (brain infarction) and/or they had a high minor allele frequency in their respective populations.

To examine the apelin system separately in SCD patients, the following study explored the associations between the SNPs, rs9943582, rs746886, and rs2282623 of the *APLNR* gene and different pain measures in a pediatric population of SCD patients. In addition to the different pain measures, associations between HbF and hematocrit and the different SNPs were also explored.

6.2 Methods

6.2.1 Patient medical history and sample collection

Saliva samples were obtained from a total of 76 children with SCD recruited through the Children's Center for Cancer & Blood Disorders at Palmetto Richland Health Children's Hospital in Columbia, SC. Children were excluded from the study if they had major developmental disorders or neurologic diseases that would result in severe limitations of cognitive ability. Children were also excluded if they were experiencing pain that required opioid usage on the day of sample collection. Children undergoing transfusion therapy were also excluded. Demographic as well as pain and disease information was obtained for each participant. The pain history interview consisted of recent pain status (pain frequency over the previous year, duration in days, and intensity on a scale of 1 to 10) and health care utilization (the number of emergency room, hospitalizations, or other health care contacts within the last year for patient report and past two years for the medical records) was conducted for the child and also recorded from the medical records. A review of the medical records was also used to obtain the most recent lab results for HbF and hematocrit.

6.2.2 DNA extraction

DNAzol was added to each sample after collection for preservation and stored at room temperature until ready for DNA extraction. Samples were incubated with Proteinase K (20 mg/mL) for at least 12 hours at room temperature before extraction. After incubation with Proteinase K, the mixture was transferred to a microfuge tube and centrifuged at 13,000 rpm for 15 minutes

at room temperature. The supernatant was collected and 100% ethanol was added, then the mixture was gently mixed. Samples were allowed to stand at room temperature for 10 minutes, and then they were centrifuged at 13,000 rpm for 3.5 minutes. The pellet was collected and 70% ethanol was carefully added to the pellet, then the supernatant was removed and discarded. Deionized water was added to the DNA, mixed, then incubated at room temperature for one day, then stored at -20°C until PCR amplification.

6.2.3 Polymerase chain reaction

The National Center for Biotechnology Information (NCBI) SNP database was used to obtain sequencing information for the SNPs found in the 5'-flanking and 3'-UTR regions of the *APLNR* gene. The primer sequences (19-20 bases) were chosen based on the average GC content and the specific nucleotide compositions. The final primer sequences were chosen after analysis for T_m, self-dimerization, hetero-dimerization, and hairpin formation using OligoAnalyzer software (Integrated DNA Technologies). Final primers were purchased from Eurofins MWG Operon. Since the SNPs in the 3'-UTR region were within no more than 285 bases of each other, one primer set was designed to amplify multiple SNPs within this region (Table 6.1). The primers designed for the 3'-untranslated region were originally designed for two SNPs (rs746886 and rs2282623); two additional SNPs (rs2282624 and rs2282625) were found in these amplified sequences.

Each amplification reaction of the SNP in the 5'-flanking region contained 0.2 µM of each primer, 2.5 mM of each dNTP, 25 mM or 37.5 mM of $MgCl_2$, 1U

Taq DNA polymerase, 2.5 µL of ThermoPol buffer (New England Biolabs), 10 mg/mL bovine serum albumin (BSA), and 1, 2, or 3 µL of genomic DNA in 25, 26, or 27 µL reaction volumes. The PCR amplification conditions consisted of an initial denaturation step of 2 minutes at 94°C followed by 37 cycles of 94°C for 15 seconds, annealing at 53°C for 25 seconds and extension at 72°C for 30 seconds. The final extension was at 72°C for 1 minute. PCR products were run on a 0.8% agarose gel to confirm amplification, then stored at 4°C until sequencing.

Each amplification reaction for the 3'-UTR region contained 0.2 µM of each primer, 2.5 mM of each dNTP, 25 mM or 12.5 mM of $MgCl_2$, 1U of Taq DNA polymerase, 2.5 µL of ThermoPol buffer, 10 mg/mL BSA, and 1, 2, or 3 µL of genomic DNA in 25, 26, or 27 reaction volumes. The amplification was performed at 94°C for 2 minutes followed by 40 or 43 cycles of 94°C for 15 seconds, 53°C for 25 seconds, and 72°C for 30 seconds. The final extension was set at 72°C for 1 minute.

6.2.4 DNA sequencing

The unpurified PCR products of both the 5'-flanking and 3'-UTR regions were sent to High Throughput Genomics Center for Sanger sequencing. Sequencher 5.0 (Gene Codes Corporation) or DNA Baser Sequence Assembly Software (Heracle BioSoft S.R.L.) was used to determine genotypes from sequences.

6.2.5 Data analysis

GraphPad Prism was used for all statistical analyses. Chi-square tests were used to calculate Hardy-Weinberg equilibrium for each SNP. A dominant model of inheritance, which compares individuals with at least one copy of an allele to individuals who are homozygous for the other allele, was used to evaluate the associations between the SNPs and the different pain measures and a t-test was used to calculate statistical significance. A t-test was also used to determine significance for the associations between HbF and the over-dominant model, which compares heterozygous individuals to a combined pool of homozygous individuals. A one-way ANOVA followed by a Bonferroni post-hoc test was used to determine statistical significance between the different genotypes and the pain measures and hematocrit. A Fisher's exact test was used to determine statistical significance for the associations between ACS and the dominant model.

6.3 Results

In our participant population, the rs7446886 and rs2282623 SNPs only had a minor allele frequency of 0.0547, so association for these SNPs were not pursued. The following results focus on the rs9943582 SNP found in the 5'-flanking region and two additional SNPs found in the 3'UTR region (rs2282624 and rs2282625). The demographics for these SNPs can be found in Table 6.2. These three remaining SNPs were calculated to be in Hardy-Weinberg equilibrium (rs9943582, $p = 0.9809$; rs2282624, $p = 1.0000$; rs2282625, $p = 0.5088$).

6.3.1 Associations for the SNPs found in 3'-UTR (rs2282624 and rs2282625)

No associations were found between the genotypes of SNPs found in the 3'-UTR of the *APLNR* gene and the different pain measures (Figure 6.1). There were also no significant associations found when using a dominant allele model for the SNPs (Figure 6.2). No significant associations were found between the presence of at least one copy of the T or A allele and either measure of health care utilization (Figure 6.3). However, there was a trend for an association between having at least one copy of the A allele and participant report of health care utilization for the rs2282625 SNP where participants with at least one copy of the A allele had fewer health care contact than participants who were homozygous G ($p=0.0852$) (Figure 6.3 C).

No significant associations were found between the SNPs and the history of ACS. However, there was a strong trend for an association between having at least on copy of the T allele for the rs2282624 SNP and ACS, where a lower percentage of participants with at least one copy of this allele having a history of ACS ($p=0.0617$) (Figure 6.4).

No significant associations were found between hematocrit levels and these SNPs (data not shown). For both SNPs, there was a trend for a decrease in HbF levels in the heterozygous individuals (Figure 6.5 A, C). When an over-dominant model was applied, there were significant lower levels of HbF in heterozygous individuals compared to homozygous individuals for the rs2282624 SNP ($p<0.05$) (Figure 6.5 B). No significant association was found for the rs2282625 SNP when this model was applied (Figure 6.5 D).

6.3.2 Associations for the SNP in 5'-flanking region (rs9943582)

Similar to the previously mentioned SNPs, the SNP found in the 5'-flanking region of the *APLNR* gene did not have any associations with any of the pain measures using either the three genotypes or the dominant allele model (Figure 6.6). There was a trend for less health care utilization according to participant report in homozygous individuals for this SNP (p=0.0993) (Figure 6.7 A). This trend was weaker for the health care utilization according to the medical records (p=0.1576) (Figure 6.7 B).

No significant associations were found between this SNP and the history of ACS (data not shown). There were also no significant associations for the levels of hematocrit (data not shown). There was, however, a trend for an association between HbF levels and this SNP with heterozygous individuals displaying lower levels of HbF (Figure 6.8 A) and this trend became stronger when an over-dominant model of inheritance was applied (p=0.0557) (Figure 6.8 B).

6.4 Discussion

The results of this study show that while none of the SNPs were significantly associated with the direct pain measures of pain intensity, frequency, and duration, they seemed to be associated with indirect measures of pain correlates such as HbF levels and health care utilization.

The APJ receptor gene is found on chromosome 11 in humans and encodes a 380-amino acid protein that shares close homology to the Ang II receptor type 1_a (O'Dowd et al., 1993). Both the vasodilator effect and anti-

nociceptive effect come from, at least in part, by the activation of the APJ receptor (Ishida et al., 2004). The rs9943582 SNP located in the 5'-flanking region of the gene has been associated with brain infarction in a Japanese population (Hata et al., 2007). This group found that the G allele (the C allele in the forward direction) exhibited a higher binding affinity to the Sp1 transcription factor and patients who were homozygous for the G allele had an increased incidence of brain infarction compared to patient with at least one copy of the A (or T) allele. They also found that the *APLNR* gene is likely regulated by Sp1 and postulate that individuals with the G allele have a higher activation of the apelin system through the binding of Sp1 to the gene. The Sp1 transcription factor is a DNA binding protein that can either negatively of positively affect DNA binding and transcription when phosphorylated (Tan and Khachigian, 2009). For the rat APJ receptor, which shares 90% homology with the human receptor, the Sp1 motif in the 5'-flanking region of the gene has been indicated as an important regulator of the promoter (O'Carroll et al., 2013).

Specifically with the rs2282624 SNP found in the 3'-UTR, there was a strong trend for an association with ACS where participants with at least one copy of the T allele seemed to be more protected from having ACS compared to participants who were homozygous for the C allele. The 3'-UTR of a gene can play an important role in regulating gene expression and this area of the gene has been found to be rich in SNPs (Chen et al., 2006). Even though this SNP is not located in a protein coding area of the gene, it may affect the transcription of

the APJ receptor in the lungs, thereby having an effect on the development of ACS.

There was also a significant association for the rs2282624 SNP and HbF levels where heterozygous individuals exhibited lower levels of HbF suggesting that the heterozygotes are more at a disadvantage than the homozygotes in regards to risk factors associated with VOEs. This association was also seen as a strong trend for the 5'-flanking region SNP (rs9943582) and as a weaker trend in the other SNP found in the 3'-UTR (rs2282625). In children, low levels of HbF have been associated with an increased risk of ACS, dactylitis, which is painful swelling of the hands and feet, and painful crisis, while those with higher levels are at a significantly lower risk for these complications (Bailey et al., 1992). It is interesting to note that although these SNPs are located on different regions of the gene and do not appear to be linked together, they all display an over-dominant model of inheritance for this association.

Participant report of the frequency of health care utilization within the past 2 years had somewhat strong trends for association with the rs2282625 SNP where individuals with at least one copy of the A allele exhibited a lower frequency of health care contacts. This trend was also seen for the individuals who were homozygous for the C allele of the rs9943582 SNP. Health care utilization for patients with SCD is highest among 18-30 year olds, with an average 3.61 health care contacts per patient year, compared to those between the ages of 31 and 45 (Brousseau et al., 2010). It has been shown that average pain intensity is predictive of health care utilization in SCD patients with higher

ratings of pain intensity relating to higher health care utilization (Ezenwa et al., 2014). Being able to predict which children will be at an increased risk for having greater health care utilization would be of benefit in developing pain management plans for when these children leave pediatric care, thereby reducing the high costs of hospitalizations associated with this patient population.

One important limitation of this association study is the small sample size. With a larger sample size, stronger and significant associations may have been discovered for the three SNPs and health care utilization and HbF levels. This study would have to be replicated in order to further interpret these results; however, these preliminary results suggest that polymorphisms in the *APLNR* gene may be associated with health care utilization and HbF levels in children with SCD.

Table 6.1 Forward and reverse primers for the SNPs found in the 5'-flanking and 3'-untranslated region of the *APLNR* gene.

Region	SNP	Polymorphism	Primer Name	Primer Sequence	PCR Product
5' flanking region	rs9943582	C→T	APJR-Forward	GGCTGAACATTATCTGTGGT	279 bp
			APJR-Reverse	CCATCCTGCGAAATCTTACA	
3'-UTR	rs746886	G→A	APJR-3UTR-Forward	TAGACATCATGCTATCTGC	718 bp
	rs2282623	C→T	APJR-3UTR-Reverse	CTTACCCCATCATACTGAT	
	rs2282624				
	rs2282625	G→A			

Table 6.2 Demographics for rs2282624, rs2282625, and rs9943582 SNPs. **(A)** SNPs (rs2282624 and rs2282625) in the 3'-untranslated region. **(B)** SNP (rs9943582) in the 5'-flanking region of the *APLNR* gene.

A

Characteristics	N=63
Age (M, SD)	14.0, 3.3
Gender (n)	
Male	37
Female	26
SCD Phenotype (n)	
HbSS	39
HbSC	16
HbSb+	4
HbSb°	4

B

Characteristics	N=60
Age (M, SD)	14.3, 3.3
Gender (n)	
Male	35
Female	25
SCD Phenotype (n)	
HbSS	38
HbSC	15
HbSb+	4
HbSb°	3

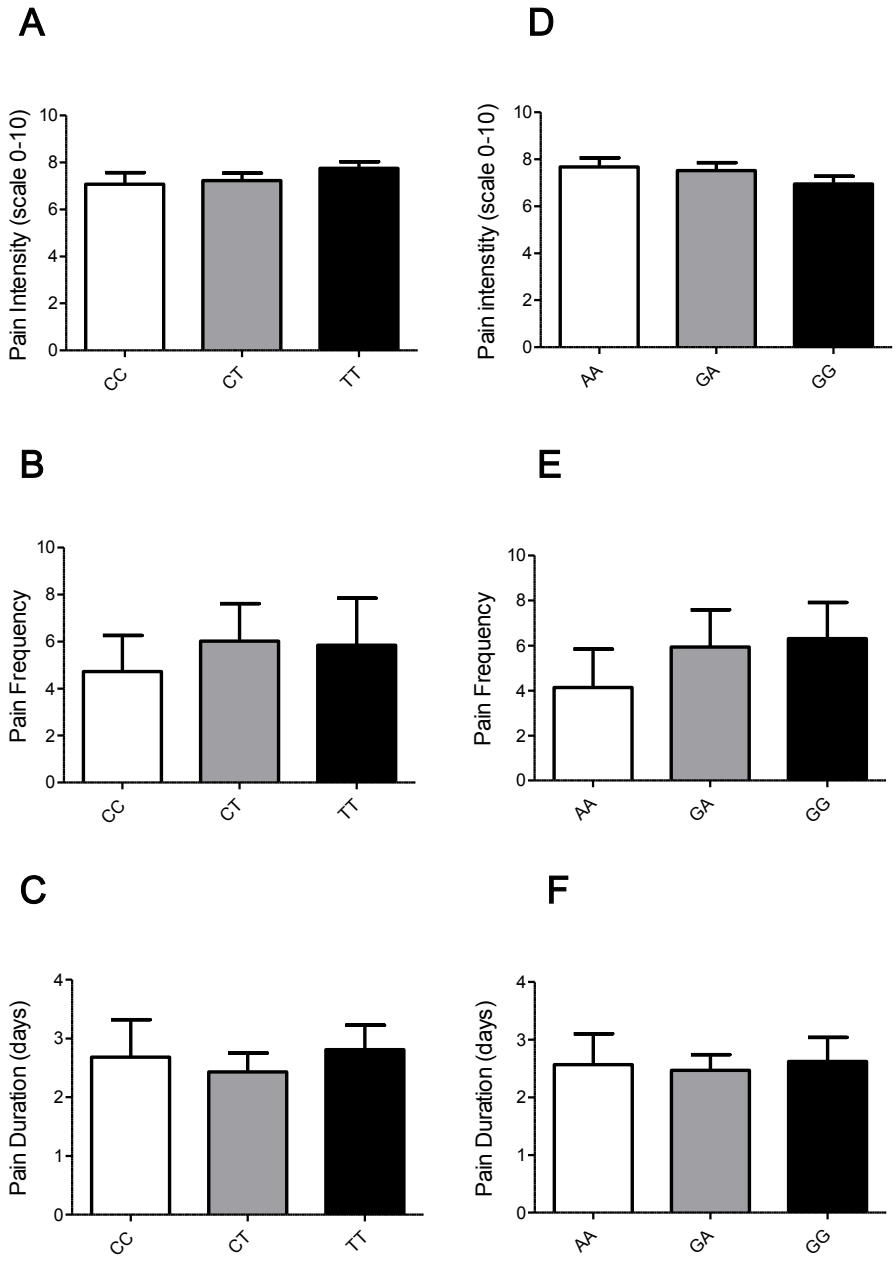

Figure 6.1 Associations between pain measures and genotypes for 3'-UTR SNPs (rs2282624 and rs2282625). No associations were found between pain intensity **(A)**, frequency **(B)**, and duration **(C)** and the CC, CT, and TT genotypes for the rs2282624 SNPs. There were also no associations between pain intensity **(D)**, frequency **(E)**, and duration **(F)** for the rs2282625 SNP.

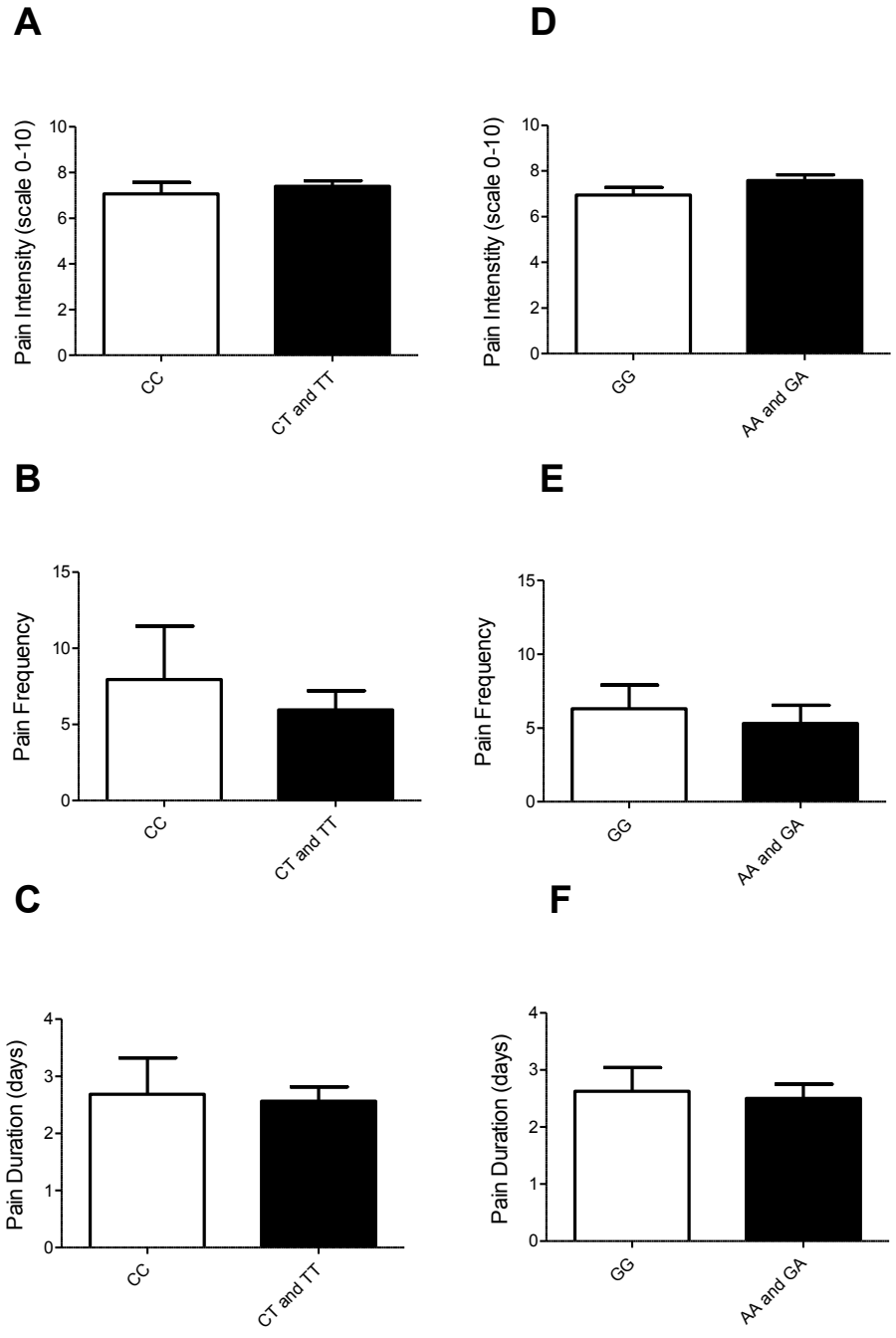

Figure 6.2 Dominant T/A model for 3'-UTR SNPs and different pain measures. No associations were found for pain intensity **(A)**, frequency **(B)**, and duration **(C)** and the different genotypes using the dominant T model for the rs2282624 SNP. No associations were for pain intensity **(D)**, frequency **(E)**, and duration **(F)** and the genotypes using the dominant A model for the rs2282625 SNP.

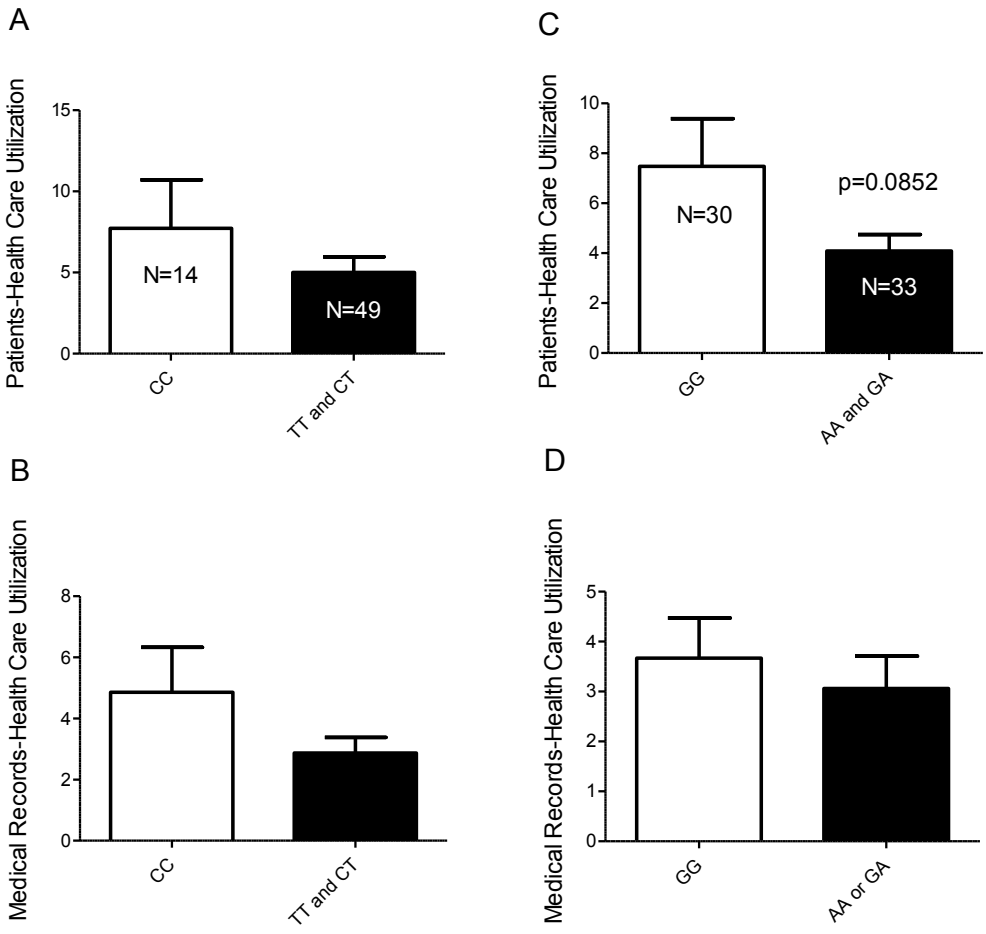

Figure 6.3 Associations for health care utilization (patient report and medical records) and 3'UTR SNPs. No associations were found for health care utilization either for **(A)** patient report or **(B)** medical records for the rs2282624 SNP. There was a trend for an association between patient report of **(C)** health care contacts over previous 2 years and the presence of at least one copy of the A allele for the rs2282625 SNP (p = 0.0852). No significant associations were found for this allele and the number of health care contacts from the medical records **(D)**.

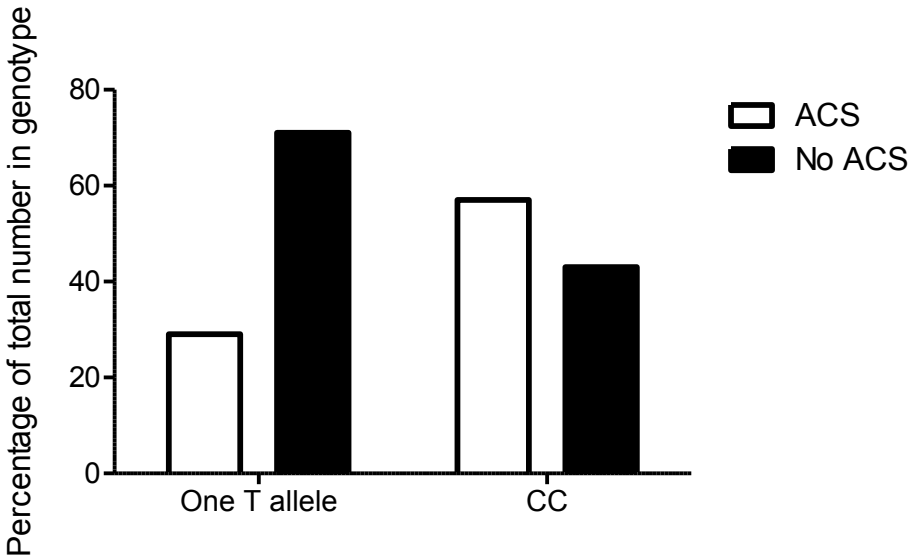

Figure 6.4 Association between history of ACS and the presence of the T allele for the rs2282624 SNP. There was a strong trend for an association between the history of ACS and the genotypes for the rs2282624 SNP where having the at least one copy of the T allele appeared to be associated with not having a history of ACS (p=0.0617).

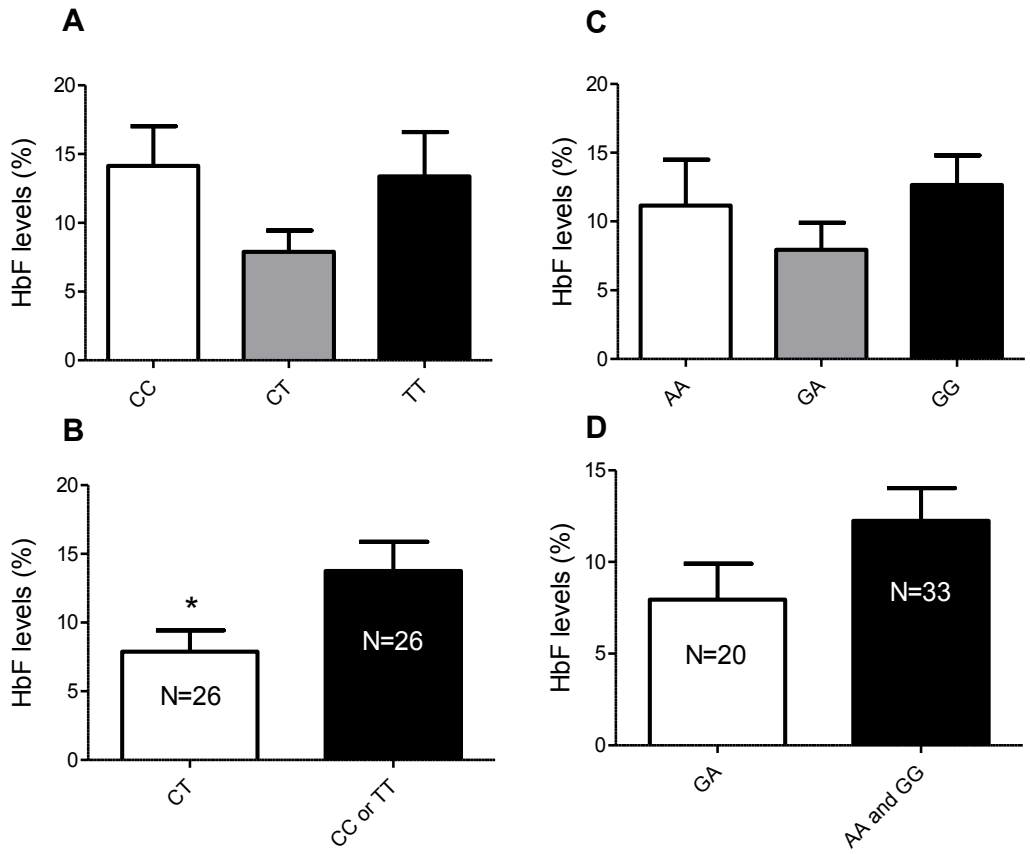

Figure 6.5 Associations between HbF levels and the genotypes or the over-dominant model for the rs2282624 and rs2282625 SNPs. (A) No significant differences were found between the different genotypes for the rs2282624 SNP, but there was a trend for lower levels of HbF in participants with the CT genotype ($p=0.0962$). **(B)** When an over-dominant model was employed, there was a significant difference between the heterozygous participants and the homozygous participants with the heterozygous individuals having lower HbF levels for the rs2282624 SNP ($*p<0.05$). No significant associations were found between the genotypes and HbF levels for the rs2282625 SNP **(C)**. This was true even when an over-dominant model was employed **(D)**.

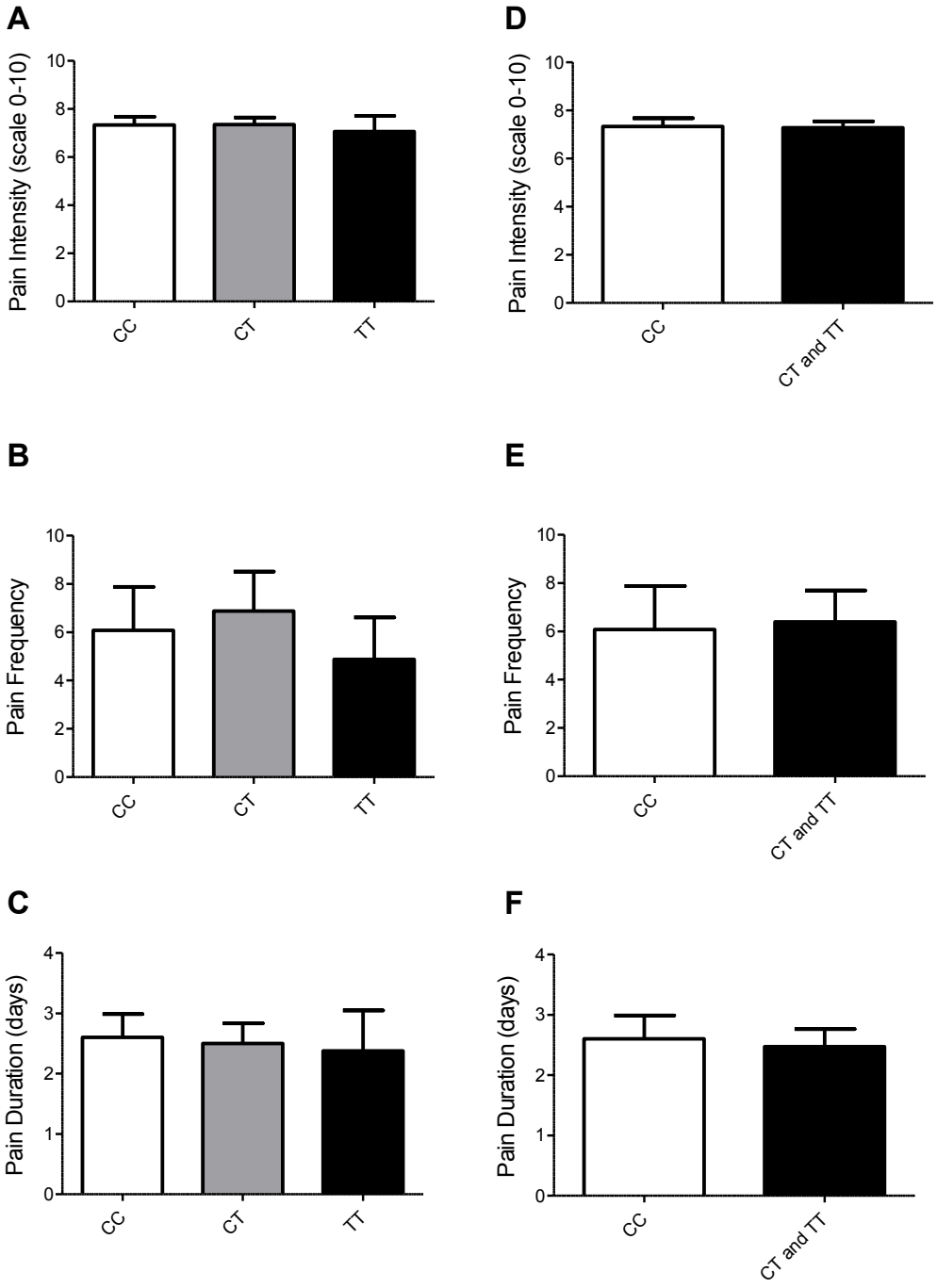

Figure 6.6 Associations between genotypes or dominant model for rs9943582 SNP and pain measures. No significant associations were found for either of the pain measures, intensity **(A)**, frequency **(B)**, and duration **(C)** and the genotypes. No significant associations were found for the pain measures, intensity **(D)**, frequency **(E)**, and duration **(F)** and the dominant T model.

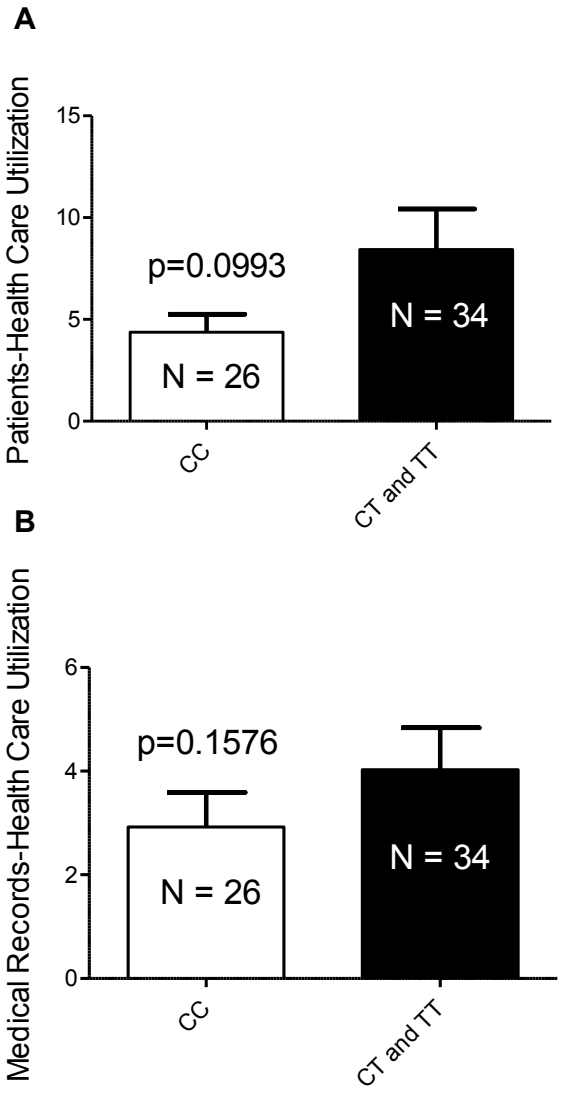

Figure 6.7 Associations of health care utilization and dominant T allele model for rs9943582 SNP. Although there were no statistically significant associations between health care utilization and the genotypes for the rs9943582 SNP in the 5'-flanking region, there was a trend for and association for homozygous CC participants having lower health care contact per the report of the participants **(A)** compared to the participants with at least one copy of the T allele (p = 0.0993). There was less of a trend for this association using the medical records of health care utilization **(B)** (p = 0.1576).

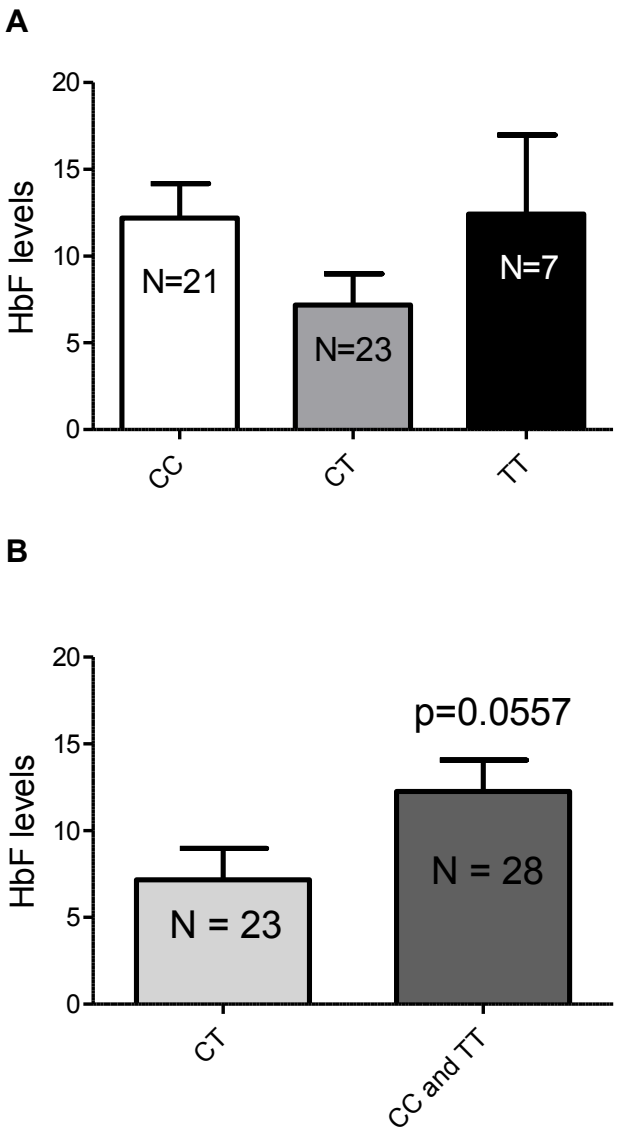

Figure 6.8 Associations for HbF levels and genotype or over-dominant model for rs9943582 SNP. (A) There was a trend for lower levels of HbF in heterozygous individuals compared to homozygous individuals. **(B)** When an over-dominant model was applied, this association became stronger (p=0.0557).

CHAPTER 7:

Conclusions

7.1 Summary of findings

7.1.1 Specific Aim 1

Children with sickle cell disease, a genetic condition characterized by changes in hemoglobin which leads to sickling of red blood cells, have frequent painful vaso-occlusive episodes which begin early in life and reoccur often across the lifespan of the individual. A major player in these painful vaso-occlusive episodes are the balance between vaso-constrictive and vaso-dilatory molecules. Endothelin-1 is a major vasoconstrictor that has been shown to be released during vaso-occlusive episodes and correlated with pain history. Apelin is a recently identified vasodilatory peptide that also has a modulatory role in pain processing. The goal of this specific aim was to determine the impact of the balance between vasoconstrictors (ET-1 and Ang II) and vasodilators (apelin) on the pain associated with SCD. We hypothesized that the ratio between vasodilatory (apelin) and vasoconstrictive (ET-1) tone in the vasculature of children with SCD may be a marker of pain sensitization and vaso-occlusion and that this relationship would be specific for the ratio between apelin and ET-1. Plasma endothelin, Ang II and apelin levels were measured following venipuncture at routine health visits in children with SCD (ages 2 to 18). Procedural pain was assessed via child- and caregiver-reports and observational distress. Pain history was assessed using retrospective chart review. The results of these studies suggest that an imbalance between apelin and endothelin systems may be involved in underlying baseline pain and the frequency of VOEs. In contrast, Ang II seems to be correlated with acute

procedural pain. Additional research is needed to understand the role of apelin in SCD associated sensitization and Ang II in acute procedural pain in children with SCD.

7.1.2 Specific Aim 2

Endothelin-1 is a known algogen that causes acute pain and sensitization in humans and spontaneous nociceptive behaviors when injected into the periphery in rats, and is elevated during VOEs in SCD patients. The first study of this specific aim sought to examine the effect of ET-1 exposure in the neonatal period on subsequent contralateral capsaicin-induced secondary mechanical hyperalgesia. ET-1 or saline was injected into the left plantar hindpaw on postnatal day 7 (P7). On postnatal day 11 (P11), capsaicin cream or control lotion was applied to the right dorsum hind paw and mechanical paw withdrawal thresholds were measured in the plantar hind paw. In saline control males, P11 administration of capsaicin produced a secondary mechanical hyperalgesia that was still present at 2 hrs. Neonatal priming with ET-1 did not alter the magnitude or the duration of secondary mechanical hyperalgesia in males. In contrast, in control females, P11 administration of capsaicin produced less than 40 minutes of mechanical hyperalgesia. Neonatal priming with ET-1 prolonged the duration of secondary mechanical hyperalgesia in females. Priming with ET-1 on P7 led to a significant increase in capsaicin-induced Fos expression in the dorsal horn of the spinal cord in both males and females compared to controls ($p<0.001$) . These findings further suggest that pain in early life may alter future responses to painful stimuli at both the behavioral and neuronal level. It also suggest that

exposure to ET-1 early in life may alter the processing of future painful insults in pathologies that exhibit increased levels of ET-1 such as VOEs experienced by SCD patients.

Previously, our lab has shown that repeated administration of ET-1 results in a sex-dependent priming effect, where males display a sensitizing effect to a repeat exposure to ET-1 while females display a desensitized effect (McKelvy and Sweitzer, 2009). The second part of this specific aim focused on determining if the sensitizing/desensitizing effect of ET-1 occurs at the level of the primary afferent neuron. Calcium imaging using DRG neurons was utilized to examine the effects of ET-1 on primary afferent neurons. DRG neurons were isolated from adult male rats and kept in culture for four days to mimic the timeline (P7-P11) of our *in vivo* model. On the day of culture, cells were primed with ET-1 or vehicle, and then on the day of imaging, cells were challenged with ET-1, capsaicin, or vehicle. We found that ET-1 causes a large increase in $[Ca^{2+}]_i$ in unprimed cells; however, this effect is not seen in ET-1 primed cells challenged with ET-1. This result suggests that at the level of the primary afferent neuron, ET-1 priming has a desensitizing effect on subsequent exposures to ET-1. Priming with ET-1 also causes a desensitized response to capsaicin compared to unprimed cells challenged with capsaicin, suggesting that this priming effect of ET-1 can also extend to other algogens. This result also lends further evidence to the idea that the enhancing effect of ET-1 priming on capsaicin-induced secondary hyperalgesia seen in our animal model is likely occurring at the level of the spinal cord.

The last part of this specific aim sought to determine the effect of endothelin system activation on the apelin system in our *in vivo* as well as our *in vitro* model. Western blot analysis was used to measure the expression of the APJ receptor in our rodent model of an acute VOE in the hindpaw using ET-1. Male animals that had been administered ET-1 showed a decrease, although not statistically significant, in APJ receptor expression in the hindpaw, while APJ receptor expression increased in the spinal cord of ET-1 treated male animals. Activation of the endothelin system using an ET_B receptor agonist in hindpaw skin from male rats causes a decrease in the release apelin; this effect is not seen in female animals. These results suggest that activation of the endothelin system causes a decrease in the peripheral apelin system and a concomitant increase in the central (spinal cord) apelin system in male animals. In the preliminary study examining the effect of apelin on ET-1-induced calcium transients, at the doses used, apelin does not appear to have an effect on ET-1-induced increases in $[Ca^{2+}]_i$ or the desensitizing effect of ET-1 priming.

7.1.3 Specific Aim 3

The purpose of this specific aim was to examine the apelin system in a pediatric population of SCD focusing on genetic variability in the APJ receptor gene and the association of this variability with pain measures. To execute this aim, DNA was isolated from saliva samples were collected from children with SCD, which was then amplified and sequenced. Three SNPs, two in the 3'-UTR and one in the 5'-flanking region were the focus of this association study. None of the SNPs had significant associations with the direct pain measures of pain

intensity, frequency, and duration. However, all of the SNPs showed trends for associations between health care utilization and HbF. The 3'-UTR SNP rs2282624 showed a strong trend for an association with a history of ACS. Although significant associations were not found between these SNPs and direct pain measures, the potential associations with the indirect pain measures of health care utilization and HbF levels suggest that further explorations using a larger sample size is warranted to help identify potentially vulnerable patients.

7.2 Limitations

7.2.1 Specific Aim 1

Similar to many human studies, the interpretation for the results in this thesis is somewhat limited due to the small sample size studied. Statistical significance was approached for the relationship between several factors for this specific aim, and with larger sample sizes, true statistically significant and clinically relevant relationships may be revealed. It is also important to recognize that the plasma levels of apelin and endothelin do not necessarily reveal accurate tissue levels, which may be more relevant for determining the relationship between these peptides and SCD pain.

7.2.2 Specific Aim 2

For the first part of this specific aim (Chapter Four) that examined contralateral ET-1-induced sensitization to capsaicin, we recognize that our findings are somewhat limited by the lack of knowledge of the specific cell types that were found to be activated in the dorsal horn of the spinal cord. Determining these cell types and the mediators involved in this effect of ET-1 may help

explain the sex differences found in the effect of contralateral ET-1 priming on capsaicin-induced secondary hyperalgesia. We also cannot rule out the possibility that this effect is mediated by supraspinal influences on cells in the dorsal horn.

For similar reasons listed above, the results of the Ca^{2+} imaging *in vitro* studies in Chapter Five are somewhat limited because the mechanism of the priming effect of ET-1 on calcium transients is currently unknown. Finding the mechanism and receptors responsible for this effect will be important for possibly exploring therapeutic strategies in diseases in which ET-1 is known to play a role. The interpretation of the results for the study examining the effects of apelin on the priming effect of ET-1 is also limited because unlike determining the doses for ET-1 and capsaicin, a dose response for apelin was not performed before examining its effects. It is possible that the doses of apelin used in these very preliminary experiments may not have been the appropriate doses needed for these cell types and assay to reveal the true effects of apelin on ET-1-induced calcium transients. It should also be noted that while our animal models focused on neonatal animals of both sexes, our *in vitro* model only utilized the DRG neurons from adult male rats. Since it has been reported that neonatal and adult rat DRG neurons display different calcium currents (Kostyuk et al., 1993), it will important to determine if there are age differences in our *in vitro* model.

7.2.3 Specific Aim 3

The major limitation for this aim was the small sample size used for the genetic associations. Many of the associations found between the SNPs and the

different measures approached significance, but did not meet the limit to be considered statistically significant. With a larger sample size, we may be able to find more meaningful associations between the SNPs located in the *APLNR* gene and the different measures examined. Although the SNPs explored in this aim were carefully chosen, it is possible that we missed or overlooked SNPs that have more of an impact in the pain associated with SCD.

7.3 Future Directions

7.3.1 Specific Aim 1

This specific aim presented preliminary evidence that suggests that an imbalance in the apelin (vasodilatory) and endothelin (vasoconstrictive) systems may contribute to the occurrence of VOEs and SCD pain. This aim also provides evidence that both apelin and Ang II significantly decrease with age. Currently it is unknown if the age-dependent decrease in these systems is only seen in this disease or if this is a normal occurrence in children without SCD. It will be important to compare the plasma apelin levels found in our pediatric sample to race and age-matched controls without SCD to determine if these levels compare to the controls. In adult SCD patients (aged 18-34 years old), during both acute crisis and the steady state, there is severe impairment of endothelial function and endothelium-independent vasodilation compared to healthy controls (Blum et al., 2005). Since adult patients experience these complications as well as experiencing more frequent SCD pain compared to children and adolescents with SCD (Smith et al., 2008), future studies should examine the levels of apelin, endothelin, and Ang II in an adult population of SCD to see if there are further

relationships between these mediators in SCD pain. For future studies, it would also be interesting to compare these apelin and endothelin levels found in plasma taken during the steady state with levels from healthy controls as well as from patients having a VOE to see if levels and ratios change during vaso-occlusion.

7.3.2 Specific Aim 2

Previous results from our lab examining the effect of repeat exposure to ET-1 on localized changes in the skin found that ET-1 causes an increase in ET_B receptor expression in plantar hindpaw skin of female rats while it decreased ET_B receptor expression in male rats (McKelvy and Sweitzer, 2009). The results in this specific aim show that at the level of the primary afferent neuron, repeat exposures to ET-1 cause desensitization, but the mechanism that mediates this effect specifically in our model is unknown. A previous study using a repeat exposure model of ET-1 examined the effects in a very short time span (seconds) and found that in this time span, this desensitizing effect is mediated by the ET_B receptor (Yamamoto et al., 2006). For future studies, it will be important to characterize this mechanism according to receptor and cell types involved since our model involves a long term window (days) for this effect.

In our animal model, the contralateral sensitizing effect of ET-1 resulted in an increase in capsaicin-induced c-Fos expression. Future studies should determine the particular cell type exhibiting this increase in c-Fos activation in ET-1 primed animals treated with capsaicin in order to characterize this mechanism. Also in our animal model, the apelin system should be fully

characterized. Although this specific aim measured the expression level of the APJ receptor in both the spinal cord and hindpaw skin, the location of the receptor in these two locations was not examined. Future studies should focus on examining the expression levels of apelin in hindpaw skin, DRG, and spinal cords as well as the location of the APJ receptor in the skin and spinal cord in our animal model of acute VOEs.

7.3.3 Specific Aim 3

Single nucleotide polymorphisms in the *APLNR* gene have been associated with the risk of stroke (Hata et al., 2007), blood pressure response to salt intervention (Zhao et al., 2010a), and the progression of heart failure (Sarzani et al., 2007). The results presented in this thesis present promising associations between SNPs in the *APLNR* gene and health care utilization, HbF levels, and ACS. Future studies could begin to explore SNPs located in the apelin gene (*APLN*), which is located on the X chromosome, to see if they play a role in the complications associated with SCD. Single nucleotide polymorphisms in the *APLN* gene have been found to be associated with blood pressure response to potassium (Zhao et al., 2010b) and fasting glucose levels (Zhang et al., 2009). It would also be interesting to do an association study for the angiotensin I converting enzyme 2 (*ACE2*) gene since this is the only known enzyme to degrade apelin fragments (Zhao et al., 2010b).

7.4 Clinical Implications

Sickle cell disease is characterized by many complications, but one of the most life altering complications is VOEs, which are responsible for 90% of

hospital admissions for SCD patients (Ballas, 2005). It is thought that the repetitive VOEs that occur during the first two decades of life is what may serve as the precursor to the development of chronic pain later in life in some patients (Smith and Scherer, 2010). It is also thought that neuropathic pain can develop in some patients due to previous painful episodes, which may involve either peripheral or central sensitization (Smith and Scherer, 2010). The findings from Chapter Four of this thesis provide evidence that exposure to ET-1 during the early neonatal period can cause contralateral sensitization later in the neonatal period to capsaicin, an acute pain stimulus, through centrally (spinal cord) mediated mechanisms, which may involve the process of central sensitization. Central sensitization involves neuroplastic changes in the dorsal horn of the spinal cord and is thought to be an important process in the transition from acute to chronic pain in some pathological conditions (Voscopoulos and Lema, 2010; Woolf, 2011). The findings from Chapter Four also show that pain in early life can alter the response to pain later in life, which highlights the importance of finding more effective therapies for treating pain during this critical period in children with SCD, who may experience repeated VOEs throughout childhood.

Since relationships were found between the ratio plasma levels of apelin to endothelin and underlying baseline pain and the frequency of VOEs, potential treatment strategies for pain in SCD should possibly be targeting both the apelin and endothelin systems together rather than targeting either system alone. One surprising finding from these studies is the differential involvements of the vasoconstrictors, endothelin and Ang II, in pain in children with SCD. While

endothelin is involved in SCD related pain, Ang II appears to be more involved in acute pain, suggesting that pain management plans for children should discriminate the cause of pain since these different forms of pain may respond to different pharmacotherapy approaches. The results from the apelin receptor gene association study suggest that polymorphisms in this gene may be a predictive tool by which to determine which children will be at an increased risk for having greater health care utilization, which would be of benefit in developing pain management plans for when these children leave pediatric care, thereby reducing the high costs of hospitalizations associated with SCD.

7.5 Conclusions

The findings in this thesis present preliminary, but novel evidence that the newly discovered vasodilatory peptide, apelin may play a role in the underlying pain associated with SCD and it is the balance between the apelin and endothelin systems that may be one of the mechanisms responsible for this role. The involvement of the balance between apelin and endothelin in SCD pain and VOEs appears to be specific for endothelin since Ang II is more related to acute pain other than the pain associated with SCD and the vasculopathy/endothelial dysfunction sub-phenotype of SCD. The results in this thesis also present evidence that activation of the endothelin system causes a decrease in the peripheral apelin system while also causing an increase in the central apelin system, suggesting that these two systems may interact in our models, but further studies are needed to identify these interactions. Near associations found between polymorphisms in the apelin receptor gene and health care utilization

and HbF levels in children with SCD further suggest that the apelin system may be involved in the SCD related complications. The results of this thesis support the postulates that ET-1 induces sensitization through central mechanisms in the spinal cord and that an imbalance between the vasoconstrictive and pro-nociceptive systems and vasodilatory and anti-nociceptive systems contributes to pain associated with SCD.

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