MECHANISMS OF CUTANEOUS WOUND HEALING ARE MEDIATED VIA PERIPHERAL NEUROPEPTIDE ACTIVITY

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ABSTRACT

Topically applied morphine is routinely used to alleviate pain in cutaneous wounds such as burns and pressure sores, yet evidence suggests the topical administration of exogenous opioid drugs may impair wound closure. This dissertation research was designed to test the hypothesis that topical morphine application delays cutaneous wound healing via mechanisms dependent upon peripheral neuropeptide activity. Results demonstrate that topical morphine application delays cutaneous wound closure rates. The delay occurs in a concentration-dependent manner (consistent with opioid-receptor mediated effects), is mimicked by NK-1 and NK-2 receptor antagonists, and can be reversed by the exogenous application of either substance P or neurokinin A. The results indicate that morphine acts presynaptically, delaying wound closure by activating opioid receptors located on primary afferent nerve terminals and subsequently inhibiting the antidromic release of neuropeptides into the wound. The temporal pattern of the effects of topical morphine treatment can be attributed to alterations in the initiation and duration of essential, early processes during wound healing. The delay in closure evoked by topical morphine not only leaves the cutaneous wound open longer, increasing the risk of infection, but also results in long-term architectural deficits, compromising the integrity of the healed skin. Furthermore, dysregulation of neurokinin receptor-expressing cells essential for normal wound healing emerges as a

mechanism capable of significantly disrupting the dynamic processes involved in wound healing.

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ABBREVIATIONS

α-smooth muscle actin (α-SMA)

Analysis of variance (ANOVA)

Angiotensin-converting enzyme (ACE)

Bovine serum albumin (BSA)

Calcitonin gene-related peptide (CGRP)

Central nervous system (CNS)

Extracellular matrix (ECM)

G protein-coupled receptor (GPCR)

Hemokinin-1 (HK-1)

Immunoreactive (-ir)

Nerve growth factor (NGF)

Neurokinin A (NKA)

Neurokinin B (NKB)

Neurokinin-1 (NK-1)

Neurokinin-2 (NK-2)

Neurokinin-3 (NK-3)

Neutral endopeptidase (NEP)

Polymorphonuclear cells (PMNs)

Preprotachykinin (PPT)

Substance P (SP)

CHAPTER ONE

BACKGROUND AND SIGNIFICANCE

1.1 Introduction

Accumulating evidence suggests that sensory neuropeptides play an important role in wound repair, as healing is enhanced by their exogenous application (Kjartansson et al., 1987a; Engin, 1998; Delgado et al., 2005) and impaired by their depletion (Kjartansson et al., 1987b; Peskar et al., 1995; Khalil et al., 1996; Smith et al., 2002). Neuropeptides such as substance P (SP) and neurokinin A (NKA) facilitate wound healing by regulating blood flow and modulating the function of immunocompetent and inflammatory cells, as well as epithelial and endothelial cells within the wound.

Prominent medical factors contributing to the incidence of cutaneous wounds include diabetes mellitus, obesity, and aging. Chronic cutaneous wounds, such as burns and skin ulcers, result in prolonged hospitalization and considerable morbidity and remain a significant burden on our health care system. Treatment of such wounds costs health services billions of dollars per year. Despite the obvious clinical importance, the basic molecular and cellular mechanisms underlying cutaneous wound healing; specifically, the role of neuropeptides remains unclear.

Cutaneous wounds can be extremely painful. Systemic opioid drugs, such as morphine, are highly effective and widely used analgesic agents. However, the pain associated with chronic wounds can be particularly difficult to manage and many patients continue to experience considerable pain despite the use of systemic analgesics. Oral and parenteral administration of opioids provides robust analgesia for both acute pain and long-term severe pain, yet they are commonly accompanied by dose-limiting side-effects including constipation, sedation, nausea and vomiting,

respiratory depression, or confusion and hallucinations. In addition, opioid analgesics have a potential risk of addiction and are often administered in escalating doses due to the development of tolerance to the effects of the drug. Many individuals suffering from painful cutaneous wounds such as pressure ulcers also have serious co-morbid illnesses, (e.g. cardiovascular disease, renal dysfunction related to diabetes) or other risk factors associated with prolonged immobility or old age. Pain management for these patients can be problematic given that side-effects associated with systemic use of opioids can create severe complications in their current medical conditions.

Systemic opioid drugs, such as morphine, remain the standard course of care in providing analgesia to patients with cutaneous wounds, though recent studies have focused on the activation of peripherally-located opioid receptors. Opioid analgesia was initially believed to originate exclusively from the activation of opioid receptors within the central nervous system. Accumulating evidence, however, demonstrates the analgesic efficacy of peripheral opioids. The topical application of morphine has been shown to successfully reduce the pain associated with cutaneous wounds (Long et al., 2001). Christoph Stein et al. (Stein, 1993) showed that analgesia can be obtained topically via the activation of opioid receptors on afferent sensory nerve terminals located in peripheral tissues. The mode of action is believed to be local rather than systemic, avoiding negative effects seen upon stimulation of opioid receptors located within the central nervous system. Furthermore, Stein et al. found that while all opioid receptors are present to some degree on peripheral nerve terminals, activation of mu receptors is primarily responsible for peripheral analgesia.

Although topical opioid compounds offer a promising alternative therapeutic strategy for alleviating pain, they may also adversely effect wound healing limiting the usefulness of this approach. Opioid peptides are known to inhibit action potential generation within nerves and, consequently, suppress the release of neuropeptides from sensory nerve terminals (Werz et al., 1983b; Schroeder et al., 1991) disrupting the connection between the nervous and immune systems. Thus, the studies described in this dissertation were designed to identify the mechanisms that delay cutaneous wound healing during topical morphine application and provide new and important information concerning both the basic science and role of sensory neurons and opioids in cutaneous wound healing.

1.2 Mechanisms of Wound Healing

The skin is an organ that serves as a protective physical barrier to environmental insults and is essential for maintaining homeostasis. Any breach of the skin's integrity can potentially expose a patient to a number of pathological conditions including infection and fluid loss. The responses to injury include a vital, innate host immune response to restore tissue integrity. Wound healing proceeds via a complex overlapping pattern of events including coagulation, inflammation, formation of granulation tissue, reepithelialization, and matrix and tissue remodeling (Baum et al., 2005). Underlying these events are numerous, complex processes involving various cells, growth factors, cytokines, and components of the extracellular matrix. The three phases of wound healing, including the inflammatory

phase, proliferative phase, and maturation phase, are explained below in further detail.

The first phase of wound healing is the inflammatory phase, which begins with hemostasis resulting from blood clot formation and vasoconstriction. At the cellular level, clot formation is mediated by fibrin and platelets and occurs within minutes. Upon injury, the protease thrombin cleaves fibrinogen producing fibrin. Fibrin monomers then bind directly to platelets forming a clot (Blomback et al., 1978; Mosesson et al., 2001). Thrombin is also responsible for the initiation of platelet aggregation (Ofosu et al., 2000). Vasoconstriction occurs immediately following injury and persists for approximately 15 minutes. Multiple factors contribute to this phenomenon including the release of prostaglandins and thromboxanes from injured cells (Becker et al., 2000) and circulating epinephrine from the adrenal medulla and release of norepinephrine from sympathetic nerves (Lawrence, 1998).

Inflammation is manifested by erythema, heat, swelling, and pain, which are consequences of vasodilation and increased vascular permeability. Vasodilation and increased vascular permeability are stimulated by numerous factors. Antidromic activation of sensory nerves results in the release of their contents, including the neuropeptides, SP, NKA, and calcitonin gene-related peptide (CGRP), which cause vasodilation and increased vascular permeability respectfully (Lundberg et al., 1983; Payan, 1989; Khalil et al., 1996). Another substance responsible for vasodilation is histamine. Substance P and NKA cause the degranulation of mast cells, resulting in the release of histamine, a potent vasodilator, into the extracellular matrix (Carter,

1970; Hebda et al., 1993). Histamine also indirectly produces vasodilation by increasing the production of prostaglandins (Hebda et al., 1993). Another substance responsible for stimulating increased vascular permeability is thrombin (Stiernberg et al., 1993).

These alterations in vascular function facilitate the extravasation of serum proteins and migration of inflammatory cells into the wound site. The first inflammatory cells to infiltrate the wound area are polymorphonuclear cells (PMNs) (Lawrence, 1998). The concentration of PMNs peaks within 24 and 48 hours and are responsible for clearing the wound area of cellular debris, bacteria, and foreign matter (Steed, 1997; Singer et al., 1999). Moreover, PMNs are an important source of proinflammatory cytokines (Werner et al., 2003). Circulating monocytes arrive subsequent to PMNs (48 to 72 hours) where they are activated and converted into macrophages (Provvedini et al., 1986). Macrophages serve as scavengers within the wound as well; however, they remain longer (up to weeks) and play a more complex role in wound healing. Macrophages maintain the inflammatory process via production of proinflammatory cytokines and growth factors. These factors facilitate the ability of macrophages to stimulate the production of collagen from fibroblasts, the differentiation of fibroblasts to myofibroblasts, angiogenesis, and reepithelialization (Werner et al., 2003). Macrophages are essential for proper wound healing as their depletion results in poor debridement, delayed activation and inhibited proliferation of fibroblasts, and impaired wound closure (Leibovich et al., 1975). Other subsets of immune cells that participate in wound healing are T

lymphocytes and eosinophils. Studies have shown that depletion of T cells results in impaired would healing (Peterson et al., 1987; Barbul et al., 1989; Efron et al., 1990), demonstrating the regulatory effects of these cells. Eosinophils produce growth factors which contribute to reepithelialization by keratinocytes (Werner et al., 2003).

The inflammatory phase of wound healing is followed by the proliferative phase, which commences approximately four days post-injury. The proliferative phase is initiated by the replacement of the extracellular matrix (ECM) by granulation tissue. Invading capillaries constitute the granular appearance. At the cellular level this process is marked by the entrance of permanent residents of the repaired dermis (Singer et al., 1999). Comprising the granulation tissue are fibroblasts, collagen, blood vessels, and macrophages (Steed, 1997).

Fibroblasts provide the largest contribution to the wound healing process of any mesenchymal cell (Lawrence, 1998). During the proliferative phase, temporary fibrin-based matrix is replaced by collagen-based ECM, a product of fibroblasts. Increased fibroblast population within the wound is due to both the migration and proliferation of cells, which is primarily initiated by the various growth factors released by macrophages (Ross et al., 1970; Lawrence, 1998). Production of various cytokines and growth factors by fibroblasts results in the regulation of several processes in multiple stages of wound healing. Fibroblasts have been linked to the regulation of inflammation, angiogenesis, cell-matrix interactions, and matrix remodeling. Keratinocyte proliferation, nerve innervation, and angiogenesis are stimulated by nerve growth factor (NGF), which is produced by fibroblasts (Pincelli

et al., 1994; Hasan et al., 2000). Furthermore, the contractile properties of fibroblasts promote the redefining of wound edges during wound maturation.

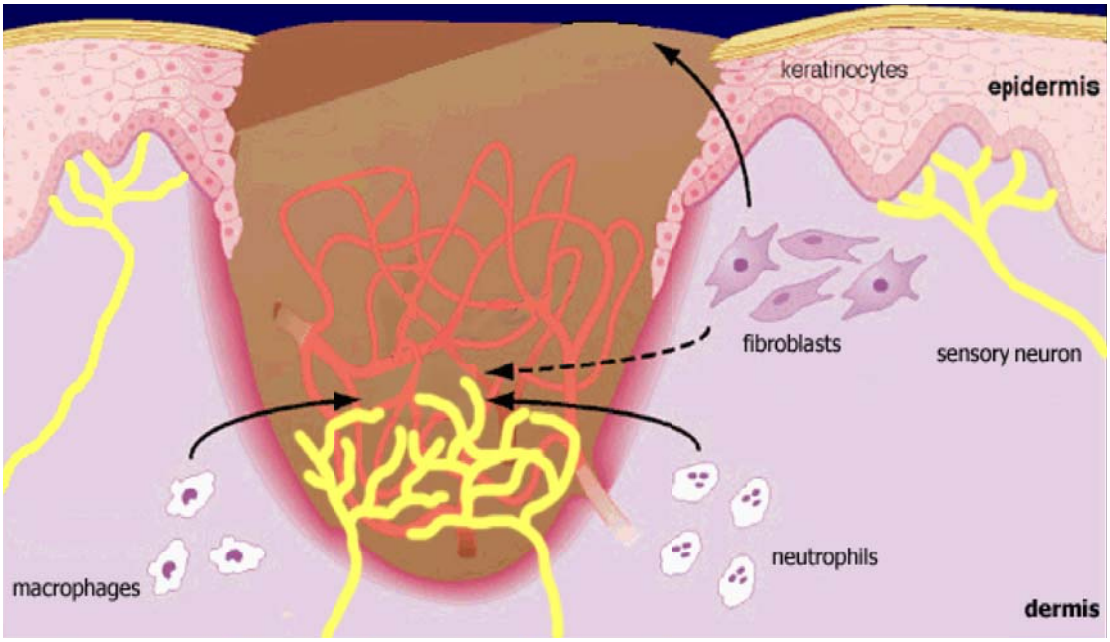


Figure 1. Illustration depicting the sprouting of sensory neurons (yellow) and blood vessels (red), and infiltration of important inflammatory and mesenchymal cells into a cutaneous wound. Adapted from (Grose et al., 2004)

The reestablishment of epidermis over granulation tissue and sloughing off of residual eschar occurs during reepithelialization. Reepithelialization progresses with keratinocytes migrating along the leading edge of the wound across the granulation tissue leaving a stratified layer of proliferating cells in their wake. Keratinocytes continue traveling until opposing sides reestablish contact in a process known as contact guidance (Beck et al., 1961).

Wound healing concludes with the maturation phase defined by contraction, decreased redness, decreased thickness, and increased strength. These changes begin within days of the initial injury and can continue over a period of weeks, months, or years depending on the severity of the wound. The initiation of maturation overlaps the proliferation phase as fibroblasts and their products and blood vessels comprise the main participants in wound maturation. Contraction is a cell-directed process in which wound edges move inward at a rate of 0.6 to 0.75 mm/day (Lawrence, 1998). This process is attributed to the contractile properties of myofibroblasts generated from the phenotypic transformation from fibroblasts (Werner et al., 2003). Nerve growth factor also contributes to wound contraction by upregulating the production of α-smooth muscle actin in myofibroblasts (Schmitt-Graff et al., 1994; Hasan et al., 2000). The inverse relationship between decreased wound thickness and increased wound strength can be attributed to remodeling of collagen-based ECM. This process is characterized by a balance between collagen production, breakdown, and remodeling (Lawrence, 1998; Monaco et al., 2003). The redness located within wounds is attributed to increased capillary density. While young wounds are characterized by capillary-rich granulation tissue, mature wounds are much less vascular (DiPietro et al., 1996). Together these alterations result in a relatively acellular mature scar.

1.3 Peripheral Nerves in Cutaneous Healing

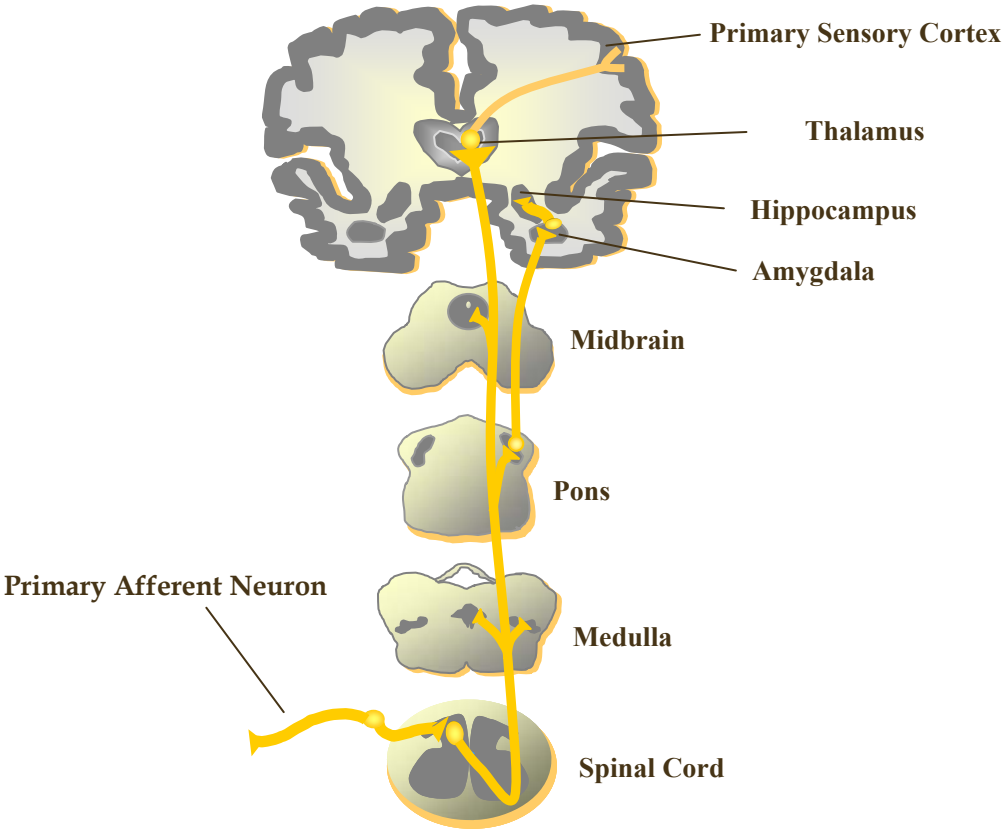


Figure 2. Role of primary afferent neurons in pain transmission. Pain signals originate from primary afferent sensory neurons located within the skin. The cell bodies of these first order neurons are contained within the dorsal root ganglia and their central processes enter the spinal corn via the dorsal horn where they synapse on second order neurons. The second order neurons then cross the midline and ascend to nuclei in regions of the limbic system including the thalamus. Third order neurons then transmit the signal to the somatosensory cortex where the location and intensity of the noxious stimulus is interpreted.

More than a century ago, the observation was made that activation of dorsal root ganglia neurons results in peripheral vasodilation, suggesting that these neurons not only conduct *afferent* information into the spinal cord, but also have an *efferent* function (Bayliss, 1901). Since that time, abundant evidence has accumulated supporting the idea that activation of peripheral terminals of sensory neurons by local depolarization or axonal reflexes stimulates the release of their contents including neuropeptides. These neuropeptides, in turn, act on target cells in the periphery such as mast cells, immune cells, and vascular smooth muscle producing inflammation. The inflammation is characterized by redness and warmth caused by vasodilation, swelling secondary to plasma extravasation, and hypersensitivity resulting from alterations in the excitability of sensory neurons. This phenomenon is known as "neurogenic inflammation".

Thinly myelinated (Aδ) and unmyelinated (C) small diameter sensory nerve fibers are of significant importance in the generation of neurogenic inflammation (Kjartansson et al., 1987b; Holzer, 1991). Both subsets of nerve fibers respond to a variety of exogenous stimuli including heat, cold, mechanical distention, UV light, chemical substances, and tissue damage. Endogenous stimuli such as ATP, protons, hormones, cytokines, and kinins released from local cells and tissues following injury which are involved in the inflammatory response are also capable of activating primary sensory afferent neurons. Activation of peripheral sensory nerves mediates early components of neurogenic inflammation, specifically immune cell migration (Eglezos et al., 1988; Eglezos et al., 1991), vasodilation (Khalil et al., 1989a, ,

1990b), plasma extravasation (Khalil et al., 1989b, , 1990a), and modulation of cellular constituents of the immune response (Eglezos et al., 1991).

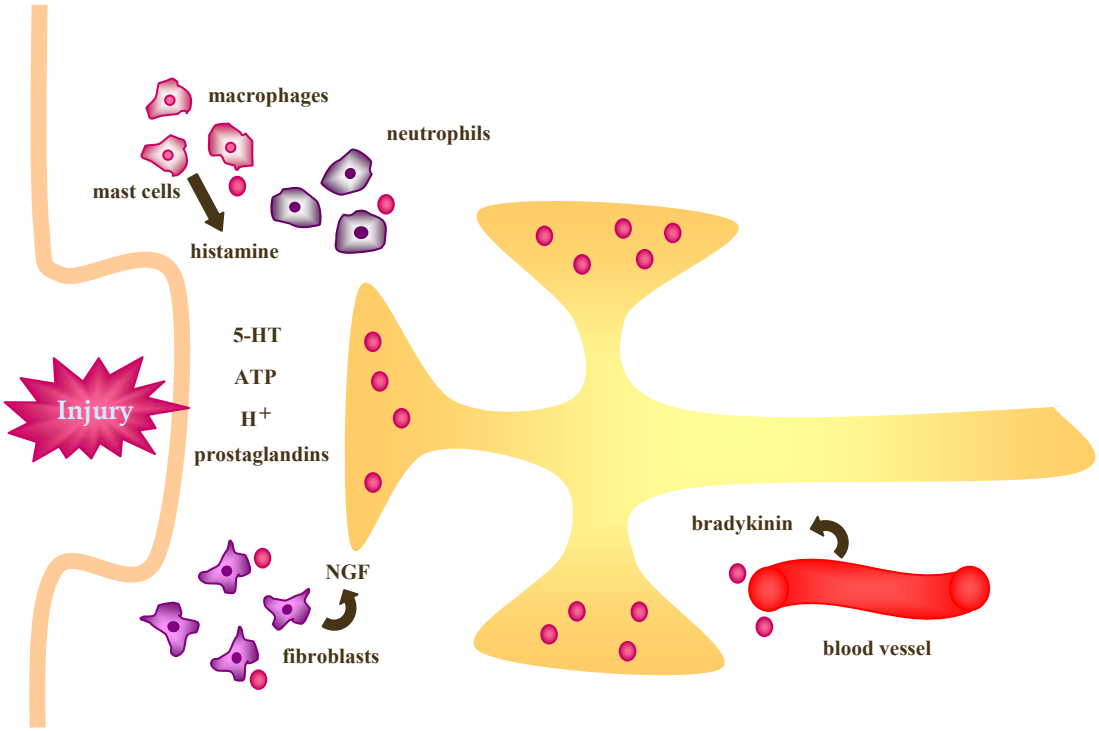


Figure 3. Illustration depicting the role of sensory neurons in neurogenic inflammation. Upon injury, cells release their contents into the extracellular matrix. These substances, such as serotonin, ATP, prostaglandins, and protons, activate primary afferent neurons. The action potentials generated not only travel centrally, but also antidromically along branching nerve terminals. Neuropeptides, such as SP and NKA, stored within these terminals are then released into the periphery. Neuropeptides cause the degranulation of mast cells and subsequent release of histamine, as well as the release of NGF from fibroblasts. In addition, they induce vasodilation and increased vascular permeability which results in the synthesis of

bradykinin. These chemical mediators released activate surrounding sensory neurons resulting in neurogenic inflammation.

Many studies have been conducted to investigate the role of peripheral sensory nerves in cutaneous wound healing. Pretreatment with the neurotoxin capsaicin, the pungent vanilloid found in hot chili peppers, results in depletion of sensory neuropeptides and destruction of primary afferent neurons (Lynn, 1990). Functional denervation utilizing capsaicin results in permanent sensory and functional deficits (Holzer, 1991), reduced inflammatory responses, and ultimately diminished wound healing (Kjartansson et al., 1987b; Smith et al., 2002). In addition, denervation by dissection of the spinal hemicord demonstrated a delay in both wound contraction and reepithelialization (Fukai et al., 2005). The primary reason for delayed wound healing in the absence of neuropeptides appears to be due to a lag phase between injury and the initiation of wound contraction (Khalil et al., 1996).

1.4 Neuropeptides & Peripheral Targets

Neuropeptides can function as neurotransmitters, hormones, and paracrine factors, and comprise one of the largest families of extracellular messengers. The most recognized and extensively studied neuropeptides in the skin are SP, NKA, and CGRP. In the periphery, neuropeptides are located in both noradrenergic and cholinergic autonomic nerve fibers as well as in free nerve endings of afferent sensory nerves (Felten et al., 1987; Holzer, 1988). Stimulation of these nerve fibers causes the release of neuropeptides stored within the peripheral terminals. Effects

induced by neuropeptides, such as vasodilation, increased vascular permeability, and edema are not restricted to the site of initial injury, suggesting that nerve signals not only travel centrally but also spread antidromically along collateral neuron terminal branches, causing the release of neuropeptides from unstimulated nerve terminals (Holzer, 1988). Increasing evidence has demonstrated non-neuronal expression of neuropeptides, particularly SP. Immunoreactivity studies have identified SP contained within human mast cells (Toyoda et al., 2000) and endothelial cells (Ralevic et al., 1990). Additionally, SP expression has been reported in eosinophils (Metwali et al., 1994), T lymphocytes (Lai et al., 1998), and monocytes (Bost et al., 1992; Ho et al., 1997).

Neuropeptides serve as a link between the immune and nervous system. This has been validated by the demonstration of direct peptidergic innervation of primary and secondary lymphoid organs, the close proximity between sensory nerve endings and immune cells, and specific neuropeptide receptors located on immune effector cells (Felten et al., 1985; Payan, 1989; Felten et al., 1992). Furthermore, most cells expressing neuropeptide receptors also make neuropeptide-degrading enzymes such as neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE), providing a feedback mechanism to terminate the inflammatory effects of neuropeptides (Damas et al., 1996). Neuropeptide interactions are seen with virtually all components of the immune system. Abundant evidence exists demonstrating that neuropeptides contribute toward many of the processes involved in inflammation that are essential for normal wound healing.

A primary impact of neuropeptides on wound healing is the modulation of inflammatory cell proliferation and function. Tachykinins stimulate PMN chemotaxis (Roch-Arveiller et al., 1986; Carolan et al., 1993) and induce adhesion of PMNs to the endothelium (Thureson-Klein et al., 1987; Zimmerman et al., 1992; Perretti et al., 1993). SP exhibits further proinflammatory actions by promoting macrophage chemotaxis and increased function (Hartung et al., 1986). In addition, reports have shown that SP induces the synthesis and release of cytokines from human monocytes (Lotz et al., 1988; Rameshwar et al., 1994). Tachykinins also have a direct impact on T-cell activation (Scicchitano et al., 1988). For example, HIV-1 enhances SP expression in human monocytes and lymphocytes (Ho et al., 2002), and SP, in turn, can amplify viral replication in human monocytes (Li et al., 2001). Mast cells play a key role in the modulation of the latter phase of inflammation by mediating vascular permeability, fibrin deposition, edema, and leukocyte infiltration (Wershil et al., 1987). Moreover, mast cells have been proposed to be one of the major effector cells influenced by neuropeptides given that neuronal stimulation results in mast cell degranulation and histamine release (Goetzl et al., 1985).

Observation of improved wound healing with exogenous neuropeptide application (Delgado et al., 2005) and increased proliferation of target cells (Ansel et al., 1996) also supports a regulatory role of neuropeptides in tissue repair. Permanent scar tissue is comprised mainly of keratinocytes and fibroblasts. Tachykinins stimulate the proliferation of both keratinocytes (Tanaka et al., 1988) and fibroblasts (Nilsson et al., 1985; Ziche et al., 1990a). Sprouting of new blood vessels and

adequate blood flow within a wound is crucial for the healing process. In vivo, both tachykinins stimulate angiogenesis in inflammation and wound healing (Ziche et al., 1990b; Fan et al., 1993). These data indicate that neuropeptides are not only important for the inflammatory response but may also play a significant role in the proliferation of epithelial, connective tissue, and vascular cells.

1.5 Tachykinins

Tachykinins are a family of peptides that share a common carboxyl-terminal amino acid sequence, Phe-X-Gly-Leu-Met-NH_2 (Hershey et al., 1990). The classic family of tachykinins consists of substance P, neurokinin A, and neurokinin B with differential splicing and posttranslational processing also producing the neurokinin A-like peptides, neuropeptide gamma and neuropeptide K. They are traditionally viewed as neuropeptides due to their wide distribution throughout both the central and peripheral nervous systems where they act as excitatory neurotransmitters. However, the concept that tachykinins act exclusively as neuropeptides is being challenged given that the best known members of the family, SP, NKA, and neurokinin B (NKB) are also expressed in non-neuronal cells and in non-innervated tissues (Nelson et al., 2004). In addition, new members to the family, hemokinin-1 (HK-1) and the endokinins, are primarily expressed in non-neuronal cells (Zhang et al., 2000; Kurtz et al., 2002) suggesting a widespread distribution and important role as intercellular signaling molecules within the periphery.

1.5.1 Tachykinin Biosynthesis

Tachykinins are relatively short peptides that are produced as preproproteins, processed, and secreted via the classical secretory pathway. Substance P and neurokinin A are encoded by the preprotachykinin-A (PPT-A) gene which is located on chromosome 7 (Table 1) (Nawa et al., 1984; Carter et al., 1990). The human gene consists of seven exons with the sequence that encodes SP and NKA contained in exon 3 and exon 6, respectively (Fig. 1). The PPT-A gene undergoes transcription producing a primary transcript that can be alternatively spliced resulting in four different mRNA isoforms (α, β, γ, and δ) (Nawa et al., 1984; Kawaguchi et al., 1986; Krause et al., 1987). SP is synthesized from all four isoforms, whereas NKA can only be synthesized from the β and γ PPT-A mRNAs. Hence, SP can be expressed alone while NKA expression is always accompanied by SP. These mRNAs are then translated, generating a preprotachykinin comprised of a signal peptide, the neuropeptides, and spacers. The signal peptide allows the forming peptide to attach to and pass into the endoplasmic reticulum and is quickly cleaved off following synthesis. The resulting preprotachykinin is then transported to the Golgi apparatus where the spacers are cleaved giving rise to the active peptide (Nawa et al., 1984; Kawaguchi et al., 1986). Active peptides are packaged into secretory granules, which bud off from the Golgi apparatus and are transported through the axon to the nerve terminals (Krause et al., 1987).

Neurokinin B is the only tachykinin derived from the preprotachykinin-B (PPT-B) gene (Kotani et al., 1986). The human gene consists of 7 exons, and the

sequence that encodes NKB is located in exon 5. Neurokinin B is primarily found within the CNS (Kangawa et al., 1983; Moussaoui et al., 1992), however, recent studies have discovered the tachykinin in various female reproductive organs including the placenta (Page et al., 2000) and uterus (Cintado et al., 2001).

The recent discovery of a third preprotachykinin gene (PPT-C) has led to the identification of several novel tachykinins (Zhang et al., 2000; Kurtz et al., 2002). This group includes hemokinin-1 (HK-1) in mouse and rat, endokinin-1 in rabbit, and endokinin A, endokinin B, human HK-1 and human HK(4–11) (Page, 2004). Hemokinin-1 was first cloned in hematopoietic cells from mice and named for its important role in maturation of B cells (Zhang et al., 2000). The PPT-C gene has subsequently been cloned in both rat and human (Kurtz et al., 2002; Page et al., 2003). The human gene consists of five exons and is located within exon 2 (Fig. 2).

The human tachykinin peptide genes

Name	Gene	Alternative name	Chromosome location
SP/NKA NPK/NPγ	TAC1	PPT-A or PPT-I	7q21-q22
NKB	TAC3	PPT-B or PPT-II	12q13-q21
HK-1/EKA, EKB, EKC, EKD	TAC4	PPT-C	17q21.33

Table 1. Human tachykinin genes. Adapted from (Pennefather et al., 2004)

The amino acid sequences of SP, NKA, and NKB are identical across all mammalian species, though many more orthologues and other related peptides exist

in mollusks and amphibians. These closely related peptides are characterized by the presence of a common C-terminal amino acid sequence. However, the amino acid sequence of rat and mouse HK-1 only shares 45% homology with the human peptide (Zhang et al., 2000; Kurtz et al., 2002; Page et al., 2003). In addition, the recently discovered endokinin C and endokinin D have a slightly modified C-terminal tachykinin recognition sequence (Page et al., 2003).

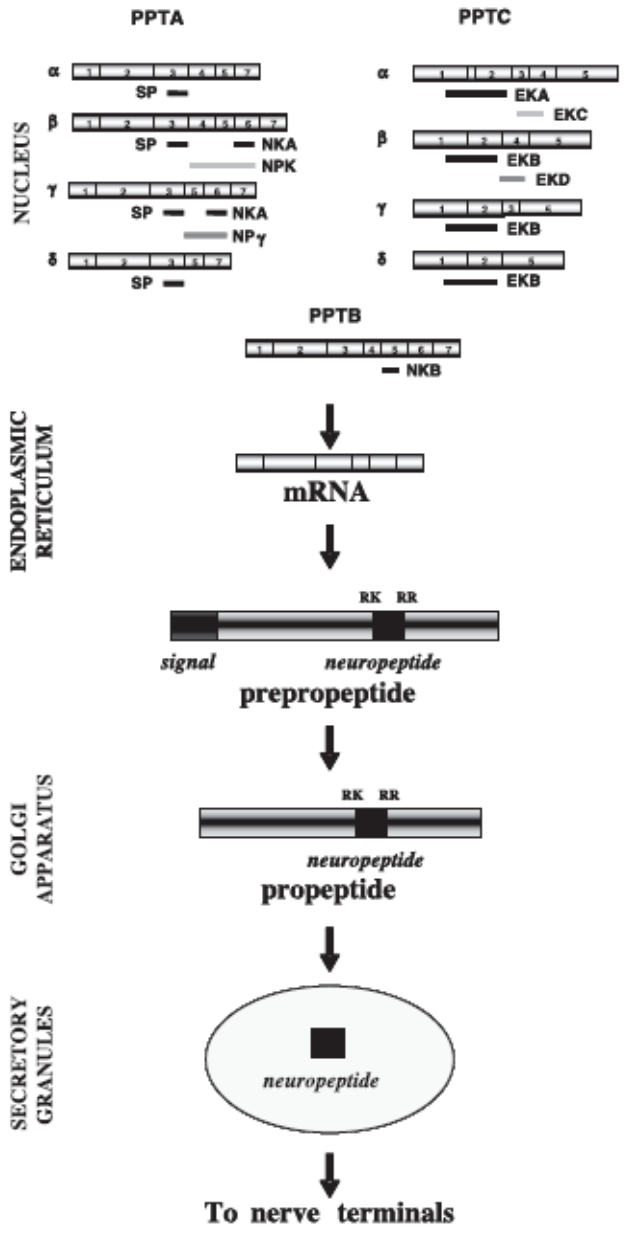


Figure 4. Synthesis of tachykinins from PPT-A, PPT-B and PPT-C genes in neurons (Pennefather et al., 2004).

1.5.2 Substance P

Substance P is an 11-amino acid peptide (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) that is present in many areas of the central and peripheral nervous system. SP is primarily synthesized within the dorsal root ganglia of small thinly myelinated A-delta and unmyelinated C primary afferent sensory fibers where it migrates either centrally to the dorsal horn of the spinal cord or peripherally to nerve terminals (Hokfelt et al., 1975b; Barber et al., 1979). Historically, the main role of SP was believed to be the transmission of nociceptive signaling. However, in the 1980s, SP was proposed to have proinflammatory properties as well (Payan, 1989). Upon activation of primary afferent neurons, 4-5 times as much substance P is transferred via axonal transport toward the peripheral terminals as is directed toward central terminals (Harmar et al., 1982). Although the primary source of SP within the periphery is peripheral sensory nerves, recent studies have identified peripheral expression of SP, especially in cells of immunologic importance, such as eosinophils (Aliakbari et al., 1987), macrophages (Pascual et al., 1990; Bost et al., 1992), dendritic cells (Lambrecht et al., 1999), lymphocytes(Lai et al., 1998; Qian et al., 2001), and fetal microglia (Lai et al., 2000; Lai et al., 2002).

Evidence supporting the involvement of SP in the pathophysiology of inflammatory disease stems from data demonstrating the heightened levels of SP in diseased tissue (Koch et al., 1987; Kaltreider et al., 1997), the beneficial effect of SP receptor antagonists (Lofgren et al., 1999; Tough et al., 2003), as well as the altered expression of the receptor for SP in diseased tissue (Krause et al., 1995; Kaltreider et

al., 1997). In addition, SP receptor deletion significantly reduced both inflammation and hyperalgesia in animal models of inflammatory disease (Bhatia et al., 1998; Kidd et al., 2003).

The principal effects of SP within the periphery impact blood flow and vascular permeability, the inflammatory response, and cellular proliferation. Vasodilation is induced by a direct action on vascular smooth muscle as well as enhanced production of nitric oxide by the endothelium (Hokfelt et al., 1975a; Bolton et al., 1986). SP-evoked increases in blood flow are transient compared to its more prominent effect on endothelial cells in mediating increased vascular permeability and protein extravasation resulting in edema formation (Lundberg et al., 1983; Pernow, 1983). Substance P directly enhances inflammatory cell activation, migration, and proliferation (Hartung et al., 1989). Substance P also has the ability to magnify the inflammatory response by stimulating mast cell degranulation and subsequent release of mediators such as histamine, prostaglandins, and serotonin. These mediators not only modulate the late-phase of inflammation but can also directly stimulate nerve terminals and initiate an axon reflex (Goetzl et al., 1985).

Substance P further participates in the inflammatory response by enhancing lymphocyte proliferation, immunoglobulin production, and cytokine secretion from lymphocytes, monocytes, macrophages, and mast cells (Stanisz et al., 1986; Lotz et al., 1988; Scicchitano et al., 1988; Bost et al., 1992; Ho et al., 1997; Maggi, 1997). SP stimulates the chemotaxis of lymphocytes, monocytes, and fibroblasts (Haines et al., 1993; Kahler et al., 1993; Schratzberger et al., 1997) and induces rapid

recruitment of neutrophils and eosinophils in the wound (Smith et al., 1993). SP-induced release of inflammatory mediators such as cytokines, oxygen radicals, arachidonic acid derivatives, and histamine stimulates further leukocyte recruitment, thus amplifying the inflammatory response (Holzer et al., 1997).

Substance P has also been implicated in the resolution of the inflammatory response. Its connection with tissue repair has been primarily derived from studies describing its proliferative effect on a variety of cells. Substance P functions as a mitogen for smooth muscle cells, fibroblasts, keratinocytes, and endothelial cells (Nilsson et al., 1985; Tanaka et al., 1988; Ziche et al., 1990a; Ziche et al., 1990b; Rameshwar et al., 1997). A role in angiogenesis has also been proposed (Fan et al., 1993). DNA synthesis in cultured arterial smooth muscle cells can also be stimulated by SP (Nilsson et al., 1985). Moreover, SP has been shown to enhance angiogenesis during neurogenic inflammation in the rat (Seegers et al., 2003).

1.5.3 Neurokinin A

Neurokinin A is also a small polypeptide consisting of ten amino acids (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2). Similar to SP, the primary source of origin of NKA is neuronal, however it is co-localized with SP in enteric non-neuronal cells as well. Neurokinin A is released from nerve endings in the peripheral and central nervous systems and functions similarly as an excitatory neurotransmitter (Lembeck et al., 1979; Maggi, 1991; Otsuka et al., 1993). Neurokinin A activates epithelial cells (Chu et al., 2000), fibroblasts (Bae et al., 2002), and intestinal and

airway smooth muscle cells (Khan et al., 1994; Maghni et al., 2003). Additionally, NKA stimulates production of proinflammatory cytokines in keratinocytes (McGovern et al., 1995; Song et al., 2000). Many of the experiments investigating the role of SP in the periphery were conducted using non-specific neurokinin receptor antagonists. Given that NKA is always co-localized with SP, many of the known effects of tachykinins in wound healing are non-specific for SP and NKA. Although NKA has been far less investigated than its counterpart SP, observations that its expression is increased in various inflammatory diseases (Kennedy et al., 2003; Lecci et al., 2003) suggests that NKA may also function as a paracrine or endocrine factor and play a role in neuroimmunomodulation.

1.5.4 Hemokinin-1

The recently discovered tachykinin, HK-1, is an eleven amino acid peptide (Thr-Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2) primarily expressed in non-neuronal cells (Zhang et al., 2000; Kurtz et al., 2002; Page et al., 2003). Expression of HK-1 was initially demonstrated in mouse bone marrow and thymus with additional evidence that in bone marrow, HK-1 expression was largely restricted to B cells (Zhang et al., 2000). Further data, however, have illustrated moderate to strong expression in the brain, spleen, stomach, skin, lactating breast, and uterus (Kurtz et al., 2002; Patak et al., 2003). Unfortunately, lack of a specific anti-HK-1 antibody has hindered the study of cell- and tissue-specific production and expression of the HK-1 peptide to date.

Receptor binding assays demonstrate a similar high affinity, and therefore specificity of HK-1 for the SP-specific receptor, neurokinin-1 (Morteau et al., 2001; Kurtz et al., 2002; Duffy et al., 2003). Pharmacological studies also illustrate a similar pharmacological profile, where HK-1 behaves as a full agonist displaying similar potencies for neurokinin receptors as SP. Neurokinin-1 receptor-specific antagonists demonstrated the same inhibitory effects with similar potencies on HK-1 and SP as well (Camarda et al., 2002). *In vivo* studies show HK-1 capable of generating the same dose-dependent salivary secretion and reduction in blood pressure as SP (Duffy et al., 2003). Moreover, HK-1 promotes proliferation of B cells and T cells. And similarly, neurokinin-1 receptor antagonists decrease cell survival for both B cells and T cells, which can be partially reversed by the addition of HK-1 (Zhang et al., 2000; Zhang et al., 2003). One would assume the restorative effects of HK-1 could be mimicked by SP. However, SP was not able to enhance B cell proliferation as seen with HK-1 (Zhang et al., 2000; Zhang et al., 2003). These data provide compelling evidence for HK-1's role in modulating inflammation and raise questions regarding the previously understood role of SP in the periphery.

1.6 Tachykinin Receptors

The biological actions of tachykinins are mediated via membrane-bound receptors belonging to the superfamily of G protein-coupled receptors (GPCRs) (Gerard et al., 1993). Three distinct tachykinin receptors have been cloned: neurokinin-1 (NK-1), neurokinin-2 (NK-2), and neurokinin-3 (NK-3). These

receptors share the same structural motif, seven hydrophobic transmembrane domains with three intracellular and three extracellular loops (Krause et al., 1993; Maggi, 1995). While each tachykinin (SP, NKA, and NKB) act as full agonists at all three receptors, the receptors are recognized with moderate selectivity showing preferentially binding to the NK-1, NK-2, and NK-3 receptor, respectively (Mussap et al., 1993; Regoli et al., 1994). Ligand binding to the receptor results in a cascade of events promoting the formation of intracellular second messengers such as cAMP, inositol phosphates, diacylglycerol, and calcium and ultimately providing their biological actions. Persistent activation of the receptors triggers rapid internalization, recycling, and degradation of the receptor, which may be a contributing factor to the rapid desensitization of GPCRs (Collins et al., 1992; Garland et al., 1994; Grady et al., 1995).

Neurokinin-1 receptors, the preferred receptor of SP and HK-1, are expressed by neurons and glia in the central nervous system, neurons within the mesenteric plexus, smooth muscle cells, acinar cells, endothelial cells, fibroblasts, keratinocytes, and various circulating immune cells and inflammation-activated immune cells (Krause et al., 1992; Ansel et al., 1996; Bowden et al., 1996). Immune cells expressing NK-1 mRNA are monocytes (Li et al., 2000), macrophages (Ho et al., 1997), lymphocytes (Lai et al., 1998; Li et al., 2000; Qian et al., 2001), and fetal microglia (Lai et al., 2000). Using anti-NK-1 antibodies, receptor protein has been detected in macrophages (Marriott et al., 2000), dendritic cells (Marriott et al., 2001), and microglia (Rasley et al., 2002) as well.

Much of the emphasis to date has been on the detection of SP and NK-1 receptors within various cell types, and therefore the location of NK-2 and NK-3 receptors in the periphery is largely unknown. However, NK-2 receptors have been localized to specific nuclei within the brain (Pinto et al., 2004), smooth muscle cells (Haley et al., 2001), keratinocytes (Song et al., 2000), and eosinophils (Renzi et al., 2000). Activation of NK-2 receptors on smooth muscle cells results in vasodilation. These effects have been well documented within the airways (Chapman et al., 1998) and lungs (Haley et al., 2001). The expression and release of cytokines from keratinocytes are modulated by NK-2 receptors as well (Song et al., 2000). The tachykinin NK-3 receptor is mainly expressed in the CNS, and has only been detected in certain peripheral tissues such as the uterus, skeletal muscle, lung, and liver (Tsuchida et al., 1990; Massi et al., 2000; Lecci et al., 2003; Patak et al., 2003).

1.7 Opioid Peptides

Opioid peptides are endogenous neuromodulators that play a pivotal role in nociceptive pathways within the central nervous system. Opioid peptides evoke potent analgesia via inhibition of ascending excitatory transmissions and activation of descending inhibitory systems (Fields et al., 1999; Yaksh, 1999). However, opioid drugs also produce undesirable side-effects such as gastrointestinal problems, respiratory depression, and tolerance and dependence when administered systemically restricting their effectiveness. The actions of opioid compounds are induced by activation of opioid receptors located both centrally and peripherally. The dose-

limiting side-effects of centrally-acting opioid drugs prompted the investigation of peripheral opioid analgesia as an alternative therapeutic approach. Administered topically during peripheral inflammation, opioid drugs exert powerful antinociceptive effects (Antonijevic et al., 1995) via activation of peripheral opioid receptors on primary sensory nerve terminals (Zhou et al., 1998). The mode of action is believed to be local rather than systemic, avoiding any central effects. In addition, while all opioid receptor subtypes (mu, delta, kappa) are present to some degree on peripheral nerve terminals, mu receptors primarily generate peripheral analgesia (Stein et al., 1989). Thus, exogenous opioid drugs with specific affinity for mu receptors are most effective in providing analgesia (e.g., morphine, fentanyl, sufentanil, methadone, and hydromorphone).

Local administration of exogenous opioid drugs at the site of inflammation has demonstrated therapeutic benefits in humans. Intra-articular administration of morphine reverses the hyperalgesia associated with osteoarthritis (Stein et al., 1999) or seen following arthroscopic knee surgery (Kalso et al., 1997). Researchers have also investigated the use of topically applied opioid compounds for pain associated with open wounds and burns. Patients receiving exogenous opioid-infused gel treatments reported lower pain ratings and used less oral morphine than those in placebo groups (Back et al., 1995; Twillman et al., 1999; Long et al., 2001; Cerchietti et al., 2002; Zeppetella et al., 2003). These studies indicate that the topical application of morphine or other opioid analgesics to open cutaneous wounds offers a promising analgesic strategy without eliciting centrally-mediated adverse effects.

In addition to the beneficial antinociceptive effects, opioid drugs also exhibit anti-immune and anti-inflammatory effects which may limit their usefulness in this approach of alleviating pain. Opioid binding to receptors at peripheral sensory nerve terminals inhibits action potential generation due to an increase in potassium and decrease in calcium currents (Werz et al., 1983b, 1983a; Schroeder et al., 1991). The inhibition of neuronal firing suppresses the release of sensory neuropeptides including SP, NKA, and CGRP (Yaksh et al., 1980; Aimone et al., 1989; Collin et al., 1993). Consequently, peripheral administration of opioid compounds during inflammation blocks edema (Binder et al., 2001) and plasma extravasation (Green et al., 1992; Taylor et al., 2000).

1.8 Conclusion

Neuropeptides, such as SP and NKA facilitate wound healing by regulating blood flow and modulating the migration and function of immunocompetent and inflammatory cells, as well as epithelial and endothelial cells within the wound. Neuropeptides serve as a link between the immune and nervous system. This has been validated by the demonstration of direct peptidergic innervation of primary and secondary lymphoid organs, the close proximity between sensory nerve endings and immune cells, and specific neuropeptide receptors located on immune effector cells. Abundant evidence exists demonstrating that neuropeptides contribute toward many of the processes involved in inflammation that are essential for normal wound healing.

The topical application of opioids successfully reduces the pain associated with cutaneous wounds. Although topical opioid compounds offer a promising new therapeutic strategy for alleviating pain, there is little information available regarding the effects of opioid drugs on wound healing, which could complicate their topical use in some circumstances. The present study was undertaken to address this issue.

CHAPTER TWO

STATEMENT OF PURPOSE

Chronic cutaneous wounds, such as burns and skin ulcers, result in prolonged hospitalization and considerable morbidity and remain a significant burden on our health care system. Pain associated with chronic wounds can be particularly difficult to manage and many patients continue to experience considerable pain despite the use of systemic analgesics. Topical application of opioid peptides can successfully reduce the pain associated with cutaneous wounds (Long et al., 2001) likely through activation of opioid receptors located on peripheral afferent sensory neuron terminals (Stein et al., 1993). Topical application of opioid compounds avoids negative central effects, but may adversely affect wound healing as they are known to suppress the release of neuropeptides from sensory neuron terminals. Smith et al. (2002) demonstrated that sensory neurons are essential for normal cutaneous wound healing. Neuropeptides such as SP and NKA facilitate wound healing by modulating the function of immunocompetent and inflammatory cells, as well as epithelial and endothelial cells within the wound. **Thus, the studies in this dissertation were designed to test the hypothesis that topical morphine application delays cutaneous wound healing via mechanisms dependent upon peripheral neuropeptide activity.** The objective of this research program was to address the impact morphine and sensory neuropeptides have on cutaneous wound healing. The resulting data present novel and significant details concerning both the basic science and role of sensory neurons and opioid peptides in wound healing.

2.1 Specific Aim #1: *Determine morphine's ability to activate opioid receptors within the peri-wound area and inhibit neuropeptide actions, resulting in delayed wound closure.*

Analgesia during topical morphine administration is obtained via activation of opioid receptors located on primary afferent nerve terminals in peripheral tissues. Activation of these receptors inhibits action potential generation within pain-sensing fibers, blocking transmission of the noxious signal to the central nervous system. Consequently, the general inhibition of primary afferent neurons also suppresses the release of neuropeptides into peripheral tissues. During inflammation, neuropeptides (such as SP and NKA) are released from peripheral terminals of primary afferent nerves where they mediate the early components of the neurogenic inflammatory response (e.g. vasodilation, plasma extravasation, and immune cell infiltration). Previous studies demonstrate that topical morphine application delays cutaneous wound healing. Therefore, a standardized model of cutaneous wound healing was used to determine wound closure rates in rats treated topically with increasing concentrations of morphine sulfate. The goal of this study was two-fold: to determine an optimal morphine concentration for future studies, and to determine whether any morphine-induced delays in wound closure were concentration-dependent, supporting a receptor-mediated effect.

The biological actions of SP and NKA are mediated primarily via NK-1 and NK-2 receptors. If morphine-induced delays in wound closure are a consequence of diminished neuropeptide release from primary afferent neurons, then functional

blockade of peripheral neuropeptide receptors should display similar effects as morphine application on wound closure. Therefore, wounds were treated topically with selective, non-peptide NK-1 and NK-2 receptor antagonists and wound closure rates assessed. In addition, inclusion of exogenous SP or NKA in morphine-infused gel treatments was used to determined gel to determine whether deficits in wound healing induced by morphine can be overcome by exogenous application of the sensory neuropeptide. Studies have shown that cutaneous wound closure rates are increased by application of exogenous neuropeptides. Morphine potently inhibits neuropeptide release. Therefore, replacement of the diminished neuropeptides may potentially restore normal wound healing rates and help identify the role of peripheral neuropeptides in wound healing and mechanisms by which morphine delays wound closure.

2.2 Specific Aim #2: *Determine whether morphine's site of action is presynaptic or postsynaptic.*

Morphine exerts its effect via activation of opioid receptors. Peripheral opioid receptors have been characterized not only on pre-synaptic sensory neuron terminals, but also on post-synaptic, non-neuronal neuropeptide targets including fibroblasts, keratinocytes, immune and inflammatory cells, and vascular endothelium. It has been proposed that topical morphine might delay wound closure by pre-synaptic inhibition of neuropeptide release. However, morphine may be directly inhibiting non-neuronal cells and structures necessary for normal wound healing. Therefore, studies within

this aim utilized pretreatments of the neurotoxin capsaicin to selectively deplete the skin of adult rats of small-diameter sensory neurons and/or the neuropeptides contained within their terminals. Denervated rats were treated topically with morphine, SP or NKA, or morphine combined with SP or NKA and wound closure rates analyzed. Functionally removing morphine's pre-synaptic target provides a method for determining morphine's site of action.

2.3 Specific Aim #3: *Determine the temporal effects of morphine on wound closure rates, wound inflammatory and parenchymal cells, and healed skin architecture.*

The studies in aims 1 and 2 revealed that topical application of morphine significantly delays cutaneous wound closure rates in rats. Interestingly, morphine-induced delays in wound closure are immediately apparent, occurring only during the early phase of wound healing. This transient delay is followed by acceleration in wound closure, resulting in morphine-treated wounds closing at times similar to controls. Wounds were treated with morphine for either the first four days of healing only or beginning three days post-wounding and continuing through the course of closure, and wound closure rates were measured. The experiments in aim 3 were designed primarily to assess the temporal effects of topical morphine application on wound closure rates. Additionally, immunohistochemistry was conducted on wounds treated throughout the entire time course with morphine. Wound tissue was taken on days 0, 1, 3, 5, and 8 post-wounding and stained with antibodies against macrophages and myofibroblasts to determine what impact morphine has on inflammatory and

parenchymal cells essential in the healing process and when cellular alterations occur during the course of healing.

Although morphine-treated wounds ultimately closed at times similar to controls, disruption of normal closure may produce long-term effects that outlast the duration of topical morphine administration. Accordingly, the structural architecture of healed skin was evaluated after closure of cutaneous wounds treated with topical morphine. Residual scar area was determined on wound day 18. In addition, the dermal and epidermal thickness of the skin was determined. Wounds of gel-only treated control rats close on average nine days following wounding. Therefore, healed skin was analyzed approximately nine days following closure. Based on calculations using average life spans for rats and humans (2 and 75 years, respectively) the 18-day time point correlates to approximately one year in humans.

Studies have demonstrated that an endogenous peripheral opioid tone exists during states of inflammation. This endogenous tone, produced primarily from peripheral immune cells, is sufficient to reduce nociceptive behaviors in rodents and, therefore, may alter wound healing. Wounds were treated with the non-selective opioid receptor antagonist, naltrexone, and wound closure rates were assessed. Blocking the actions of endogenous opioid receptors should reveal what, if any, effect endogenous opioid tone has on wound closure.

2.4 Specific Aim #4: *Examine the regulation of neurokinin receptors in peripheral targets through which the effects of sensory neuropeptides are mediated.*

The biological actions of SP and NKA are mediated via neurokinin-1 and neurokinin-2 receptors, respectively. Neurokinin receptors are located on several cell types essential for normal wound healing, including vascular smooth muscle and endothelial cells, fibroblasts, keratinocytes, monocytes, macrophages, and lymphocytes. Studies using NK-1 receptor knock-out mice demonstrate that blocking the biological responses of the neurokinin receptors to their respective neuropeptides results in delayed wound healing. In addition, previous work within this laboratory has indicated a significant increase in neurokinin-1 receptor mRNA levels in the peri-wound area of homogenized tissue by day 7 post-wounding. This up-regulation is inhibited by exogenous application of either morphine or a selective neurokinin-1 antagonist. Therefore, this aim was designed to better characterize the mechanisms by which topical morphine inhibits wound closure by defining the expression patterns of neurokinin receptors in the closing wound and their regulation by morphine. Wound tissue was dissected on days 0, 1, 3, 5, and 8 post-wounding. To assess neurokinin receptor protein distribution and regulation during wound healing, double-labeling, fluorescence immunohistochemistry experiments were performed. Tissue sections were incubated with either NK-1 or NK-2 receptor antibodies in combination with markers for macrophages, vasculature, or myofibroblasts. The results of these experiments revealed alterations in the availability of neuropeptide receptors during topical morphine treatment, as well as the localization of the receptors in peripheral targets during cutaneous wound healing.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animal Handling and Housing

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) at approximately 8 weeks of age (200-220 grams body weight) were utilized for all experiments. All rats were housed individually to prevent cage mates from grooming or otherwise perturbing the wounds. Handling of animals was unchanged to reduce any variation between treatment groups. Animal facilities were temperature- and humidity-controlled with a 12-h dark–light cycle and food and water *ad libitum*. All surgical procedures and animal handling were performed in accordance with National Institutes of Health laboratory care standards and approved by the University of Kansas Medical Center Animal Care and Use Committee. Every effort was made to ensure minimal animal suffering and reduce animal numbers used for each study.

3.2 Drug Preparation and Gel Administration

IntraSite® Gel was utilized as a vehicle for the topical application of all compounds. The amorphous, hydrogel is a transparent aqueous gel containing a cellulose polymer with propylene glycol as a humicant. IntraSite® Gel maintains hydration of the wound promoting rapid debridement, autolysis of necrotic tissue, and reepithelialization. Wounds commonly treated with IntraSite® Gel include burns, ulcers, and pressure sores. IntraSite® Gel is also frequently utilized as a vehicle for the topical application of antimicrobial agents.

Drug solutions were made the day of or the day prior to use and were stored at 4^{o} C. Morphine sulfate (25 mg/mL) (Abbott Laboratories, Inc., North Chicago, IL)

was directly infused into IntraSite® Gel (amorphous hydrogel; Smith+Nephew, England). The peptides, SP and NKA (Sigma-Aldrich, St. Louis, MO), NK-1 and NK-2 receptor antagonists, RP 67580 and GR 159897 (Tocris, Ellisville, Missouri), and naltrexone were solubilized in 0.9% saline and then infused into IntraSite® Gel to their final concentrations. Control animals received IntraSite® Gel alone treatments. Saline was added to control gel to match the consistency of the drug-infused gels. Beginning one hour post-surgery, 150 µL IntraSite® Gel alone or IntraSite® Gel infused with a drug was applied to the wounds every 12 hours for the duration of the study. Gel and drug were combined in 3 cc syringes by repeated passage through a Luer-lock stopcock.

3.3 Capsaicin Pretreatment

Systemic administration of the neurotoxin capsaicin results in the depletion of sensory neuropeptides and permanent degeneration of small-diameter C-fibers and was used as a chemical method of sensory denervation. A 10 mg/mL capsaicin (Sigma-Aldrich, St. Louis, MO) solution containing 20% ethanol and 10% Tween 80 was prepared in physiological saline. Vehicle or capsaicin was injected subcutaneously on three consecutive days (30 mg/kg on day 1, 50 mg/kg on day 2, 70 mg/kg on day 3) (Zhou et al., 1998). Animals were wounded 96 hours following the last injection. Solutions were prepared each day prior to treatment, and injections were performed under inhaled isoflurane anesthesia. The efficiency of the capsaicin pretreatment in depleting and/or destroying primary afferent neurons was determined

utilizing immunohistochemistry to assess the number of CGRP-immunoreactive (-ir) intraepidermal nerve fibers in mid-periscapular skin.

3.4 Wounding Procedure

A cutaneous wound healing model was utilized to evaluate wound closure rates in rats. This model provides sensitivity sufficient to detect age-related variations in wound closure, as well as the negative impact of partial sensory denervation on wound healing (Liu et al., 1999; Smith et al., 2002). All surgical procedures were conducted under sterile conditions. Rats were anesthetized by intraperitoneal administration of 65 mg/kg ketamine hydrochloride and 5.5 mg/kg xylazine hydrochloride and the mid-periscapular region clipped and shaved. Using a skin biopsy punch, a 4-mm diameter (12.6 mm^2) full-thickness circular skin flap was excised from the midline just below the scapulae to a depth just above the panniculus carnosus muscle (Liu et al., 1999). The wounding procedure causes minimal bleeding. Any excess blood is removed using sterile gauze and wounds are left uncovered.

3.5 Wound Imaging and Data Analysis

Wound images were captured each morning prior to treatment using a hand-held digital camera. A bar attached to the camera provided a fixed focal distance target for wound imaging. A size standard with known surface area was attached to the target bar and included in each image. Wound area was measured using a

computerized planimetric program (Scion Image, Fredrick, MD). The area occupied by the wound was defined by the boundary created by the granulation tissue or scab/intact tissue interface. Wound area data generated by Scion Image software were converted from pixels to area units of mm^2 by comparison to the known area of the fixed size standard and are reported as area (mm^2) mean ± SEM.

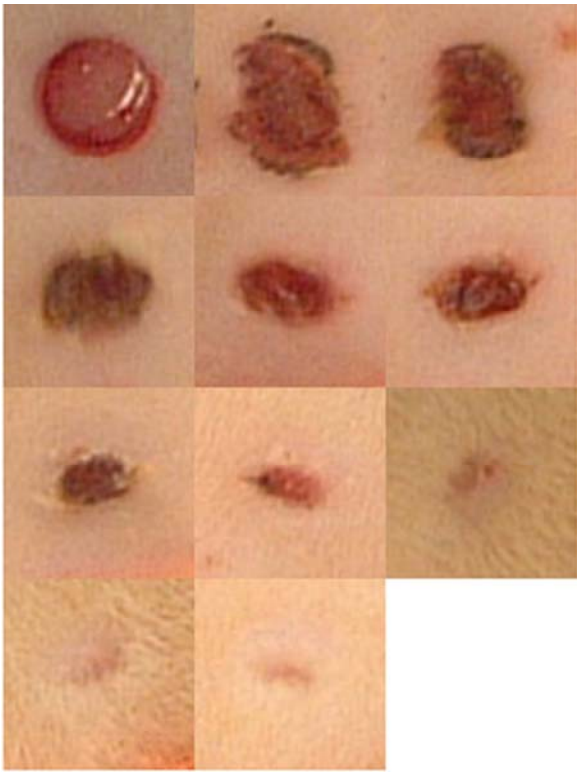


Figure 5. Wound healing in a rat receiving topical gel-only treatment. Images of the wound were captured daily on days 0 – 10 post-wounding.

3.6 Tissue Harvesting

Rats were decapitated and wound tissue including approximately 1.0 cm of surrounding intact skin was dissected. Tissue was embedded in tissue freezing medium (Electron Microscopy Sciences, Hatfield, PA), frozen on dry ice, and cryosectioned serially throughout the center of the wound at 14 μm thickness. Sections at ten-section intervals were placed on adjacent slides and stored at -80ºC until staining.

3.7 Histochemistry

Sections were post-fixed at room temperature in 4% paraformaldehyde for 5 minutes and subsequently stained with hematoxylin and eosin. Slides were mounted with Permount (Fisher, St. Louis, MO) and images were captured using light microscopy at a magnification of 20x (Nikon Eclipse 80i microscope, Nikon Digital Sight Fi1 camera, Melville, NY). Epidermal and full-skin thicknesses were quantified in 3 sections equally spaced throughout each wound. In each analyzed section, two regions were selected randomly. All measurements were obtained by a blinded observer using ImageJ (NIH, Bethesda, MD) analysis software.

3.8 Immunohistochemistry

Tissue sections stained for macrosialin and α-smooth muscle actin (α-SMA) were post-fixed in 4% paraformaldehyde for 5 minutes, blocked in 5% goat serum for 1 hour, and directly immunostained (90 minutes at room temperature) for the

macrophage marker macrosialin (rat polyclonal IgG, fluorescein isothiocyanate conjugated, 1:200, clone FA-11, Serotec, Oxford, United Kingdom) or the myofibroblast marker α-SMA (mouse monoclonal IgG 2A, Cy3 conjugated, 1:200, clone 1A4, Sigma, St. Louis, MO)]. Rat serum was added to the blocking serum of sections stained with macrosialin antisera to negate the impact of non-specific staining. In addition, a rat IgG antibody conjugated to fluorescein isothiocyanate (1:100, Serotec, clone YTH71.3) was utilized as a negative control, and no specific staining was detected with this approach. In pilot studies, ED1 mouse monoclonal IgG (Chemicon, Temecula, CA), CD68 goat polyclonal (Santa Cruz, Santa Cruz, CA), ED1 mouse monoclonal IgG (Serotec), and CD68 mouse monoclonal IgG (Biomeda, Foster City, CA) antibodies were evaluated and they demonstrated similar staining patterns. However, they exhibited reduced sensitivity compared to the antibody selected and were thus not utilized for quantification.

For indirect single staining of CGRP-positive neurons, tissue sections were post-fixed in Zamboni's fixative for 5 minutes, blocked in 5% goat serum for 1 hour, and incubated overnight at room temperature with a polyclonal antibody (1:500 CGRP rabbit IgG, Chemicon, Temecula, CA), followed by incubation for 90 minutes at room temperature with a cy3-conjugated goat anti-rabbit IgG secondary antibody (1:200, Jackson Immunoresearch Labs, West Grove, PA).

For double-labeling studies, sections were post-fixed in 4% paraformaldehyde for 5 minutes, blocked in 5% goat serum for 1 hour, and incubated overnight at room temperature with a polyclonal antibody for the NK-1 receptor (rabbit polyclonal IgG,

1:200, Novus Biologicals, Littleton, CO) or the NK-2 receptor (rabbit polyclonal IgG, 1:200, Novus) in combination with either the macrophage marker macrosialin (rat polyclonal IgG, FITC conjugated, 1:200, clone FA-11, Serotec, Oxford, United Kingdom) or the myofibroblast marker α-SMA (mouse monoclonal IgG, 1:500, clone 1A4, Sigma). NK-1 receptor and NK-2 receptor labeling was visualized by subsequent incubation for 90 minutes at room temperature with a cy3-conjugated goat anti-rabbit IgG secondary antibody (1:200, Jackson), while α-SMA visualization was achieved with goat anti-mouse IgG secondary antibody conjugated to cy2 (1:500, Jackson). Primary antiserum omission controls for each non-conjugated antibody demonstrated no specific staining.

3.9 Imaging and Quantification

Slides were mounted with Fluoromount G (Fisher) and viewed using a Nikon Eclipse 80i microscope. Images were captured with a Nikon Digital Sight Fi1 camera and adjusted for brightness and contrast. Immunoreactive morphological structures were quantified in 3 sections equally spaced throughout each wound. In each analyzed section, four regions, each equaling an area of 0.35 mm^2, were selected randomly; two images from within the wound and two at the edge of the lesion were analyzed.

Numbers of cells macrosialin-ir were averaged and expressed as counts per unit area. The α-SMA-ir area was obtained by threshold discrimination, divided by the total area analyzed and expressed as a percent of field area. α-SMA-ir vasculature

was excluded from analysis of myofibroblasts and only cells demonstrating a spindle-shaped morphology were included. Neurokinin receptor-ir area of keratinocytes was obtained by threshold discrimination and then divided by the total area analyzed and expressed as a percent of field area. CGRP-ir intraepidermal nerve fibers were assessed utilizing the European Federation of Neurological Societies guidelines for quantification (Lauria et al., 2005). For double-labeling studies, numbers of cells macrosialin- and NK-1 or NK-2 receptor-ir were averaged and expressed as percentage of neurokinin receptor-positive macrophages per unit area. Neurokinin receptor- and α-SMA-ir area of blood vessels and myofibroblasts were also obtained by threshold discrimination. All images were evaluated by a blind observer using Metamorph (Molecular Devices, Downingtown, PA) analysis software.

3.10 Statistical Analyses

Statistical analyses were performed using SigmaStat (San Jose, CA). Data are reported as mean ± SEM. For wound time course data, the effect of drug treatment and time on wound closure, macrophage infiltration, myofibroblast activation, and neurokinin receptor regulation were evaluated using separate two-way repeated measures analyses of variance (ANOVA). Differences between treatment groups and within treatment groups over time were identified using Tukey post-hoc tests. The long-term effects of morphine application on skin thickness and residual scar area were analyzed using unpaired t-tests. Differences between means were considered significant when $p < 0.05$.

CHAPTER FOUR

SPECIFIC AIM # 1

4.1 Hypothesis (SA #1)

Delayed cutaneous wound healing during topical morphine application is mediated via the activation of opioid receptors located on peripheral sensory nerve terminals, resulting in the inhibition of neuropeptide actions within the peri-wound area.

4.2 Rationale (SA #1)

Tissue damage results in intracellular contents being released into the interstitial fluid. Included are substances (e.g., prostaglandins, serotonin, ATP, and protons) that are capable of stimulating sensory afferent nerve terminals. Stimulation of these nerve fibers induces the peripheral release of neuropeptides such as SP and NKA into the periphery. Neuropeptide-induced activation of peripheral targets including blood vessels, epithelial cells, and immune cells initiates and perpetuates inflammation and tissue repair, which are key components in the healing process. Previous studies have demonstrated that sensory neurons, and more specifically the neuropeptides stored within them, are essential for normal wound healing. The biological actions of SP and NKA are mediated via the membrane-bound GPCRs, NK-1 and NK-2 respectively. Activation of neurokinin receptors results in vasodilation, increased vascular permeability, magnification of the inflammatory response, as well as the recruitment, proliferation, and activation of various immune and parenchymal cells essential for normal wound healing.

Morphine exerts its analgesic effects via activation of opioid receptors. In the periphery these effects are mediated via receptors located on sensory afferent nerve terminals. Anti-nociception can be attained by inhibiting the generation of action potentials and, consequently, the transmission of nociceptive signals. However, the analgesia attained by topical opioid compounds is accompanied by a more general suppression of sensory afferent nerve function. Opioid drugs are known to prevent the peripheral release of pro-inflammatory neuropeptides from sensory afferent nerves, thus inhibiting the progression of cutaneous wound healing processes. Experiments within this aim addressed morphine's ability to activate opioid receptors within the peri-wound area resulting in a delay in wound closure. In addition, the role SP and NKA play in wound healing and how their effects are altered by topical morphine administration was explored.

4.3 Experimental Design (SA #1)

A standardized model of cutaneous wound healing will be utilized to determine the wound healing rate in rats. This model of wound healing provides sensitivity sufficient to detect age-related differences in wound healing capacity, as well as the negative impact of partial sensory denervation (Liu et al., 1999; Smith et al., 2002). Ninety-nine male Sprague-Dawley rats (Harlan, Indianapolis, IN) at approximately 8 weeks of age (200-220 grams body weight) were randomly assigned to one of fifteen treatment groups. Rats were then anesthetized by intraperitoneal administration of 65 mg/kg ketamine HCl and 5.5 mg/kg xylazine HCl and the mid-

periscapular region shaved. A 4-mm diameter (12.6 mm^2) full-thickness circular skin flap was excised from the midline just below the scapulae using a skin biopsy punch to a depth just above the panniculus carnosus muscle (Liu et al., 1999). All rats were subsequently housed individually to prevent cage mates from grooming or otherwise perturbing the wound. Animal facilities were temperature- and humidity-controlled with a 12-h dark–light cycle and food and water ad libitum. All surgical procedures and animal handling were performed in accordance with National Institutes of Health laboratory care standards and approved by the University of Kansas Medical Center Animal Care and Use Committee. Eight treatment groups were established (Table 2).

Treatment	**n**
IntraSite® Gel Only	25
Gel + 0.5 mM Morphine Sulfate	7
Gel + 1.5 mM Morphine Sulfate	7
Gel + 5 mM Morphine Sulfate	11
Gel + 15 mM Morphine Sulfate	7
Gel + 1 mM NK-1 Antagonist RP67580	8
Gel + 1 mM NK-2 Antagonist GR159897	6
Gel + 1 mM SP	5
Gel + 1 mM NKA	5
Gel + 5 mM Morphine Sulfate (Abbott) + 1 mM SP	5
Gel + 5 mM Morphine Sulfate (Abbott) + 1 mM NKA	5

Table 2. IntraSite® Gel treatments for Specific Aim 1.

Beginning one hour post-surgery, ~150 μL IntraSite® Gel (amorphous hydrogel; Smith+Nephew, England) alone or IntraSite® Gel infused with a drug was applied twice daily for 10-14 days. Each morning prior to the first treatment for that day, wound images were captured using a hand-held digital camera. A bar attached to the camera provided a fixed focal distance target for wound imaging. A size standard with known surface area was fastened to the target bar and included in each image. Wound area was determined daily using a computerized planimetric program (Scion Image, Fredrick, MD). The area occupied by the wound was defined by the boundary created by the granulation tissue or scab/intact tissue interface. Wound area data generated by Scion Image were converted from pixels to area units of mm^2 by comparison to the known area of the fixed size standard and are reported as area (mm^2) mean ± SEM.

The first experiment utilize this standardized model of cutaneous wound healing to determine a concentration-response curve for morphine sulfate on wound healing rate. Animals were divided into five groups **(Table 2)**: gel only (n = 6), gel + 0.5 mM morphine sulfate (n = 7), gel + 1.5 mM morphine sulfate (n = 7), gel + 5 mM morphine sulfate (n = 6) and gel + 15 mM morphine sulfate (n = 7). The following three experiments used the same cutaneous wound healing model. The second study contained four treatment groups consisting of two separate gel only (n = 14), gel + NK-1 antagonist RP67580 (n = 8), gel + NK-2 antagonist GR159897 (n = 6). In the next experiment, rats were randomly divided into six treatment groups: gel only, gel

+ 5 mM morphine sulfate, gel + 1 mM SP, gel + 1 mM NKA, gel + 5 mM morphine sulfate + 1 mM SP, gel + 5 mM morphine sulfate + 1 mM NKA (n = 5 each).

A pilot study with gel containing peptidase inhibitors was utilized to determine the stability of the peptides within the hydrogel as well as the effects endogenous peptidases have on wound closure. Peptidase inhibitor-infused gel was prepared by combining 0.17 mg/mL bacitracin, 0.02 mg/mL leupeptin, 0.02 mg/mL chymostatin and 0.85 mg/mL BSA in IntraSite® Gel. This study contained two groups consisting of gel only and gel + peptidase inhibitors (n = 4 each) and data was collected through day 5 post-wounding.

4.4 Statistical Analysis (SA #1)

Statistical analyses were performed using SigmaStat (San Jose, CA). The effects of drug treatment and time on wound closure were evaluated using separate two-way repeated measures analyses of variance. Differences between treatment groups and within treatment groups over time were identified using Tukey post-hoc tests. Differences between means were considered significant when $p < 0.05$.

4.5 Results (SA #1)

Effects of topical administration of morphine sulfate on cutaneous wound closure rates

The impact of increasing concentrations of topical morphine sulfate application on cutaneous wound closure rates in rats was assessed using a standardized model of cutaneous wound healing. Animals receiving topical morphine

sulfate treatment demonstrated a significant delay in wound closure rates when compared to gel-only treated controls. In animals receiving 0.5 mM morphine sulfate applications, wound area was significantly larger on days 1 and 6 post-wounding (**Figure 6**). The wound area of animals receiving 1.5 mM morphine sulfate treatments was significantly larger on wound days 1-7. Topical application of 5 mM morphine sulfate significantly increased wound area on wound days 1-9, whereas 15 mM morphine sulfate treatment produced significantly larger wounds on days 1-10 post-wounding. In addition, the delay in wound contraction observed in morphine-treated animals increased in a concentration-dependent manner. Total wound area over the complete time course of animals receiving 0.5, 1.5, 5, and 15 mM morphine sulfate treatments was significantly larger (approximately 6, 16, 26, and 33% respectively) than gel-only treated control rats. In addition, the total wound area of animals treated with 1.5 mM morphine-sulfate was significantly larger than animals in the 0.5 mM morphine sulfate-treated group, and the total wound area of animals treated with 5 mM morphine sulfate was significantly larger than animals in the 1.5 mM morphine sulfate-treated group.

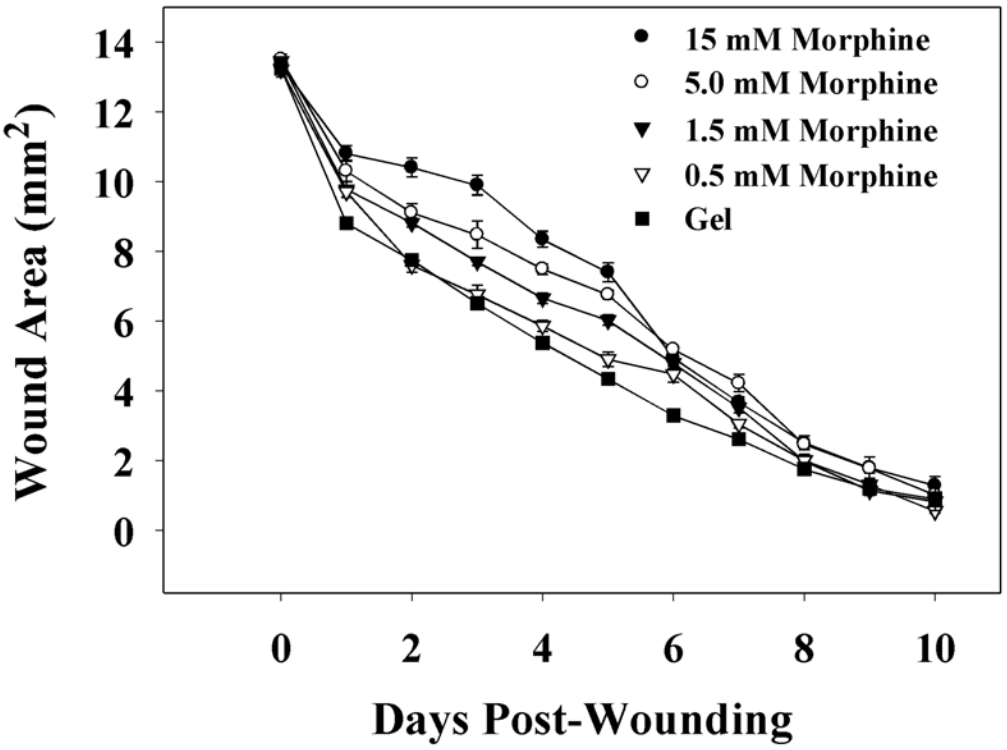
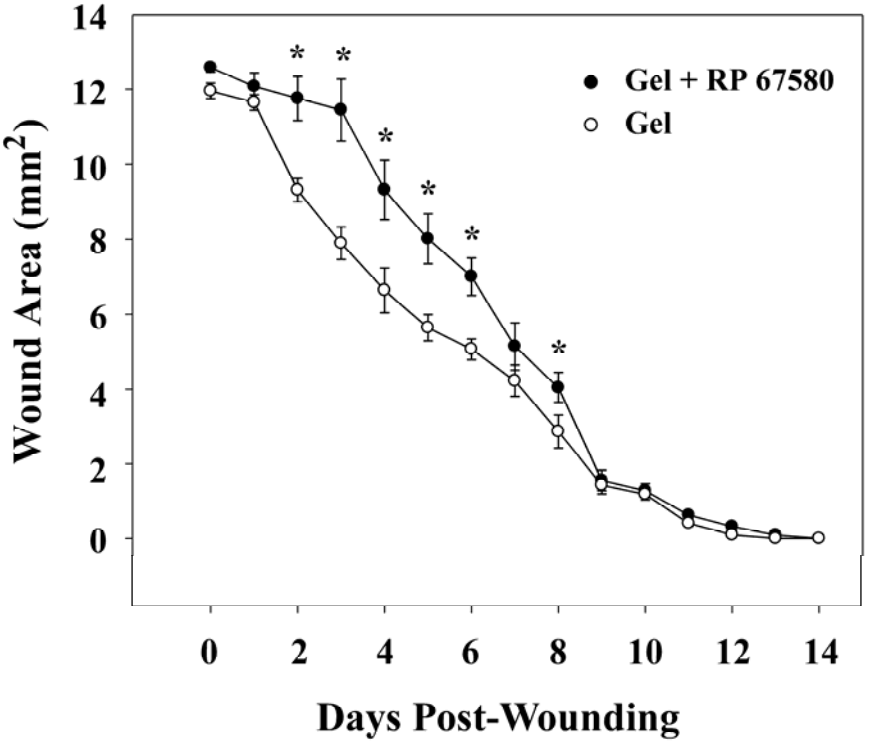


Figure 6 - Time course of wound closure for rats receiving increasing concentrations of morphine sulfate-infused gel treatments. IntraSite® gel (150 µl) was applied to the wound twice daily through wound day 10. Wound size is presented as area (mm^2) mean ± SEM and was determined by analysis of digital images. Note that IntraSite® gel infused with 0.5, 1.5, 5, or 15 mM morphine sulfate significantly delayed wound closure compared to gel-only treatment. Additionally, the delay in wound closure seen in gel + morphine treated animals increased in a concentration-dependent manner (n = 6 to 7; $p < 0.05$; two-way ANOVA, Tukey's post hoc test).

Effects of topical application of selective, non-peptide neurokinin-1 and neurokinin-2 receptor antagonists on cutaneous wound closure rates

Selective, non-peptide NK-1 and NK-2 receptor antagonists were utilized to determine the effects their topical administration have on cutaneous wound closure rates in rats. Animals receiving topical NK-1 or NK-2 receptor antagonists demonstrated a significant delay in wound closure rates when compared to gel-only treated controls. Wound area of animals treated with gel infused with 1 mM RP 67580, a selective NK-1 receptor antagonist, was significantly larger on days 2, 3, 4, 5, 6, and 8 post-wounding when compared to gel-only treated control animals (**Figure 7A**). A 25% increase in the total wound area over the complete time course of animals receiving the NK-1 receptor antagonist was seen when compared to controls. Similar results were observed in the wounds of animals receiving topical treatment with 3mM of the selective, non-peptide NK-2 receptor antagonist GR 159897. A significant increase in the area of the wounds was seen on wound days 1-8 (**Figure 7B**) with a 19% increase in the total wound area.

A


Gel + RP 67580
Gel
Wound Area (mm^2)
Days Post-Wounding
0
2
4
6
8
10
12
14

B

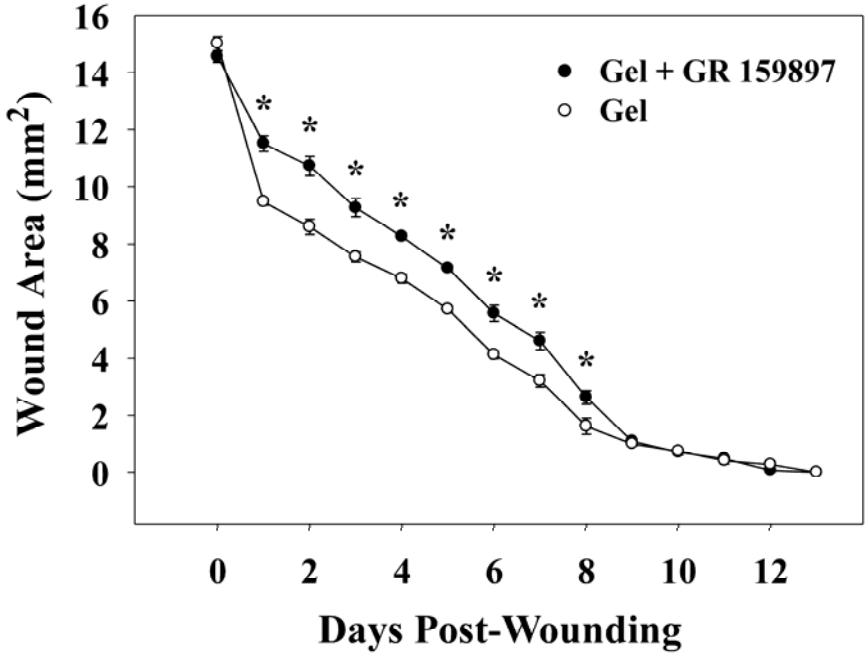
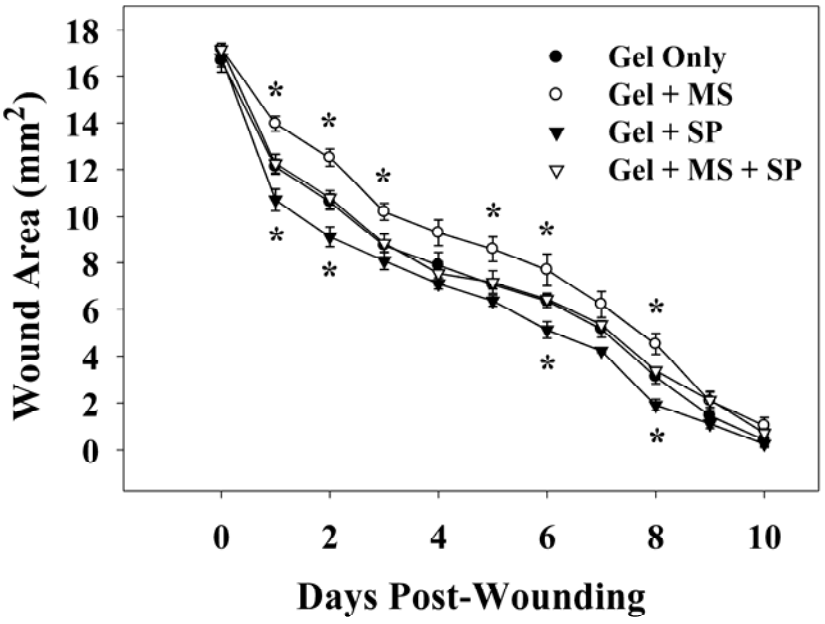

Gel + GR 159897
Gel
Wound Area (mm^2)
Days Post-Wounding
0
2
4
6
8
10
12
14
16

Figure 7 - Wound closure time course for rats receiving IntraSite® gel infused with the selective, nonpeptide NK-1 or NK-2 receptor antagonist, RP 67580 or GR 159897. Data are presented as area (mm^2) mean $\pm$ SEM and were determined by analysis of digital images. Rats received applications of IntraSite® gel (150 μL) to the wound twice daily through wound day 13 or 14. **(A)** IntraSite® gel infused with 1 mM RP 67580 (n = 8) significantly delayed wound closure compared to gel-only controls (n = 8). Gel + RP 67580 treated rats had significantly larger wound areas when compared to gel-only controls on wound days 2-6 and 8. **(B)** Treatment with 3 mM GR 159897 (n = 6) significantly delayed wound closure compared to gel-only controls (n = 6) with significant increases in wound area compared to control on days 1-8 post-wounding (*$p < 0.05$; two-way ANOVA, Tukey's post hoc test).

Effects of neuropeptide replacement in morphine sulfate-infused gel on cutaneous wound closure rates

The effects of the addition of SP or NKA into morphine sulfate-infused gel applications on wound closure rates in rats were assessed. As previously demonstrated, 5 mM morphine sulfate significantly increased the area of healing wounds. In this experiment, significant increases in wound area of morphine sulfate treated rats were seen on days 1, 2, 3, 5, 6 and 8 post-wounding (**Figure 8A & B**). A 17% increase in the total wound area was seen for animals in this treatment group. In addition, topical application of 1 mM SP significantly decreased the wound area on wound days 1, 2, 6, and 8 (**Figure 8A**), with an 11% decrease in the total wound area over the entire time course demonstrating acceleration in wound closure. However, a significant difference was not seen between topical treatment of 1 mM NKA and control (**Figure 8B**). Wounds treated with a combination of either 1 mM SP or 1 mM NKA and 5 mM morphine sulfate did not exhibit significant changes in wound area when compared to gel-only treated controls (**Figure 8A & B**). Furthermore, no noticeable erythema or pain-related behaviors were observed in rats receiving topical application of either peptide.

A


Wound Area (mm^2)
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Days Post-Wounding
Gel Only
Gel + MS
Gel + SP
Gel + MS + SP
*

B

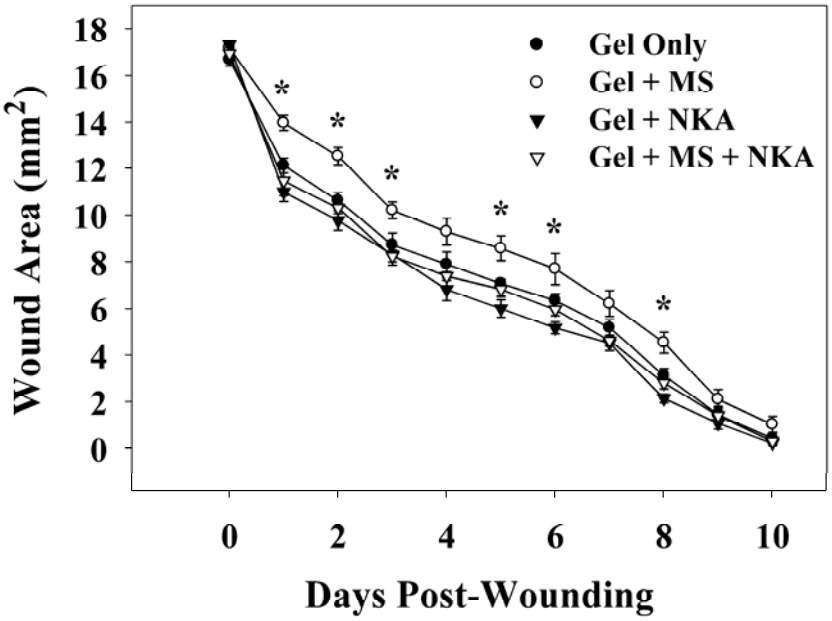

Wound Area (mm^2)
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Days Post-Wounding
Gel Only
Gel + MS
Gel + NKA
Gel + MS + NKA
*

Figure 8 - Wound closure time course for rats receiving IntraSite® gel treatments infused with morphine and/or neuropeptides. Rats were treated with IntraSite® gel (150 μL) twice daily through wound day 10. Wound size is presented as area (mm^2) mean ± SEM and was determined by analysis of digital images. IntraSite® gel infused with 5 mM morphine sulfate significantly delayed wound closure compared to gel-only treated controls. Wound area was significantly larger in the morphine treated rats when compared to the gel-only treated rats on wound days 1,2,3,5, and 8. **(A)** Rats were treated with 5 mM morphine sulfate, 1 mM SP, or 5 mM morphine sulfate + 1 mM SP. IntraSite® gel infused with SP significantly increased wound closure compared to gel-only treatment. In animals treated with SP wound area was significantly smaller than wounds of rats receiving gel-only on wound days 1, 2, 6, and 8. Note that a significant difference was not seen in rats receiving morphine + SP treatment when compared to controls. **(B)** IntraSite® gel treatments were infused with 5 mM morphine sulfate, 1 mM NKA, or 5 mM morphine sulfate + 1 mM NKA. Note a significant difference was not seen in either NKA or morphine + NKA treated groups when compared to controls (*$p < 0.05$; two-way ANOVA, Tukey's post hoc test; n = 5).

Effects of peptidase inhibitors on wound closure rates

Peptidase inhibitors were utilized to determine the effects of local peptidases and the stability of peptides within the healing wound. Rats were treated with peptidase inhibitor-infused gel on days 0 – 5 post-wounding and the area of the wounds quantified. The closure of wounds treated with peptidase inhibitors did not differ significantly from control wounds **(Figure 9)**.

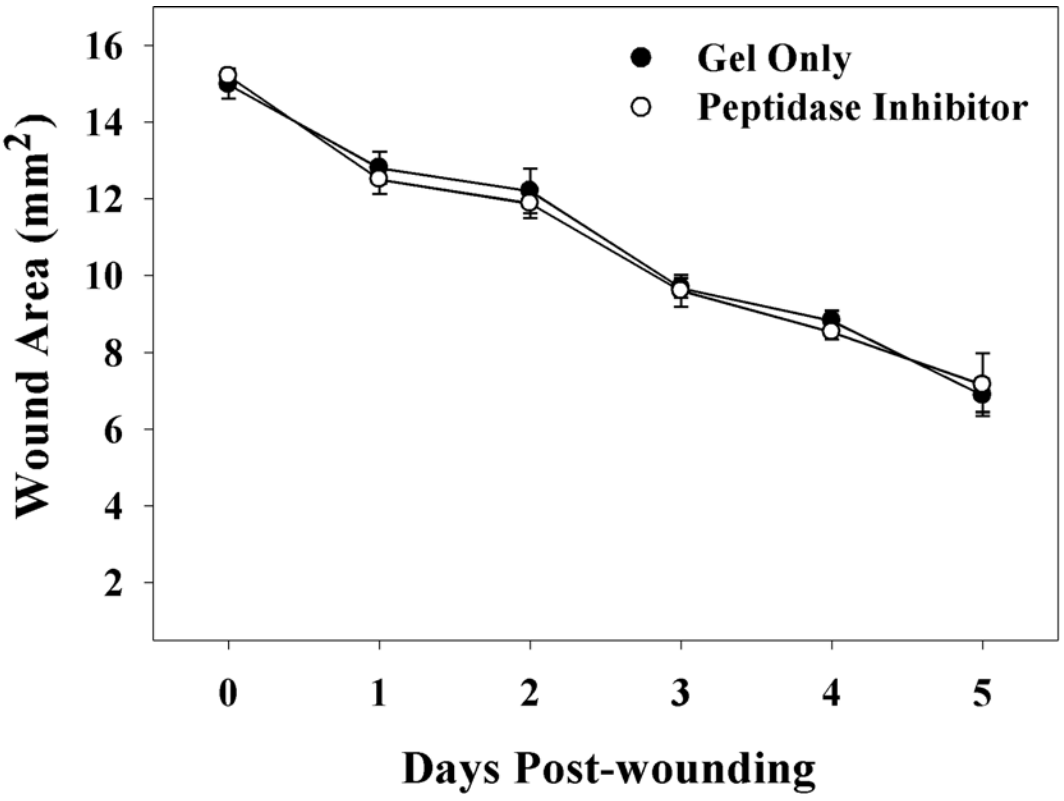


Figure 9 - Peptidase inhibitors' effect on wound closure. Rats were treated with IntraSite® gel (150 μL) twice daily through wound day 5. IntraSite® gel infused with peptidase inhibitors did not significantly alter wound closure rates compared to gel-only treated controls (two-way ANOVA; n = 4).

4.6 *Discussion (SA #1)*

Topical morphine is currently being used clinically to provide analgesia to patients with painful cutaneous wounds (Twillman et al., 1999; Long et al., 2001). However, this therapeutic strategy may negatively impact wound healing. Unmyelinated, capsaicin-sensitive primary afferent neurons are primarily responsible for the peripheral analgesic effects of topical morphine application (Bartho et al., 1990; Zhou et al., 1998). Opioids provide analgesia in part by inhibiting the generation of action potentials within these neurons, which subsequently results in blocking the antidromic release of important pro-inflammatory neuropeptides such as SP and NKA into peripheral tissues (Brodin et al., 1983; Werz et al., 1983a; Schroeder et al., 1991). This study investigated the effects that topical morphine application and neuropeptide replacement have on the closure rate of cutaneous wounds.

A standardized model of cutaneous wound healing was utilized to examine the impact increasing concentrations of topical morphine-sulfate application have on cutaneous wound closure rates in rats. This model of wound healing provides sensitivity sufficient to detect age-related variations in wound closure, as well as the negative impact of partial sensory denervation on wound healing (Liu et al., 1999; Smith et al., 2002). The results of this study demonstrate that the rate of closure is significantly slower in animals receiving topical morphine treatment when compared to control animals. This delay occurs in a concentration-dependent manner consistent with an opioid receptor-mediated effect.

If morphine is delaying wound closure rates by inhibiting the release of neuropeptides from primary afferent neurons, then functional blockade of peripheral neuropeptide receptors should mimic the effect seen with morphine application. When cutaneous wounds were treated with a selective, non-peptide NK-1 or NK-2 receptor antagonist, RP 67580 or GR 159897 respectively, wound closure rates were significantly delayed when compared to control. This observation suggests that topical morphine application slows wound closure by inhibiting the release and peripheral action of SP and NKA at NK-1 and NK-2 receptors and confirms the importance of SP/NK-1 receptor and NKA/NK-2 receptor interactions during wound healing. Both RP 67580 and GR 159897 are high-affinity antagonists for the NK-1 or NK-2 receptor, respectively, and exhibit at least 3,000-fold selectivity between these receptors. However, the degree of penetration of drug from the gel into the peri-wound skin and the actual concentration of antagonist drug at the receptor in these studies are unknown. The similarity of magnitude of delay in wound closure evoked by the NK-1 and NK-2 receptor antagonists does not reveal whether one of the two receptors is more important, mechanistically, in promoting wound closure. Extensive concentration-response relationships would need to be established to determine the relative potency of NK-1 and NK-2 receptor agents in modifying cutaneous wound closure.

Neuropeptides mediate early components of the cutaneous neurogenic inflammatory response. Previous studies have demonstrated that denervation with the neurotoxin capsaicin results in diminished wound healing (Kjartansson et al., 1987b;

Smith et al., 2002). The primary reason for delayed wound healing in the absence of neuropeptides appears to be due to a lag phase between injury and the initiation of wound contraction (Khalil et al., 1996). Similar to morphine treatment, the delay in closure seen with selective receptor antagonists is most evident within the first few days of the time course, suggesting an essential neurokinin receptor-mediated neuromodulation by SP and NKA early in the time course of wound healing.

Morphine slows wound closure by blocking the release of neuropeptides into the healing wound. Replacement of the neuropeptides should be able to attenuate the deleterious effects of morphine. Therefore, the ability of SP or NKA to restore normal wound closure in morphine-treated animals was determined by addition of the neuropeptides in morphine-infused gel. In this study, twice-daily topical administration of morphine significantly delayed wound closure, while treatment of wounds with SP accelerated wound closure. The delay in wound closure seen with morphine treatment was fully reversed by the addition of either SP or NKA into morphine-infused gel, demonstrating the neuropeptides' capacity to restore normal wound closure rates. While peri-wound pain thresholds were not directly quantified, treatment of the wounds with either SP or NKA did not produce any overt algesic effects (e.g., enhanced wound-directed biting or scratching behaviors). The results demonstrate the potential use of the neuropeptides in reversing the detrimental effect of topical morphine therapy in wound healing.

Most cells expressing neuropeptide receptors also express neuropeptide-degrading enzymes such as NEP, potentially providing a feedback mechanism to

effectively control the bioavailability of neuropeptides and regulate their inflammatory effects (Damas et al., 1996). Studies have shown that chronic, non-healing diabetic ulcers have increased NEP localization and activity (Spenny et al., 2002). Therefore, the role of local peptidases on normal wound closure rates was determined. Wounds receiving twice-daily application of gel infused with a cocktail of peptidase inhibitors were not significantly different in size when compared to control wounds. This result suggests that enzymatic degradation of neuropeptides within the wound does not impact normal healing rates. In addition, treatment with either SP- or NKA-infused gel significantly increased wound closure rates, suggesting that the peptides are stable in the hydrogel.

Data acquired in experiments from Specific Aim #1 suggest that topical morphine alters wound healing by activating opioid receptors on primary afferent neurons, thereby inhibiting the release of neuropeptides such as SP and NKA. Although morphine-treated wounds do ultimately close at times similar to controls, the area of these wounds is significantly larger during earlier days of the time course. Wound healing is a dynamic process consisting of multiple overlapping phases, which rely heavily on the orchestrated movement of various inflammatory and parenchymal cells into the wound. Data from this study suggest morphine treatment may be disrupting cellular processes which occur early in wound healing. In addition, interruption in the normal progression of healing by topical morphine administration may result in long-term detrimental alterations in the cellular architecture of healed skin.

CHAPTER FIVE

SPECIFIC AIM # 2

5.1 Hypothesis (SA #2)

Activation of pre-synaptic opioid receptors located on peripheral sensory nerve terminals delays cutaneous wound healing by inhibiting the release of neuropeptides from the nerve terminals into the peri-wound area.

5.2 Rationale (SA #2)

Systemic opioid drugs remain the standard course of care in providing analgesia to patients with cutaneous wounds. However, recent studies have focused on the activation of peripherally-located opioid receptors. Opioid analgesia was initially believed to originate exclusively from the activation of opioid receptors within the central nervous system. Accumulating evidence demonstrates the analgesic efficacy of peripherally-administered opioids. Analgesia obtained during peripheral opioid administration results from the activation of opioid receptors located on primary afferent sensory nerve terminals in peripheral tissues (Stein, 1993). Peripheral opioid analgesia is particularly attractive since it lacks the typical central, dose-limiting side effects that can accompany systemic opioid administration. Furthermore, analgesics applied locally provide optimal drug concentrations at the site of the noxious stimulus, avoiding the need to titrate systemic doses into a therapeutic range and offering a new alternative in the treatment of chronic pain.

Several clinical studies have explored the peripheral application of opioids as a strategy for pain management. The most extensive studies evaluating the analgesic effect of peripheral opioid drugs involved intra-articular morphine for postoperative

pain relief in patients undergoing arthroscopic knee surgery (Stein et al., 1991; Ho et al., 2000). Intra-articular injections of morphine have also been used to successfully alleviate the pain associated with rheumatoid and osteoarthritis (Likar et al., 1997; Stein et al., 1999). In addition, the topical application of morphine has been explored as a strategy for reducing the pain associated with skin ulcers, burns, and oral mucositis (Long et al., 2001; Cerchietti et al., 2002; Flock, 2003; Zeppetella et al., 2003; Watterson et al., 2004).

Although topical opioid compounds offer a promising new therapeutic strategy for alleviating pain, they may also adversely effect wound healing limiting the usefulness of this approach. Opioid receptor agonists exhibit anti-inflammatory effects (Jin et al., 1999; Khalil et al., 1999). Activation of opioid receptors on primary afferent neurons reduces the excitability of these neurons suppressing the antidromic release of pro-inflammatory neuropeptides (Aimone et al., 1989; Schroeder et al., 1991; Stein, 1993). Sensory neuropeptides such as SP and NKA are essential for normal wound healing. Exogenous application of neuropeptides enhances wound repair (Kjartansson et al., 1987a; Engin, 1998; Delgado et al., 2005) while their depletion impairs the process (Kjartansson et al., 1987b; Peskar et al., 1995; Khalil et al., 1996; Smith et al., 2002). Neuropeptides facilitate wound healing by mediating early components of neurogenic inflammation, specifically enhancing inflammatory cell migration and function (Eglezos et al., 1988; Eglezos et al., 1991), vasodilation (Khalil et al., 1990a), and plasma extravasation (Khalil et al., 1989b). In addition, neuropeptides function as mitogens for smooth muscle cells, fibroblasts,

keratinocytes, and endothelial cells in the closing wound (Nilsson et al., 1985; Tanaka et al., 1988; Ziche et al., 1990a; Ziche et al., 1990b; Rameshwar et al., 1997).

The biological actions of SP and NKA are mediated via neurokinin receptors belonging to the GPCR superfamily. While SP and NKA can act as full agonists at all of the neurokinin receptors, the receptors are recognized with moderate selectivity showing preferential binding to the NK-1 and NK-2 receptor, respectively (Mussap et al., 1993; Regoli et al., 1994). NK-1 receptors are located on smooth muscle cells, endothelial cells, fibroblasts, keratinocytes, monocytes, macrophages, and lymphocytes, while NK-2 receptors have been identified on all of the previously mentioned with the exception of fibroblasts (Krause et al., 1992; Ansel et al., 1996; Bowden et al., 1996; Ho et al., 1997; Lai et al., 1998; Song et al., 2000; Haley et al., 2001; Qian et al., 2001). Blocking the biological responses of the neurokinin receptors to their respective neuropeptides results in delayed wound healing (Schmassmann et al., 2004; Rook et al., 2007).

Although topical morphine offers a promising new therapeutic strategy for alleviating pain, opioid drugs appear to adversely effect wound healing, possibly limiting the overall therapeutic usefulness of this approach. Experiments from Specific Aim #1 have revealed that topical application of hydrogel infused with morphine sulfate significantly delays cutaneous wound closure rates in rats. This delay occurs in a concentration-dependent manner (consistent with opioid-receptor mediated effects), is mimicked by NK-1 and NK-2 receptor antagonists, and can be reversed by the addition of the neuropeptides SP or NKA (Rook et al., 2007). These

data suggest that topical morphine impairs wound closure by inhibiting the peripheral release of SP and NKA into the healing wound. However, many non-neuronal cells important in wound healing that express neurokinin receptors and potentially respond to neuropeptides also express opioid receptors. Consequently, topical morphine application may delay wound closure as a result of direct, or postsynaptic inhibition of these cells and structures. Therefore, this aim was designed to better characterize the site of action through which topical morphine inhibits wound closure.

5.3 Experimental Design (SA #2)

Thirty-seven male Sprague Dawley rats (200 g; Harlan) were divided into seven treatment groups **(Table 3)**. Animals were pretreated systemically with either vehicle or increasing doses of capsaicin for three consecutive days (s.c. 30 mg/kg, 50 mg/kg, 70 mg/kg). Animals were wounded 96 hours following the last injection. The experiment in this aim used the same standardized model of cutaneous wound healing as described in Specific Aim 1 to determine wound closure rates. The optimal concentration of morphine sulfate used was determined as a result of data acquired in the concentration-response study in Specific Aim #1.

Pretreatment	Topical Treatment	n
Vehicle	IntraSite® Gel Only	5
Capsaicin	IntraSite® Gel Only	5
Capsaicin	Gel + 5 mM Morphine Sulfate	6
Capsaicin	Gel + 1 mM SP	5
Capsaicin	Gel + 1 mM NKA	5
Capsaicin	Gel + 5 mM Morphine Sulfate + 1 mM SP	6
Capsaicin	Gel + 5 mM Morphine Sulfate + 1 mM NKA	5

Table 3. IntraSite® Gel treatments for Specific Aim 2.

5.4 Statistical Analysis (SA #2)

Statistical analyses were performed using SigmaStat (San Jose, CA). The effects of drug treatment and time on wound closure were evaluated using separate two-way repeated measures analyses of variance. The effect of drug treatment on the area under the time course curve was assessed using one-way analyses of variance. Differences between treatment groups and within treatment groups over time were identified using Tukey post-hoc tests. Differences between means were considered significant when $p < 0.05$.

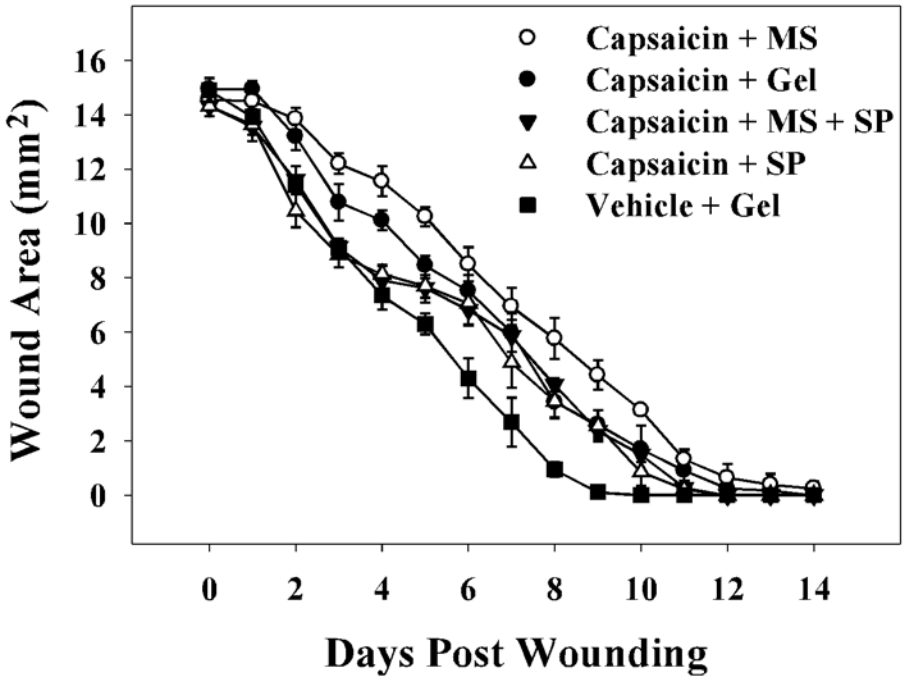
5.5 Results (SA #2)

Neuropeptide replacement in morphine sulfate-infused gel restores cutaneous wound closure rates in denervated rats

The impact of systemic capsaicin pretreatment on cutaneous wound closure rates in rats was assessed using a standardized model of cutaneous wound healing. Primary sensory afferent denervation resulted in a significant delay in time required for full closure of cutaneous wounds ($p = 0.02$). Vehicle-treated wounds closed 9.2 days post-wounding **(Figure 10)**. Wounds of rats pretreated with capsaicin receiving applications of gel alone completely closed by post-wound days 11.75, correlating to a 28% increase in time to closure over controls. A 34% increase in the total wound area throughout the time course was also seen in denervated wounds when compared to controls ($p = 0.001$; **Figure 11**).

In contrast, topical application of either 1 mM SP or NKA to wounds of capsaicin-treated rats resulted in a significant decrease (17% and 15%) in total wound area relative to denervated wounds treated with gel alone ($p = 0.01$ and $p = 0.02$; **Figure 11**). Treatment of denervated wounds with 5 mM morphine did not further delay wound closure compared to gel-only treated controls. However, combining either 1mM SP or NKA with morphine-infused gel did significantly reduce the total wound area compared to morphine treatment of denervated wounds demonstrating acceleration in wound closure ($p = 0.0002$ and $p = 0.04$). Furthermore, addition of either neuropeptide into morphine-infused gel returned closure rates to those not significantly different from those in vehicle-treated rats.

A


Capsaicin + MS
Capsaicin + Gel
Capsaicin + MS + SP
Capsaicin + SP
Vehicle + Gel
Wound Area (mm2)
0
2
4
6
8
10
12
14
16
0
2
4
6
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12
14
Days Post Wounding

B

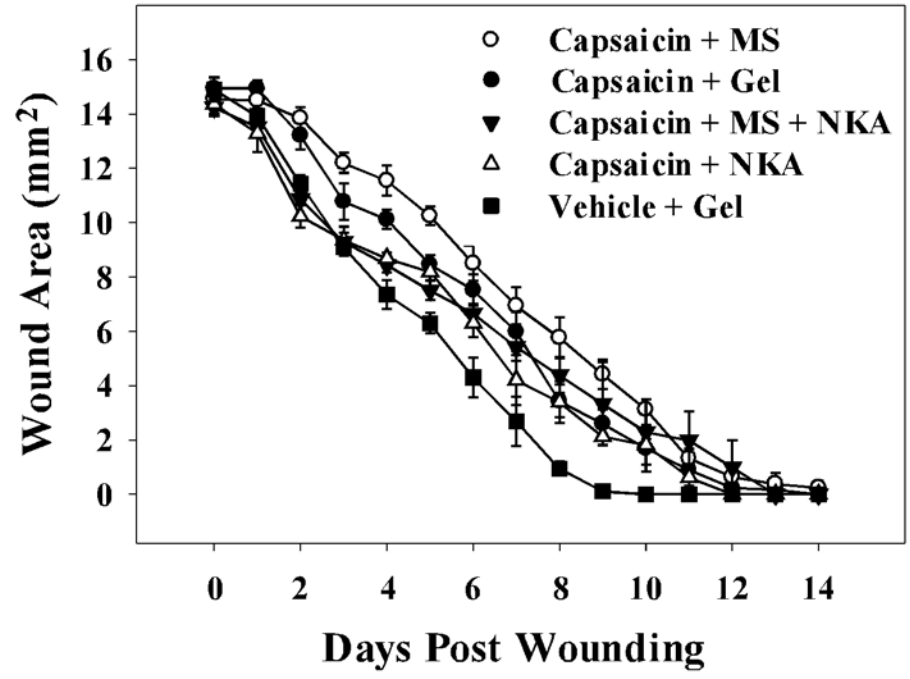

Capsaicin + MS
Capsaicin + Gel
Capsaicin + MS + NKA
Capsaicin + NKA
Vehicle + Gel
Wound Area (mm2)
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
12
14
Days Post Wounding

Figure 10. Wound closure time course for capsaicin-treated rats receiving IntraSite® gel treatments infused with morphine and/or neuropeptides. IntraSite® gel was applied to the wound twice daily through wound day 10. Wound size is presented as area (mm^2) mean ± SEM and was determined by analysis of digital images. Note that the wounds of chemically denervated rats treated with gel-only fully closed significantly later than vehicle-only treated controls (n = 5 or 6; $p < 0.05$; two-way ANOVA, Tukey's post hoc test).

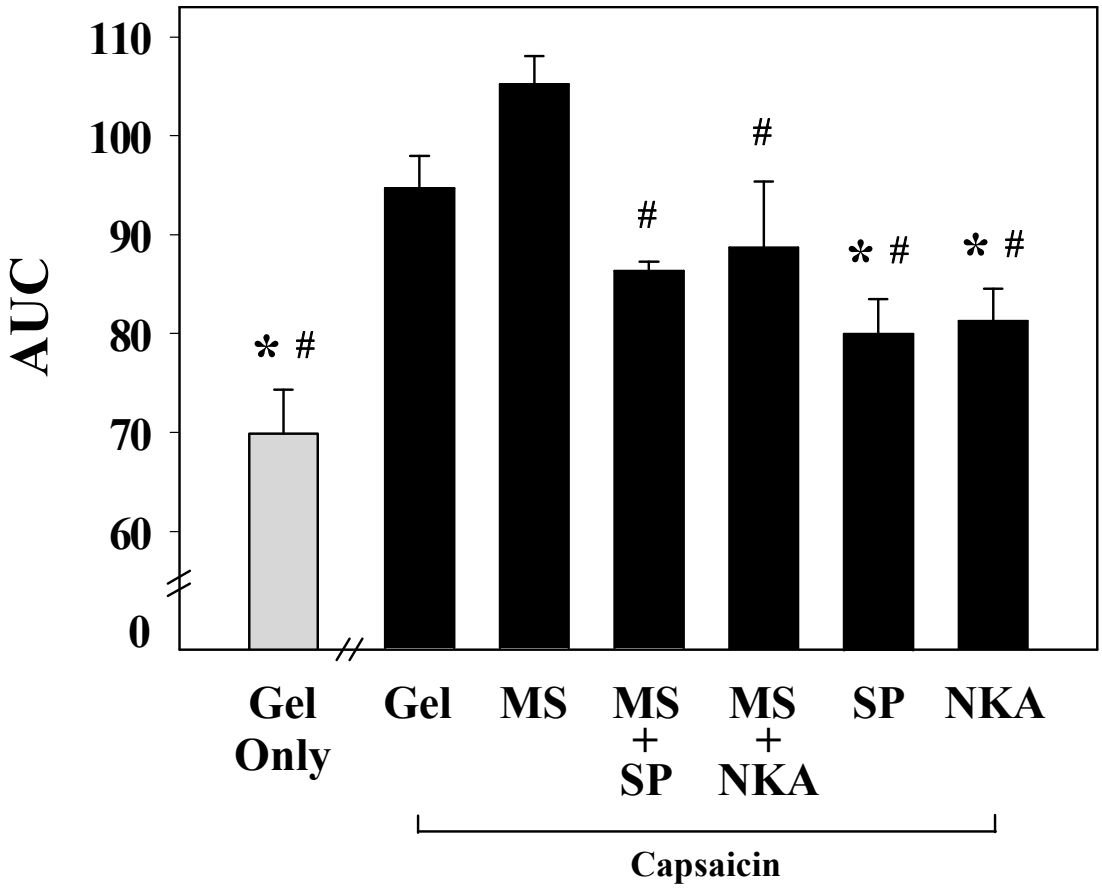


Figure 11. Area under the wound closure time course curve for capsaicin-treated rats receiving IntraSite® gel infused with morphine and/or neuropeptides. Data are presented as area (mm^2) mean $\pm$ SEM and were determined by analysis of digital images. Rats received applications of IntraSite® gel to the wound twice daily through wound day 10. Sensory denervation with capsaicin significantly delayed wound closure compared to vehicle-treated controls. Topical application of either 1 mM SP or NKA to chemically denervated wounds significantly accelerated wound closure compared to gel-only treated control. Addition of either 1 mM SP or NKA into gel infused with 5 mM morphine accelerated wound closure in capsaicin-treated rats (n = 5 or 6; *$p < 0.05$ when compared to capsaicin + gel-only treatment, # $p < 0.05$ compared to capsaicin + morphine treatment; one-way ANOVA, Tukey's post hoc test).

5.6 Discussion (SA #2)

The present study investigated the mechanisms underlying morphine-induced delays in wound closure. Topically applied morphine delays cutaneous wound closure in the rat. Previous data suggest this delay occurs via inhibition of the peripheral release of neuropeptides from primary afferent neurons since morphine's actions are concentration-dependent, emulated by neurokinin receptor antagonists, and reversed by exogenous application of the neuropeptides (Rook et al., 2007). However, opioid receptors are not only located on peripheral nerve terminals but also on other neuropeptide-targeted cells essential in the healing process. Therefore, this study was performed to determine whether morphine impairs healing directly through activation of opioid receptors on immune and/or parenchymal cells within the wound or by inhibiting the activation of primary afferent neurons thereby inhibiting the peripheral actions of neuropeptides.

Systemic administration of the neurotoxin capsaicin, the pungent vanilloid found in hot peppers, results in the depletion of sensory neuropeptides and permanent degeneration of small-diameter C-fibers, reducing inflammatory responses and ultimately diminishing wound healing (Kjartansson et al., 1987b; Lynn, 1990; Holzer, 1991; Smith et al., 2002). Capsaicin pretreatment was utilized in this study to produce chemically "denervated" skin and thereby assess the impact opioid receptors located on primary afferent neurons have on morphine-induced delays in wound closure. The protocol used produces a 29% decrease in CGRP-ir neurons located in the dorsal root ganglion (Zhou et al., 1998) and a 74% reduction in inflammation-

induced c-fos staining in the dorsal horn of the spinal cord (Zhang et al., 1998). Data from this study confirm significant sensory denervation, demonstrating an 80% reduction in cutaneous CGRP-positive neurons following capsaicin pretreatment.

Neuropeptides peripherally released by primary afferent neurons following injury are essential for mediating the early components of neurogenic inflammation and initiating the healing process (Schaffer et al., 1998). Consistent with previous reports, our data demonstrated that sensory denervation with capsaicin significantly impairs wound healing, with wounds fully closing at times significantly later than those in rats treated with vehicle. However, exogenous application of either SP or NKA topically to the healing wound was able to restore wound closure rates in denervated rats to those seen in intact animals, emphasizing the importance of neuropeptides in the wound closure process. While opioid receptors are located on various cells and structures within the healing wound, topical morphine treatment of wounds in sensory denervated rats did not further delay wound closure, suggesting a presynaptic effect of morphine on primary afferent neurons. Furthermore, the addition of SP or NKA to morphine-infused gel accelerated wound closure to rates not significantly different from vehicle-treated controls. The results of this aim support an indirect action of morphine in delaying wound closure via primary afferent neurons, rather than a direct inhibitory effect of morphine on non-neuronal target cells activated by the neurokinins.

CHAPTER SIX

SPECIFIC AIM # 3

6.1 Hypothesis (SA #3)

Physiological events in wound healing that facilitate morphine-induced delays in closure occur early in the healing process.

6.2 Rationale (SA #3)

The topical application of opioids has been explored as a strategy for reducing the pain associated with cutaneous wounds (Long et al., 2001; Zeppetella et al., 2003; Tran et al., 2007). However, studies within this laboratory have revealed that topical application of morphine sulfate significantly delays cutaneous wound closure rates in rats. The delay occurs in a concentration-dependent manner (consistent with opioid-receptor mediated effects) and can be reversed by the addition of the tachykinins SP or NKA (Rook et al., 2007). Interestingly, morphine-induced delays in wound closure were immediately apparent, occurring only during the early phases of wound healing. This transient delay was followed by acceleration in wound closure, resulting in morphine-treated wounds closing at times similar to controls. Therefore, the primary goal of this aim was to assess the temporal effects of topical morphine application on wound closure rates and the impact morphine has on inflammatory and parenchymal cells essential in the healing process. This study also addressed whether endogenous opioids play a direct role in wound closure, since their production by peripheral immune cells has been implicated in cutaneous analgesia (Stein et al., 1990; Labuz et al., 2006). Finally, although morphine-treated wounds ultimately closed at times similar to controls, disruption of normal closure may produce long-

term effects that outlast the duration of topical morphine administration. Accordingly, the structural architecture of healed skin was evaluated after closure of cutaneous wounds treated with morphine.

6.3 Experimental Design (SA #3)

Seventy-eight male Sprague Dawley rats (200 g; Harlan) were divided into five treatment groups **(Table 4)**. Experiments in this aim utilized the same cutaneous wound healing model as described in Specific Aim 1 to determine wound closure rates. In the first study, rats were treated topically with either gel alone through the entire time course (n = 6), gel + 5 mM morphine sulfate for the first three days followed by gel alone for the remainder of the time course (n = 5), or gel alone for the first three days followed by gel + 5 mM morphine sulfate for the remainder of the time course (n = 6). A second study was performed to evaluate the effects endogenous opioids have on wound closure rates. Rats were treated with gel only (n = 6) or 1 mM naltrexone (n = 5) throughout the entire time course. Wound area was determined each day prior to treatment for both studies.

During the following two experiments, rats were wounded and treated topically with either gel alone or gel + 5 mM morphine sulfate throughout the entire course of healing. In the third study, animals were sacrificed by decapitation on wound days 0, 1, 3, 5, and 8 (n = 3 or 4). Wound tissue including approximately 1 cm of surrounding intake tissue was dissected, frozen, and cryosectioned. Sections were then incubated with antibodies against the macrophage marker macrosialin or

the myofibroblast marker α-SMA. In the final study of this aim, residual scar area was measured and animals were sacrificed 18 days post-wounding. Wound tissue was again dissected, frozen, and cryosectioned. Sections from these tissues were then stained with hematoxylin and eosin.

Treatment	n
IntraSite® Gel Only	37
Gel + 5 mM Morphine Sulfate (Wound Days 0 – 3)	5
Gel + 5 mM Morphine Sulfate (Wound Days 4 – 10)	6
Gel + 5 mM Morphine Sulfate	25
Gel + 1 mM Naltrexone	5

Table 4. IntraSite® Gel treatments for Specific Aim 3.

6.4 Statistical Analysis (SA #3)

Statistical analyses were performed using SigmaStat (San Jose, CA). The effects of drug treatment and time on wound closure, macrophage infiltration, and myofibroblast activation were evaluated using separate two-way repeated measures analyses of variance. Differences between treatment groups and within treatment groups over time were identified using Tukey post-hoc tests. The long-term effects of morphine application on skin thickness and residual scar area were analyzed using unpaired t-tests. Differences between means were considered significant when $p < 0.05$.

6.5 Results

Time-dependent effects of topical morphine sulfate administration on cutaneous wound closure rates

The effects of topical morphine sulfate application at different times throughout healing on cutaneous wounds were assessed using a standardized model of cutaneous wound healing in rats. The previous concentration-response study demonstrated that 5 mM morphine sulfate causes a significant, but not maximal, delay in wound closure and was, therefore chosen as the concentration to be used in subsequent experiments. Topical morphine treatment on wound days 0-3 significantly delayed wound closure when compared to controls ($p = 0.017$). Animals treated with 5 mM morphine sulfate on days 0-3 post-wounding had significantly larger wounds on days 1, 2, 3, 4, 6, and 7 when compared to gel-only treated controls (**Figure 12**). Total wound area over the complete time course of animals receiving 5 mM morphine sulfate treatments for the first 4 days was 14% larger than both gel-only treated control rats and rats treated with morphine sulfate for the last 7 days. In addition, no significant differences were seen in animals treated topically with 5 mM morphine sulfate on days 4-10 post-wounding when compared to gel-only treated controls.

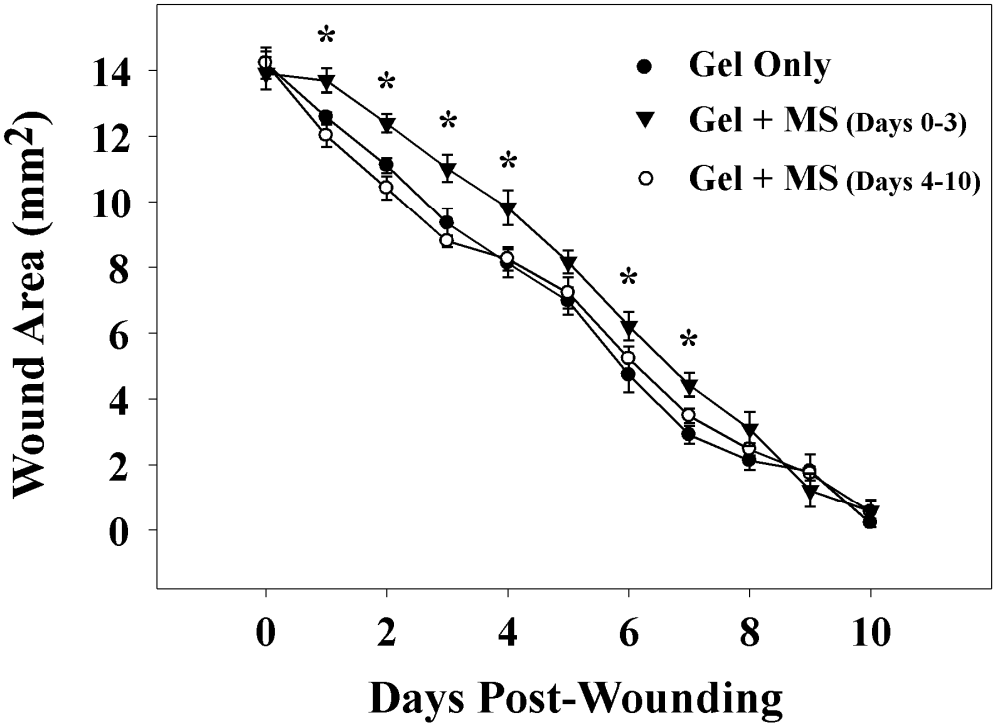


Figure 12. Wound closure time course for rats receiving morphine sulfate-infused gel treatments. IntraSite Gel (Smith+Nephew, Hull, United Kingdom) alone or containing 5 mM morphine sulfate was applied to the wound twice daily through wound day 10. Wound size is presented as area (mm^2) mean ± SEM and was determined by analysis of digital images. Note that wounds treated with morphine on wound days 0-3 were significantly larger compared to gel-only controls, while those treated with morphine on days 4-10 post-wounding showed no change (n = 5). * $p <$ 0.05 comparison between morphine 0-3 group and gel-only treated controls (two-way ANOVA, Tukey's post-hoc test).

Effect of topical naltrexone treatment on cutaneous wound closure rates in rats

The impact endogenous opioids originating from immune cells within the healing wound have on wound closure rates were assessed utilizing topical treatment of the non-selective opioid receptor antagonist, naltrexone. In rats treated topically with gel infused with 1 mM naltrexone, the area of the wounds treated with the opioid receptor antagonist did not differ significantly from control wounds **(Figure 13)**.

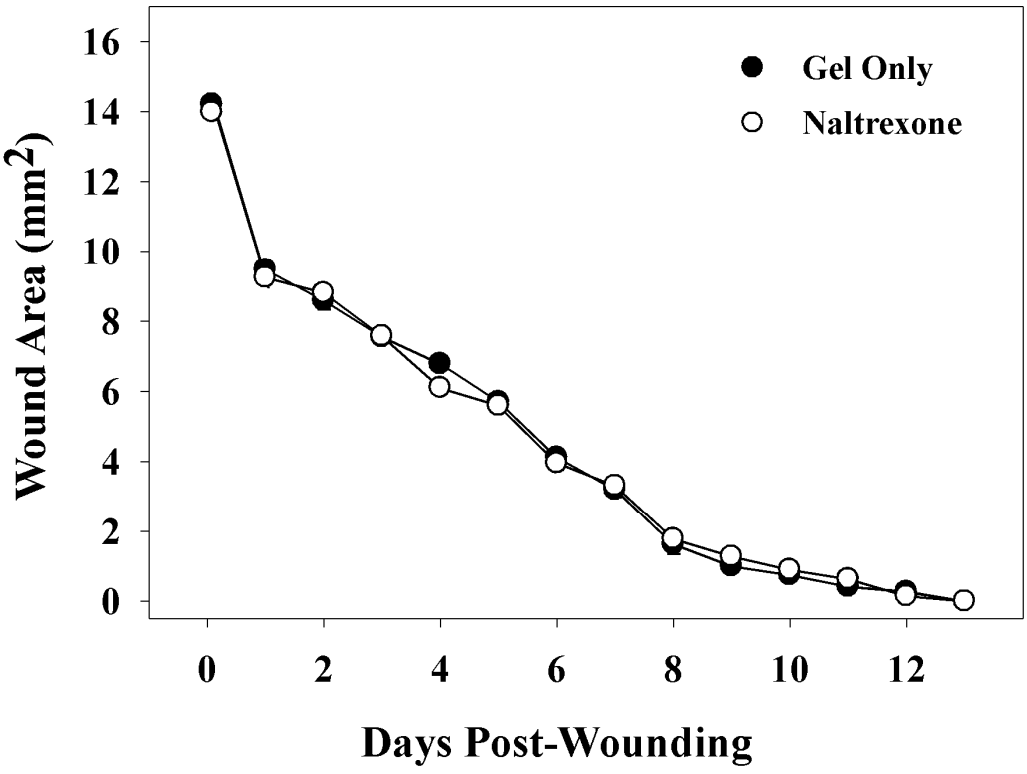


Figure 13. Wound closure time course for rats receiving naltrexone-infused gel treatments. IntraSite Gel alone or containing 1 mM naltrexone was applied to the wound twice daily through wound day 10. Wound size was determined by analysis of digital images. Wounds treated with naltrexone were not significantly different when compared to gel-only controls (n = 5 or 6; two-way ANOVA).

Impact of topical morphine sulfate treatment on the infiltration of macrophages into the healing wound

Macrophage infiltration into the closing wound was assessed with immunohistochemistry using the macrophage marker macrosialin. Very few macrosialin-ir cells were observed in control skin adjacent to the peri-wound area. Macrosialin-ir cells in gel-only treated wounds were greatest on day 1 post-wounding **(Figure 14)**. A slight decrease in the number of macrophages in the wound was seen on wound day 3 followed by a return to near-baseline levels on day 5 and 8 post-wounding. Appearance of macrosialin-ir cells was significantly delayed in morphine-treated wounds ($p = 0.01$). Macrophage concentrations in wounds treated with morphine sulfate did not peak until wound day 3. A significant decrease in macrosialin-ir cells numbers of 51% in morphine-treated wounds was observed on wound day 1 when compared to gel-only treated controls. While macrosialin-ir cell numbers declined in morphine-treated wounds on day 5 post-wounding, they were significantly higher than control levels and remained elevated through wound day 8 compared to gel-only treated controls.

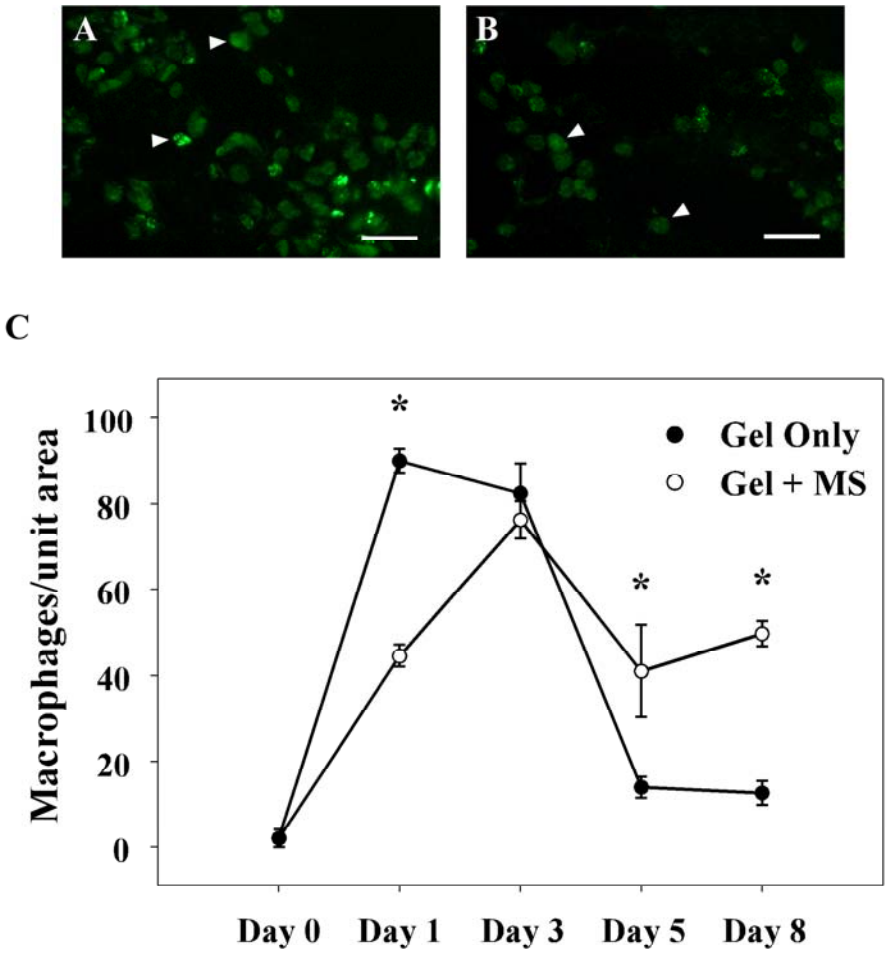


Figure 14. Effect of morphine on immunolabeled macrophages in the healing wound. Cutaneous wounds were treated twice daily with IntraSite Gel alone or gel infused with 5 mM morphine sulfate. **(A, B)** Macrosialin-ir cells (arrows) in granulation tissue of healing wounds. Fluorescence photomicrographs of gel-only **(A)** and morphine-treated **(B)** wounds were obtained 1 day post-wounding. Morphine-treated wounds have a significantly fewer macrophages present compared to controls. Scale bar = 100 μm. **(C)** Time course of macrophages present in the peri-wound area. Data are presented as number of macrosialin-ir cells per unit area (mm^2) of histological sections analyzed. Macrophage infiltration is delayed in

morphine-treated wounds (n = 3 or 4). * $p < 0.05$ comparison between morphine and gel-only treated controls (two-way ANOVA, Tukey's post-hoc test).

Myofibroblast density in control versus morphine-treated wounds

α-SMA-immunoreactivity was utilized to measure myofibroblast density in the healing wounds. α-SMA-ir cells first appear on wound day 3, although at negligible levels. In gel-only treated wounds, α-SMA-ir cell density increased rapidly at day 5 post-wounding and still further at wound day 8 **(Figure 15)**. However, this increase was markedly reduced in wounds treated with topical morphine sulfate ($p < 0.001$). α-SMA-ir cell density was significantly lower in morphine-treated wounds on days 5 and 8 post-wounding compared to controls, with an 84% and 79% reduction seen on days 5 and 8, respectively.

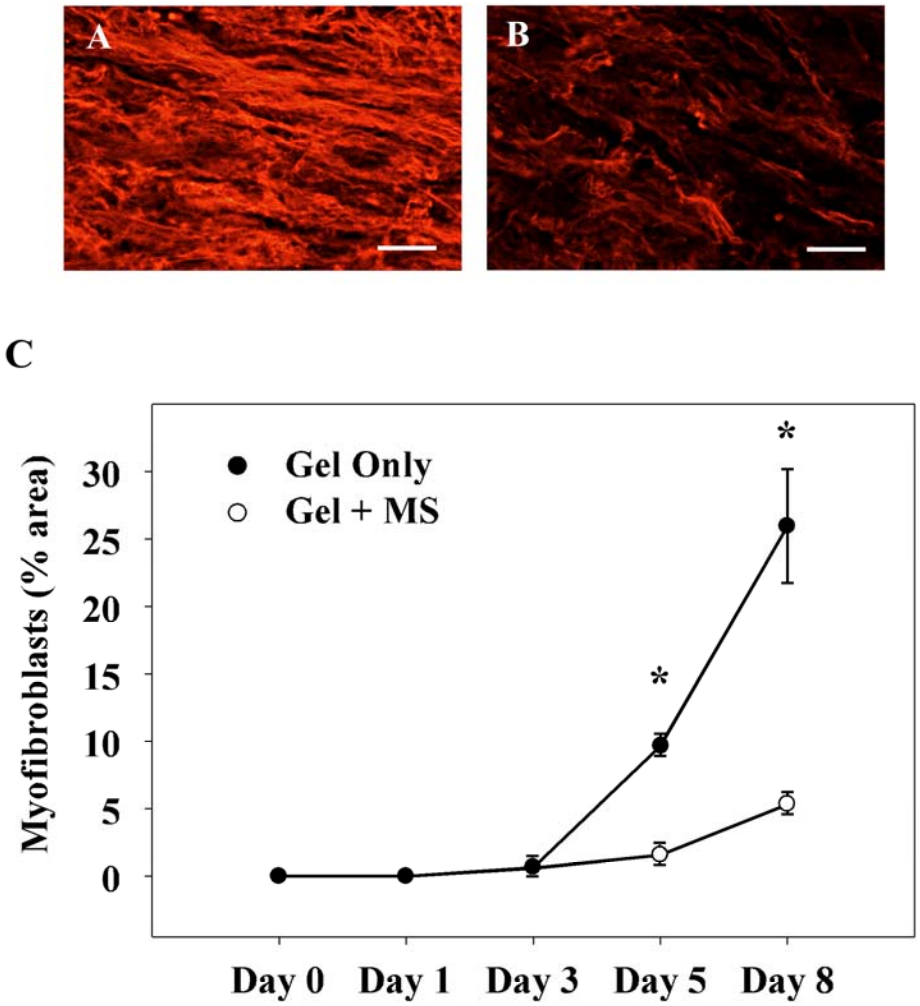


Figure 15. Myofibroblast accumulation in the healing cutaneous wound. Wounds received twice-daily treatment with IntraSite Gel alone or gel infused with 5 mM morphine sulfate. **(A, B)** α-SMA-ir cells in granulation tissue of healing wounds 8 days following wounding. Fluorescence photomicrographs of gel-only **(A)** and morphine-treated **(B)** wounds. Note a significant decrease in myofibroblast density in morphine-treated wounds as compared to controls. Scale bar = 100 μm. **(C)** Time course of myofibroblasts present in the peri-wound area. Data are presented as a percent field area occupied by α-SMA immunoreactive cells (mm^2).

Myofibroblast density in wounds treated with morphine was significantly reduced compared to gel-only treated controls (n = 3 or 4). * $p < 0.05$ comparison between morphine and gel-only treated controls (two-way ANOVA, Tukey's post-hoc test).

Architectural changes of rat skin following topical application of morphine sulfate during wound healing

The long-term impact of topical morphine sulfate treatment during cutaneous wound healing on the structural architecture of healed skin was assessed at post-wound day 18 using measures of epidermal and dermal thickness and the extent of scar tissue formation in rats receiving topical 5 mM morphine sulfate applications days 0-18 post-wounding. Control wounds treated with gel alone had an average epidermal thickness of 71.5 μm, while the full thickness of the skin (epidermis + dermis) was 1.6 mm. Closed wounds of rats treated with topical morphine had significantly thinner (35% $p = 0.019$) epidermis over the wound compared to gel-only treated controls **(Figure 16A)**. The full-skin thickness was 19% ($p = 0.014$) less in closed morphine-treated wounds as well **(Figure 16B)**. In addition, topical morphine treatment resulted in significantly larger (41% $p = 0.023$, **Figure 16C**) scar tissue surface area on day 18 post-wounding.

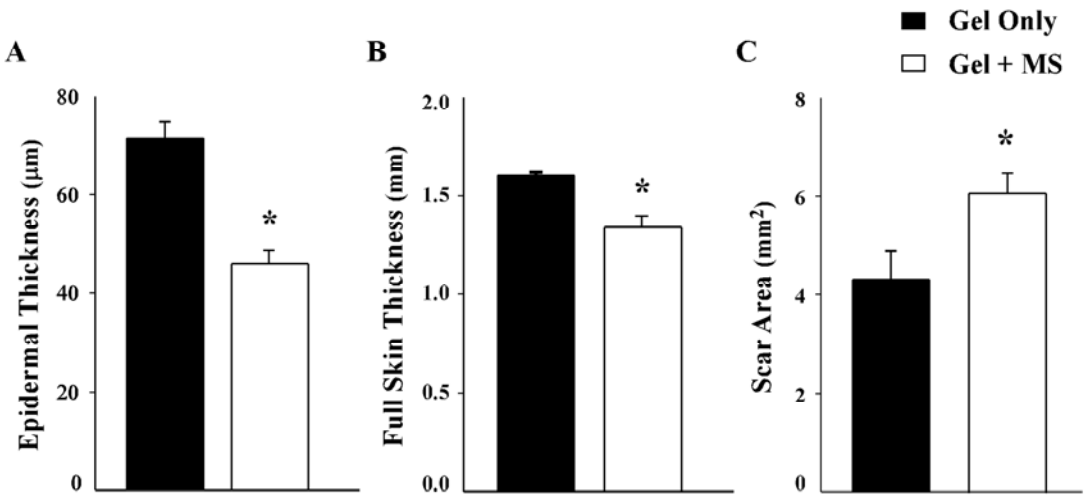


Figure 16. Effects of topical morphine on skin architecture of fully-closed cutaneous wounds. Rats were treated with IntraSite Gel (Smith+Nephew, Hull, United Kingdom) alone or gel infused with 5 mM morphine sulfate (MS) twice daily through wound day 18. **(A)** Epidermal thickness of new skin in wounds. Note that topical morphine treatment resulted in significantly thinner epidermis than that in control wounds. **(B)** Full thickness of the new skin covering closed cutaneous wounds following topical morphine treatment. Similarly, the full skin thickness was diminished following topical morphine administration. **(C)** Area of scar tissue covering closed cutaneous wounds following topical morphine treatment. Scar tissue was defined as the visibly distinct new epithelium covering the original wound site. Scar size is presented as area (mm^2) mean ± SEM and was determined by analysis of digital images of closed wounds on day 18 after wounding. Treatment of the wounds with 5 mM morphine sulfate significantly increased the scar area compared to gel-only controls (n = 4-7). * $p < 0.05$ comparison between morphine and gel-only treated controls (unpaired t test).

6.6 Discussion (SA #3)

Previous experimental studies in rats have demonstrated that treatment with topical morphine results in a delay in the closure of cutaneous wounds via inhibition of peripheral neuropeptide release from primary afferent neurons (Rook et al., 2007). The onset of the delay begins within the first 24 hours after wounding and continues for the first few days of healing. After approximately 4 days following wounding, wound closure accelerates with wounds ultimately closing fully at times not significantly different than controls. These results suggest a time-dependent effect of topical morphine treatment on wound closure. Therefore, the studies within this aim were designed to begin to characterize the temporal characteristics of morphine's impact on wound healing.

The results of the current study demonstrate that the closure of wounds treated with morphine on days 0-3 post-wounding only is nearly identical to that of wounds treated with morphine throughout the entire healing process (Rook et al., 2007), with an immediate delay in closure followed by acceleration and full closure at approximately day 10 post-wounding. However, wounds treated with morphine beginning on day 4 post-wounding and continuing through the remainder of the time course were not significantly different from controls treated with gel alone throughout the entire healing process. These results demonstrate that physiological events in wound healing that facilitate morphine-induced delays in closure occur early in the healing process.

Neuropeptides have proinflammatory actions and serve as a link between the nervous and immune systems. For example, SP promotes chemotaxis and increased function of macrophages (Hartung et al., 1986), as well as cytokine release from human monocytes (Lotz et al., 1988; Rameshwar et al., 1994). Macrophages are essential for normal wound healing and are the predominant cell type in the wound in the early inflammatory phase of wound healing (DiPietro, 1995). Macrophages not only clear the wound of bacteria and cellular debris but also secrete a number of growth factors and other cytokines that induce angiogenesis and stimulate keratinocytes and fibroblasts (Sunderkotter et al., 1994; Gharaee-Kermani et al., 1996; Belperio et al., 2000; Yamamoto et al., 2000; Werner et al., 2003). Accordingly, the effect of topical morphine treatment on macrophage density within the healing wound was assessed.

Activation of opioid receptors located on primary afferent neurons inhibits the antidromic release of neuropeptides from these terminals. Neuropeptides both attract and activate macrophages in states of inflammation. Therefore, decreased concentrations of neuropeptides within the peri-wound area may result in reduced macrophage infiltration. Results from this study demonstrate the inhibitory effect of topical morphine on macrophage infiltration into the closing wound. Macrophage density within control wounds peaked 1 day following injury. The number of macrophages in the wound began to decline on day 3 post-wounding and continued through days 5 and 8 after wounding. The number of macrophages in the wound decreased to near-baseline levels by day 5 after wounding, a time consistent with the

resolution of the inflammatory phase in normal wound healing. However, macrophage migration was significantly delayed in morphine-treated wounds. In this group, macrophage numbers were significantly less on day 1 post-wounding and a maximum was not reached until wound day 3. Decreased macrophage density would result in reduced induction of the parenchymal cells essential for wound closure (Diegelmann et al., 1986; Gharaee-Kermani et al., 1996; Yamamoto et al., 2000). Hence, the results of this study suggest that topical morphine delays wound closure, at least in part, by inhibiting the recruitment of macrophages into the closing wound. In addition to delaying macrophage infiltration, topical morphine application prolonged the presence of macrophages within the wound. Macrophage density in morphine-treated wounds was significantly higher at later time points (wound days 5 and 8) than in gel-only treated control wounds. This finding suggests that morphine inhibits resolution of the inflammatory phase, which may result in aberrant healing, propagate chronic inflammation, and result in undue tissue damage.

Wound healing is a dynamic process consisting of a complex, overlapping pattern of events that have been artificially divided into three phases: the inflammatory phase, including coagulation and inflammation; the proliferative phase, consisting of angiogenesis, the formation of granulation tissue, and reepithelialization; and the maturation phase, involving matrix formation and tissue remodeling (Baum et al., 2005). Myofibroblasts are transdifferentiated fibroblasts that express α-SMA, giving them contractile capabilities (Gabbiani et al., 1971; Gabbiani, 1992). Myofibroblasts are the predominant cell recruited by macrophages

to initiate the proliferative phase of wound healing (Ross et al., 1970; Lawrence, 1998). Closure of excision wounds occurs primarily through the contractile actions of myofibroblasts. The presence of α-SMA-immunoreactive myofibroblasts appeared within the wound in this study on day 3 post-wounding in control animals. The density of myofibroblasts was significantly increased through day 8. This trend is consistent with the pattern of normal wound healing. However, the presence of myofibroblasts was drastically reduced in morphine-treated wounds, with only a small increase occurring by wound day 8. These data are consistent with the idea that topical morphine application prolongs the inflammatory phase of wound closure and concomitantly delays progression into the proliferation phase of wound healing.

Examining the effects of exogenous opioids on the closure of cutaneous wounds led us to questions whether endogenous opioids play a role in regulating their closure. Previous studies have demonstrated that endogenous opioids produced by resident immune cells produce antinociceptive effects in inflamed tissues (Stein et al., 1990; Przewlocki et al., 1992). Accordingly, production of local endogenous opioids could impact wound closure rates. The possibility of delayed wound closure by activation of peripheral opioid receptors by endogenous opioids during normal wound closure was tested in this study when the opioid antagonist naltrexone was applied topically to wounds at a concentration of 1 mM; however, no significant differences in wound closure rates resulted from this treatment. This suggests that while local endogenous opioids can contribute to peripheral analgesia, their actions do not

significantly impact wound closure and, therefore, do not modulate the effects of topical morphine on wound closure dynamics.

Although morphine-treated wounds ultimately closed at times similar to control wounds, it was reasonable to suspect that morphine could have longer-term effects than that of delaying wound closure. Alterations in the cellular content of the healing wound or modulation of other mechanisms of wound closure by morphine could result in aberrant organization of newly-formed skin despite apparent wound closure. Evaluation of the architecture of healed wound skin on day 18 demonstrated a significant decrease in the thickness of the healed skin over the wound following morphine treatment. Topical morphine applied throughout the entire time course of wound closure also resulted in a significant increase in the residual scar area. Analysis of the myofibroblast density during the healing process revealed a significant decrease in the numbers of activated myofibroblasts in morphine-treated wounds, which supports the likelihood that a larger residual scar is formed due to the lack of contractility of the newly-formed tissue. These results demonstrate that topical morphine has lasting effects beyond the duration of its application to the healing wound and suggest that its use as a topical analgesic agent could produce persistent detrimental effects that would reduce the strength of healed skin.

Taken together, the studies related to aim 3 provide evidence that the delays in wound closure seen with topical morphine application can be attributed to alterations in the initiation and duration of essential, early processes during wound healing. Morphine-induced delays in the onset of inflammation produce a shift in the timing of

essential subsequent events (such as myofibroblast activation) in the healing process. Alterations in the temporal processes of wound healing not only results in delayed wound closure but also results in long-term architectural deficits, jeopardizing the integrity of the healed skin following topical morphine administration.

CHAPTER SEVEN

SPECIFIC AIM # 4

7.1 Hypothesis (SA #4)

Increased neuropeptide receptor density facilitates the ability of neuropeptides to accelerate wound closure.

7.2 Rationale (SA #4)

The pro-inflammatory effects of SP and NKA are mediated via activation of their respective receptors (NK-1 and NK-2) located on peripheral target cells. The primary effects of these neuropeptides are vasodilation, increased vascular permeability, and proliferation and migration of inflammatory and immune cells. Neurokinin-1 receptors are expressed in smooth muscle cells, endothelial cells, fibroblasts, keratinocytes, and various circulating immune cells and inflammation-activated immune cells. Few studies have examined the localization of NK-2 receptors. NK-2 receptors have, however, been localized to cells important in wound healing such as smooth muscle cells, keratinocytes, and eosinophils. Currently, little is known regarding the activity-dependent regulation of neuropeptide receptor expression in the periphery. The experiments related to aim 4 explored the location of neurokinin receptors in peripheral targets as well as their regulation by morphine during cutaneous wound healing.

7.3 Experimental Design (SA #4)

The same cutaneous wound healing model as described in Specific Aim 1 was used to determine wound closure rates. Forty-one male Sprague Dawley rats (200 g;

Harlan) were divided into two treatment groups. Rats were wounded and treated topically with either gel alone or gel + 5 mM morphine sulfate throughout the entire course of healing. Animals were sacrificed by decapitation on wound days 0, 1, 3, 5, and 8 (n = 3 or 4). Wound tissue including approximately 1 cm of surrounding intake tissue was dissected, frozen, and cryosectioned. Sections were then incubated with antibodies against the NK-1 or NK-2 receptor. Additional sections were used for double-labeling studies combining antibodies against the NK-1 or NK-2 receptor and the macrophage marker macrosialin or α-SMA for myofibroblasts and vasculature.

7.4 Statistical Analyses (SA #4)

Statistical analyses were performed using SigmaStat (San Jose, CA). Data are reported as mean ± SEM. The effects of drug treatment and time on neurokinin receptor expressing macrophages, vasculature, and myofibroblasts were evaluated using separate two-way repeated measures analyses of variance. Differences between treatment groups and within treatment groups over time were identified using Tukey post-hoc tests. Differences between means were considered significant when $p < 0.05$.

7.5 Results (SA #4)

Topical morphine sulfate treatment reduces the number of NK-1 and NK-2 receptor-positive macrophages in the healing wound

The effect of topical morphine on the number of NK-1 and NK-2 receptor-positive macrophages in healing wounds was assessed with immunohistochemistry using NK-1 and NK-2 receptor antiserum and the macrophage marker macrosialin. Numbers of macrophages expressing NK-1 and NK-2 receptor immunoreactivity increased significantly in gel-only treated controls following wounding with maximum numbers seen on wound day 1 **(Figure 18A, B)**. Conversely, an increase in NK-1 or NK-2 receptor-ir macrophages did not develop in wounds treated with topical morphine. Morphine administration significantly decreased macrosialin-ir cells positive for the NK-1 receptor ($p = 0.02$). Differences were seen on days 1 and 3 post-wounding (26% and 20%, respectively) **(Figure 18A)**. Macrophages positive for the NK-2 receptor decreased significantly in number ($p = 0.01$). Significant differences were observed on wound days 1 (30%), 3 (30%), and 5 (31%) with topical morphine treatment **(Figure 18B)**.

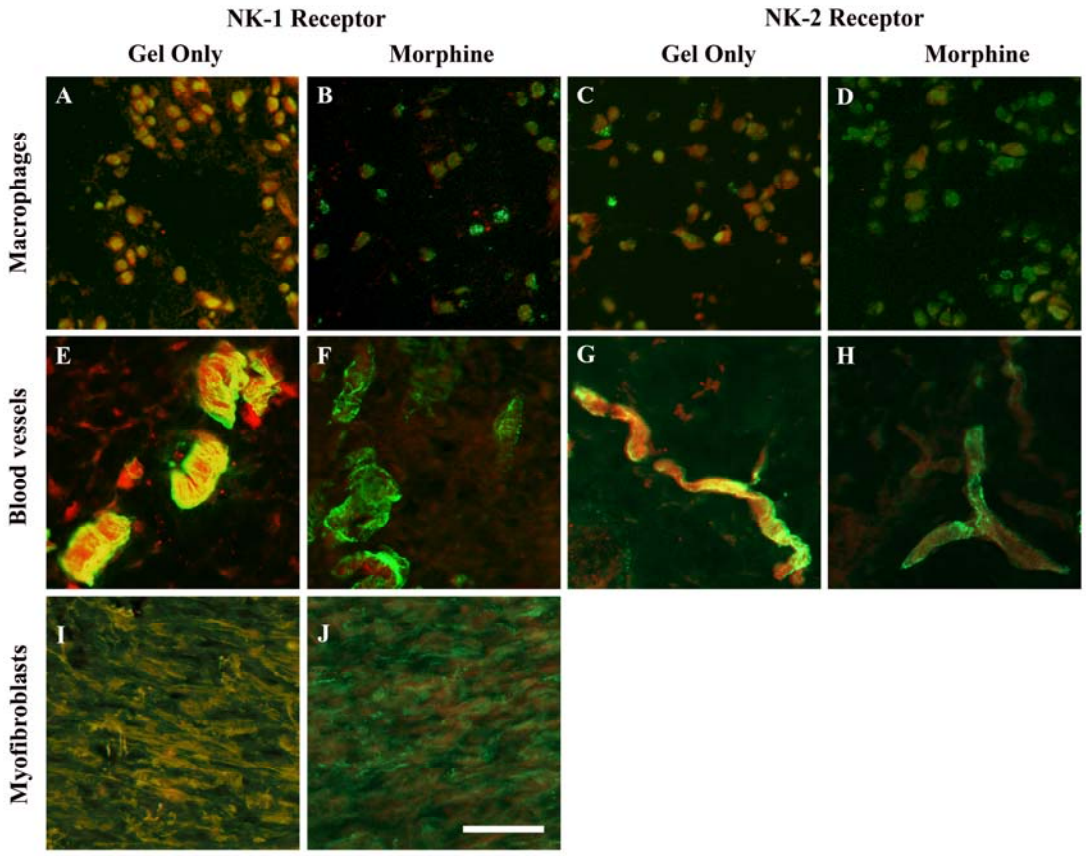
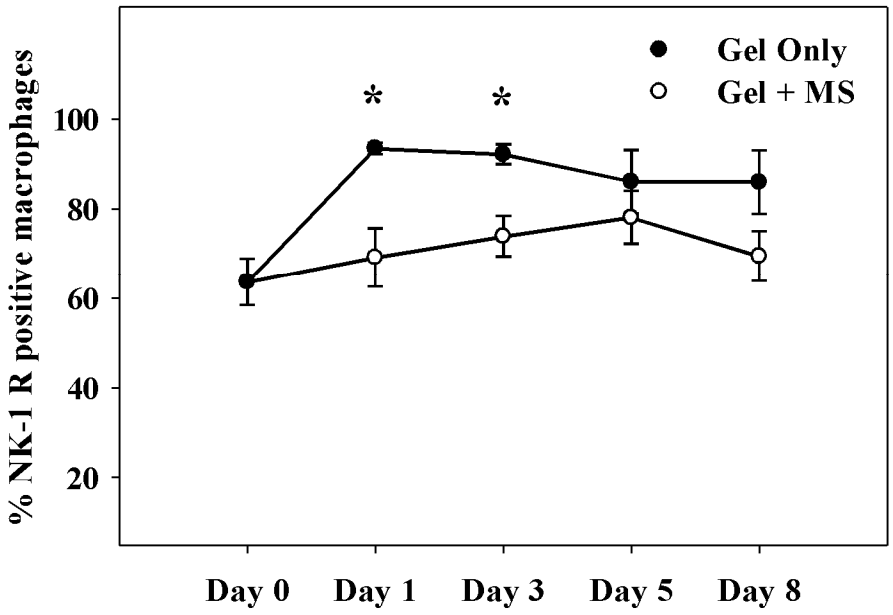
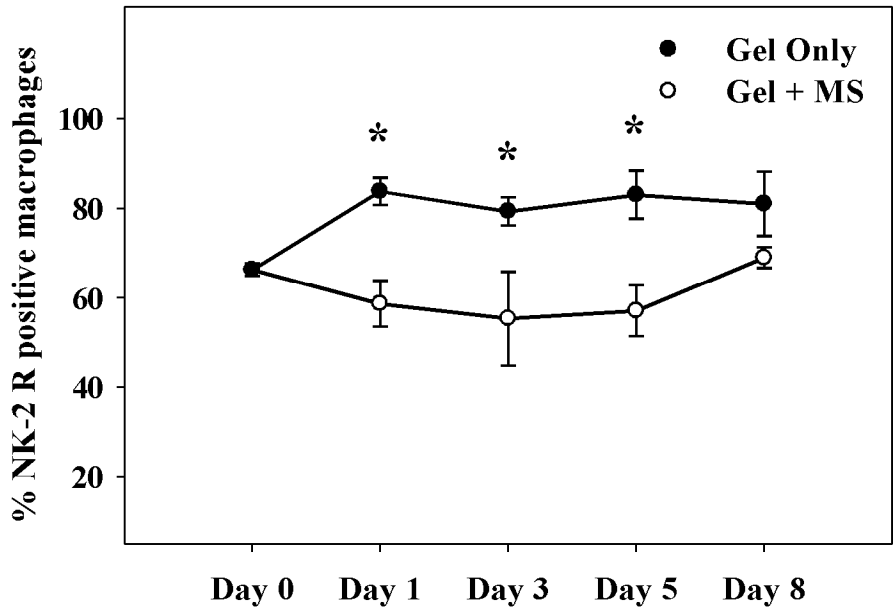


Figure 17. Photomicrographs of immunostaining in wound tissue during topical morphine treatment. Cutaneous wounds were treated twice daily with IntraSite® gel alone **(a, c, e, g, i)** or gel infused with 5 mM morphine sulfate **(b, d, f, h, j)**. Images represent wound tissue dissected from rats on wound days 1 **(a, b, c, d)**, 3 **(e, f, g, h)**, and 8 **(i, j)**. Sections were stained by direct immunofluorescence for macrosialin **(a, b, c, d)** or α-smooth muscle actin **(e, f, g h, I j)** conjugated to cy2 (green) in combination with indirect markers for the neurokinin-1 **(a, b, e, f, i, j)** or neurokinin-2 receptor **(c, d, g, h)** visualized with cy3-conjugated (red) secondary antibodies. Scale bar = 100 μm.

A


Gel Only
Gel + MS
% NK-1 R positive macrophages
100
80
60
40
20
*
*
Day 0
Day 1
Day 3
Day 5
Day 8

B


Gel Only
Gel + MS
% NK-2 R positive macrophages
100
80
60
40
20
*
*
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Day 0
Day 1
Day 3
Day 5
Day 8

Figure 18. Time course of NK-1 and NK-2 receptor-positive macrophages in morphine treated wounds. Cutaneous wounds were treated twice daily with IntraSite® gel alone or gel infused with 5 mM morphine sulfate. Data are presented as percentage of neurokinin receptor-positive macrophages per unit area. Note the number of NK-1 and NK-2 receptor-expressing macrophages increased following wounding. **(A)** Gel infused with 5 mM morphine sulfate significantly reduced the number of NK-1 receptor positive macrophages within the healing wound. **(B)** Wounds treated with 5 mM morphine sulfate had significantly fewer NK-2 receptor positive macrophages (n = 3 or 4; $*p < 0.05$; two-way ANOVA, Tukey's post hoc test).

Topical morphine application decreases NK-1 and NK-2 receptor expression in vasculature in the healing wound

The density of blood vessels in the healing wounds was measured utilizing α-SMA-immunoreactivity. In control wounds, a significant difference was not observed in NK-1 or NK-2 receptor-positive blood vessels across time **(Figure 19A, B)**. Topical morphine application significantly decreased NK-1 and NK-2 receptor expression in blood vessels when compared to gel-only treated controls ($p = 0.04$ and 0.03, respectively). A 34% and 62% reduction was observed in NK-1 receptor-ir blood vessels on wound days 3 and 8 while morphine decreased NK-2 expressing blood vessels by 30% and 45% on days 3 and 8.

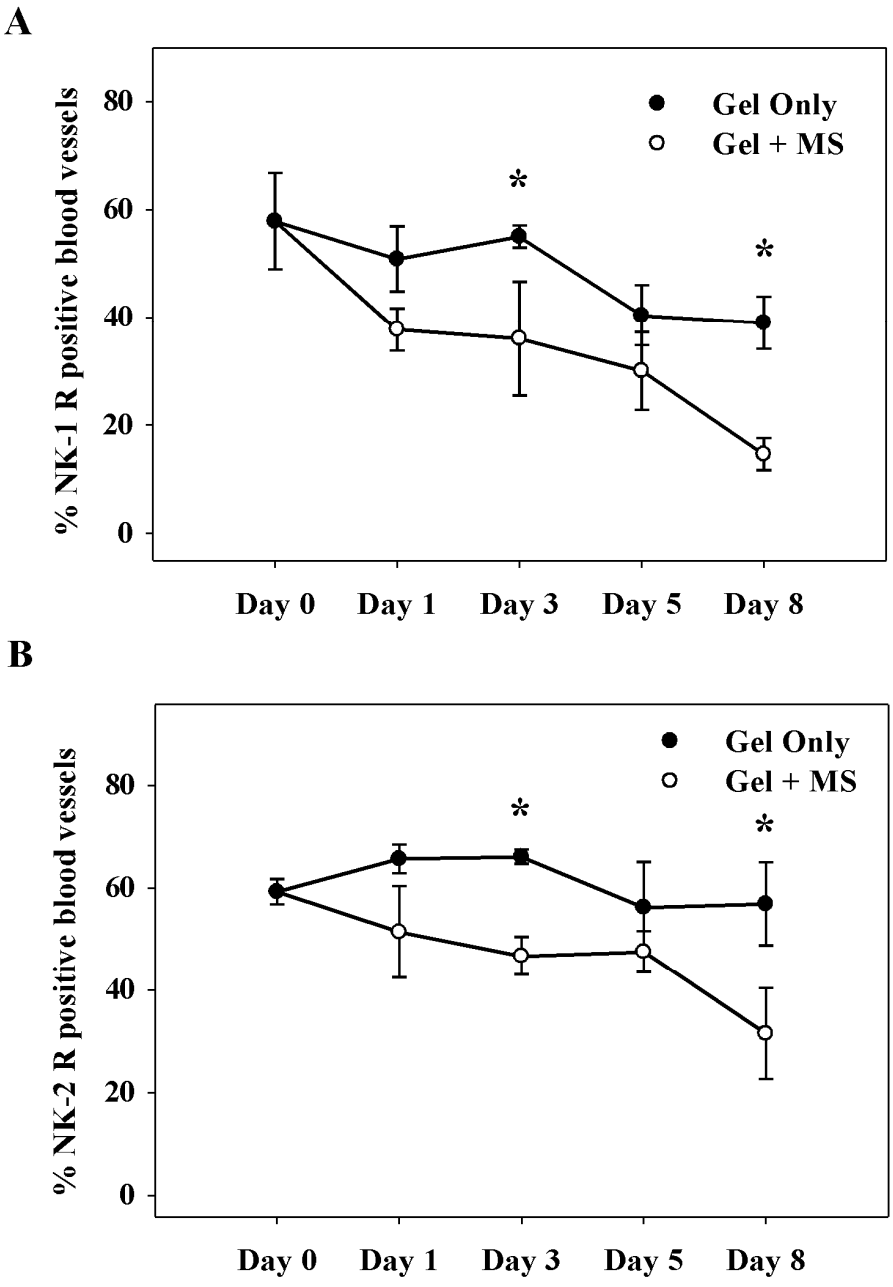


Figure 19. Time course of NK-1 and NK-2 receptor-positive vasculature in morphine treated wounds. Rats received twice daily treatments of IntraSite® gel. Data are presented as percentage of neurokinin receptor-positive blood vessels per unit area. **(A & B)** Application of 5 mM morphine sulfate to healing wounds

significantly decreased NK-1 and NK-2 receptor levels in blood vessels within the wound (n = 3 or 4; *$p < 0.05$; two-way ANOVA, Tukey's post hoc test).

Fewer NK-1 receptor-ir myofibroblasts are present in morphine-treated wounds versus control

Myofibroblast density within the wound was measured using α-SMA-immunoreactivity. For the quantification of myofibroblasts, α-SMA-ir vasculature was excluded and only cells demonstrating a spindle-shaped morphology and organized in a sheath-like mass were quantified. NK-1 receptor-ir myofibroblasts levels were measurable beginning on day 5 post-wounding. NK-1 receptor-expressing myofibroblasts increased in number from wound day 5 to 8 in gel-only treated controls **(Figure 20)**. However, topical morphine treatment significantly inhibited this increase in expression ($p = 0.002$) resulting in a 74% decrease on day 8 post-wounding relative to gel-only treated controls.

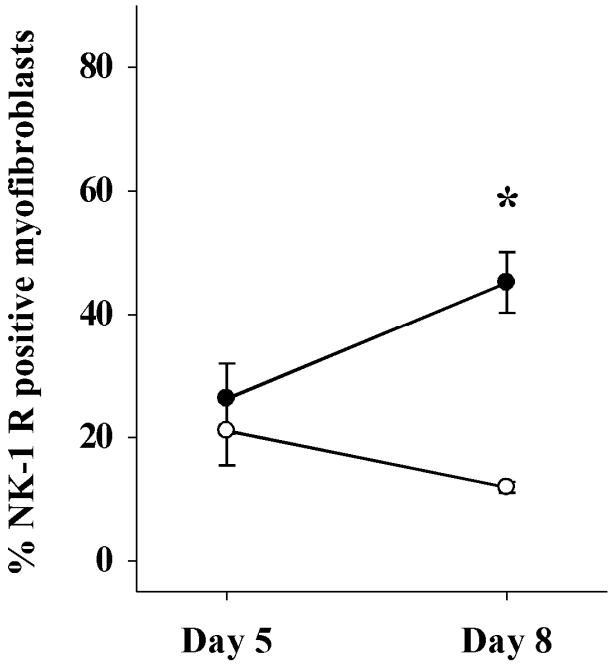
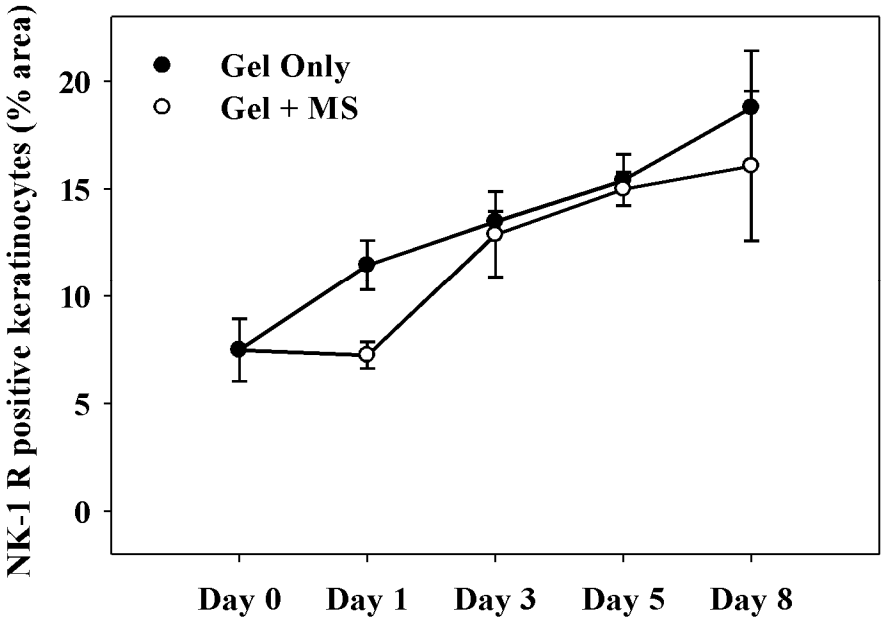


Figure 20. NK-1 receptor-positive myofibroblasts in morphine treated wounds. Wounds were treated twice daily with IntraSite® gel alone or gel infused with 5 mM morphine sulfate. Data are presented as percentage of neurokinin receptor-positive myofibroblasts per unit area. NK-1 receptor-positive myofibroblasts numbers were detectable on wound day 5. Gel infused with 5 mM morphine sulfate significantly reduced the number of NK-1 receptor-positive myofibroblasts within the healing wound (n = 3 or 4; $*p < 0.05$; two-way ANOVA, Tukey's post hoc test).

Number of NK-1 and NK-2 receptor-ir keratinocytes in control versus morphine-treated wounds are unchanged.

NK1 and NK-2 receptor-immunoreactivity was assessed in keratinocytes in the healing wounds. NK-1 and NK-2 receptor-ir keratinocytes increased throughout the course of healing in both control and morphine-treated wounds with maximum percent area seen on day 8 post-wounding **(Figure 21A & B)**. Morphine treatment did not significantly alter NK-1 or NK-2 receptor expression in keratinocytes in the healing wound.

A


Gel Only
Gel + MS
NK-1 R positive keratinocytes (% area)
0
5
10
15
20
Day 0
Day 1
Day 3
Day 5
Day 8

B

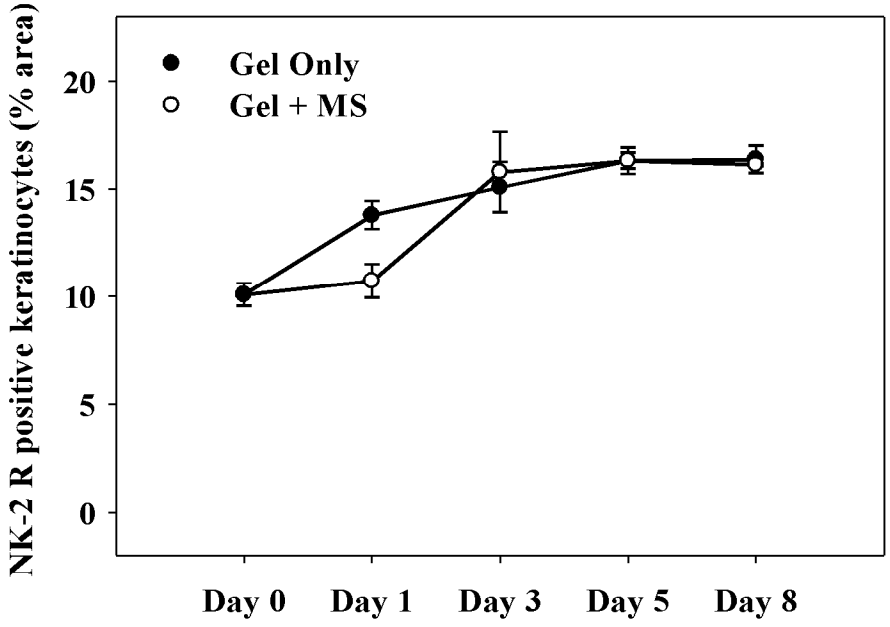

Gel Only
Gel + MS
NK-2 R positive keratinocytes (% area)
0
5
10
15
20
Day 0
Day 1
Day 3
Day 5
Day 8

Figure 21. Time course of NK-1 and NK-2 receptor-positive keratinocytes in wounds treated with topical morphine. Rats were treated with IntraSite® gel alone or gel infused with 5 mM morphine sulfate twice daily. Results are expressed as percent area of neurokinin receptor-ir keratinocytes. The area of NK-1 and NK-2 receptor-ir keratinocytes increased over the time course of wound closure. **(A & B)** A significant difference was not detected between treatment groups (n = 3 or 4; $p < 0.05$; two-way ANOVA).

7.6 Discussion (SA #4)

Activation of neurokinin receptors located in peripheral tissues by SP and NKA initiates vasodilation and increased vascular permeability, stimulates the migration of immune cells and the production and release of inflammatory mediators from these cells, and induces proliferation of parenchymal cells within the healing wound (Levine et al., 1993). The results of this and previous studies implicate the actions of neuropeptides in the inhibition of wound closure by topical morphine application. Accordingly, the regulation of neurokinin receptor-expressing target cells in the closing wound was evaluated as a relevant mechanism underlying the effect morphine treatment has within the healing wound.

Macrophages are the predominant cell infiltrating the wound during the early, inflammatory phase of healing and are essential for the progression of normal wound healing (DiPietro, 1995). Depletion of macrophages results in poor debridement, delayed fibroblast activation and inhibited fibroblast proliferation, and impaired wound closure (Leibovich et al., 1975). We have previously demonstrated that topical morphine application both reduces and delays macrophage migration into the healing wound (Dinda et al., 2005) (Rook et al., 2008 in press). Current results demonstrate a significant decrease in the number of NK-1 and NK-2 receptor-positive blood vessels in morphine-treated wounds throughout the healing process. This finding may account for the reduction in macrophage numbers reported previously, and possibly results from decreased vasodilation and vascular permeability in the closing wound when treated topically with morphine.

In addition to mediating the inflammatory phase of wound healing, macrophages also initiate the second, proliferative phase (Diegelmann et al., 1986; Gharaee-Kermani et al., 1996; Yamamoto et al., 2000). During the proliferative phase the extracellular matrix is replaced by granulation tissue, which consists of macrophages, fibroblasts, collagen, and blood vessels (Baum et al., 2005). Closure of full-thickness excision wounds occurs primarily via contraction of myofibroblasts. Macrophages release growth factors and cytokines responsible for the activation and proliferation of fibroblasts (Gharaee-Kermani et al., 1996; Werner et al., 2003). Activated fibroblasts transdifferentiate into myofibroblasts expressing α-SMA, which provides their contractile potential (Gabbiani et al., 1971; Gabbiani, 1992). Additionally, tissue remodeling from granulation tissue to scar is dependent on continued synthesis and catabolism of collagen. The degradation of collagen is carried out by matrix metalloproteinases secreted by macrophages (Goetzl et al., 1996). The results of the current study demonstrate an increase in the numbers of NK-1 and NK-2 receptor-expressing macrophages in the wound during closure. However, topical morphine application inhibits this increase in the proportion of neurokinin receptor-expressing macrophages. These data strongly suggest that the chemotactic and mitogenic effects on these cells in the healing wound, contributed in part by the actions of neuropeptides released from sensory nerve endings, would thereby be diminished by topical morphine application. Neuropeptides also directly initiate the activation, migration, and proliferation of fibroblasts (Nilsson et al., 1985; Tanaka et al., 1988; Ziche et al., 1990a). Current observations show a significant

decrease in numbers of NK-1 receptor-positive fibroblasts in morphine-treated wounds. Data from this aim demonstrate the dysregulation of neurokinin receptor-expressing inflammatory and parenchymal cells, and suggest that the dysregulation contributes to impaired wound closure. In conclusion, dysregulation of neurokinin receptor-expressing vasculature, macrophages and fibroblasts (but not keratinocytes) secondary to blockade of the peripheral release of neuropeptides emerge as mechanisms that can significantly disrupt the dynamic processes involved in wound healing.

CHAPTER EIGHT

CONCLUSIONS

The number of patients suffering from chronic cutaneous wounds, including those with pressure ulcers, ulcers that develop as a result of diabetes mellitus or peripheral vascular disease, or severe burns is substantial. Approximately 26% of hospice patients (Galvin, 2002) and as many as 40,000 hospital inpatients per year suffer from pressure ulcers, while thousands more experience ulcers due to chronic medical conditions (Twillman et al., 1999). In addition, an estimated 100,000 burn injuries per year result in hospitalization (Brigham et al., 1996). Chronic cutaneous wounds result in prolonged hospitalization and considerable morbidity. Treatment of such wounds costs billions of dollars per year and remains a significant burden on our health care system.

Pain is undeniably the most distressing symptom experienced by patients suffering from chronic cutaneous wounds. Systemic opioid compounds are the most effective and widely used drugs in the treatment of severe pain and remain the standard course of care in providing analgesia to patients with chronic cutaneous wounds. Although oral and parenteral administration of opioid drugs provides robust analgesia for both acute and long-term severe pain, the management of pain associated with chronic wounds can be particularly difficult. Many patients report considerable pain despite the use of systemic analgesics. In addition, systemic opioid drugs are frequently accompanied by dose-limiting side-effects mediated via activation of centrally-located opioid receptors. Consequently, pain research over the past two decades has focused on locally-applied opioid drugs and the development of peripherally-restricted opioid compounds.

In the early 90s, Christoph Stein et al. (Stein, 1993) demonstrated the efficacy of peripheral opioid drugs in providing analgesia during injury and/or inflammation via the activation of opioid receptors on primary afferent sensory neurons located in peripheral tissues. Several clinical studies support the findings of Stein et al., confirming analgesia obtained by peripherally-administered opioid drugs (Stein et al., 1991; Likar et al., 1997; Stein et al., 1999; Ho et al., 2000; Long et al., 2001; Cerchietti et al., 2002; Zeppetella et al., 2003; Watterson et al., 2004). The analgesic effects of peripherally-applied opioid drugs are believed to act locally, avoiding the typical systemic opioid-related side-effects. A pharmacokinetics study in humans assessing the bioavailability of morphine and its metabolites, morphine-6-glucuronide and morphine-3-glucuronide, following topical application of morphine sulfate to skin ulcers in hospice patients showed little to no systemic absorption (Ribeiro et al., 2004). In five of the six patients, morphine and its metabolites were undetectable. One patient with detectable levels (only 20% bioavailability of morphine and morphine-3-glucuronide) had a significantly larger ulceration than the other individuals treated likely increasing the systemic absorption of the drug. In addition to avoiding negative, central side-effects, peripheral opioid drugs act at the site of injury, preventing the need to titrate systemic doses. Therefore, peripheral opioid drugs are currently being pursued as a new alternative in the treatment of chronic cutaneous wound pain.

However studies designed for this dissertation research revealed possible detrimental effects of topical morphine application on cutaneous wounds,

demonstrating a delay in the closure of full-thickness cutaneous wounds in rats treated topically with morphine sulfate-infused hydrogel (Rook et al., 2007). Analgesia during peripheral opioid drug administration is achieved via activation of opioid receptors located on primary afferent nerve terminals. Activation of these receptors not only inhibits the transmission of afferent action potentials but also results in general suppression of nerve function. Opioid drugs, therefore, block the antidromic release of essential neuromodulators stored within the sensory nerve terminals, disrupting the link between the immune and nervous systems. Neuropeptides, such as SP and NKA, are essential for normal wound healing. They initiate the early components of neurogenic inflammation by facilitating increased blood flow and enhancing the migration and activation of inflammatory cells within the healing wound. Neuropeptides also function as mitogens for parenchymal cells within the wound facilitating its closure. The anti-inflammatory effects of opioids result in alterations in the normal healing process, which ultimately result in diminished wound healing. While substantial evidence has accumulated that the cutaneous peripheral nervous system plays a pivotal role in skin homeostasis and pathophysiology, our knowledge regarding the exact role of neuropeptides in cutaneous wound healing is far from complete. Thus, the primary goal of these studies was to elucidate the function of sensory neuropeptides, SP and NKA, in cutaneous wound healing and the mechanisms that delay cutaneous wound healing during topical morphine application.

Studies exhibit delays in wound closure during topically-applied morphine occur in a concentration-dependent manner. As the concentration of morphine in the hydrogel increased, the surface area of the wounds increased. While a concentration-dependent response of morphine supports a receptor-mediated phenomenon, it does not confirm an opioid receptor-mediated effect. Therefore, additional studies are necessary to further strengthen this hypothesis. One proposed future study would be the addition of an opioid receptor antagonist to morphine-infused gel. An opioid receptor-mediated effect should be blocked by introducing an antagonist.

Topical application of selective non-peptide NK-1 and NK-2 receptor antagonists mimicked the effects of morphine, displaying similar temporal patterns in delayed closure. These data support the hypothesis that morphine-induced delays in wound closure are a result of inhibited release of peripheral neuropeptides, as functional blockade of their respective receptors provides similar results. Additionally, the replacement of exogenous SP or NKA in morphine-infused gel significantly reversed the delay in wound closure seen with topical morphine treatment alone, further indicating morphine slows wound closure by blocking the release of neuropeptides into the healing wound.

The effects of SP and NKA are mediated via neurokinin receptors, which results in the activation of intracellular cascades promoting the formation of second messengers, such cAMP, inositol phosphates, diacylglycerol, and calcium. An interesting question is whether the actions of SP and NKA are mediated through a convergent or divergent mechanism. A future study in which the NK-1 and NK-2

receptor antagonists are applied to wounds in combination could resolve this issue. A concentration-response experiment for each antagonist would need to be performed as a pilot study to determine the appropriate concentration to provide a sub-maximal effect. An additive delay in closure by the combination of both receptor antagonists would suggest a divergent mechanism of SP and NKA in the facilitation of would closure. Along the same lines, a study combining SP and NKA would provide similar evidence in respect to a parallel versus deviating mechanism. However, the neuropeptides are significantly more expensive than the antagonists and, therefore, would optimally be utilized following definitive results from an antagonist study.

The biological actions of SP and NKA are primarily mediated via the activation of NK-1 and NK-2 receptors, respectively. Neurokinin receptors have been localized on smooth muscle cells, endothelial cells, fibroblasts, keratinocytes, monocytes, macrophages, and lymphocytes. Inhibiting the biological responses of the neurokinin receptors to their respective neuropeptides, either by blocking the receptor with an antagonists or removing the neuronal source of their endogenous ligands, results in delayed wound healing. Previous data generated in association with this dissertation work suggest that topical morphine impairs wound closure by inhibiting the peripheral release of SP and NKA into the healing wound. However, many non-neuronal cells important in wound healing that express neurokinin receptors and potentially respond to neuropeptides also express opioid receptors. Consequently, topical morphine application may delay wound closure as a result of direct, or postsynaptic, inhibition of these neuropeptide-target cells and structures.

Therefore, the neurotoxin capsaicin was used to chemically "denervate" the skin, functionally removing the primary source of neuropeptides within the wound providing a model to assess the impact opioid receptors located on primary afferent neurons have on morphine-induced delays in wound closure.

CGRP-immunoreactivity is commonly used as a marker for small fiber sensory innervation and was, therefore, utilized to determine the effects of systemic capsaicin on cutaneous primary afferent neuron innervation. Quantification of cutaneous CGRP-positive neurons confirmed significant sensory denervation following capsaicin pretreatment. Sensory neurons are essential for normal wound healing. Our data supported previous studies, demonstrating that sensory denervation with capsaicin significantly impairs wound healing, as denervated wounds fully closed significantly later than those in vehicle-treated rats. The treatment of wounds in sensory denervated rats with topical morphine did not further delay wound closure, suggesting a presynaptic site of action of morphine on primary afferent neurons. The addition of SP or NKA to morphine-infused gel accelerated wound closure to rates not significantly different from vehicle-treated controls, further supporting an indirect action of morphine in delaying wound closure via primary afferent neurons, rather than a direct inhibitory effect of morphine on non-neuronal target cells activated by the neurokinins. Furthermore, exogenous application of either SP or NKA topically to the healing wound was able to restore wound closure rates in denervated rats to those seen in intact animals, emphasizing the importance of neuropeptides in the closure of wounds.

While results from this study support a presynaptic site of action of morphine in delayed wound closure, questions remain regarding the mechanism of this effect. The delays in wound closure seen with capsaicin pretreatment are greater than those seen in topical morphine treatment alone. Therefore, it is possible that the chemical denervation of sensory neurons in the skin maximizes potential delays in wound closure. Additionally, while statistical analysis of the data verified no significant difference between the surface area of denervated wounds treated with gel only and those treated with morphine, a small increase in the area throughout the entire time course was seen. This phenomenon can be explained by at least two different mechanisms. First, it is possible that our statistical power is not great enough to expose a significant difference. In this instance, a power analysis would be necessary to determine if an increase in animal numbers is required. However, the slight increase may be explained by incomplete denervation of the skin. Assessment of the effectiveness of sensory denervation utilizing systemic capsaicin in adult rats resulted in an 80% decrease in CGRP-ir neurons. Therefore, it is plausible that the increase is due to incomplete denervation of the skin. Pretreatment of neonates, as opposed to adult animals, with systemic doses of capsaicin results in a more complete denervation of sensory neurons in the skin and should be a consideration in future studies.

Delays in wound closure seen with both morphine and neurokinin receptor antagonists are most evident within the first few days of healing, suggesting an essential neurokinin receptor-mediated modulation by SP and NKA during

physiological events occurring early in the wound healing process. A study designed to assess the temporal effects of topical morphine on wound closure demonstrated that wounds treated with morphine for the first four days of healing only displayed identical patterns in delayed healing as wounds that were treated with morphine throughout the entire time course. Conversely, wounds treated with morphine beginning on day 4 post-wounding and continuing through closure exhibited identical closure rates as those seen in gel-only treated control wounds.

While the temporal pattern of morphine-induced delays in wound closure suggests that physiological events important in wound healing and disrupted by topical application of the opioid drug occur early in the course of healing, it can be argued that the acceleration in wound closure rates later in the healing process could also be due to the development of tolerance at the opioid receptor. Tolerance is the decreased efficacy of an agonist at its receptor with prolonged administration. While the development of tolerance at opioid receptors in the central nervous system is well-known, it remains uncertain whether tolerance develops at peripheral opioid receptors. Peripheral opioid tolerance has been observed in mice during non-inflammatory conditions (Kolesnikov et al., 1999a; Kolesnikov et al., 1999b). Conversely, a lack of tolerance development has been described in models of inflammatory pain (Stein et al., 1996; Tokuyama et al., 1998; Nozaki-Taguchi et al., 1999; Ueda et al., 1999). The lack of effect on wound closure rates in rats receiving topical morphine treatment beginning on day 4 post-wounding would suggest that the acceleration in wound closure rates is not due to the development of tolerance.

Further evidence for a lack of tolerance development is observed in the neurokinin receptor antagonist studies in which the data demonstrate temporal patterns similar to those exhibited by morphine.

Alternatively, studies have demonstrated that endogenous opioids produced by resident immune cells have antinociceptive effects in inflamed tissues (Stein et al., 1990; Przewlocki et al., 1992). These endogenous opioids could initiate the development of tolerance at peripheral opioid receptors, decreasing the effect of morphine administration beginning on day 4 post-wounding. However, when the opioid receptor antagonist naltrexone was applied topically to wounds, no significant differences were seen in wound closure rates. This suggests that while local endogenous opioids contribute to peripheral analgesia, their presence does not impact wound closure and, therefore, is not impacting morphine's effects on wound closure. Naltrexone was only applied at one concentration (1 mM). While the concentration used for the opioid receptor antagonist correlates to a relative binding affinity at the opioid receptor as the concentration of morphine used, a concentration-response study is necessary to deny the impact of endogenous opioids in wound healing.

The temporal pattern of morphine-induced delays led to the investigation of cells essential for the initiation and propagation of the inflammatory response. Macrophages are one of the first invading immune cells and are the predominant cell type in the wound in the early inflammatory phase of wound healing. They are not only responsible for debridement of the wound, but also promote chemotaxis and increased function of keratinocytes and fibroblasts. Neuropeptides have

proinflammatory actions as they promote infiltration and activation of macrophages within the wound. Inhibition of the antidromic release of neuropeptides from primary afferent nerve terminals by morphine resulted in a significant delay in macrophage migration into the wound as well as a decrease in the proportion of neurokinin receptor-expressing macrophages. Additionally, topical morphine application prolonged the presence of macrophages within the wound, inhibiting the resolution of the inflammatory phase. The activation of neurokinin receptors located in vascular endothelium and smooth muscle cells initiates vasodilation and increased vascular permeability. Results demonstrated a significant decrease in the number of NK-1 and NK-2 receptor-positive blood vessels in morphine-treated wounds throughout the healing process. This observation may account for the reduction in macrophage numbers, and may result from decreased vasodilation and vascular permeability in the closing wound when treated topically with morphine.

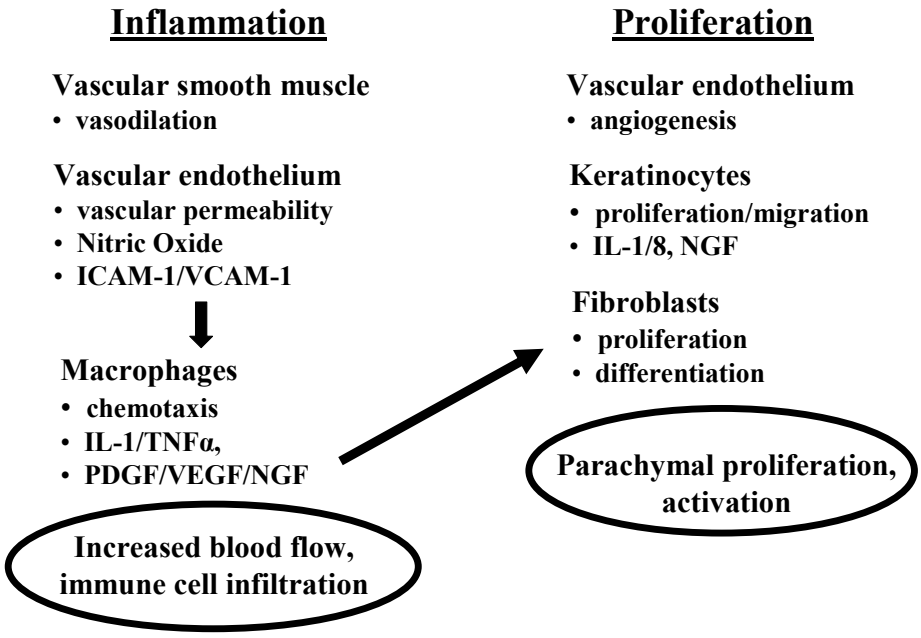


Figure 22 . Overall effects of neurokinin receptor activation in the healing wound.

Closure of excision wounds occurs primarily through the contraction achieved by myofibroblasts, transdifferentiated fibroblasts that express α-SMA, providing their contractile abilities. Myofibroblasts are the principal cell recruited by macrophages to initiate the proliferative phase of wound healing. Neuropeptides also directly initiate the activation, migration, and proliferation of fibroblasts. Myofibroblast density was drastically reduced in morphine-treated wounds, consistent with the idea that topical morphine application prolongs the inflammatory phase of wound closure and concomitantly delays progression into the proliferation phase of wound healing.

A significant decrease in numbers of NK-1 receptor-positive myofibroblasts was also detected in morphine-treated wounds. Data demonstrating the dysregulation of neurokinin receptor-expressing inflammatory and parenchymal cells during topical morphine application suggest that the alterations in cellular composition as well as expression of receptor protein contribute to impaired wound closure.

While the topical application of morphine does result in slow wound closure rates, the delays observed are transient and, therefore, the clinical relevance of the phenomenon has been questioned. Although morphine-treated wounds ultimately closed at times similar to control wounds, evaluation of healed wound skin architecture on day 18 post-wounding demonstrated a significant decrease in the thickness of the healed skin over the wound following morphine treatment. In addition, topical morphine applied throughout the entire time course of wound closure also resulted in a significant increase in the residual scar area. Decreased myofibroblast density during morphine treatment supports the likelihood that a larger residual scar would arise due to the lack of contractility of the newly-formed tissue. Although these results demonstrate a lasting effect of topical morphine on cutaneous architecture beyond the duration of its application to the healing wound, these observations do not provide insight as to the integrity of the healed skin. Further investigation is required to determine if topical morphine application during wound healing produces persistent detrimental effects. To determine if the alterations in skin anatomy are enduring, the scar area and thickness of the healed skin should be assessed at a later time point. An additional proposed future experiment would be to

test the tensile strength of healed skin to resolve whether the architectural changes observed translate into biological effects.

Topical morphine application is most widely used clinically on wounds of elderly patients with pressure ulcers or burn patients with highly inflamed wounds. It is important to acknowledge that the current studies were conducted on young, healthy animals with relatively aseptic wounds. Further study is required to characterize fully the effects of topical morphine on wounds with co-morbid disease states. Future studies should be conducted on burn wounds, wounds in aged animals, or wounds of animals in disease states, such as diabetes mellitus, to interpret clinical relevance in the delay of closure seen in morphine-treated wounds.

Data obtained in pursuing this line of investigation have advanced significantly the understanding of the role neuropeptides and their respective receptors play in cutaneous wound healing as well as how these processes are altered by local administration of morphine. Taken together, the data presented herein suggest that topical morphine alters wound healing by activating opioid receptors on primary afferent neurons, thereby inhibiting the release of neuropeptides such as SP and NKA and their subsequent actions at NK-1 and NK-2 receptors. These studies provide evidence that the delays in wound closure seen with topical morphine application can be attributed to alterations in the initiation and duration of essential, early processes during wound healing. Morphine-induced delays in the onset of inflammation produce a shift in the timing of essential subsequent events (such as myofibroblast activation) in the healing process. Alterations in the temporal

processes of wound healing not only results in delayed wound closure but also results in long-term architectural deficits, which may jeopardize the integrity of the healed skin following topical morphine administration. Additionally, alterations in neurokinin receptor-expressing vasculature, macrophages and fibroblasts (but not keratinocytes) secondary to inhibited peripheral neuropeptides release has emerged as a mechanism by which morphine disrupts the dynamic processes involved in wound healing.

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