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Early-In-Life Bladder Inflammation Alters Kappa-Opioid Modulation Of Inflammatory Bladder Pain

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EARLY-IN-LIFE BLADDER INFLAMMATION ALTERS K-OPIOID MODULATION OF INFLAMMATORY BLADDER PAIN

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ABSTRACT

In adult female rats, zymosan-induced inflammation of the bladder leads to bladder hypersensitivity, as reflected in augmented visceromotor reflex (VMR) responses to urinary bladder distension (UBD). Prior early-in-life (EIL) experience with bladder inflammation further increases bladder hypersensitivity. Naloxone increases bladder hypersensitivity in animals inflamed only as adults, but not in animals inflamed EIL, indicating that bladder inflammation recruits an inhibitory opioid system which may be impaired by EIL inflammation. This thesis examined what role the dynorphin/κ-opioid system played in mediating bladder hypersensitivity through the impairment of opioid inhibition. In all experiments, animals were treated EIL (P14-P16) and as adults with anesthesia only or zymosan. In Experiment 1, responses to a κ-opioid agonist were examined by measuring the VMR to UBD after i.v. administration of 1 mg/kg and 4 mg/kg of U50,488H. In most animals 1 mg/kg of U50,488H inhibited bladder sensitivity. However, when animals received bladder inflammation both EIL and as adults, 1 mg/kg of U50,488H increased bladder sensitivity. 4 mg/kg of U50,488H inhibited bladder sensitivity in all animals, but to a lesser extent in animals that received inflammation both EIL and as adults. In Experiment 2, spinal cord, serum, and bladder content of dynorphin were measured using enzyme-linked immunosorbent assay (ELISA). EIL bladder inflammation increased dynorphin content in the lumbosacral spinal cord. In contrast, EIL bladder inflammation and adult re-inflammation decreased bladder content of

dynorphin relative to animals that received EIL inflammation alone. In Experiment 3, intrathecal (i.t.) administration of the κ-opioid receptor antagonist nor-BNI occurred 24 hours prior to U50,488H administration to determine whether the effects of U50,488H were due to a spinal site of action. Animals receiving i.t. saline vehicle had attenuated responses to U50,488H. Therefore, it was difficult to determine if i.t. administration of nor-BNI prevented the effects of U50,488H. In conclusion, EIL bladder inflammation followed by adult re-inflammation reduced the inhibitory effect of U50,488H and revealed a facilitatory effect. Changes in spinal and bladder dynorphin content may contribute to this reduction in inhibition. Therefore, alterations in the dynorphin/κ-opioid system may participate in bladder hypersensitivity and impairment of opioid inhibition after EIL inflammation and adult re-inflammation.

Keywords: Bladder Pain Syndrome, Inflammation, Interstitial Cystitis, Neonatal, Opioid, Visceral Pain

DEDICATION

To my fantastically amazing husband, Mark, without whom none of this would have any meaning. And to my precious son, Jack. Words cannot express how excited I am to meet you.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table *Page*

LIST OF FIGURES

Figure *Page*

CHAPTER 1

INTRODUCTION

1.1. The Clinical Problem

Pain originating in the hollow organs, or visceral pain, is a leading cause for physician visits and hospitalizations. It is unique from somatic pain in that it is diffuse, poorly-localized, and not always linked to tissue injury. One of many sources of visceral pain is the urinary bladder. Generally, treatment of bladder pain focuses on the underlying etiology, such as infection, outlet obstruction, and bladder cancer. However, patients with disorders called bladder pain syndrome (BPS) and interstitial cystitis (IC) experience bladder pain in the absence of obvious pathology.

1.1.1. Diagnosis

Of the two disorders, IC is the older and more exclusionary diagnosis. Defining the constellation of symptoms that comprise IC has been a difficult task since Skene coined the term in 1887 (Bogart, Berry, & Clemens, 2007; Parsons & Parsons, 2004; for a review see Hanno, 2008). Hunner (1915) first described IC in a group of young women who displayed symptoms of frequency, nocturia, urgency, and suprapubic pain, and in whom mucosal ulcers were observed upon endoscopic examination (Duncan & Schaeffer, 1997). The diagnostic criteria of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) are those criteria which must be met for inclusion in research

studies of IC funded by the National Institute of Health (NIH; Jones & Nyberg, 1997). These criteria include the finding of glomerulations and/or a Hunner's ulcer on cystoscopy, the presence of pain associated with the bladder and/or urinary urgency, and the absence of exclusion criteria used to eliminate other possible causes of the symptoms (Jones and Nyberg, 1997). Criteria for enrollment in the Interstitial Cystitis Data Base (ICDB) include being at least 18 years of age, urinary frequency or urgency for the past 6 months or longer, and a score on the frequency, pain, or urgency scales of at least 1 (Jones & Nyberg, 1997).

The criteria used in clinical practice are much less stringent in order to prevent the misdiagnosis of individuals who may benefit from treatment. To simplify and expedite diagnosis and treatment, several committees including the International Interstitial Cystitis Patient Network Foundation and the NIDDK Interstitial Cystitis Epidemiology Task Force have suggested that IC and a more inclusive diagnosis, BPS, be referred to as one syndrome (Bogart et al., 2007). The International Continence Society defined BPS in 2002 as "the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as daytime and nighttime frequency, in the absence of proven urinary infections or other obvious pathology" (Abrams et al., 2002). They also indicated that the definition of IC should be a syndrome that shares the symptoms of BPS but must be confirmed by the presence of typical cystopic and histological features including Hunner's ulcers and glomerulations (Abrams et al., 2002). However, even this distinction between BPS and IC is controversial, and the two syndromes are often treated as one disorder because symptom severity is often not associated with cystopic findings (Tomaszewski et al., 2001) and glomerulations have been observed after hydrodistension in

asymptomatic women (Waxman, Sulak, & Kuehl, 1998). The American Urological Association (AUA) has recently proposed especially inclusive diagnostic criteria. They recommend that for clinical practice BPS/IC be diagnosed based on the definition proposed by the Society for Urodynamics and Female Urology, "An unpleasant sensation (pain, pressure, discomfort) perceived to be related to the urinary bladder, associated with lower urinary tract symptoms of more than six weeks duration, in the absence of infection or other identifiable causes" (Hanno et al., 2011). In subsequent introductory material, the term BPS/IC will be used unless the study referred to specifically addresses patients diagnosed with IC or BPS.

1.1.2. Prevalence

Estimates of the prevalence of BPS/IC vary widely due to the aforementioned differences in diagnostic criteria and the methodology used for the estimate (for reviews, see Jones & Nyberg, 1997; Parsons, Kurth, & Sant, 2007). Estimates are usually made either on the basis of physician diagnosis or patient response on validated surveys such as the Pelvic Pain and Urgency/Frequency Questionnaire or the O'Leary-Sant Symptom and Problem Indexes. One estimate based on physician diagnoses found a prevalence of 197 in 100,000 women (Clemens et al., 2005). Estimates based on validated symptom questionnaires range from 450 in 100,000 women (Leppilahti, Tammela, Huhtala, & Auvinen, 2002) to 11,200 in 100,000 women (Clemens et al., 2005). The female to male ratio is estimated to be approximately 10:1 in most studies (Nickel et al., 2005), but misdiagnosis is more likely in men, contributing to this gender bias. High rates of misdiagnosis in males is supported by Clemens et al. (2005) who found a lower female to male ratio than

generally reported (approximately 5:1) with an estimated prevalence of IC in men based on physician diagnosis of 41 in 100,000. Also, an annual incidence estimate by Roberts et al. (2003) revealed a female to male ratio of 3:1. One factor that may lead to high rates of misdiagnosis in males is the difficulty in distinguishing symptoms of BPS/IC from those of chronic prostatitis, since these disorders are often comorbid (Roberts et al., 2003).

1.1.3. Treatment

Since the etiology of BPS/IC is poorly understood, treatment focuses on symptom control. Such treatment is usually multimodal and includes pharmacological agents, changes in diet and lifestyle, and other nonpharmacological therapeutics. According to recently-published AUA guidelines (Hanno et al., 2011), nonpharmacological therapeutics are the first line of treatment. These treatments include diet changes, stress reduction techniques, and bladder training. The second line of treatment includes pharmaceutical therapy. Dimethyl sulfoxide (DMSO) instillation into the bladder was the only Food and Drug Administration (FDA) approved treatment for BPS/IC until 1996 when the FDA approved the use of oral pentosan polysulfate sodium (PPS). PPS is a sulfated polysaccharide similar to heparin and to glycosaminoglycans (GAGs) produced by bladder epithelial cells. Therefore, PPS is thought to act by repairing the damaged GAG layer lining the bladder epithelium. In two randomized, double-blind, placebo-controlled, multicenter studies (n=110-148), improvement occurred in 28-32% of IC patients receiving PPS and 13-16% of patients receiving placebo (Mulholland, Hanno, Parsons, Sant, & Staskin, 1990; Parsons et al., 1993). These studies indicate that PPS provides significant relief in

only a small proportion of IC patients. Very poor bioavailability (<1%) may contribute to this limited efficacy (e.g. Simon, McClanahan, Shah, Repko, & Modi, 2005). In addition to oral administration, PPS has been administered intravesically either independent of or in combination with oral PPS. Both treatment regimens have demonstrated some efficacy for the treatment of IC symptoms (e.g. Bade, Laseur, Nieuwenburg, van der Weele, & Mensink, 1997; Davis, El Khoudary, Talbott, Davis, & Regan, 2008; Parsons et al., 2002). Intravesical heparin has also been used for the treatment of IC symptoms. Because pain and functional symptoms (urinary frequency and urgency) are often poorly controlled with any single pharmaceutical treatment, oral PPS is often combined with other oral treatments such as antihistamines, analgesics, antidepressants, or antispasmatics. Similarly, PPS has been combined with intravesical treatments such as PPS, DMSO, heparin, sodium bicarbonate, or lidocaine (Dell & Parsons, 2004). Third through sixth line treatments are increasingly invasive, culminating with major surgery (substitution cystoplasty or urinary diversion with or without cystectomy), though even such drastic procedures do not guarantee symptom relief (Rossberger, Fall, Jonsson, & Peeker, 2007).

1.1.4. Etiology

The etiology of BPS/IC is unclear. Chronic infection, defects in the bladder epithelium, and increased mast cell infiltration are some of the major factors that have been proposed to contribute to BPS/IC. The idea that BPS/IC is due to a chronic infection is the oldest proposed etiology, dating back as far as Hunner (1915), but efforts to isolate either bacteria or fungi from urine or biopsy specimens or otherwise detect bacterial or viral activity have not indicated that any microorganism(s) is responsible for BPS/IC (e.g.

Hanish & Pool, 1970; Fall, Johansson, & Vahlne; 1985; for a review, see Duncan & Schaeffer, 1997). The use of media that support the growth of fastidious microorganisms has allowed for the identification of bacteria, fungi, and viruses in either urine or biopsy specimens from individuals diagnosed with IC (Keay et al., 1995; Wilkins, Payne, Pead, Moss, & Maskell, 1989). However, these studies still have provided no evidence of an ongoing infection in BPS/IC patients because even under these conditions no one microorganism was found to be consistently and uniquely isolated. Recent efforts to identify infectious agents in BPS/IC have involved isolation of 16S ribonucleic acid (RNA) and amplification of this molecule by polymerase chain reaction (PCR) for sequencing and identification of the organism. This method has yielded similarly disappointing results. One study amplified bacterial 16S rRNA in 4 out of 14 bladder specimens from IC patients and none from the 15 control subjects (Domingue, Ghoniem, Bost, Fermin, & Human, 1995). However, even in this study the 16S rRNAs amplified were from different species. Therefore, no microorganism has yet to be identified as the causative agent of BPS/IC. These negative results indicate that a chronic bacterial infection is unlikely to be directly involved in the etiology of BPS/IC in the majority of patients.

Another view is that a defect in the normally impenetrable GAG layer of the bladder mucosa allows for exposure of the bladder epithelium to cytotoxic factors in the urine of BPS/IC patients (Rajasekaran, Stein, & Parsons, 2006). Enhanced sensitivity to potassium (Parsons, Greenberger, Gabal, Bidair, & Barme, 1998) and enhanced extravasation of fluorescein (Buffington & Woodworth, 1997) indicate increased permeability in the bladders of IC patients. A GAG layer defect may be accompanied by damage to the bladder epithelium. There are histological signs of damage to the epithelial wall of the

bladders of some patients including tearing and thinning (for reviews, see Kusek & Nyberg, 2001; Oravisto, Alfthan, & Jokinen, 1970; Ruggieri, Chelsky, Rosen, Shickley, & Hanno, 1994). This damage may persist because of suppression of bladder epithelial repair by anti-proliferative factor (APF) in BPS/IC patients. APF is a factor purified from the urine of IC patients that decreases heparin-binding epidermal growth factor-like growth factor (HB-EGF) and inhibits bladder epithelial cell proliferation in vitro (Keay et al., 1996, 1998; for a review, see Chai & Keay, 2004). APF is much more commonly found in IC patients (86%) than asymptomatic controls (8%) and women with bacterial cystitis (12%), indicating that APF may be selectively produced or that APF production may be increased to detectable levels much more commonly in IC patients (Keay et al., 1998). APF appears to be produced by bladder epithelial cells (Keay, Kleinberg, Zhang, Hise, & Warren, 2000), and by inhibiting proliferation it may contribute to impaired repair of bladder epithelia in IC patients. This is especially important because explanted bladder epithelial cells from IC patients display a decrease in proliferation (Keay, Zhang, Shoenfelt, & Chai, 2003). However, additional experiments are required to determine the role, if any, that APF plays in the etiology of IC. For example, results obtained by Keay et al. (1996, 1998, 2000) in cell culture should be replicated in other laboratories to determine the reproducibility of these findings. In addition, APF should be administered in vivo to determine if such treatment produces symptoms reminiscent of IC.

Neural-immune interactions have been proposed to play a role in the etiology of BPS/IC. Simmons and Bunce (1958) first proposed that mast cell infiltration may be a critical component of this interaction. The number of mast cells is increased in the lamina propria and urine of IC patients, and the degree of mast cell infiltration is correlated

with IC symptoms (Boucher, el-Mansoury, Pang, Sant, & Theoharides, 1995; Christmas & Rode, 1991; Leiby, Landis, Propert, Tomaszewski, & Interstitial Cystitis Data Base Study Group, 2007; Tomaszewski et al., 2001). Also, a much greater proportion (90%) of bladder mast cells of IC patients is activated compared to controls in which nearly all bladder mast cells were intact (Theoharides et al., 1995). Metabolites of histamine, a major inflammatory mediator released by mast cells, are increased in the urine of IC patients (el-Mansoury, Boucher, Sant, & Theoharides, 1994; Holm-Bentzen, Sondergaard, & Hald, 1987). In addition, blocking histamine activation of H2 receptors using cimetidine significantly decreased pain and nocturia in BPS patients (Thilagarajah, Witherow, & Walker, 2001). The mast cell theory of BPS/IC provides a link through which neurogenic inflammation may contribute to the development of symptoms. IC patients display an increase in substance P (SP) and vasoactive intestinal peptide (VIP) containing nerve fibers (Hohenfellner et al., 1992; Pang, Marchand, Sant, Kream, & Theoharides, 1995a). Both of these neuropeptides can trigger mast cell secretion. Exposure of bladder epithelial cells to irritants in the urine may cause the initial mast cell infiltration and activation and secretion of inflammatory mediators such as histamine in IC patients. Inflammation may also sensitize primary afferents, which in turn may release neuropeptides from peripheral terminals in the bladder itself. Neuropeptide release then may increase mast cell secretion, forming a positive feedback loop for bladder inflammation.

In addition to linking immunological (mast cell) and neural (neuropeptide) changes that occur during BPS/IC, abnormal mast cell infiltration and activation also provides a potential link between BPS/IC symptoms and gonadal steroid hormones and stress. Gonadal steroid hormones are thought to play an important role in BPS/IC be-

cause, as mentioned previously, BPS/IC is much more common in females than males. In addition, affected women often experience a worsening of symptoms during the perimenstrual period (Powell-Boone et al., 2005). Expression of high affinity estrogen receptors is increased in bladder mast cells of IC patients (Pang, Cotreau-Bibbo, Sant, & Theoharides, 1995b), and in rats estradiol has been shown to increase rat bladder mast cell secretion in response to carbachol (Spanos et al., 1996). Therefore, estradiol-induced mast cell secretion is one way in which gonadal hormone fluctuations may enhance bladder symptoms. Mast cells also provide a link between stress and BPS/IC. As well as having a profound impact on the immune system, stress can exacerbate BPS/IC (Koziol, Clark, Gittes, & Tan, 1993; Lutgendorf, Kreder, Rothrock, Ratliff, & Zimmerman, 2000) as well as other functional urinary tract disorders (Baldoni, Ercolani, Baldaro, & Trombini, 1995; Macaulay, Stern, Holmes, & Stanton, 1987) and comorbid conditions such as irritable bowel syndrome (IBS; Dancey, Taghavi, & Fox, 1998). One piece of evidence supporting involvement of mast cells in the relationship between stress and bladder symptoms includes the finding that immobilization stress in rats leads to bladder mast cell activation (Spanos et al., 1997).

Mast cells may also mediate the relationship between immune abnormalities, such as autoimmune disorders and allergies, and BPS/IC. Although efforts to identify an autoimmune disorder responsible for BPS/IC have been unsuccessful, certain autoimmune disorders have been associated with BPS/IC. Sjogren's syndrome (SS), a disorder of the salivary and lachrimal glands, is the autoimmune disorder most strongly associated with IC. Approximately 25% of IC patients display symptoms of SS (Van de Merwe & Arendsen, 2000). Van de Merwe and Arendsen (2000) propose that in this disorder au-

toantibodies against the muscarinic M3 acetylcholine receptor may block the receptor and lead to contraction of the bladder. Systemic lupus erythematosis (SLE) has also been associated with IC, but cystitis in these patients, called lupus cystitis, may be a focal manifestation of SLE rather than a separate, comorbid disorder (Boye et al., 1979). In addition, IC patients are more likely to experience allergies than the general population. Alagiri, Chottiner, Ratner, Slade, and Hanno (1997) reported that allergies are the most common IC-associated symptom, and the prevalence in IC patients (40%) is greater than that of the general population (22.5%; Diepgen and Fartasch, 1992). In either an autoimmune or allergic reaction, the initial immune response to one's own tissue or an environmental trigger may lead to mast cell recruitment. This could potentially enhance bladder mast cells infiltration and activation and contribute to symptoms of BPS/IC.

In summary, an increase in mast cell infiltration and activation has been demonstrated in IC, and this increase is postulated to mediate the relationship between BPS/IC and bladder neuropeptide content, gonadal steroid hormones, stress, autoimmune disorders, and allergies. However, there is evidence that mast cells are not increased in all IC patients. Therefore, increased mast cells may only account for a subset of BPS/IC patients. Also, excluding the limited trial of the H2 receptor antagonist cimetidine (Thilagarajah et al., 2001), no effective treatment has been developed based on a mast cell etiology. Perhaps the most important reservation regarding mast cells as an etiology for BPS/IC is that the increase in mast cell number and release observed in patients is likely secondary to an initial insult that damages the bladder or otherwise permanently changes bladder pain processing. Mast cells may contribute to bladder pain and functional symp-

toms, but the underlying cause of the disorder is still unknown. Therefore, evidence is far from conclusive that mast cells play a causal role in the etiology of PBS/IC.

While there is some evidence for the contribution of factors described thus far in the etiology of BPS/IC, one process that more clearly contributes to symptoms in all BPS/IC patients is altered activation or function of neuronal pathways underlying pain processing since pain is a defining feature of the disorder. Neuronal changes that may contribute to abnormal pain processing from the bladder have been demonstrated in BPS/IC patients. For example, staining with protein gene product 9.5 (PGP 9.5) revealed nerve fiber proliferation in the subepithelial and muscle layers of bladders of IC patients (Christmas, Rode, Chapple, Milroy, & Turner-Warwick, 1990). Nerve fiber proliferation may serve as a basis for augmented bladder input to the spinal cord and thus contribute to bladder pain. In addition, IC patients display more SP-positive nerve fibers (Pang et al., 1995a) and increased levels of mRNA for the corresponding receptor, neurokinin-1 (NK1), in the vascular endothelium (Marchand, Sant, & Kream, 1998). SP is a vasodilatory neuropeptide expressed in a subset of unmyelinated (C-fiber) primary afferents that project to the superficial dorsal horn of the spinal cord (lamina I and II_o). SP has been proposed to be a principal neurotransmitter mediating nociception from the bladder because it is found in bladder primary afferents in both humans (e.g. Gu et al., 1984) and rats (e.g. Lasanen, Tammela, Liesi, Waris, & Polak, 1992). In addition, intravenous (i.v.) or intravesical administration of SP, as well as release of endogenous SP after stimulation of primary afferents or intravesical administration of capsaicin, produce signs of inflammation such as increased vascular permeability (Abelli et al., 1992; Bjorling, Saban, & Saban, 1994; Gu et al., 1984; Lasanen et al., 1992; Lundberg, Brodin, Hua, & Saria,

1984). Neurogenic inflammation due to SP release is associated with bladder hypersensitivity, because release of SP after pelvic nerve stimulation or intra-arterial (i.a.) or intrathecal (i.t.) administration of SP increases bladder contractions during cystometrogram (CMG) testing (Chien, Yu, Lin, Lai, & Hsu, 2003). Also, mustard oil and cyclophosphamide administration, which lead to bladder inflammation and hypersensitivity, increase primary afferent and spinal dorsal horn SP content (Callsen-Cencic & Mense, 1997; Vizzard, 2001; see section 1.2. *Animal Models using Bladder Inflammation*). Finally, knockout of the receptor for SP, neurokinin-1 (NK-1), reduces nociceptive behaviors after cyclophosphamide-induced bladder inflammation (Laird et al., 2000). Because SP may mediate bladder nociception, an increase in the number of submucosal fibers expressing SP in IC patients may amplify bladder input to the spinal cord. VIP and neuropeptide Y positive fibers are also increased in IC patients and may contribute to bladder hypersensitivity (Hohenfellner et al., 1992).

From the factors discussed, the following model of the pathogenesis of BPS/IC can be devised (Goins et al., 2009). A triggering event, such as infection of the bladder in susceptible individuals, may disrupt the GAG layer, exposing the bladder epithelium to irritants. The defective GAG layer may persist because of suppressed epithelial repair due to APF release. An immune reaction to irritants may lead to mast cell infiltration and activation and secretion of inflammatory mediators such as histamines. Bladder afferents may also be sensitized by irritants. SP released by these afferents may initiate more mast cell secretion, forming a positive feedback loop and amplifying inflammation and primary afferent activation. This would lead to a painful, hypersensitive bladder.

1.2. Animal Models using Bladder Inflammation

1.2.1. Mustard Oil and Other Irritants

While not directly related to BPS/IC, several animal models using inflammation to produce bladder pain have been developed. For example, Koltzenburg and McMahon (1986) and McMahon and Abel (1987) found that 2.5% mustard oil, 25% turpentine, or 2% croton oil induced significant Evans blue plasma extravasation, edema, and leukocyte infiltration indicative of the presence of neurogenic inflammation. Moreover, all these agents produced a hyper-reflexive bladder, as indicated by increased baseline intravesical pressures and lowered micturition thresholds. 2.5% mustard oil also increased the magnitude of the visceromotor reflex (VMR) and decreased the VMR threshold during graded, phasic distension of the bladder in mice (Ness & Elhefni, 2004). The VMR is a primary response measure of bladder nociception and is used in the current study. It will be explained in detail in section *1.2.4. Quantification of Bladder Nociception: the VMR to UBD.*

Intravesical administration of chemical irritants such as turpentine, mustard oil, croton oil, and xylene also increased the basal and evoked activity of bladder afferents and L6/S1 spinal dorsal horn neurons (McMahon, 1988; Su, Sengupta, & Gebhart, 1997a). Moreover, mustard oil (2.5%) altered primary afferent neuropeptide expression, increasing the proportion of bladder afferent neurons expressing calcitonin gene-related peptide (CGRP) and SP immunoreactivity in rostral lumbar (L1, L2) and lumbosacral (L6, S1) dorsal root ganglia (DRG; Callsen-Cencic & Mense, 1997). CGRP, like SP, is a vasodilatory neuropeptide expressed in peptidergic bladder primary afferents that is thought to be a primary mediator of bladder nociception (Gabella & Davis, 1998). In ad-

dition, intravesical administration of mustard oil increased the proportion of galanin-positive lumbosacral DRG and nitric oxide synthase (NOS)-positive rostral lumbar DRG (Callsen-Cencic & Mense, 1997). One additional irritant, 1% acetic acid, produced a hyper-reflexive bladder, increased spinal neuronal activation, and increased immediate-early gene product (c-Fos) immunoreactivity in lumbosacral (L6-S1) spinal cord (Birder & DeGroat, 1992, 1993). All these changes indicate that intravesical administration of bladder irritants enhances nociceptive input to the central nervous system (CNS) from the bladder.

1.2.2. Cyclophosphamide

The chemotherapeutic agent cyclophosphamide is metabolized and excreted as acrolein. Acrolein is a potent bladder irritant. Cyclophosphamide administration has been widely studied for its capacity to produce inflammation, bladder hyperreflexia, and bladder hypersensitivity through the action of this metabolite on the bladder. For example, cyclophosphamide (100 mg/kg i.p.) induces a characteristic set of nocifensive behaviors including lacrimation, piloerection, a rounded-back posture, head immobility, and brief crises involving tail hyperextension, abdominal retractions, licking of the lower abdomen, and backward withdrawal movements (Lanteri-Minet, Bon, de Pommery, Michiels, & Menetrey, 1995). These behaviors are accompanied by gross histological changes of the bladder that progress from chorionic edema to complete cystitis including edema, mucosal abrasion, fibrin deposit, leukocyte infiltration, and petechial hemorrhage. These changes also correspond with spinal activation as indicated by immediate-early gene product (c-Fos and Krox-24) expression.

Cyclophosphamide-induced cystitis has become the most commonly-used model of inflammatory bladder pain. It has been found to increase the excitability of L6 and S1 bladder afferent neurons (Yoshimura & de Groat, 1999). Also, pERK expression and ERK activation are increased in the DRG of bladder afferent neurons after inflammation indicating increased primary afferent activation. For example, cyclophosphamide-induced bladder inflammation increases the percentage of pERK positive bladder afferent neurons in the L6 and S1 DRG (Corrow & Vizzard, 2009) and increases pERK levels in homogenates of L1, L2, L6, and S1 DRG (Qiao & Gulick, 2007). At the spinal cord, spinal pERK levels are increased in homogenates of L1, L2, L6, and S1 spinal cord after cyclophosphamide (Qiao & Gulick, 2007), though increases are not found when L1, L2, and L5-S1 segments are examined separately (Corrow & Vizzard, 2009). Also, immediate-early gene product (c-Fos and Krox-24) expression is increased after cyclophosphamide (Lanteri-Minet, 1995). Finally, cyclophosphamide leads to increases in spinal neuropeptide content. For example, CGRP and SP immunoreactivity is increased in the L6 and S1 dorsal horn and DRG after cyclophosphamide (Vizzard, 2001). One drawback to the use of cyclophosphamide is that it is poorly tolerated in some regimens because it can produce extensive damage to bladder tissue. Therefore, it is a less than desirable model to study the mechanisms underlying physiological processes that normally result in bladder pain, such as UTI. One model that may be more useful for this purpose is zymosan-induced bladder inflammation.

1.2.3. Zymosan

The pro-nociceptive effects of zymosan have been characterized in the skin (Kunz et al., 2005; Randich, Meller, & Gebhart, 1997) and colon (Coutinho, Meller, & Gebhart, 1996; Coutinho & Gebhart, 1999; Honore, Kamp, Rogers, Gebhart, & Mantyh, 2002), and have now been extensively characterized in the rat bladder (DeBerry, Ness, Robbins, & Randich, 2007; DeBerry, Randich, Shaffer, Robbins, & Ness, 2010; Ness & Randich, 2010; Randich, Uzzell, Cannon, & Ness, 2006a; Randich, Uzzell, DeBerry, & Ness 2006b; Randich, Mebane, DeBerry, & Ness, 2008a; Randich, Shaffer, Ball, & Mebane, 2008b; Randich, Mebane, & Ness, 2009). Zymosan is a glucan purified from the cell wall of the yeast *Saccharomyces cerevisiae*. It binds to toll-like receptor 2 (TRL2) and activates the NF-κB pathway, culminating in inflammation (Sato et al., 2003). Unlike cyclophosphamide, zymosan is well-tolerated and produces receptor-mediated inflammation similar to that which occurs during urinary tract infection (UTI). When instilled intravesically, Evans blue plasma extravasation indicated that zymosan produces bladder inflammation that peaks 24 hours after administration (Randich et al., 2006a). Inflammation is accompanied by a modest bladder hypersensitivity to mechanical distension of the bladder, as measured by an increase in mean arterial pressure (MAP) and the VMR during urinary bladder distension (UBD). The translational potential of this model of bladder inflammation is important because a number of UTI are mycotic (for a review, see Malani & Kauffman, 2007). Moreover, BPS/IC patients often describe their symptoms as similar to those associated with UTI, and therefore similar neuronal mechanisms might underly the transmission of bladder pain during BPS/IC, UTI, and zymosan-induced bladder inflammation.

1.2.4. Quantification of Bladder Nociception: the VMR to UBD

Quantifying changes in visceral, as compared to somatic, nociceptive function has been a challenge. As noted previously, one well-characterized method is the VMR to UBD and colorectal distension (CRD). The VMR is a pseudo-affective reflex to noxious stimulation of the viscera and involves contractions of the hindlimb and abdominal musculature (Woodworth & Sherrington, 1904). Ness and Gebhart (1998) determined that the VMR to CRD is a spino-bulbo-spinal reflex since it is present after midcollicular transection (decerebrate rat) but not after C1 or T6 transection (spinalized rat). The VMR to CRD has been used extensively to determine the effects of pharmaceuticals (Banner & Sanger, 1995; Danzebrink & Gebhart, 1991; Greenwood-Van Meerveld, Johnson, Cochrane, Schulkin, & Meyers, 2005; Greenwood-Van Meerveld, Venkova, Hicks, Dennis, & Crowell, 2006a; Gschossmann et al., 2001; Kiso et al., 2001; Miranda et al., 2007; Ness, 2000; Ness & Gebhart, 1988a; Shen, Yang, Qian, & Hou, 2010; Traub, Zhai, Ji, & Kovalenko, 2002), gonadal steroid hormones (Ji, Murphy, & Traub, 2003; Ji, Tang, & Traub, 2008; Lu et al., 2007), surgical lesion (Palecek & Willis, 2003; Traub, 2000), excitation of elements of the pain pathway (Qin, Greenwood-Van Mejerveld, & Foreman, 2003; Tsuruoka, Maeda, & Inoue, 2005), stress (Bradesi et al., 2006, 2007; Gunter, Shepard, Foreman, Myers, & Greenwood-Van Meerveld, 2000; Ren et al., 2007; Shen et al., 2010), and inflammation (Coutinho et al., 1996; Greenwood-Van Meerveld, Johnson, Schulkin, & Meyers, 2006b; Lamb, Zhong, Gebhart, & Bielefeldt, 2006; Miranda et al., 2007) on the processing of nociceptive input from the colon. The VMR to UBD has also been extensively used as an index of bladder nociception (Ball, Ness, & Randich, 2010; Blatt, Lashinger, Laping, & Su, 2009; Castroman & Ness, 2001; DeBerry et al., 2007; Hu

et al., 2009; Randich et al., 2006 a,b, 2008 a,b; Su et al., 2008a; Su, Leon, & Laping, 2008b; Su et al., 2008c; Su, Riedel, Leon, & Laping, 2008d). The VMR to UBD satisfies the three criteria for a valid model of visceral nociception as proposed by Ness (1999) in that it is reliable, not nonspecifically inhibited by known nonanalgesics, and inhibited by known analgesics. Responses are reproducible within animals after an initial sensitization period (Castromann & Ness, 2001; Ness, Lewis-Sides, & Castroman, 2001). The nonanalgesic atropine does not alter the incidence of VMR to UBD, whereas the VMR to UBD is inhibited by commonly used analgesics including morphine, fentanyl, and lidocaine (Castromann & Ness, 2001; Ness et al., 2001). The validity of using the VMR as a measure of bladder nociception in animals is also supported by several parallels that exist between the VMR observed in response to UBD in animals and verbal reports of sensation intensity during UBD in women. For example, the threshold to evoke a VMR to UBD in rats occurs at similar intravesical pressures (between 20 and 40 mmHg) to that which produce discomfort during UBD via saline infusion in healthy female volunteers (24.4 ± 2.4 cm H_20; Ness, Richter, Varner, & Fillingim, 1998). This indicates that the VMR may be selective for noxious distending pressures. In addition, the strength of the VMR to UBD increases with repeated distensions (Castromann & Ness, 2001), and sensitization also occurs with repeated UBD in women (Ness et al., 1998). Finally, UBD has been shown to evoke visceromotor activity during UBD in healthy human volunteers (Fagius & Karhuvaara, 1989).

Although initial analysis of the VMR to UBD was restricted to reporting incidence rate and threshold for evoking a VMR (Ness et al., 2001), technological advances have allowed for better quantification of the VMR, which is now typically reported as

area under the curve of rectified electromyographic (EMG) activity. Observation of EMG activity has revealed that the VMR to UBD increases in a graded manner with increasing distension pressure. Therefore, it is possible to observe how a manipulation alters responses to innocuous (10-30 mmHg), noxious (30-60 mmHg), and super-physiological (60 mmHg-80 mmHg) pressures. The capacity for quantification, reliability, sensitivity to analgesics, and parallels to findings in humans support the use of the VMR to UBD as a measure of bladder nociception in studies of the mechanisms underlying bladder hypersensitivity.

1.3. Early-in-Life (EIL) Inflammation

The animal models described previously utilized adult bladder inflammation to examine the basic mechanisms underlying bladder pain. Understanding these basic mechanisms is essential for understanding the nature of pain pathways activated by acute bladder inflammation and is a first step in developing therapeutic targets. However, animal models must be developed to address the long-term changes in pain processing that occur in chronic pain states such as BPS/IC where there is no clear-cut bladder pathology. One model that has the potential to produce such long-term changes utilizes EIL inflammation of the bladder. This model proposes that an EIL insult to the bladder, such as a UTI, may prime or predispose an individual to experiencing bladder pain as an adult, especially after a second insult to the bladder (Randich et al., 2006b). This model is supported by data showing that women with BPS/IC are more likely to recall recurrent childhood UTI and antibiotic use (Peters, Killinger, & Ibrahim, 2009). The focus of this

thesis is alterations in bladder nociceptive sensitivity produced by EIL (P14-P16) bladder inflammation as it relates to κ-opioid function.

1.3.1. Development of Nociceptive Pathways

The postnatal period is a time when noxious stimuli are uncommon, but if they occur such encounters have the potential to alter developing pain pathways. In rats, and to a lesser extent in humans, nociceptive pathways do not become functionally mature until several weeks after birth (for a review, see Fitzgerald, 1995). For example, organization of somatic peripheral nerve terminals increases in complexity after birth (e.g. Payne, Middleton, & Fitzgerald, 1991; Reynolds, Fitzgerald, & Benowitz, 1991). The ability of C-fibers to produce neurogenic inflammation does not develop until postnatal days 11-14 (Fitzgerald & Gibson, 1984). Maturation of C-fibers may be required to restrict A-fibers, which initially occupy laminae I-V, to their adult targets in laminae III-V (Fitzgerald, Butcher, & Shortland, 1994). Central dorsal horn neurons are also immature at birth, with axo-dendritic growth in the substantia gelatinosa (SG) delayed until after birth (Bicknell & Beal, 1984). Connections between C-fibers and SG neurons require a prolonged postnatal maturation process. Functionally, this process leads to a delay in the ability of C-fiber stimulation to activate dorsal horn neurons because of a lack of the required C-fiber action potential bursts (Fitzgerald, 1988; Hori & Kanda, 1994; Hori & Watanabe, 1987; Soyguder, Schmidt, & Morris, 1994; Williams, Evan, & Hunt, 1990). For instance, mustard oil, a C-fiber irritant, is unable to evoke a flexion response or evoke c-Fos or nitric oxide synthase expression until the second postnatal week when applied to hindlimb skin (Fitzgerald & Gibson, 1984; Soyguder et al., 1994; Williams et al., 1990).

Concurrent with development of anatomical connections between primary afferents and second order neurons, neurochemical identity also matures postnatally. For example, somatostatin, CGRP, SP, galanin, and VIP are not trafficked to axons until after birth, and the number of peptidergic neurons begins to decline after birth (Jonakit, Ni, Walker, & Hart, 1991; Marti et al., 1987). NK-1 and N-Methyl-D-aspartate (NMDA) receptors are also restricted to specific nuclei postnatally (Charlton & Helke, 1986). In addition, functional vasopressin receptors are eliminated from nociceptive pathways (Tribollet, Goumaz, Raggenbas, Dubois-Dauphin, & Dreifuss, 1991). The affinity of NMDA receptors and NMDA-induced elevation of $[Ca^{2+}]_i$ also declines postnatally (Hori & Kanda, 1994).

Postnatal modifications discussed thus far primarily involve the development of excitatory neurotransmission. Inhibitory neurotransmission also develops during the early postnatal period. Behaviorally, inhibition of reflex responses to noxious stimuli and restriction of responses to the injured area occur postnatally (P10-P15) (Bicknell & Beal, 1984; Guy & Abbott, 1992). This is accompanied by a decrease in dorsal horn neuron receptive field size during the first 2 postnatal weeks (Fitzgerald, 1985). Maturation of SG interneurons, which are largely inhibitory, also occurs postnatally (Bicknell & Beal, 1984). The postnatal shift from depolarizing to hyperpolarizing action of GABA and glycine must also play an important role in the development of spinal inhibition (Reichling, Kyrozis, Wang, & MacDermott, 1994). Finally, descending modulatory pathways, which are also critical for inhibition of noxious inputs, are not functionally mature until P21 (Boucher, Jennings, & Fitzgerald, 1998; Fitzgerald & Koltzenburg, 1986; for a review, see Fitzgerald & Jennings, 1999).

One specific system important for inhibition of nociceptive transmission, the opioid system, also undergoes substantial postnatal modifications. In the spinal cord, μ- and κ-opioid receptor binding is present at birth (McDowell & Kitchen, 1987). Binding peaks between P1 and P7, after which binding decreases and becomes concentrated to neurons located in the spinal dorsal horn (Attali, Saya, & Vogel 1990; Kar & Quirion, 1995; McDowell & Kitchen, 1987; Rahman, Dashwood, Fitzgerald, Aynsley-Green, & Dickenson, 1998). δ-opioid receptors appear later, within the first two weeks of postnatal development (McDowell & Kitchen., 1987; Rahman et al., 1998). Opioid receptor function also changes during the early postnatal period, with the sensitivity to morphine and κ-opioid receptor agonists being greater during development than in adult rats. The ED_{50} for morphine to inhibit C-fiber evoked responses of dorsal horn neurons is much less at P21 than in adults (Rahman et al., 1998). Also, the ability of morphine to inhibit responses on the tail flick and hot plate tests is greater in neontates than adult rats (McLaughlin & Dewey, 1994). Enadoline, a κ-opioid agonist, is a more potent inhibitor of responses on the formalin test in neonatal compared to adult rats (McLaughlin et al., 1995). In addition, i.t. administration of U50,488H and U69,593, κ-opioid receptor agonists, produce selective inhibition of C-fiber evoked responses of spinal dorsal horn neurons at lower doses in P21 rat pups than in adult rats (Sullivan & Dickenson, 1991). Interestingly, the effects of these κ-opioid receptor agonists are reversible by the κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI) only in rat pups (Sullivan & Dickenson, 1991). In contrast, naloxone is able to reverse inhibition by U50,488H and U69,593 in both adults and pups (Sullivan & Dickenson, 1991). Reversibility by the selective antagonist only in rat pups suggests that as κ-opioid receptor binding decreases during devel-

opment, κ-opioid receptor agonists may become less selective for κ-opioid receptors relative to other opioid receptor subtypes. Postnatal development of the opioid system is especially important in relation to the EIL bladder inflammation model because noxious insults during this period of development may disrupt normal modifications that occur during this period and thereby induce long-lasting changes in opioid modulation of nociception.

1.3.2. Long-Term Effects of EIL Exposure to Noxious Stimuli

There is a mounting interest in the long-term effects of exposure to EIL cutaneous noxious insults because of the growing number of premature infants who often receive repeated heal lancing and other painful procedures. These individuals have especially underdeveloped pain pathways. For example, repeated heel lancing produces a prolonged decrease in the flexor reflex threshold of the damaged heel (Fitzgerald, Millard, & McIntosh, 1989) and pre-term infants display lower mechanical withdrawal thresholds than full-term infants (Andrews & Fitzgerald, 1994).

The impact of EIL noxious stimuli has been examined in detail in animal models. In rats, EIL hindpaw inflammation has been shown to produce hypersensitivity and neuroanatomical changes that last into adulthood. For example, Ruda, Ling, Hohmann, Peng, & Tachibana. (2000) found that persistent (5-7 day) hindpaw inflammation induced by injection of Complete Freund's Adjuvant (CFA) on P1 followed by adult (8-10 weeks) re-inflammation with CFA decreased thermal paw withdrawal latencies compared to adult inflammation alone. Neonatal hindpaw inflammation by itself shifted the late phase of the formalin test to an earlier phase (Ruda et al., 2000). In studies of spinal dorsal

horn neurons, neonatal hindpaw inflammation alone increased their spontaneous activity and responses to both innocuous brushing and noxious pinch (Ruda et al., 2000). A subsequent study replicated these findings and found that neonatal CFA also increased receptive field size and responses of spinal dorsal horn neurons to noxious (49ºC) heat in adult rats (Peng, Ling, Ruda, & Kenshalo, 2003). Retrograde tracing of nociceptive, non-myelinated and thinly myelinated neurons with WGA-HRP injected into the sciatic nerves revealed an increased density and caudal distribution of dorsal horn primary afferents after neonatal (P0, P1, P3, but not P14) hindpaw inflammation (Ruda et al., 2000). The increase in primary afferent density corresponded with an increase in CGRP staining (Ruda et al., 2000). Adult inflammation was not required to observe increases in primary afferent density or neuropeptide content after EIL inflammation. However, adult re-inflammation does appear to be required for hypersensitivity to develop in EIL-treated rats, since in a separate study no changes in mechanical or thermal thresholds were observed in adult (8+ weeks) rats after neonatal (P1) hindpaw inflammation with carrageenan or CFA by itself (Walker, Meredith-Middleton, Cooke-Yarborough, & Fitzgerald, 2003).

Ren et al. (2004) systematically examined the critical period for development of changes in thermal and mechanical sensitivity after neonatal hindpaw inflammation. They found that injection of carrageenan on P0, P1, P3, or P5 (but not at P8, P10, P12, or P14) by itself *increased* thermal and mechanical withdrawal latencies when rats reach P40 (Ren et al., 2004). Surprisingly, these data indicate that animals were less sensitive to noxious stimuli, or hypoalgesic, at P40 after an EIL encounter with hindpaw inflammation. In contrast, postnatal carrageenan injection occurring on or before P8 followed

by adult (P40) re-inflammation with carrageenan *decreased* these withdrawal latencies (Ren et al., 2004). Therefore, when EIL hindpaw inflammation was followed by adult re-inflammation, animals were more sensitive to noxious stimuli, or hyperalgesic. The time course for manifestation of hyperalgesia differed from that for hypoalgesia. Hypoalgesia was manifested starting on P34, while re-inflammation induced hyperalgesia was present when re-inflammation occurred as early as 4 days after the initial inflammation (P7; Ren et al., 2004). The hypoalgesia observed after EIL inflammation alone was due to bilateral changes in nociceptive transmission because it was observed in both hindpaws (Ren et al., 2004). In contrast, hyperalgesia after re-inflammation was restricted to the inflamed hindpaw and therefore reflected unilateral changes in nociceptive transmission (Ren et al., 2004).

EIL noxious stimulation of the viscera also has been proposed as a contributing factor to functional bowel or bladder disorders in adults such as IBS or BPS/IC. Because of this theory, the effects of EIL inflammation of the colon or bladder have been examined in rats. Al-Chaer, Kawasaki, and Pasricha (2000) demonstrated that neonatal irritation of the colon from P8 through P21 with repeated mechanical distension or administration of mustard oil decreased the threshold and increased the magnitude of the abdominal withdrawal reflex response to CRD when animals were tested as adults. Hypersensitivity to adult CRD was accompanied by increases in the baseline activity and responses of CRD-sensitive spinal dorsal horn neurons to graded CRD, pinch, and deep nerve stimulation (Al-Chaer et al., 2000). No such changes were observed when colon irritation occurred during the periadolescent (beginning on P21) or young adult (beginning on P45) period (Al-Chaer et al., 2000). Hematoxylin and eosin (H&E) staining of the colon re-

vealed no significant structural damage, loss of crypts, mucin depletion, or increase in intraepithelial lymphocytes (Al-Chaer et al., 2000). These data suggest that, by adulthood, any pathological changes to the colon had resolved and that long-term increases in the excitability of spinal dorsal horn neurons (central sensitization) persisted in the absence of gross colon pathology. A subsequent study (Lin & Al-Chaer, 2003) revealed that neonatal colon irritation also increases the excitability of primary afferents. Repeated CRD increased the responses of thoracolumbar and lumbosacral afferents to CRD and the number of lumbosacral and thoracolumbar afferents activated by CRD. In addition, repeated CRD increased the spontaneous activity of lumbosacral afferents while decreasing the response threshold for activation of lumbosacral afferents (Lin & Al-Chaer, 2003). Increases in primary afferent input may drive central sensitization and ultimately behavioral hypersensitivity.

Randich et al. (2006b) and DeBerry et al. (2010) showed that, similar to colon irritation, EIL bladder inflammation produced adult bladder hypersensitivity in the absence of any of gross histological changes of the bladder. Once daily intravesical treatment with zymosan (P14-P16; 1%) increased VMR and cardiovascular responses to graded UBD compared to animals treated with anesthesia only from P14-P16 when both groups received zymosan as adults (12+ weeks of age). This EIL treatment also increased adult baseline micturition frequency and decreased micturition thresholds compared to saline or anesthesia-treated controls (Randich et al., 2006b; DeBerry et al., 2010). EIL bladder inflammation also altered the responses of L6-S1 dorsal horn neurons after adult treatment with zymosan or anesthesia only (Ness & Randich, 2010). In this study, neurons were classified by responses to a heterotopic noxious conditioning stimulus (HNCS).

Type I neurons were defined as being inhibited (>20% inhibition) by HNCS and type II neurons were defined as not being inhibited by HNCS. When animals were exposed to anesthesia only EIL, an adult intravesical zymosan treatment increased the spontaneous and UBD-evoked activity of only type II neurons (Ness & Randich, 2010). In contrast, when animals were exposed to zymosan EIL, the adult zymosan treatment increased the spontaneous and UBD-evoked activity of both types of neurons (Ness & Randich, 2010).

Animals inflamed EIL also display enhanced plasma extravasation produced by intravesical mustard oil (DeBerry et al., 2010), which reflects neurogenic inflammation of the bladder due to the release of neuropeptides such as SP and CGRP from bladder afferents. Bladder SP and CGRP content presumably reflects primary afferent content and was increased after EIL inflammation (DeBerry et al., 2010; Shaffer, Ball, Robbins, Ness, & Randich, 2011). Importantly EIL inflammation alone does not produce gross histological changes in the adult bladder, such as changes in thickness, as assessed using hematoxylin and eosin staining, fibrosis, measured using Gomori's trichrome, or mast cell infiltration, observed using toluidine blue (DeBerry et al., 2010). In summary, EIL bladder inflammation produces long-term neuronal changes that may contribute to an increase in bladder hypersensitivity after adult re-inflammation in the absence of gross pathology of the adult bladder. Processes that contribute to bladder hypersensitivity in this model could potentially be similar to those contributing to the symptoms of BPS/IC, especially since BPS/IC patients recall more experiences with EIL inflammation than asymptomatic controls (Peters et al., 2009).

1.4. Opioids and their Role in Mediating Bladder Hypersensitivity

1.4.1. The Opioid System

One specific aspect of nociceptive processing that is altered by EIL bladder inflammation is the opioid system (DeBerry et al., 2007). The pain-relieving properties of opiates are well-established. Opiates act on the same seven-transmembrane domain G-protein coupled receptors (GPCRs) as a group of endogenous peptides, referred to as opioids. These GPCRs, called opioid receptors, include three well-established subtypes: the μ-opioid receptor, the δ-opioid receptor, and the κ-opioid receptor (for reviews, see Lord, Waterfield, Hughes, & Kosterlitz, 1977; Pan, 2008; Przewlocki & Przewlocka, 2001). Endogenous ligands include β-endorphins, which are relatively selective for the μ-opioid receptor (Guillemin, Ling, & Burgus, 1976); enkephalins, which bind to the μ- and δ-opioid receptor (Hughes, Smith, Morgan, & Fothergill, 1975); dynorphins, which are relatively selective for κ-opioid receptor (Goldstein, Tachibana, Lowney, Hunkapiller, & Hood, 1979; Lowney, Gentleman, & Goldstein, 1979); and endomorphins, which are highly selective for μ-opioid receptor (Zadina, Hackler, Ge, & Kastin, 1997). Activation of opioid receptors by endogenous or exogenous ligands initiates a signaling cascade. Generally, associated G_i/G_o proteins are activated, resulting in the opening of K^+ channels (Marker, Lujan, Loh, & Wickman, 2005; North, Williams, Surprenant, & Christie, 1987; Williams, Egan, & North, 1982) or inhibition of Ca^{2+} channels (Moises, Rusin, & Macdonald, 1994 a,b; Wu, Chen, & Pan, 2004). Channels are modulated either through direct interactions with the Gβγ subunit or through a signaling cascade involving decreases in cAMP. The final result of receptor activation is hyperpolarization, which decreases the excitability of the cell (Brandt et al., 1976; Kohno, Kumamoto, Higashi,

Shimoji, & Yoshimura, 1999; Light & Willcockson, 1999; for reviews, see Dickenson and Kieffer, 2005; Grudt & Williams, 1995; Pan, Li, Chan, & Pan, 2002; Pan et al., 2008; Waldhoer, Bartlett, & Whistler, 2004). Following the development of expression cloning and the subsequent cloning of the δ-opioid receptor (Evans, Keith, Morrison, Magendzo, & Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff, & Hirth, 1992), a novel receptor with very high sequence homology to the opioid receptors was discovered and named the opioid receptor like-1 (ORL-1) receptor (Uhl, Childers, & Pasternak, 1994). Shortly thereafter, its endogenous ligand, nociceptin/orphanin FQ, was identified (Meunier et al., 1995; Reinsheid et al., 1995). There is evidence that nociceptin/orphanin FQ activates the same signaling cascades as the classical opioid receptors (Meunier et al., 1995; Reinscheid et al., 1995; Connor, Vaughan, Chieng, & Christie, 1996; Vaughan & Christie, 1996; for a review, see Harrison and Grandy, 2000), and may therefore also have important implications for the modulation of nociception (for a review, see Harrison & Grandy, 2000). As discussed later, the dynorphin/κ-opioid system may be especially important for the modulation of visceral nociception. Therefore, the following introductory material concentrates on this system since it serves as the focus of this thesis.

1.4.2 Localization

Opioid peptides and receptors are present throughout the CNS including areas involved in autonomic regulation, reward, and nociception. Medullary (e.g. rostral ventral medulla; RVM), pontine (parabrachial nucleus; PBn), and midbrain (e.g. periaqueductal gray; PAG) areas are important for the antinociceptive impact of opioids. In addition to supraspinal areas, opioid peptides and receptors are also located in primary afferents,

spinal interneurons, and the spinal terminals of descending fibers. They are especially concentrated in the superficial lamina I and II of the spinal dorsal horn, where afferent inputs from the periphery and descending modulatory inputs from supraspinal areas converge. In addition to the superficial dorsal horn, laminae V, VI, and X appear to be important for control of visceral nociception (Sugiura, Terui, & Hosoya, 1989). In general, opioid peptides and receptors are relatively concentrated in the spinal cord compared to other areas of the body, and endogenous and exogenous opioid agonists acting on spinal opioid receptors have been shown to have antinociceptive effects. Therefore, the current study focused on spinal sites of action of opioids.

The dynorphin/κ-opioid system consists of dynorphin A, dynorphin B, and α-neoendorphin, which are the active peptide products of the prodynorphin gene, and κ-opioid receptors, which are encoded by opioid receptor, kappa 1 (Oprk1). In the spinal cord, dynorphin is located primarily in the dorsal horn (e.g. Arvidsson et al., 1995; Botticelli, Cox, & Goldstein, 1981; Cruz & Basbaum, 1985; Khachaturian et al., 1982; Vincent, Hokfelt, Christensson, & Terenius, 1982) and is concentrated in lamina I (Marvizon, Chen, & Murphy, 2009). Dynorphin exhibits some colocalization with CGRP and SP, markers of peptidergic primary afferents, but not IB4, a marker of non-peptidergic primary afferents (Marvizon et al., 2009; Tuchscherer & Seybold, 1989). Further evidence that some primary afferents contain dynorphin includes the finding that dorsal rhizotomy decreases dynorphin immunoreactivity in the rat dorsal horn (Tuchscherer & Seybold, 1989, but see Botticelli et al., 1981). The presence of dynorphin-containing neurons that do not also contain CGRP, SP, or IB4 and the incomplete depletion of dynorphin by dorsal rhizotomy (Tuchscherer & Seybold, 1989) or spinal transection (Mac-

Arthur, Ren, Pfaffenroth, Franklin, & Ruda, 1999) indicate that some dynorphinergic fibers are intrinsic dorsal horn neurons.

Neurons expressing κ-opioid receptors are concentrated in close proximity to dynorphin-containing neurons. κ-opioid receptors are detected in the superficial dorsal horn of the spinal cord, where they also colocalize with CGRP and SP (Belanger, Ma, Chabot, & Quirion, 2002), as well as being in lamina X (Gouarderes, Cros, & Quirion, 1985). κ-opioid receptors are predominantly localized to dendrites, axons, and axon terminals, and therefore may act both pre- and postsynaptically (Harris, Chang, & Drake, 2004). Arvidsson et al. (1995) demonstrated that prodynorphin-immunoreactive neurons exist in close apposition with κ-opioid receptor immunoreactive neurons. However, autoinhibition of a dynorphin-containing neuron through activation of κ-opioid receptors on the same neuron is unlikely because no unambiguous incidence of colocalization of prodynorphin and κ-opioid receptors was noted (Arvidsson et al., 1995). In addition, κ-opioid receptor immunoreactivity has been demonstrated in primary afferent DRG (Arvidsson et al., 1995; Mansour, Burke, Pavlic, Akil, & Watson, 1996). In conclusion, dynorphin and κ-opioid receptors are both concentrated in the superficial dorsal horn and expressed by some peptidergic primary afferents. The close apposition shown by Arvidsson et al. (1995) provides additional immunocytochemical evidence that a functional circuit exists for interactions between dynorphin and κ-opioid receptors in the spinal cord. This circuit may involve dynorphin's action on primary bladder afferents or intrinsic dorsal horn neurons activating pre- or postsynaptic κ-opioid receptors and thereby altering the excitability of that neuron.

1.4.3. κ-opioid Receptor Activation or Blockade

Antinociception as a result of μ- and δ-opioid receptor activation has been consistently noted in cutaneous nociceptive models. For example, studies using naloxone as well as selective μ-opioid receptor antagonists (e.g. Chen, Chen, & Pan, 2005; Le Bars, Menetrey, Conseiller, & Besson, 1975; Yaksh & Rudy, 1977) and knockout of the gene encoding μ-opioid receptors (Matthes et al., 1996) have revealed that μ-opioid receptors are required for morphine antinociception. In contrast, the role that dynorphin and κ-opioid receptors play in the modulation of nociception is controversial (for a review, see Yaksh & Noueihed, 1985).

Schmass and Yaksh (1984) found that i.t. administration of κ-opioid receptor agonists ethylketocyclazocine, bremazocine, and U50,488H inhibited responses to visceral chemical stimuli in the acetic acid writhing test but did not significantly alter response latencies to thermal stimuli on the hot plate and tail-flick tests. Similarly, Tung and Yaksh (1982) found no effect of i.t. administration of dynorphin on the tail flick or hot plate tests, while Przewlocki et al. (1983a) found that i.t. administration of dynorphin inhibited responses in the acetic acid writhing test (Przewlocki et al., 1983a). Several other studies have echoed this notion that κ-opioid agonists administered i.t., i.v., or subcutaneously (s.c.) in rats and mice have much weaker effects on responses to thermal stimuli (hot plate and tail-flick tests) compared to non-thermal stimuli (mechanical pressure and chemical irritants including formalin administered s.c. or acetylcholine or acetic acid administered i.p.; Abbott, Franklin, & Libman, 1986; Hayes, Sheehan, & Tyers, 1987; Leighton, Johnson, Meecham, Hill, & Hughes, 1987; Schmauss, 1987; Tyers, 1980; Upton, Sewell, & Spencer, 1982; Vonvoigtlander, Lahti, & Ludens, 1983; Ward & Takemo-

ri, 1983). In contrast, Millan, Czlonkowski, Lipkowski, and Hertz. (1989) found that i.t. administration of U50,488H or U69,592 was equally potent against mechanical and thermal stimuli, as indicated by increased tail withdrawal latencies to either noxious mechanical (pressure) or noxious thermal (radiant heat) stimuli. In addition, Parsons and Headley (1989 a,b) demonstratesd that i.v. administration of U50,488H was equally able to inhibit motoneuron responses to heat or pinch applied to the hindpaw when thermal and mechanical stimulus intensities were matched so that they produced the same pre-drug motoneuron firing rate. Vonvoigtlander et al. (1983) found that U50,488E was equipotent in the ability to inhibit responses in the tail flick (thermal), tail pinch (mechanical), and HCl writhing (visceral chemical) tests in mice. Finally, Han and Xie (1982), Przewlocki et al. (1983a), and Przewlocki, Shearman, & Herz (1983b) demonstrated a marked decrease in withdrawal latencies on the tail flick test with i.t. administration of dynorphin. These latter data indicate that κ-opioid agonists may be effective against thermal as well as mechanical and visceral stimuli. However, perceived efficacy of κ-opioid agonists to inhibit responses to thermal stimuli is especially dependent on stimulus intensity, where the ability to observe thermal antinociception decreases with increases in stimulus intensity (e.g. Millan, 1989).

The presence of both facilitatory and inhibitory effects of κ-opioid agonists on neuronal activity may account for the ambiguous effects of κ-opioid agonists observed in behavioral studies. Knox and Dickenson (1987) found that i.t. administration of U50,488H and dynorphin each gave rise to both facilitation and inhibition of dorsal horn neurons to electrical C-fiber stimulation and noxious pinch, such that no net change was detectible in the population. However, higher doses of i.t. U50,488H and a high dose of

U69,593 produced selective inhibition of dorsal horn neuron responses to electrical C-fiber stimulation and formalin (Sullivan & Dickenson, 1991). In agreement with the bidirectional effects of low dose U50,488H administration, a study by Stanfa and Dickenson (1994) found that some dorsal horn neurons excited by C-fibers displayed a decrease in firing after administration of nor-BNI while others increased firing.

An additional method that has been used to evaluate the role that κ-opioid receptors and dynorphin play in antinociception is deletion of the Oprk1 or prodynorphin genes. Mice deficient in κ-opioid receptors (Martin, Matifas, Maldonado, & Kieffer, 2003) and dynorphin (Wang et al., 2001) display increased thermal sensitivity in the tail immersion test. Also, prodynorphin knockout animals display an increase in the number of nociceptive responses in the second, inflammatory phase of the formalin test (Wang et al., 2001). These results indicate that release of dynorphin and activation of κ-opioid receptors tonically inhibits thermal nociception. In addition, endogenous dynorphin inhibits inflammatory nociception.

In contrast to its impact on acute inflammatory or thermal nociception, dynorphin may sustain nociception after nerve injury, once again reflecting the complexity of the κ-opioid system. Unlike wild type animals, the mechanical and thermal thresholds of prodynorphin knockouts return to baseline after spinal nerve ligation (Wang et al., 2001). The role of dynorphin in the maintenance of chronic inflammatory and neuropathic pain will be discussed in detail in section *1.4.6. Pro-nociceptive and Non-opioid Mediated Effects of Dynorphin*.

In addition to a possible pro-nociceptive role, the ability of the endogenous ligand, dynorphin, to produce antinociception is especially controversial because antinoci-

ception produced by dynorphin is confounded by non-opioid mediated flaccid paralysis (e.g. Przewlocki et al., 1983b; Stevens & Yaksh, 1986) and neurotoxicity (e.g. Caudle & Isaac, 1987; Stewart & Isaac, 1989) that can be produced by concentrations of dynorphin that are required to produce antinociception. Paralysis and neurotoxicity are thought to be mediated through a non-opioid receptor because several studies have found that naloxone and nor-BNI do not prevent or reverse motor dysfunction or nerve damage after i.t. administration of dynorphin (e.g. Faden & Jacobs, 1984; Herman & Goldstein, 1985; Long, Rigamonti, de Costa, Rice, Martinez-Arizala, 1989; but see Bakshi, Ni, & Faden, 1992; Przewlocki et al., 1983b). In addition, fragments of dynorphin with no opioid receptor activity such as dynorphin A (3-13) and dynorphin A (2-17) as well as the non-opioid analogue des-Tyr1-dynorphin(1-13) produce paralysis (Faden & Jacobs, 1984; Przewlocki et al., 1983b; Stevens & Yaksh, 1986). Dynorphin-induced paralysis and nerve damage may be NMDA receptor-dependent. Involvement of NMDA receptors is supported the finding that motor dysfunction and neurotoxicity after i.t. administration of dynorphin are reduced by NMDA antagonists (Bakshi et al., 1992; Skilling, Sun, Kurtz, & Larson, 1992). Dynorphin has not been clearly shown to directly activate NMDA receptors (e.g. Chen, Gu, & Huang, 1995; Brauneis, Oz, Peoples, Weight, & Zhang, 1996; but see Zhang et al., 1997; for a review, see Lai, Ossipov, Vanderah, Malan, & Porreca, 2001). However, paralysis due to spinal dynorphin administration is accompanied by release of excitatory amino acids aspartate and glutamate, which may indirectly activate NMDA receptors (Skilling et al., 1992). Therefore, dynorphin-induced paralysis and nerve damage may be due to excitotoxicity after enhanced release of excitatory amino acids and subsequent indirect activation of NMDA receptors.

1.4.4. Role of Opioid Receptor Subtypes in Visceral Nociception

κ-opioid receptors may be particularly important for the modulation of visceral pain. In addition to the well-established inhibition of the writhing test by κ-opioid agonists (for a review, see Yaksh & Noueihed, 1985), knockout of the gene encoding the κ-opioid receptor increases writhing behavior after i.p. acetic acid injections (Martin et al., 2003; Simonin et al., 1998). The writhing test provides a measure of visceral nociception but is not specific to one organ. Other studies have focused specifically on the bladder and colon, and have revealed that systemic κ-opioid receptor agonists and spinal μ- and δ-opioid receptor agonists inhibit visceral nociception (for a review, see Gebhart, Su, Joshi, Ozaki, & Sengupta, 2000). Systemic (i.a. or i.v.) administration of κ-opioid receptor agonists consistently decreases VMR, MAP, and primary afferent responses to UBD (Su, Sengupta, & Gebhart, 1997a; Su et al., 2008;), CRD (Burton & Gebhart, 1998; Danzebrink, Green, & Gebhart, 1995; Harada, Nishioka, Kitahata, Nakatani, & Collins, 1995; Sengupta, Su, & Gebhart, 1996; Su, Sengupta, & Gebhart, 1997b), and duodenal distension (Diop, Riviere, Pascaud, Dassaud, & Junien, 1994). In contrast, i.a. or i.v. administration of μ- and δ-opioid receptor agonists do not alter the CRD- or UBD-evoked increase in primary afferent activity (Sengupta et al., 1996; Su et al., 1997a). These data suggest that κ-opioid receptor agonists, but not μ- and δ-opioid receptor agonists, can inhibit nociceptive transmission from the bladder and colon by acting at the level of primary afferents. I.t. administration of μ- and δ-, but not κ-opioid receptor agonists decrease VMR and MAP responses to CRD (Danzebrink et al., 1995; Harada et al., 1995). These data indicate μ and δ-opioid receptor agonists inhibit nociception from the colon through activation of spinal opioid receptors and not receptors located on primary afferents.

Morphine administered i.a. or i.v. has also been shown to decrease VMR and MAP responses to UBD (Blatt et al., 2009; Ness et al., 2001; Su et al., 2008d) and CRD (Burton & Gebhart, 1998), presumably by acting at central μ-opioid receptors. In summary, systemic administration of κ-opioid receptor agonists can inhibit responses to UBD and CRD by acting at primary afferents. It is also possible that though i.t. administration of κ-opioid receptor agonists did not inhibit the VMR to CRD, in the bladder it may inhibit the VMR to UBD by acting at the spinal level. However, this hypothesis has not been tested.

1.4.5. Alterations in the Opioid System after Inflammation

In general, the potency of opioid receptor agonists and antagonists increases after inflammation in rats. For example, the antinociceptive efficacy of U50,488H is greater after joint or CFA-induced hindpaw inflammation compared with healthy controls (Neil, Kayser, Gacel, Besson, & Guilbaud, 1986; Stein, Millan, Yassouridis, & Hertz, 1988). Regarding antagonists, naloxone does not change neuronal firing (Le Bars, Chitour, Kraus, Dickenson, & Besson, 1981) or nociceptive responses, including vocalization (Kayser & Guilbaud, 1991) and the VMR to UBD (DeBerry et al., 2007), under basal (non-inflamed) conditions. However, the potency of naloxone increases after cutaneous, joint, or bladder inflammation. For example, when a dose of naloxone that non-selectively blocks the three classical opioid receptors (μ-, δ-, and κ-opioid receptors) is administered i.v., it potentiates nociceptive responses such as vocalization and paw withdrawal to noxious pressure and the VMR to UBD (DeBerry et al., 2007; Kayser & Guilbaud, 1990; Kayser, & Guilbaud, 1991; Millan & Colpaert, 1990, 1991; Millan et al., 1988). This dose of naloxone also increases the firing of dorsal horn neurons (Lombard

& Besson, 1989; Stanfa, Sullivan, & Dickenson, 1992). Interestingly, doses that block μ- and/or δ-opioid receptors, but not κ-opioid receptors, do not appear to change nociceptive responses (Kayser & Guilbaud 1990; Kayser, Chen, & Guilbaud, 1991; Millan & Colpaert, 1991) or dorsal horn firing (Stanfa et al., 1992), suggesting that κ-opioid receptors may be particularly important for the modulation of inflammatory nociception.

The use of selective κ-opioid receptor antagonists provides further support for an important role of κ-opioid receptors in inflammatory nociception. I.t. administration of the κ-opioid receptor antagonists nor-BNI and MR2266 decreases mechanical thresholds for paw withdrawal after cutaneous (Millan et al., 1988; Schepers, Mahoney, Gehrke, & Shippenberg, 2008) or joint (Millan et al., 1987) inflammation, but not in normal, non-inflamed controls. Increases in antagonist efficacy can also be seen at the neuronal level. Nor-BNI increases the activity of more spinal neurons after ankle inflammation than in normal controls (Stiller, Grubb, & Schaible, 1993). Nor-BNI also increases the magnitude of C-fiber evoked responses of dorsal horn neurons in animals that received carragennnan-induced hindpaw inflammation (Stanfa & Dickenson, 1994).

1.4.6. Pro-nociceptive and Non-opioid Mediated Effects of Dynorphin

Changes in nor-BNI efficacy may reflect spinal dynorphin release after inflammation. Though inflammation-induced dynorphin release has not been directly measured, dynorphin mRNA and peptide have been consistently shown to increase in different models of arthritis and hindpaw inflammation (Calza et al., 1998; Draisci & Iadarola 1989; Draisci, Kajander, Dubner, Bennett, & Iadarola, 1991; Hollt, Haarmann, Millan, & Hertz, , 1987; Iadarola, Brady, Draisci, & Dubner, 1988a; Iadarola, Douglass, Civelli, &

Naranjo, 1988b; MacArthur et al., 1999; Millan et al., 1985, 1986, 1987, 1988; Nahin, Hylden, Iadarola, & Dubner, 1989; Nakamura, 1994; Noguchi et al., 1991; Przewlocka, Lason, & Przewlocki, 1992; Ruda, Iadarola, Cohen, & Young, 1988; Weihe, Millan, Hollt, Nohr, & Herz, 1989). Dynorphin may act like a traditional opioid peptide and inhibit nociception during acute release. However, long-lasting increases in dynorphin due to nerve injury or chronic inflammation may instead maintain hyperalgesia or allodynia, possibly through non-opioid receptor-related mechanisms.

Most research in this area has been conducted in neuropathic pain models, and there are converging lines of evidence that dynorphin plays a pro-nociceptive role after nerve injury (for a review, see Lai et al., 2001). Increases in dynorphin mRNA (Draisci et al., 1991) and protein (Kajander, Sahara, Iadarola, & Bennett, 1990; Malan et al., 2000; Wagner, DeLeo, Coombs, Willenbring, & Fromm, 1993) occur after injury to spinal or sciatic nerves. The increase in dynorphin protein peaks at 10 days and is sustained for weeks, coinciding with the time frame during which thermal hyperalgesia and mechanical allodynia are manifested after nerve injury. Removal of dynorphin by prodynorphin gene knockout reveals that it is important for the maintenance rather than the induction of neuropathic pain. Mechanical and thermal thresholds of both wild-type and knockout mice are decreased after spinal nerve ligation, but only the thresholds of knockout animals return to baseline by 10 days after injury (Wang et al., 2001). Similarly, dynorphin removal by absorption with anti-dynorphin antiserum on day 10 after nerve injury reduces neuropathic behaviors (Malan et al., 2000; Wang et al., 2001). As mentioned previously, dynorphin administration by itself can be neurotoxic (e.g. Caudle & Isaac, 1987; Stewart & Isaac, 1989). At concentrations below those which produce paralysis, i.t. administration

of dynorphin can produce tactile, cold, and thermal hypersensitivities, similar to those which occur after nerve injury, that are reduced by NMDA receptor antagonists MK-801 and LY235959 and bradykinin antagonists HOE 140 and DALBK but not naloxone (Lai et al., 2006; Laughlin et al., 1997; Vanderah et al., 1996). As mentioned previously, though the mechanism underlying the dynorphin-NMDA receptor interaction is unknown, there is evidence that dynorphin could indirectly excite NMDA receptors by promoting the release of excitatory amino acids (Faden, 1992; Skilling et al., 1992) or otherwise promoting Ca^{2+} influx into NMDA receptor-containing neurons (Tang, Lynch, Porreca, & Lai, 2000).

Dynorphin may play a similar role in the maintenance of chronic inflammatory nociception. As indicated previously, dynorphin is consistently increased by inflammation. For example, lumbosacral dynorphin is increased 3 days after CFA-induced hindpaw inflammation (Luo et al., 2008). The function of this upregulation was revealed by absorption of dynorphin by antiserum, which reversed thermal hyperalgesia. Bradykinin antagonists also reversed thermal hyperalgesia, indicating that dynorphin may exert pro-nociceptive effects by acting at bradykinin receptors (Luo et al., 2008). Administration of dynorphin antiserum after bladder inflammation could reveal a similar pro-nociceptive role for dynorphin in visceral nociception.

1.4.7. Impact of EIL Bladder Inflammation on the Opioid System

Alterations in the opioid system may contribute to changes in nociceptive processing observed in adults after EIL inflammation, and this topic is the focus of this thesis. For example, carrageenan-induced inflammation of the hindpaw on P0 produces

naloxone-reversible thermal hypoalgesia, assessed using the Hargreaves test, in adults (LaPrairie & Murphy, 2009). This hypoalgesia appears to be mediated by increases in β-endorphin and met- and leu-enkephalin immunoreactivity in the PAG; in contrast, opioid receptor binding in this area decreased (LaPrairie & Murphy, 2009).

In the urinary system, when normal rats receive zymosan as adults, administration of the non-selective opioid receptor antagonist naloxone (1 mg/kg, i.p.) increases VMRs to UBD (DeBerry et al., 2007). There is no tonic effect of opioids in non-inflamed animals because the VMRs of controls that receive anesthesia only are not altered by naloxone (DeBerry et al., 2007). These data indicate that adult bladder inflammation normally recruits a reactive opioid inhibitory system. As mentioned previously, an encounter with an EIL inflammatory insult to the bladder and re-inflammation of the bladder as an adult leads to VMRs to UBD that are significantly greater than those of animals that did not encounter EIL inflammation (Randich et al., 2006b). Naloxone is not able to increase responses in animals that received zymosan both EIL and as adults, in contrast to the increase observed in animals inflamed only as adults (DeBerry et al., 2007). These data indicate that EIL bladder inflammation leads to the inability of an opioid inhibitory system(s) to operate properly.

1.5. Conceptual Model

The existing literature suggests an important role for dynorphin in the modulation of inflammatory nociception and for κ-opioid receptor agonists in the modulation of visceral nociception. Therefore, the current study examined the role the κ-opioid system plays in mediating inflammatory bladder nociception and how this role may be altered by

EIL bladder inflammation. Figure 1.1 is a conceptual model that summarizes the proposed circuit underlying bladder nociception and changes that may occur to this circuit after adult and EIL bladder inflammation. It was developed to demonstrate how changes in the κ-opioid system may interact with other components of the circuit to create bladder hypersensitivity.

In normal adult (non-inflamed) animals, UBD activates primary afferents and may lead to release of CGRP and SP from central terminals in the spinal cord. These peptides may activate or sensitize spinal transmission neurons by binding to their corresponding receptors, the NK-1 receptor and the CGRP receptor, composed of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 1 (RAMP1). Axons of these spinal transmission neurons ascend through white matter tracts, activating supraspinal areas and leading to the sensation of nociception.

After adult bladder inflammation, bladder content of CGRP and SP are increased (Shaffer et al., 2011), which possibly corresponds to increased release of CGRP and SP from central terminals of primary afferents during UBD (1 in Figure 1.1) resulting in increased spinal neuronal activation (2 in Figure 1.1). Increased spinal neuronal activation may lead to increased activation of supraspinal areas (3 in Figure 1.1) and contribute to increased VMRs to UBD (Randich et al., 2006b). Activation of supraspinal areas may also impact the magnitude of VMRs by engaging descending modulatory systems. VMRs of animals that experience acute adult bladder inflammation are facilitated (+) by norepinephrine (NE) acting at spinal α_1 receptors (4 in Figure 1.1) (Randich et al., 2008a). This NE may originate in noradrenergic brainstem nuclei such as A5, A6, and A7. VMRs of animals inflamed as adults are also facilitated (+) by serotonin (5HT) act-

ing at spinal $5HT_{1A}$ and $5HT_3$ receptors (5 in Figure 1.1) (Randich et al., 2008a). This 5HT may originate in cells located in the rostral ventral medulla (RVM).

In addition to facilitatory influences, adult inflammation also recruits a reactive opioid inhibitory system (6 in Figure 1.1) mentioned previously (DeBerry et al., 2007). This system is promoted by RVM activation (Randich et al., 2008b) and involves a spinal component (DeBerry et al., 2007). The subtype of opioid receptor (OR) activated after bladder inflammation is unknown. What is known is that when animals receive a prior inflammatory insult to the bladder EIL, opioid inhibition no longer occurs (indicated by the red lines, 6 in Figure 1.1) (DeBerry et al., 2007). In summary, after adult bladder inflammation, VMRs to UBD are increased due to increased input from primary afferents and further increased by spinal 5HT and NE. At the same time, responses are attenuated by an endogenous opioid ligand acting at a spinal opioid receptor(s). However, when animals receive a prior inflammatory insult to the bladder EIL, this opioid inhibition is impaired.

This impairment could occur through several mechanisms. First, primary afferent input may increase without a corresponding increase in opioid inhibition. Second, the available content or release of endogenous opioid ligands with inhibitory actions could be decreased. Third, the number or activity of the corresponding opioid receptors could decrease. However, the documented pro-nociceptive activity of dynorphin (Lai et al., 2001) introduces additional possibilities; the opioid inhibition may be impaired through the addition of an opioid facilitatory system such as the dynorphin/κ-opioid receptor system that counteracts the opioid inhibitory system. If this were the case, then spinal dynorphin may be increased by EIL bladder inflammation. Dynorphin may normally inhibit bladder

sensitivity by activating κ-opioid receptors (7 in Figure 1.1). However, κ-opioid receptor function could become pro-nociceptive after EIL inflammation, in which case dynorphin may contribute to bladder hypersensitivity (8 in Figure 1.1). Alternatively, dynorphin could act independently of opioid receptors to maintain nociception. To begin to examine changes in the dynorphin/κ-opioid system after adult and EIL bladder inflammation, the following Specific Aims were proposed.

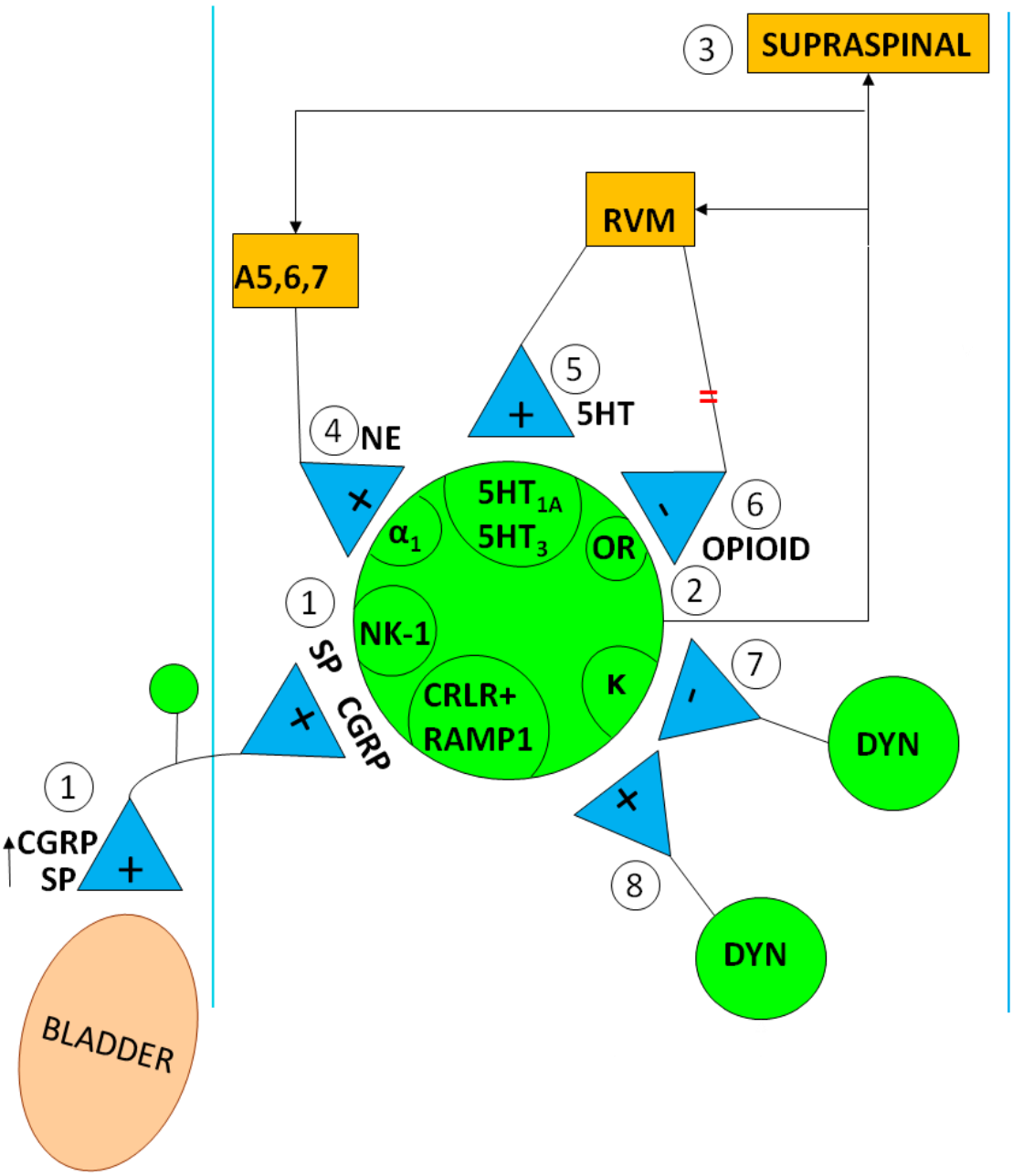

Figure 1.1. A conceptual model that summarizes the proposed circuit underlying bladder nociception and changes that occur to this circuit after adult and EIL bladder inflammation. CGRP, Calcitonin Gene Related Peptide; CRLR, Calcitonin Receptor Like Receptor; DYN, Dynorphin; NE, Norepinephrine; NK-1, Neurokinin-1; OR, Opioid Receptor; RAMP1, Receptor Activity Modifying Protein 1; RVM, Rostral Ventral Medulla; SP, Substance P; 5HT, Serotonin; κ, κ-opioid receptor

1.6. Specific Aims

The general hypothesis of this thesis is that inflammation of the bladder engages an endogenous opioid inhibitory system. The operation of this system may be altered by an EIL experience with bladder inflammation. These alterations could include changes in spinal κ-opioid receptor function. For example, in normal adult animals, activation of spinal κ-opioid receptors by endogenous opioids or exogenous agonists could decrease nociceptive responses to UBD. In contrast, spinal κ-opioid receptor activation could increase nociceptive responses in animals that have received an EIL inflammatory insult. In addition, EIL inflammation may produce long-lasting changes in central and peripheral content of the endogeous ligand for the κ-opioid receptor, dynorphin. This alteration in the κ-opioid system after EIL inflammation could reduce the net inhibitory effect of opioids. If the opioid inhibitory system is similarly impaired in individuals suffering from BPS/IC, understanding the nature of this impairment could highlight avenues for more efficacious treatments.

The following sub-hypotheses expanded on and systematically refined this hypothesis.

1. U50,488H acts at spinal κ-opioid receptors to both attenuate the hypersensitivity to mechanical distension of the bladder resulting from inflammation in normal adult animals and facilitate responses in animals inflamed EIL.
2. The spinal and peripheral concentration of dynorphin, the endogenous ligand for κ-opioid receptors, are altered by EIL inflammation.

In part A of Specific Aim 1, changes in κ-opioid receptor function after EIL inflammation were examined through i.v. administration of U50,488H after EIL and adult bladder

inflammation. In part B of Specific Aim 1, the involvement of spinal κ-opioid receptors in the effects of U50,488H was determined by i.t. administration of nor-BNI 24 hours prior to U50,488H administration and testing. In Specific Aim 2, changes in dynorphin content after EIL inflammation were examined by measuring central (spinal cord) and peripheral (serum and bladder) content of dynorphin after EIL and adult bladder inflammation using ELISA.

CHAPTER 2

METHODS

2.1 General Methodology

2.1.1. Experimental Design

Specific Aims were examined in 3 experiments. In all experiments, animals were treated EIL (P14-P16) with either anesthesia only or zymosan. These groups were each divided in half. As adults (12-17 weeks of age) these groups were again exposed to either anesthesia only or zymosan. Experiments 1 and 2 also included a group naïve to any treatments.

2.1.1.1. Experiment 1

Experiment 1 addressed part A of Specific Aim 1. Changes in κ-opioid receptor function after EIL and adult inflammation were examined through i.v. administration of U50,488H. The bladder was distended via air pressure, and nociceptive responses were quantified as electromyographic (EMG) activity resulting from the VMR.

2.1.1.2. Experiment 2

Experiment 2 addressed Specific Aim 2. Changes in central (spinal cord) and peripheral (serum and bladder) content of dynorphin were measured after adult and EIL bladder inflammation. Blood, bladders, and thoracolumbar (T13-L2) and lumbosacral

(L4-S2) spinal cord segments were removed, and spinal, circulating, and bladder content of dynorphin was measured by ELISA.

2.1.1.3. Experiment 3

Experiment 3 addressed part B of Specific Aim 1. It examined if the effects of U50,488H were due to activation of spinal κ-opioid receptors. Animals were pre-treated with i.t. administration of the selective κ-opioid receptor antagonist nor-BNI 24 hours prior to U50,488H administration and testing.

2.1.1.4. Rationale

Experiments are presented in the order in which they were conducted. Experiment 1 revealed that bladder inflammation significantly altered responses to U50,488H. Experiment 2 was conducted next and revealed that bladder inflammation altered both spinal and peripheral content of dynorphin. Because the endogenous ligand for κ-opioid receptors was altered at the spinal level and a spinal site of action was readily testable using i.t. administration of an antagonist, Experiment 3 was conducted, in which i.t. administration of nor-BNI preceded U50,488H administration and testing.

2.1.2. Selection of Species

Adult, virgin, female, Sprague-Dawley rats were tested at 12-17 weeks of age (200-280 g). Rats were chosen because of the numerous similarities between the nociceptive systems of rats and primates, as well as an extensive literature characterizing nociceptive systems in rats (Willis, 1985). Only female rats were used in these studies be-

cause the translational goal was better understanding of bladder pain in women and there is a gender bias in chronic bladder pain, with females constituting at least 90% of patients with the BPS/IC (for a review, see Jones & Nyberg, 1997). Also, non-painful urinary bladder catheterization is much more feasible in female compared to male rats.

2.1.3. EIL Intravesical Treatments

Treatments with either anesthesia only or intravesical zymosan began on P14. Anesthesia was induced with 5% isoflurane and oxygen and the external urethra of each rat was swabbed with Betadine. The urinary bladders of animals in the zymosan group were catheterized transurethrally with a 24-gauge angiocatheter (Cardinal Health, Dublin, OH). Rats were then maintained on 2% inhaled halothane and administered zymosan (0.1 ml, 1% in saline; Sigma-Aldrich, St. Louis, MO). The solution was allowed to dwell in the bladder for 30 minutes and drained. Rats in the anesthesia only group were maintained on 2% halothane for 30 minutes immediately after induction with 5% isoflurane. Body temperature was maintained with a warmed heating pad. All rats received subcutaneous (s.c.) ampicillin (0.05 ml, 100 mg/ml) at the end of the procedure and were allowed to awaken. Three successive daily treatments were performed (P14-P16).

A control group with animals administered intravesical saline EIL was not included in this study. In the initial study examining the effects of EIL treatment with zymosan (Randich et al., 2006b), both saline and anesthesia only controls were used. There were no significant differences in the VMR to UBD, micturition frequency, or Evans blue extravasation between animals treated EIL with saline and those treated EIL with anesthesia only when all animals received zymosan as adults. To reduce the number of ani-

mals required for subsequent studies, animals exposed to anesthesia only were chosen as the control group rather than those given intravesical saline. Use of anesthesia only-treated animals as the control group for this study is consistent with previous studies (DeBerry et al., 2007, 2010; Ness & Randich, 2010; Randich et al., 2009; Shaffer et al., 2011).

2.1.4. Adult Intravesical Treatments

Animals at 12-17 weeks of age were anesthetized with inhaled isoflurane and oxygen (5% for induction, 2% for maintenance). Rats were treated with anesthesia only or zymosan. Zymosan-treated animals had their urinary bladders catheterized with a 22-gauge angiocatheter via the urethra. Zymosan (0.5 ml, 1% in saline) was administered intravesically for 30 min and drained. Rats in the anesthesia only group were maintained on 2% isoflurane for 30 minutes, immediately after induction with 5% isoflurane. All animals received ampicillin at the end of the procedure (0.2 ml, 100 mg/ml, s.c.). EIL and adult intravesical treatments resulted in 5 groups. These groups consisted of animals naïve to any treatment (naïve), those receiving anesthesia both EIL and as adults (AA), those receiving anesthesia EIL and zymosan as adults (AZ), those receiving zymosan EIL and anesthesia as adults (ZA), and those receiving zymosan both EIL and as adults (ZZ). Although naïve animals were included in Experiments 1 and 2, animals exposed to anesthesia only both EIL and as adults (AA animals) are the primary control comparison used in all 3 experiments because volatile anesthetics can alter measures of nociception such as c-Fos expression (Jinks et al., 2002) and dorsal horn neuron activity (Cuellar, Dutton, Antognini, & Carstens, 2005; Jinks, Antognini, & Carstens, 2003).

2.1.5. Vaginal Cytology

Starting 1 week before testing (11-16 weeks of age), vaginal smears were obtained via vaginal lavage as previously described (Freeman, 1988) and utilized in our laboratory (Ness et al., 2001). Animals included in this study were not restricted to those with a 4-day estrous cycle in order to generate a representative sample of the female rat population. Animals were required to demonstrate at least 1 complete cycle before testing. All animals underwent adult treatment during proestrus, as characterized by a preponderance of nucleated epithelial cells. Therefore, most animals were in estrus on the day of testing/tissue collection. Testing during this phase presumably optimized the ability to detect changes in the opioid system, since the largest magnitude of opioid inhibition, as measured by potentiation of VMRs to UBD by naloxone, is present in estrus (Ball et al., 2010). To determine estrous stage at the time of testing/tissue collection, a final sample was taken after completion of testing/tissue collection. Data were inspected for variability due to differences in actual stage on day of testing/tissue collection.

2.1.6. Drugs

Nor-BNI and *trans*-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide methanesulfonate salt (U50,488H) were acquired from Sigma-Aldrich. Both drugs were dissolved in physiological saline (0.9% NaCl).

2.2. Specific Experimental Protocols

2.2.1. Experiment 1: i.v. Administration of U50,488H in EIL-Treated Rats

2.2.1.1. EIL Treatment

EIL intravesical treatments were conducted as described in the general methodology. Briefly, animals were either treated with nothing (naïve) or from P14-16 animals they were exposed to zymosan or halothane anesthesia only for 30 min once a day.

2.2.1.2. Adult Treatment

Adult intravesical treatments were conducted as described in the general methodology. Briefly, animals were either naïve or received intravesical zymosan or isoflurane anesthesia only for 30 min 24 hours before testing.

2.2.1.3. VMR Testing

2.2.1.3.1. Surgical preparation. On the day of testing, each animal was anesthetized with isoflurane and oxygen (5% for induction, 4% for maintenance), and the trachea was cannulated for artificial respiration. The external jugular vein was cannulated with PE10 tubing for i.v. drug administration. The urinary bladder was catheterized with a 22-gauge angiocatheter via the urethra. The catheter was held in place with a tight suture tied around the distal urethral orifice. The rat was moved to a recording area where it was artificially respirated and body temperature was maintained at approximately 37˚C with a heating pad. Platinum wire electrodes were inserted into the left external oblique muscle through an incision in the left abdominal skin. Differential amplification of EMG activity was achieved with a Grass P511 amplifier (Grass Technologies; West Warwick,

RI). Amplifier settings were as follows: EMG amplification factor: 1000, low frequency filter: 10 Hz, high frequency filter: 3 kHz, sample rate: 10 kHz. EMG activity was captured using the CED micro 1401 interface, after which it was recorded, rectified, and saved using Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

2.2.1.3.2. Testing protocol. Anesthesia was lowered to 1% isoflurane immediately after completion of surgery. Each animal was allowed to acclimate to anesthesia for at least 10 min, after which time the animal was tested for a hind paw withdrawal reflex. If no reflex was observed, the animal was allowed more time to acclimate to the anesthesia level before initiation of testing. If no reflex was observed after additional time, anesthesia was lowered until a reflex was observed. To establish stable VMRs before drug administration, a minimum of 6 distensions at 60 mmHg of air pressure (20 sec duration, 3 min ITI) were conducted until a measurable VMR (a distinct increase in EMG activity during UBD) was achieved and the VMRs of 2 consecutive trials varied by less than 10%. 1 min before the first test trial, 1 mg/kg of i.v. U50,488H was administered. Test trials were conducted at 1, 4, 7, 10, and 13 min after drug administration. At 15 min after drug administration, 4 mg/kg of i.v. U50,488H was administered, and 5 additional trials were conducted at 16, 19, 22, 25, and 28 min after the original drug administration. This cumulative dosing paradigm was similar to that used previously (e.g. Ness et al., 2001). The doses of U50,488H (1 mg/kg and 4 mg/kg) are slightly less than and greater than the ED_{50} (1.34 mg/kg) established by Su et al. (2008d) for decreasing the VMR to 60 mmHg UBD.

2.2.1.3.3. Data analysis. The first step in quantification of the VMR was to subtract baseline EMG activity during the 20 sec prior to UBD from EMG activity evoked by the 20 sec UBD test stimulus. To accomplish this, the area under the curve (AUC) of rectified EMG activity prior to UBD (baseline activity in mV) was subtracted from the AUC of rectified EMG during UBD (evoked activity in mV). This difference (in mV) was then normalized to background electrical noise by dividing by the lowest pre-drug baseline activity (in mV). The equation was defined as: (rectified EMG activity during UBD - rectified baseline EMG prior to UBD) / (rectified baseline EMG). This definition of the VMR results in a unitless measure of normalized EMG activity. Finally, data in the current study are presented as a percent of baseline calculated as (test trial VMR / mean VMR of last 2 pre-drug trials) x 100%.

2.2.1.4. Statistical Analysis

Data were analyzed separately at each level of dose (1 mg/kg or 4 mg/kg). Direct comparisons of VMRs at 1 mg/kg of U50,488H with those at 4 mg/kg of U50,488H were not of primary interest. A mixed between-within ANOVA was conducted with intravesical treatment group (naïve, AA, AZ, ZA, or ZZ) as a between-subjects factor and time after administration of 1 mg/kg of U50,488H as a within-subjects factor. If there was no intravesical treatment group x time interaction, subsequent analyses were conducted on data collapsed across time. If there was a significant intravesical treatment group x time interaction, ANOVAs were conducted at each trial after agonist administration. A significant effect of intravesical treatment was followed by Fisher's least significant difference test (Fisher's LSD) testing all possible pairwise comparisons between treatment groups.

This resulted in 10 comparisons. Familywise α was retained at 0.05 by Holm's correction (Holm, 1979).

A separate set of comparisons was conducted to determine if 1 mg/kg or 4 mg/kg of U50,488H significantly inhibited or facilitated VMRs relative to baseline. Percent of baseline VMRs were compared to 100 percent of baseline using one-sample t-tests. These t-tests were conducted on data collapsed across time if the mixed between-within ANOVA described in the last section at the appropriate level of dose did not reveal a significant effect of time. If there was a significant effect of time, separate t-tests were conducted at each time after agonist administration. For all comparisons, outliers (values more extreme than 1.5x the interquartile range from the first or third quartile) were excluded from analysis. Data from 1 animal in group naïve, 1 animal in group AZ, and 2 animals in group ZZ were excluded according to this criterion.

2.2.2. Experiment 2: Dynorphin Content in EIL-Treated Rats

2.2.2.1. EIL Treatment

EIL intravesical treatments were conducted as described in the general methodology. Briefly, animals were either treated with nothing (naïve) or from P14-16 animals they were exposed to zymosan or halothane anesthesia only for 30 min once a day.

2.2.2.2. Adult Treatment

Adult intravesical treatments were conducted as described in the general methodology. Briefly, animals were either naïve or received zymosan or isoflurane anesthesia only for 30 min 24 hours before testing.

2.2.2.3. Tissue collection, processing, and analysis

Cardiac blood and bladders were removed, after which animals were euthanized via decapitation. Spinal cords were removed by hydraulic extrusion, and thoracolumbar and lumbosacral sections were isolated. Spinal cords were placed on dry ice after which they were stored at -80°C until processing. Blood was centrifuged at 1600 x g for 15 min at 4°C. Serum was retained and stored at -80°C until processing. Spinal cord and bladder tissues were homogenized in 0.01M PBS. Homogenates were centrifuged at 14,000 x g for 15 min at 4°C. Supernatants were removed and protein concentrations were determined using a Pierce BCA Assay Reagent Kit (Thermo Fisher Scientific, Rockford, IL, USA). Spinal, serum, and bladder dynorphin A (1-17) content were measured using enzyme-linked immunosorbent assay (ELISA) kits from Phoenix Pharmaceuticals (Burlingame, CA) according to manufacturer's instructions. This dynorphin ELISA kit was reported by the manufacturer to have 100% cross-reactivity with porcine dynorphin A (1-17), less than 0.01% cross-reacitivity with porcine dynorphin A (1-13), and 0% cross-reactivity with porcine dynorphin A (1-8), porcine dynorphin B, human β-endorphin, porcine α-neoendorphin, and leu-enkephalin. Samples and serial dilutions of a 1,000 ng/ml dynorphin peptide standard were incubated for 2 hr at room temperature with porcine anti-dynorphin primary antibody and biotinylated dynorphin A on a plate precoated with α-porcine secondary antibody. The Fc fragment of the primary antibody was bound by the secondary antibody, while dynorphin A in samples or standards and biotinylated dynorphin A competed for binding to the Fab fragment. The plate was washed, strepavidin-horseradish peroxidase (SA-HRP) was added to each well, and the plate was incubated for 1 hr at room temperature. The strepavidin formed a complex with biotin in bio-

tinylated dynorphin. The plate was again washed and substrate (3,3',5,5'-tetramethylbenzidine; TMB) was added to each well. A final incubation of 1 hr at room temperature was performed, during which TMB was catalyzed by HRP to produce a blue color. The reaction was quenched with hydrochloric acid (HCl, 2N), producing a yellow color, and absorbance optical density of each well was read at 450 nm with a Fluostar Omega microplate reader (BMG Labtech; Ortenberg, Germany). A standard curve was constructed using MARS Data Analysis Software (BMG Labtech), and the concentration of dynorphin A in each well, which is inversely proportional to the absorbance at 450 nm, was determined using this standard curve.

2.2.2.4. Cresyl Violet Staining

To verify the segments contained in the lumbosacral and thoracolumbar sections to be used for dynorphin ELISAs, one animal was perfused with approximately 120 ml 0.01M phosphate-buffered saline (PBS) followed by approximately 180 ml Prefer fixative (Anatech LTD, Battle Creek, MI). Spinal cords were removed by hydraulic extrusion, and the thoracolumbar and lumbosacral sections were isolated in an identical manner to that to be used for analysis by ELISA. Mid-lumbar (L3-L4) sections were also collected. Tissue was post-fixed in Prefer fixative for 4 days and cryoprotected in Prefer fixative with 30% sucrose until tissue sunk. Sections were embedded in TFM Tissue Freezing Medium (Triangle Biomedical Sciences; Durham, NC), and 30 µm sections were cut using a Leica CM1850 UV cryostat (Leica Microsystems, Wetzlar, Germany). Sections were transferred to tap water before mounting on slides. Tissue was placed in 95%, 70%, and 50% ethanol, each for 1 minute. Cresyl violet stain was applied for 3

min, followed by 50% ethanol for 3 dips and then for 1 min. 70% ethanol with 4 drops acetic acid was applied for 2 minutes, followed by 95%, 95%, and 100% ethanol each for 1 minute. Tissue was cleared in xylene and coverslipped with Protocol Mounting Medium (Thermo Fisher Scientific; Waltham, MA). Sections were viewed with an Olympus BX-51 microscope (Olympus America; Center Valley, PA) and captured with a digital camera (Microfire CX900l; Optronics, Goleta, CA). The segmental identity of each section was determined by comparing to sections in *The spinal cord: a Christopher and Dana Reeve Foundation text and atlas* (Watson, Paxinos, & Kavalioglu, 2008).

1.2.2.5. Statistical analysis

The dynorphin content of each tissue type (thoracolumbar spinal cord, lumbosacral spinal cord, serum, and bladder) was analyzed separately. A one-way ANOVA was conducted with intravesical treatment group (naïve, AA, AZ, ZA, and ZZ) as a between-subjects factor. A significant effect of intravesical treatment group was followed by Fisher's LSD conducting all possible pairwise comparisons between treatment groups. This resulted in 10 comparisons. Familywise α was retained at 0.05 by Holm's correction. All outliers (values more extreme than 1.5x the interquartile range from the first or third quartile) were excluded from analysis. During analysis of thoracolumbar and lumbosacral spinal cord data, 1 animal from group ZA was excluded based on this criterion. During analysis of serum data, 1 animal from group ZZ was excluded. During analysis of bladder data, 2 animals were excluded each from groups AA and AZ, and one animal was excluded from group ZA.

2.2.3. Experiment 3: i.v. Administration of U50,488H and i.t. Administration of Nor-BNI in EIL-Treated Rats

2.2.3.1. EIL Treatment

EIL intravesical treatments were conducted as described in the general methodology. Briefly, animals were either treated from P14-16 with either zymosan or halothane anesthesia only for 30 min once a day.

2.2.3.2. Adult Treatment

Adult intravesical treatments were conducted as described in the general methodology. Briefly, animals received zymosan or isoflurane anesthesia only for 30 min 24 hours before testing.

2.2.3.4. Chronic i.t. catheter implantation.

Spinal i.t. catheters were implanted in animals at 12-16 weeks of age. Implantation of i.t. catheters occurred according to a variation of the method of Yaksh and Rudy (1976). Animals were allowed a 5 day recovery period following catheter implantation to allow time for wound healing and stabilization of the preparation. Catheters were constructed from 16 cm of polyethylene tubing (PE10). A knot was tied in the center of the tubing and secured with dental cement. One end was trimmed to 7.8 cm from the knot. Catheters were filled with 0.9% saline to maintain catheter viability. Animals were anesthetized with isoflurane (5% for induction, 4% for maintenance) and placed in a stereotaxic instrument. The catheter was threaded through a small slit in the atlanto-occipital membrane to the L5-S2 region. Skin was carefully sutured over the knot and the open

end of the catheter was plugged with 30 gauge stainless steel wire. Animals received ampicillin (0.2 ml, 100 mg/kg, s.c.) and were allowed to awaken. During the 5 day recovery period, any animals demonstrating obvious motor impairments or infection were immediately euthanized by isoflurane overdose followed by decapitation.

Nor-BNI (8 μg, i.t.) was administered 24 hours before testing concomitantly with adult intravesical treatment. Testing occurred 24 hours later, at a time when nor-BNI is most selective for κ- over μ- and δ-opioid receptors (Endoh, Matsuura, Tanaka, & Nagase, 1992; Wettstein and Grouhel, 1996). This dose of nor-BNI has been shown to decrease mechanical and thermal paw withdrawal thresholds after hindpaw inflammation (Schepers et al., 2008). VMR testing proceeded in a manner identical to that described for Experiment 1.

2.2.3.5. Statistical Analysis.

The first set of analyses addressed whether animals given different intravesical treatments (AA, AZ, ZA, or ZZ) responded differently to U50,488H. Mixed between-within ANOVAs were conducted at each level of dose and i.t. treatment, with intravesical treatment as a between-subjects factor and time after administration of 1 mg/kg of U50,488H as a within-subjects factor. If there was no intravesical treatment x time interaction, subsequent analyses were conducted on data collapsed across time. If there was a significant intravesical treatment x time interaction, subsequent analyses were conducted at each time. A significant effect of intravesical treatment was followed by all possible pairwise comparisons between intravesical treatment groups (AA, AZ, ZA, and ZZ) at each level of i.t. treatment using Fisher's LSD. This resulted in 6 comparisons at

each level of dose and i.t. treatment. Familywise α was retained at 0.05 by Holm's correction.

The second set of analyses addressed whether animals given different i.t. treatments (nor-BNI or saline) responded differently to U50,488H. Mixed between-within ANOVAs were conducted at each level of dose and intravesical treatment, with i.t. treatment as a between-subjects factor and time after administration of 1 mg/kg of U50,488H as a within-subjects factor. If there was no i.t. treatment x time interaction, subsequent analyses were conducted on data collapsed across time. If there was a significant i.t. treatment x time interaction, subsequent analyses were conducted at each time. A significant effect of i.t. treatment was followed by Student's t-tests comparing i.t. nor-BNI versus i.t. saline at each level of intravesical treatment. This resulted in only 1 comparison at each level of dose and intravesical treatment. Therefore, no correction for multiple comparisons was required.

A third set of analyses was conducted to determine if 1 mg/kg or 4 mg/kg of U50,488H significantly inhibited or facilitated VMRs relative to baseline. Percent of baseline VMRs were compared to 100 percent of baseline using one-sample t-tests. These t-tests were conducted on data collapsed across time if the mixed between-within ANOVA with intravesical treatment as a between-subjects factor and time as a within-subjects factor, at the appropriate level of dose and i.t. treatment, did not reveal a significant effect of time. If there was a significant effect of time, separate t-tests were conducted at each time after agonist administration. For all analyses, outliers (values more extreme than 1.5x the interquartile range from the first or third quartile) were excluded from analysis. Data from 1 animal from each of the following groups were excluded

based on this criterion: group AA given i.t. saline, group AZ given i.t. saline, group ZA given i.t. saline, and group ZZ given i.t. nor-BNI. In addition, data from 2 animals in group ZA given i.t. nor-BNI were excluded.

2.2.3.6. Dynorphin Content after i.t. Catheterization

In a follow-up to Experiment 3, spinal dynorphin content was measured to determine the effect of chronic i.t. catheterization. Animals at 12-16 weeks of age were either naïve or implanted with i.t. catheters in manner identical to that described previously. During proestrus, all animals were anesthetized with inhaled isoflurane and oxygen (5% for induction, 2% for maintenance) for 30 min. Half of the rats with i.t. catheters received i.t. administration of saline (8 µl followed by a 10 µl flush). The remaining half received no i.t. treatment. All animals received ampicillin at the end of the procedure (0.2 ml, 100 mg/ml, s.c.). This resulted in 3 groups: naïve animals, animals that received chronic i.t. catheterization alone, and animals that received chronic i.t. catheterization and i.t. administration of saline. Thoracolumbar and lumbosacral spinal cord sections were collected from these animals. Dynorphin was then measured in these sections as described previously.

A two-way ANOVA was conducted with group (naïve, i.t. catheter only, or i.t. saline) and section (lumbosacral or thoracolumbar) as between-subjects factors. A significant group x section interaction was followed by separate ANOVAs at each level of section. A significant effect of group was followed by Fisher's LSD conducting all possible pairwise comparisons between treatment groups. Since this resulted in only 3 comparisons, no correction for multiple comparisons was necessary. A significant effect of sec-

tion was followed by Student's t-test to compare dynorphin content in the lumbosacral spinal cord to that in the thoracolumbar spinal cord. α was set at 0.05. All outliers (values more extreme than 1.5x the interquartile range from the first or third quartile) were excluded from analysis. During analysis of lumbosacral spinal cord data, data from 1 animal in group naïve, 1 animal in group i.t. catheter only, and 1 animal in group i.t. saline were excluded based on this criterion. During analysis of thoracolumbar spinal cord data, data from 1 animal in group i.t. catheter only was excluded.

CHAPTER 3

RESULTS

3.1. Organization

This thesis consists of 3 experiments. In Experiment 1, changes in κ-opioid receptor function after adult and EIL inflammation were examined through i.v. administration of U50,488H. In Experiment 2, changes in spinal cord and peripheral (serum and bladder) content of dynorphin were measured after adult and EIL bladder inflammation using ELISA. In Experiment 3, nor-BNI was administered 24 hours prior to U50,488H administration and VMR testing to determine whether the effects of U50,488H were due to a spinal site of action.

3.2. Experiment 1: i.v. Administration of U50,488H in EIL-Treated Rats

3.2.1. Purpose

This experiment examined whether the inhibitory effects of i.v. administration of U50,488H were altered in rats that received EIL bladder inflammation. Animals were either naïve or treated EIL (P14-P16) with anesthesia only or zymosan. As adults, the anesthesia and zymosan-treated groups were divided in half and treated with either anesthesia only or zymosan. This resulted in 5 groups consisting of either animals naïve to any treatment (naïve), those receiving anesthesia both EIL and as adults (AA), those receiving anesthesia EIL and zymosan as adults (AZ), those receiving zymosan EIL and

anesthesia as adults (ZA), or those receiving zymosan both EIL and as adults (ZZ). VMR testing occurred 24 hours after adult treatment. Stable baseline VMRs to noxious (60 mmHg) UBD were established after which 1 mg/kg and 4 mg/kg of U50,488H were administered i.v. in a cumulative-dosing regimen.

3.2.2. Baseline VMRs to UBD

Baseline VMRs to 60 mmHg bladder distension obtained prior to administration of U50,488H were examined to determine if these measures were differentially affected by adult and EIL bladder inflammation. Lower baseline VMRs to UBD could increase the likelihood of observing increases in VMRs (>100 percent of baseline), and higher baseline VMRs to UBD could increase the likelihood of observing decreases in VMRs (<100 of percent baseline). Figure 3.1 depicts baseline VMRs to UBD for the various groups. Data are presented as group mean normalized EMG activity obtained from the left external oblique muscle in response to 60 mmHg bladder distension. An ANOVA revealed that baseline VMRs to UBD did not differ between intravesical treatment groups [$F(4,27)=0.834$, $p=0.516$]. These data indicate that differences between intravesical treatment groups reported later in terms of percent of baseline VMRs to UBD were not due to significant differences in baseline VMRs to UBD.

Repeated pre-drug trials may alter bladder sensitivity and therefore alter responsiveness to U50,488H. Therefore, the relationship between the number of pre-drug trials and both baseline VMRs to UBD and percent of baseline VMRs at 1 min after administration of 1 mg/kg of U50,488H was also examined. The number of pre-drug trials conducted and baseline VMRs were not significantly correlated [$r(32)=-0.179$, $p=0.327$]. In

addition, VMRs at 1 min after administration of U50,488H, expressed as percent baseline, were not significantly correlated with the number of pre-drug trials conducted [r(31)=-0.139, p=0.456]. Finally, data were examined to determine if the number of pre-drug trials conducted differed between intravesical treatment groups. An ANOVA revealed no significant effect of intravesical treatment group on the number of pre-drug trials conducted. In summary, the number of pre-drug trials conducted did not impact baseline responsiveness or VMRs to UBD at 1 min after administration of 1 mg/kg of U50,488H.

3.2.3. Percent of Baseline VMRs to UBD after U50,488H

Figure 3.2 displays VMRs to 60 mmHg UBD after 1 mg/kg and 4 mg/kg U50,488H. Responses are expressed as percent of baseline VMRs. Data were analyzed separately at each dose.

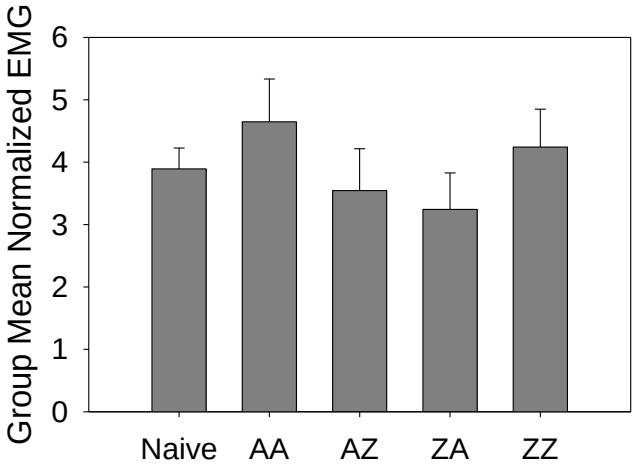

Figure 3.1. Baseline group mean normalized EMG activity (VMR; n=4-7/group). Stable baseline VMRs to a 60 mmHg UBD test stimulus were established before administration of U50,488H. Baseline VMRs did not differ between intravesical treatment groups.

3.2.3.1. 1 mg/kg of U50,488H

Responses to 1 mg/kg U50,488H differed between intravesical treatment groups. An ANOVA revealed a significant effect of intravesical treatment group [$F(4,26)=9.60$, $p<0.001$], but no significant effect of time or group x time interaction. Therefore, post-hoc comparisons using Fisher's LSD followed by Holm's correction for multiple comparisons were conducted for data collapsed across time. Post-hoc comparisons revealed that VMRs of group ZZ were significantly greater than those of all other groups ($p<0.001$). Responses of groups ZA ($p=0.004$) and AA ($p=0.004$), but not group AZ, were also significantly greater than those of naïve controls.

One-sample t-tests were also conducted testing the null hypothesis that group mean VMRs to UBD collapsed across time after administration of 1 mg/kg U50,488H were equal to 100 percent of baseline. These t-tests revealed that VMRs of groups naïve (36.87 ± 4.87), AA (60.90 ± 4.84), AZ (56.57 ± 4.94), and ZA (61.12 ± 5.78) were significantly less than 100 percent of baseline ($p<0.001$), indicating that 1 mg/kg U50,488H inhibited VMRs in these groups relative to baseline values. *In contrast, VMRs of group ZZ (120.2 ± 5.20*) *were significantly greater than 100 percent of baseline ($p<0.001$) indicating that 1 mg/kg U50,488H facilitated rather than inhibited responses in group ZZ relative to baseline values.*

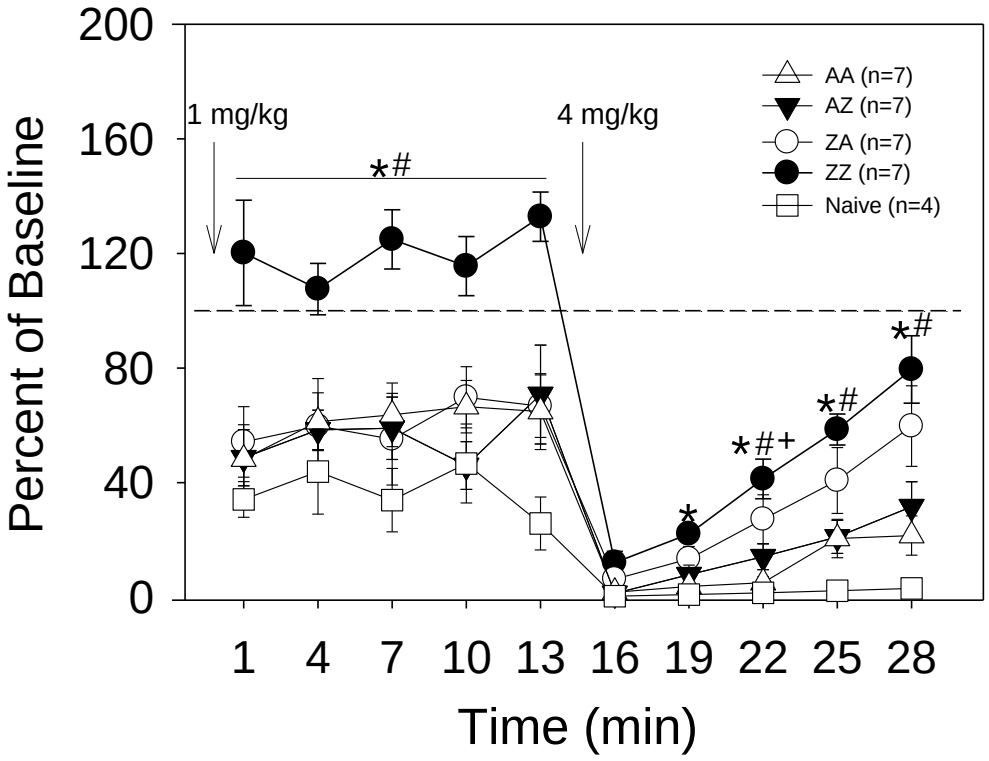

Figure 3.2. VMRs to UBD expressed as percent of baseline normalized EMG activity after i.v. administration of U50,488H (n=4-7/group). 1 mg/kg and 4 mg/kg U50,488H were administered in a cumulative dosing regimen. VMRs to a 60 mmHg UBD test stimulus were measured at 3 minute intervals and expressed as percent of pre-drug baseline. Data after 1 mg/kg U50,488H were collapsed across time after 1 mg/kg U50,488H administration. VMRs of group ZZ were significantly greater than those of all other groups (*$p<0.001$). VMRs of groups ZA and AA were significantly greater than those of naïve controls (#$p=0.004$ for both comparisons). An ANOVA of data after 4 mg/kg U50,488H administration revealed a significant intravesical treatment x time interaction, so data were analyzed individually at each time point. VMRs of group ZZ were significantly greater than those of groups naïve, AA, and AZ (*$p\leq0.005$). VMRs of group ZA were significantly greater than those of naïve controls (#$p\leq0.008$). VMRs of ZA were significantly greater than those of AA controls (+$p=0.007$).

3.2.3.2. 4 mg/kg of U50,488H

All groups showed suppression of VMRs to UBD following i.v. administration of 4 mg/kg U50,488H. An ANOVA revealed significant effects of intravesical treatment group [$F(4,25)=8.63$, $p<0.001$] and time [$F(4,100)=40.20$, $p<0.001$] and a significant group x time interaction [$F(16,100)=3.94$, $p<0.001$]. Because of the significant group x time interaction, ANOVAs were followed by post-hoc comparisons using Fisher's LSD and Holm's correction for multiple comparisons, conducted separately at each time point. Group means and p-values for specific comparisons are shown in Table 3.1. VMRs of group ZZ were significantly greater than those of groups naïve, AA, and AZ from 19 to 28 min after the first dose ($p\leq0.005$). In addition, responses of group ZA were significantly greater than those of naïve controls from 22 to 28 min after the first dose and were significantly greater than those of AA controls at one time point (22 min) after the first dose ($p\leq0.008$). *These data indicate that although VMRs of group ZZ were suppressed by 4 mg/kg of U50,488H they were generally greater than those of group naïve, AA, and AZ. Therefore 4 mg/kg U50,488H produced less inhibition in this group relative to the other groups. In addition, VMRs of group ZA were also less suppressed by 4 mg/kg U50,488H than groups naïve and AA.*

Again, one-sample t-tests were conducted comparing group mean VMRs to 100 percent of baseline. VMRs of group naïve, AA, AZ, and ZA were significantly lower than 100 percent of baseline at all time points ($p\leq0.029$), indicating that they were inhibited by 4 mg/kg U50,488H. VMRs of group ZZ were significantly lower than 100% baseline at all time points ($p\leq0.002$) except for 28 min after the first dose. This return toward baseline VMRs indicated that group ZZ had the greatest recovery of responses

after 4 mg/kg U50,488H, providing additional evidence that 4 mg/kg produces less robust inhibition in these animals.

3.2.3.3. Summary

In groups naïve, AA, AZ, and ZA, 1 mg/kg of U50,488H produced a moderate inhibition of VMRs to the 60 mmHg UBD test stimulus. In these same groups, 4 mg/kg of U50,488H strongly inhibited VMRs to UBD at the first trial after administration of this dose, which was followed by a time-dependent recovery of VMRs. In contrast, VMRs of group ZZ were facilitated by 1 mg/kg U50,488H and less inhibited than other groups by 4 mg/kg U50,488H. Similarly, VMRs of group ZA were significantly greater than control groups naïve and AA at some test trials after 4 mg/kg of U50,488H, which supports the notion that VMRs of group ZA may also be less inhibited by 4 mg/kg of U50,488H than other groups. After 1 mg/kg U50,488H, VMRs of groups AA and ZA were significantly greater than control group naïve, but this difference is not particularly salient because group AA is the most appropriate control comparison. Importantly, these data indicate that EIL inflammation alone very modestly altered the effect of U50,488H on VMRs to UBD, while EIL inflammation coupled with adult re-inflammation markedly altered the effect of U50,488H.

Table 3.1. Post-hoc comparisons at each time point after i.v. administration of 4 mg/kg of U50,488H. Intravesical treatment group, time after i.v. administration of 1 mg/kg of U50,488H, and group mean ± standard error of the mean (SEM) are shown in the first 3 columns. P-values for comparisons against groups ZZ and ZA made using Fisher's LSD and Holm's correction for multiple comparisons are shown in columns 4 and 5. Finally, P-values for one-sample t-tests comparing group means to 100 percent of baseline are shown in the last column.

Group	Time (min)	Mean ± SEM	Comparison to ZZ	Comparison to ZA	Comparison to Baseline
Naïve	16	0.35 ± 0.09	ns[1]	ns	p<0.001
	19	0.95 ± 0.89	p<0.001	ns	p<0.001
	22	1.55 ± 0.74	p<0.001	p=0.008	p<0.001
	25	2.28 ± 0.80	p<0.001	p=0.004	p<0.001
	28	3.09 ± 1.23	p<0.001	p=0.002	p<0.001
AA	16	1.74 ± 1.22	ns	ns	p<0.001
	19	3.80 ± 1.90	p<0.001	ns	p<0.001
	22	5.08 ± 2.14	p<0.001	p=0.007	p<0.001
	25	20.51 ± 6.71	p=0.003	ns	p<0.001
	28	21.53 ± 6.85	p=0.001	ns	p<0.001
AZ	16	1.34 ± 0.44	ns	ns	p<0.001
	19	7.89 ± 3.35	p=0.005	ns	p<0.001
	22	14.17 ± 4.51	p=0.003	ns	p<0.001
	25	21.06 ± 5.79	p=0.003	ns	p<0.001
	28	31.59 ± 8.73	p=0.004	ns	p<0.001
ZA	16	6.28 ± 4.01	ns	na	p<0.001
	19	13.37 ± 4.38	ns	na	p<0.001
	22	27.23 ± 8.52	ns	na	p<0.001
	25	40.75 ± 11.51	ns	na	p=0.002
	28	59.74 ± 14.07	ns	na	p=0.029
ZZ	16	12.10 ± 3.92	na[2]	ns	p<0.001
	19	22.09 ± 2.03	na	ns	p<0.001
	22	41.28 ± 6.92	na	ns	p=0.001
	25	58.54 ± 5.42	na	ns	p=0.002
	28	79.51 ± 11.76	na	ns	ns

[1]ns, not significant
[2]na, not applicable

3.3. Experiment 2: Dynorphin Content in EIL-Treated Rats

3.3.1. Purpose

VMR testing after i.v. U50,488H administration indicated that EIL bladder inflammation combined with adult re-inflammation markedly altered responses to the κ-opioid receptor agonist U50,488H. Assuming that the effects of U50,488H in Experiment 1 were mediated by κ-opioid receptors, the function of κ-opioid receptors shifted from antinociceptive to pro-nociceptive. Prolonged release of dynorphin under conditions of inflammation could alter κ-opioid receptors and contribute to this shift. In Experiment 2, animals were either naïve or treated EIL (P14-P16) with anesthesia only or zymosan as was done in Experiment 1. The latter two groups were divided in half and treated with anesthesia only or zymosan as adults. Inflamed animals (AZ, ZA, and ZZ) were expected to display increased dynorphin content because cutaneous and joint inflammation (Calza et al., 1998; Draisci and Iadarola 1989; Draisci et al., 1991; Hollt et al., 1987; Iadarola et al., 1988 a,b; MacArthur et al., 1999; Millan et al., 1985, 1986, 1987, 1988; Nahin et al., 1989; Nakamura, 1994; Noguchi et al., 1991; Przewlocka et al., 1992; Ruda et al., 1988; Weihe et al., 1989) and nerve injury (Draisci et al., 1991; Kajander et al., 1990; Malan et al., 2000; Wagner et al., 1993) consistently increase spinal dynorphin mRNA and peptide. Therefore, spinal changes in dynorphin A (1-17), a κ-opioid receptor selective 17 amino acid cleavage product of the prodynorphin gene, after EIL inflammation were measured in thoracolumbar and lumbosacral spinal cord segments using ELISA. Levels in cardiac blood and bladders were also measured.

3.3.2. Cresyl Violet Staining

Figure 3.3 shows spinal cord tissue from one animal that was stained with cresyl violet to verify the segmental identity of the lumbosacral and thoracolumbar sections to be used for dynorphin ELISAs. Thoracolumbar and lumbosacral sections were isolated in a manner identical to that used for collection of sections for ELISA. Examination of slices from the lumbosacral section revealed that the most caudal segment included was S2 (at the transition to S3), and the most rostral segment included was L4 (approaching the transition to L3). Panel A of Figure 3.3 shows a representative S2 section, and Panel B shows a section at the transition between L4 and L5. Examination of slices from the thoracolumbar section revealed that the most caudal segment included was L3, and the most rostral segment included was T13. Panel C of Figure 3.3 shows a representative L2 section, and Panel D shows a representative T13 section. Therefore, staining revealed that the lumbosacral spinal cord sections used for ELISA spanned L4-S2, while the thoracolumbar sections spanned T13-L2 and often included some of L3. These sections encompass segments know to receive afferents from and the bladder and send efferents to the bladder via the pelvic (L5-S2) and hypogastric (T13-L2) nerve (de Groat, 1986; Keast and de Groat, 1992; Sharkey, Williams, Schultzberg, & Dockray,1983; Steers, Clambotti, Etzel, Erdman, & de Groat, 1991). The region between the lumbosacral and thoracolumbar sections was also examined. This mid-lumbar section included L4 and L3 segments, indicating that most of L4, and at least part of L3, which are both regions lacking afferent input from the bladder, were excluded from ELISA analysis.

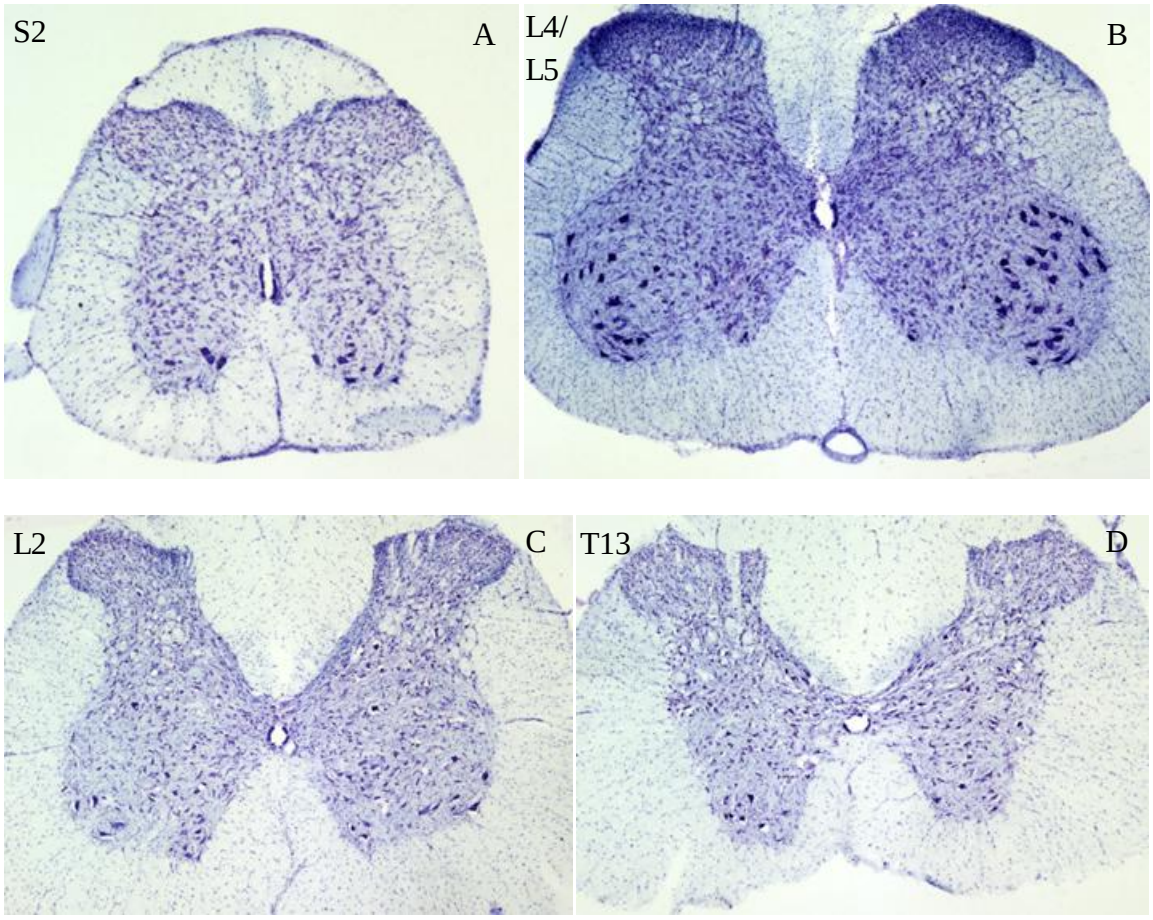

Figure 3.3. Cresyl violet staining of lumbosacral and thoracolumbar spinal sections. Cresyl violet staining was performed to confirm the segmental identity of lumbosacral and thoracolumbar sections collected for ELISA. The lumbosacral section spanned from L4-S2, while the thoracolumbar section spanned from T13-L2. Representative S2 (Panel A) and L4/L5 (Panel B) segments from the lumbosacral section, and representative L2 (Panel C) and T13 (Panel D) segments from the thoracolumbar section are shown.

3.3.3. Dynorphin Concentration Curves

Dynorphin concentration curves were constructed to determine the appropriate amount of protein to load to provide a signal within the linear range of the standard curve of the ELISA kits. These concentration curves are displayed in Figure 3.4. Panel A shows concentration curves for lumbosacral spinal cord segments, thoracolumbar spinal cord segments, and bladder. Each curve displayed a high degree of linearity ($r^2 \geq 0.995$). Based on these curves, 40 µg protein from lumbosacral spinal cord homogenates and 20 µg protein from thoracolumbar spinal cord homogenates were loaded for ELISA analysis of experimental samples. On the concentration curve, these amounts corresponded to 0.081 ng/ml and 0.089 ng/ml dynorphin, respectively (linear range 0.04-0.53 ng/ml). For bladder homogenates, 30 µg protein was loaded, corresponding to 0.231 ng/ml dynorphin (linear range 0.06-0.70 ng/ml). Panel B shows the concentration curve for serum. This curve was also highly linear ($r^2=0.998$). Based on this curve, 600 µg protein from serum was loaded, corresponding to 0.417 ng/ml dynorphin (linear range 0.06-0.7 ng/ml).

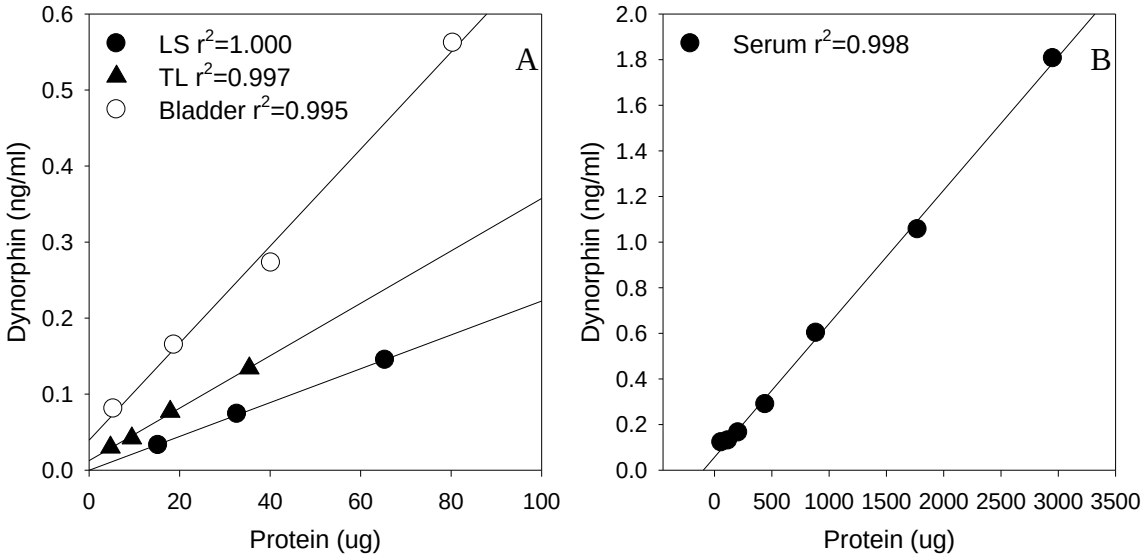

Figure 3.4. Concentration curves for dynorphin ELISAs. Dynorphin content in several different volumes of lumbosacral, thoracolumbar, serum, or bladder tissue was measured using ELISA. The amount of protein in each of these volumes was calculated and concentration curves (total protein in µg versus concentration of dynorphin in ng/ml) were constructed to determine the amount of experimental samples that should be loaded in ELISAs to provide dynorphin concentrations within the linear range of the standard curve. Panel A shows concentration curves for lumbosacral spinal cord segments, thoracolumbar spinal cord segments, and bladder tissue. Based on these curves, 40 µg protein from lumbosacral spinal cord homogenates and 20 µg protein from thoracolumbar spinal cord homogenates, corresponding to 0.081 ng/ml and 0.089 ng/ml dynorphin, were chosen for ELISA analysis of experimental samples (linear range 0.04-0.53 ng/ml). For bladder homogenates, 30 µg protein was chosen, corresponding to 0.231 ng/ml dynorphin (linear range 0.06-0.7 ng/ml). Panel B shows the concentration curve for serum. Based on this curve, 600 µg protein was chosen, corresponding to 0.417 ng/ml dynorphin (linear range 0.06-0.7 ng/ml). All curves displayed a high degree of linearity ($r^2 \geq 0.998$)

3.3.4. Impact of EIL and Adult Bladder Inflammation on Dynorphin Content

3.3.4.1. Lumbosacral Spinal Cord

Dynorphin content normalized to total protein for the lumbosacral spinal cord is shown in Panel A of Figure 3.5. An overall ANOVA revealed a significant effect of intravesical treatment group on dynorphin content in the lumbosacral spinal cord [$F(4,28)=9.28$, $p<0.001$]. Post-hoc comparisons using Fisher's LSD and Holm's correction for multiple comparisons revealed that dynorphin content of groups ZA (0.271 ± 0.013 ng/mg protein) and ZZ (0.266 ± 0.019 ng/mg protein) was increased relative to groups naïve (0.197 ± 0.016 ng/mg protein), AA (0.174 ± 0.014 ng/mg protein), and AZ (0.177 ± 0.011 ng/mg protein; $p \leq 0.003$). The latter groups were not significantly different from one another.

3.3.4.2 Thoracolumbar Spinal Cord

Dynorphin content in the thoracolumbar spinal cord is shown in Panel B of Figure 3.5. An overall ANOVA revealed no significant effect of intravesical treatment group on dynorphin content in the thoracolumbar spinal cord. Panel C of Figure 3.5 shows dynorphin content in the lumbosacral and thoracolumbar spinal cord when data were grouped across intravesical treatment. Dynorphin content in the thoracolumbar spinal cord (0.272 ± 0.008 ng/mg protein) was significantly greater than that in the lumbosacral spinal cord (0.219 ± 0.010 ng/mg protein; $p<0.001$).

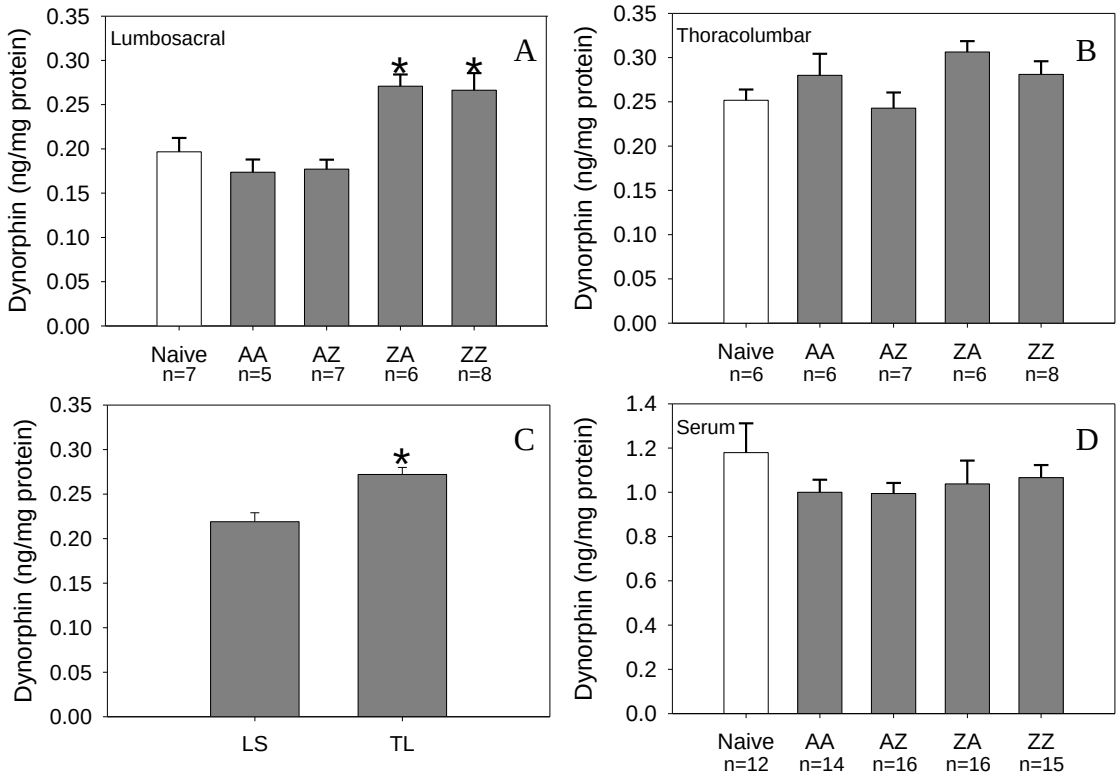

Figure 3.5. Group mean content of dynorphin in the lumbosacral and thoracolumbar spinal cord and serum. Panel A: Dynorphin content was significantly increased in the lumbosacral spinal cord of groups ZA and ZZ compared to groups naïve, AA, and AZ (*$p \leq 0.003$; n=5-8/group). Panel B: Dynorphin content in the thoracolumbar spinal cord was not significantly altered by the treatments (n=6-8/group). Panel C: When data were collapsed across intravesical treatment group, dynorphin content in the thoracolumbar spinal cord was significantly greater than in the lumbosacral spinal cord ($p<0.001$). Panel D: Dynorphin content in serum was not altered by the treatments (n=12-16/group).

3.3.4.3. Serum

Dynorphin content in the serum is shown in Panel D of Figure 3.5. As in the thoracolumbar spinal cord, an overall ANOVA revealed no significant effect of intravesical treatment group on dynorphin content in the serum.

3.3.4.4. Bladder

Dynorphin content in the bladder was expressed and analyzed in three ways: total bladder content, bladder content normalized to bladder weight, and bladder content normalized to total protein. These measures, as well as group mean bladder weights, are shown in Figure 3.6.

3.3.4.4.1. Total bladder content. Total bladder content of dynorphin is shown in Panel A of Figure 3.6. There was a significant effect of intravesical treatment group on total bladder content [$F(4,25)=13.18$, $p<0.001$]. Specifically, total bladder content of dynorphin was significantly greater in groups ZA (1.56 ± 0.08 ng, $p<0.001$) and ZZ (1.19 ± 0.07 ng, $p=0.009$) compared to naïve controls (0.91 ± 0.07 ng). Total bladder content of dynorphin was also significantly greater in groups AZ (1.15 ± 0.00 ng, $p=0.001$), ZA ($p<0.001$), and ZZ ($p=0.001$) compared to AA controls (0.75 ± 0.023 ng). Finally, total bladder content of group ZA was significantly greater than that of groups AZ ($p=0.001$) and ZZ ($p=0.003$).

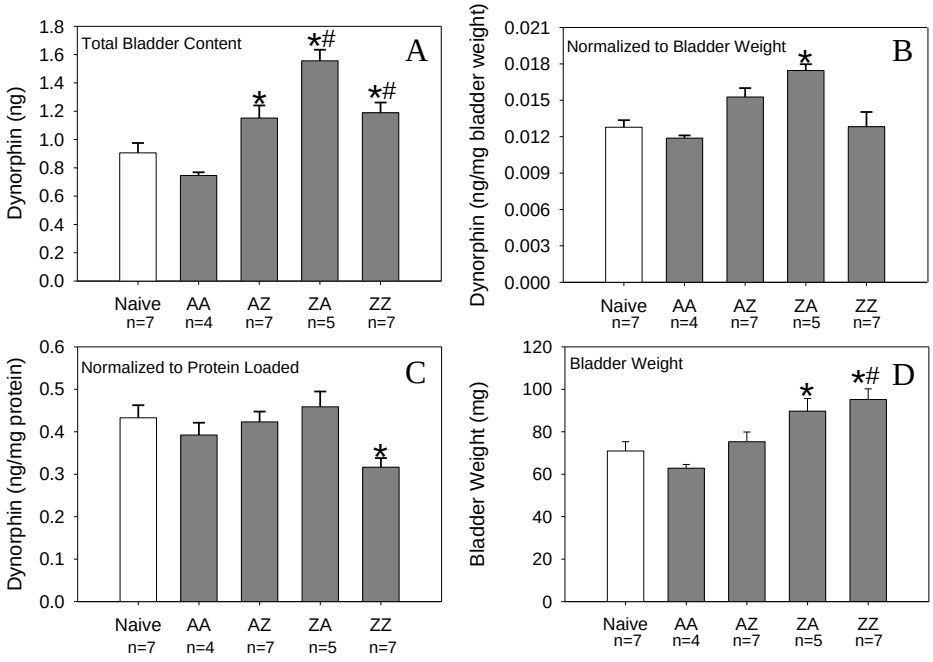

Figure 3.6. Group mean content of dynorphin in the bladder and bladder weights (n=4-7/group). Dynorphin content in the bladder was expressed and analyzed in three ways: total bladder content (A), bladder content normalized to bladder weight (B), and bladder content normalized to total protein (C). Group mean bladder weights are shown in Panel D. Panel A: Total bladder content of dynorphin was significantly increased in groups ZA and ZZ compared to naïve controls (#$p \leq 0.009$). In addition, it was significantly increased in groups AZ, ZA, and ZZ compared to AA controls (*$p \leq 0.001$). Finally, it was significantly increased in group ZA compared to groups AZ and ZZ (+$p \leq 0.003$). Panel B: Bladder dynorphin content normalized to bladder weight was significantly increased in group ZA compared to groups naïve, AA, and ZZ (*$p \leq 0.001$). Panel C: Bladder dynorphin content normalized to total protein was significantly decreased in group ZZ compared to groups naïve and ZA (*$p \leq 0.005$). Panel D: Bladder weights were significantly increased in groups ZA and ZZ compared to groups naïve and AA (*$p \leq 0.006$). Bladder weights of group ZZ were also significantly greater than those of group AZ (#$p = 0.002$).

3.3.4.4.2. Total bladder content per mg bladder weight. Total bladder content normalized to bladder weight is shown in Panel B of Figure 3.6. When total bladder content was normalized to bladder weight, there was a significant effect of intravesical treatment group [$F(4,25)=6.46$, $p=0.001$]. Dynorphin was increased in group ZA (0.0174 ± 0.0052 ng/mg) compared to groups naïve (0.0128 ± 0.0058 ng/mg, $p=0.001$), AA (0.0119 ± 0.0023 ng/mg, $p<0.001$), and ZZ (0.0128 ± 0.00122 ng/mg, $p=0.001$)

3.3.4.4.3. Total bladder content per mg protein. Total bladder content normalized total protein loaded is shown in Panel C of Figure 3.6. When total bladder content was normalized to total protein loaded, there was a significant effect of intravesical treatment group [$F(4,25)=3.75$, $p=0.016$]. Dynorphin content in group ZZ (0.317 ± 0.021 ng/mg) was significantly decreased compared to groups naïve (0.433 ± 0.030, $p=0.005$) and ZA (0.459 ± 0.036, $p=0.002$).

3.3.4.4.4. Bladder weight. Mean group bladder weights are shown in Panel D of Figure 3.6. Bladder weights were increased in groups ZA (89.7 ± 6.0 mg) and ZZ (95.2 ± 5.00 mg) compared to naïve (70.9 ± 4.35 mg) and AA (62.8 ± 1.8 mg) controls ($p\leq0.006$). Bladder weights of ZZ animals were also significantly greater than those of group AZ (75.3 ± 4.6 mg; $p=0.002$).

3.3.4.5. Summary

EIL inflammation alone (group ZA) or EIL inflammation accompanied by adult re-inflammation (group ZZ) significantly increased dynorphin in the lumbosacral spinal

cord. The increase was specific to this region because similar increases were not seen in the thoracolumbar spinal cord. Interestingly, collapsing across intravesical treatment groups revealed that the thoracolumbar spinal cord may contain more dynorphin than the lumbosacral cord.

Bladder inflammation did not alter serum content of dynorphin. However, it did alter bladder dynorphin content. Since bladder weights were known, and the amount of PBS used for homogenization was proportional to bladder weight, it was possible to express bladder content of dynorphin in three different ways: total bladder content, bladder content normalized to bladder weight, and bladder content normalized to total protein. Total bladder content was significantly increased in animals receiving EIL bladder inflammation (groups ZA and ZZ) relative to naïve controls. In addition, total bladder content of dynorphin was significantly increased after adult bladder inflammation alone (group AZ), EIL inflammation alone (group ZA), and EIL inflammation followed by adult re-inflammation (group ZZ). EIL inflammation alone produced the greatest increase in total bladder dynorphin, with group ZA having significantly greater total bladder dynorphin than groups AZ and ZZ. Bladder content per mg bladder weight was significantly increased in group ZA compared to groups naïve, AA, and ZZ. Finally, bladder content of dynorphin, normalized to total protein, was significantly decreased in group ZZ compared to groups naïve and ZA. Regardless of measure used, one difference was consistently observed. Dynorphin content of group ZZ was always significantly less than that of group ZA.

3.4. Experiment 3: i.v. Administration of U50,488H and i.t. Administration of Nor-BNI in EIL-Treated Rats

3.4.1. Purpose

VMR testing revealed that EIL inflammation significantly altered effects of i.v. U50,488H. While VMRs to the 60 mmHg test stimulus of most animals were significantly inhibited relative to baseline after 1 mg/kg U50,488H, responses of group ZZ were increased relative to baseline. This shift in responses to U50,488H may be due to alterations in κ-opioid receptors at either the peripheral or spinal level. Measurement of dynorphin using ELISA revealed that the endogenous ligand for κ-opioid receptors, dynorphin, was significantly increased in the lumbosacral spinal cord in after EIL inflammation. These data are consistent with the view that EIL inflammation alters one component of the κ-opioid system, the endogenous ligand dynorphin, at the spinal level. Therefore, a spinal site of action for U50,488H was evaluated. To determine if the altered responses to U50,488H involved altered spinal κ-opioid receptor function, nor-BNI was administered i.t. 24 hours prior to testing. VMR testing occurred in a manner identical to that used in Experiment 1. Nor-BNI was expected to prevent the inhibitory and/or facilitatory effects of U50,488H.

3.4.2. Baseline VMRs to UBD

Baseline VMRs to UBD in animals with chronic i.t. catheters are shown in Figure 3.7. Neither intravesical treatment group nor i.t. treatment significantly altered baseline VMRs. An overall ANOVA comparing baseline VMRs revealed no significant effects of intravesical treatment or i.t. treatment and no intravesical treatment x i.t. treatment inte-

raction. However, there was a trend toward an increase in the group mean VMRs to UBD in group AA given i.t. nor-BNI (6.73 ± 1.93, p=0.097) compared to group AA given i.t. saline (3.27 ± 0.67). In addition, there was a trend toward a decrease in group mean VMRs in group ZZ given nor-BNI (3.20 ± 0.78) compared to group ZZ given i.t. saline (5.72 ± 1.11, p=0.093).

As in Experiment 1, repeated pre-drug trials may alter bladder sensitivity and therefore alter responsiveness to U50,488H. Therefore, the relationship between the number of pre-drug trials and both baseline VMRs to UBD and percent of baseline VMRs at 1 min after administration of U50,488H was examined. The number of pre-drug trials conducted and baseline VMRs were inversely correlated [r(64)=-0.274, p=0.028]. Although this correlation was not significant in Experiment 1, an inverse correlation between the number of pre-drug trials conducted and baseline VMRs is not surprising because the animals with the smallest baseline VMRs required the greatest number of pre-drug trials to evoke a measurable, stable VMR to UBD. VMRs at 1 min after administration of U50,488H, expressed as percent of baseline VMRs to UBD, were not significantly correlated with the number of pre-drug trials conducted [r(59)=0.158, p=0.225]. Finally, data were examined to determine if the number of pre-drug trials conducted differed between intravesical or i.t. treatment group. An ANOVA revealed no significant effect of intravesical or i.t. treatments and no intravesical treatment group x i.t. treatment interaction on the number of pre-drug trials conducted. In summary, while the number of pre-drug trials conducted was inversely correlated with baseline VMRs to UBD, it did not impact responses to U50,488H at 1 min after administration.

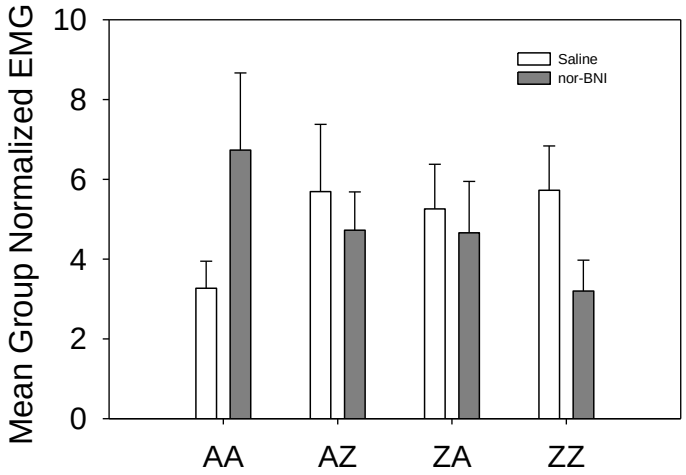

Figure 3.7. Baseline group mean normalized EMG activity (VMR) for animals with chronic i.t. catheters (n=7-9/group). Stable baseline VMRs to a 60 mmHg test stimulus were established before administration of U50,488H. Baseline VMRs to UBD did not differ between intravesical treatment groups or between i.t. treatments. However, there was a trend toward increased VMRs in group AA given i.t. nor-BNI compared to those given i.t. saline (p=0.097) and a trend toward decreased VMRs in group ZZ given i.t. nor-BNI compared to those given i.t. saline (p=0.093). Animals receiving i.t. saline are indicated by open bars, while animals receiving i.t. nor-BNI are indicated by closed bars.

3.4.3. Percent of Baseline VMRs to UBD after U50,488H

Percent of baseline VMRs after 1 mg/kg and 4 mg/kg U50,488H are shown in Figures 3.8 and 3.9. Figure 3.8 presents data as a function of the i.t. treatment to facilitate comparisons between intravesical treatment groups (AA, AZ, ZA, and ZZ) when animals were given the same i.t. treatment (i.t. saline or nor-BNI). Figure 3.9 displays data broken out by intravesical treatment group to facilitate comparisons between i.t. treatments when animals were given the same intravesical treatment.

3.4.3.1. I.t. Administration of Saline.

Panel A of Figure 3.8 displays VMRs to UBD for all animals given i.t. saline. The inhibitory and facilitatory effects of U50,488H were greatly attenuated in these animals compared to responses in animals in Experiment 1 (Figure 3.2). None-the-less, data were examined separately at each dose of U50,488H. An ANOVA was conducted with data after administration of 1 mg/kg U50,488H from animals given i.t. saline. The effects of intravesical treatment group and time and the group x time interaction were not significant. Therefore, intravesical treatment group did not significantly alter VMRs to UBD after 1 mg/kg of U50,488H. These data were not analyzed further since the overall ANOVA was not significant.

A separate ANOVA was conducted with data after administration of 4 mg/kg U50,488H from animals given i.t. saline. There was a significant effect of time [$F(4,108)=27.65$, $p<0.001$], but the effect of group, and time x group interaction, were not significant. These data indicate that intravesical treatment group did not significantly

alter VMRs to UBD after 4 mg/kg of U50,488H. Therefore, these data were also not analyzed further.

One-sample t-tests were conducted comparing group mean percent of baseline VMRs to 100 percent of baseline. As stated previously, an ANOVA revealed no significant effect of time after administration of 1 mg/kg U50,488H. Therefore, one-sample t-tests were conducted on data collapsed across time. VMRs of group AA (117.14 ± 7.37) were significantly greater than 100 percent of baseline ($p=0.025$). No other intravesical treatment groups had VMRs to UBD that differed significantly from 100 percent of baseline after 1 mg/kg of U50,488H. However, these data do not imply that group AA was significantly different from other intravesical treatment groups, because, as mentioned previously, an ANOVA revealed no significant effect of intravesical treatment group after 1 mg/kg of U50,488H. These results were very different from those in Experiment 1, in which VMRs of groups AA, AZ, and ZA were significantly lower than 100 percent of baseline, while VMRs of group ZZ were significantly greater than 100 percent of baseline. Qualitatively, both the inhibitory and facilitatory effects of 1 mg/kg of U50,488H were reduced in animals given i.t. saline vehicle in Experiment 3 compared to animals in Experiment 1.

After administration of 4 mg/kg of U50,488H, an ANOVA revealed a significant effect of time. Therefore, one-sample t-tests were conducted separately at each time point. Group means and p-values for specific comparisons are shown in Table 3.2. After 4 mg/kg of U50,488H, VMRs of group AA were significantly lower than 100 percent of baseline only at 16 min after the first dose ($p=0.010$). VMRs of group AZ were significantly lower than 100 percent of baseline at every time point ($p\leq0.030$) except for 28 min

after the first dose. VMRs of group ZA were significantly lower than 100 percent of baseline at every time point ($p \leq 0.022$) except for 25 min after the first dose. Finally, VMRs of group ZZ were significantly lower than 100 percent of baseline only at 16 and 19 min after the first dose ($p \leq 0.002$). Again, these results were very different from those of Experiment 1. In Experiment 1, VMRs of all groups were significantly lower than than 100 percent of baseline at all time points except for VMRs of group ZZ at the last trial. Qualitatively, these data indicate that VMRs of animals given i.t. saline vehicle in Experiment 3 were less inhibited by 4 mg/kg of U50,488H than animals in Experiment 1, since VMRs of the former group were significantly lower than baseline only at selected time points. This difference was especially apparent in group AA, in which VMRs were significantly lower than baseline only at the first trial after administration of 4 mg/kg of U50,488H, and in group ZZ, in which VMRs were significantly lower than baseline only at the first 2 trials after adminsitraiton of 4 mg/kg of U50,488H.

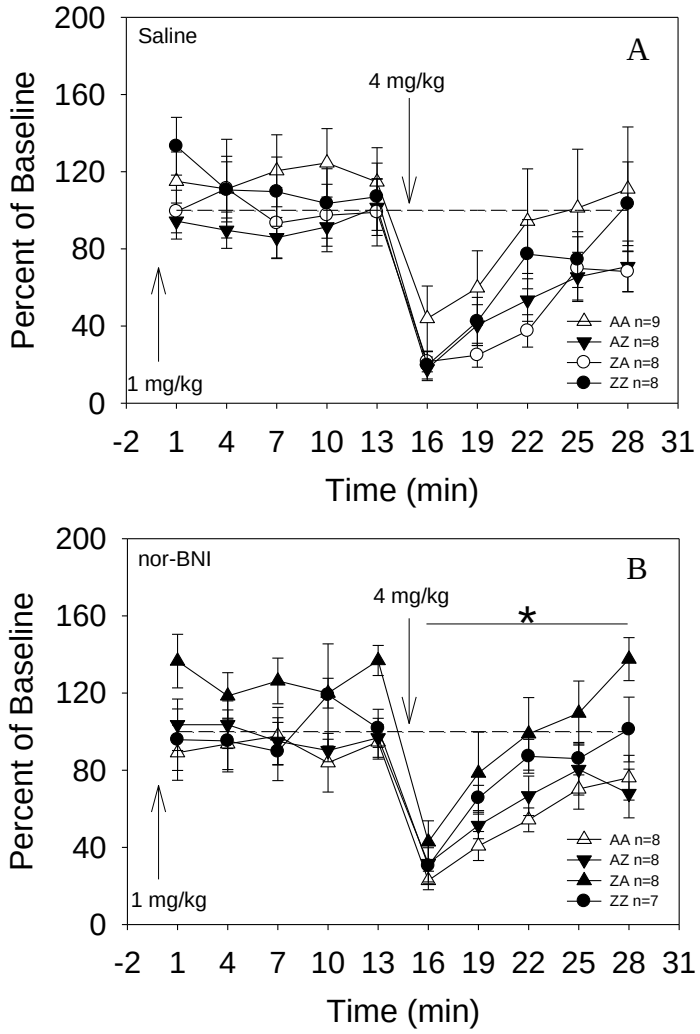

Figure 3.8. Responses to i.v. administration of U50,488H after prior administration of i.t. nor-BNI or i.t. saline (n=7-9/group). Comparisons are made between animals given different intravesical treatments (AA, AZ, ZA, or ZZ) at each level of i.t. treatment (i.t. saline or nor-BNI). Panel A compares groups AA, AZ, ZA, and ZZ given i.t. saline. Panel B shows the same comparisons in groups given i.t. nor-BNI. Panel A: In animals treated with i.t. saline, after both 1 and 4 mg/kg of U50,488H there was no significant effect of intravesical treatment group on VMRs to UBD. Panel B: In animals treated with i.t. nor-BNI, after 4 mg/kg of U50,488H VMRs of group ZA were significantly greater than those of groups AA and AZ (*$p<0.001$).

Table 3.2. Post-hoc comparisons at each time point after i.v. administration of 4 mg/kg of U50,488H after prior administration of i.t. saline. Intravesical treatment group, time after i.v. administration of 1 mg/kg of U50,488H, and group mean ± standard error of the mean (SEM) are shown in the first 3 columns. P-values for one-sample t-tests comparing group means to 100 percent of baseline are shown in the last column.

Group	Time (min)	Mean ± SEM	Comparison to Baseline
AA	16	43.84 ± 16.86	p=0.010
	19	59.77 ± 19.23	ns
	22	94.32 ± 27.11	ns
	25	101.20 ± 30.43	ns
	28	111.00 ± 32.14	ns
AZ	16	17.81 ± 6.13	p<0.001
	19	40.49 ± 10.57	p=0.001
	22	53.47 ± 11.02	p=0.004
	25	65.46 ± 12.75	p=0.030
	28	70.85 ± 13.18	ns
ZA	16	21.50 ± 5.24	p<0.001
	19	24.86 ± 6.19	p<0.001
	22	37.46 ± 8.39	p<0.001
	25	69.87 ± 16.39	ns
	28	68.20 ± 10.32	p=0.022
ZZ	16	19.65 ± 7.10	p<0.001
	19	42.35 ± 12.47	p=0.002
	22	77.22 ± 17.96	ns
	25	74.44 ± 14.44	ns
	28	103.36 ± 21.69	ns

3.4.3.2. I.t. Administration of Nor-BNI.

Panel B of Figure 3.8 displays percent of baseline VMRs to UBD for all groups given i.t. nor-BNI. An ANOVA was conducted with data after administration of 1 mg/kg of U50,488H from animals given i.t. nor-BNI. Similar to data after administration of 1 mg/kg of U50,488H from animals given i.t. saline, the effects of intravesical treatment group and time and the intravesical treatment group x time interaction were not significant. An ANOVA with data after administration of 4 mg/kg of U50,488H from animals given nor-BNI yielded significant results. There were significant effects of intravesical treatment group [$F(3,25)=3.48$, $p=0.031$] and time [$F(4,100)=45.99$, $p<0.001$]. Since the intravesical treatment group x time interaction was not significant, post-hoc analyses were conducted on data collapsed across time (16-28 min). Post-hoc comparisons using Fisher's LSD and Holm's correction for multiple comparisons revealed that VMRs of group ZA (93.46 ± 8.73) were significantly greater than those of groups AA (52.82 ± 4.76, $p<0.001$) and AZ (59.60 ± 5.29, $p<0.001$).

One-sample t-tests also were conducted comparing mean percent of baseline VMRs for each group to 100 percent of baseline. As stated previously, an ANOVA revealed no significant effect of time after administration of 1 mg/kg of U50,488H. Therefore, one-sample t-tests were conducted on data collapsed across time. 1 mg/kg U50,488H did not significantly alter VMRs of any groups from 100 percent of baseline.

After administration of 4 mg/kg of U50,488H, an ANOVA revealed a significant effect of time. Therefore, one-sample t-tests were conducted separately at each time point. Group means and p-values for specific comparisons are shown in Table 3.3. After 4 mg/kg of U50,488H, VMRs of group AA were significantly lower than 100 percent of

baseline at all time points ($p \leq 0.025$) except for 28 min after the first dose. VMRs of group AZ were significantly lower than 100 percent of baseline at every time point ($p \leq 0.039$) except for 25 min after the first dose. VMRs of group ZA were significantly lower than 100 percent of baseline only at 16 min after the first dose ($p=0.002$), and VMRs were significantly greater than 100 percent of baseline at 28 min after the first dose ($p=0.015$). Finally, VMRs of group ZZ were significantly lower than 100 percent of baseline only at 16 and 19 min after the first dose ($p \leq 0.003$). Qualitatively, these data indicate that VMRs of group ZA given i.t. nor-BNI were less inhibited by 4 mg/kg of U50,488H than those of group ZA given i.t. saline, since VMRs of the former group were significantly lower than baseline only at only one time point, and were actually greater than baseline at the last trial, while VMRs of the latter group were significantly lower than baseline at four out of five time points.

Table 3.3. Post-hoc comparisons at each time point after i.v. administration of 4 mg/kg of U50,488H after prior administration of i.t. nor-BNI. Intravesical treatment group, time after i.v. administration of 1 mg/kg of U50,488H, and group mean ± standard error of the mean (SEM) are shown in the first 3 columns. P-values for one-sample t-tests comparing group means to 100 percent of baseline are shown in the last column.

Group	Time (min)	Mean ± SEM	Comparison to Baseline
AA	16	22.85 ± 4.81	p<0.001
	19	40.69 ± 7.49	p<0.001
	22	54.24 ± 6.18	p<0.001
	25	70.25 ± 10.44	p=0.025
	28	76.09 ± 11.62	ns
AZ	16	31.61 ± 9.53	p<0.001
	19	51.35 ± 6.84	p<0.001
	22	66.73 ± 10.24	p=0.014
	25	80.40 ± 13.26	ns
	28	67.91 ± 12.65	p=0.039
ZA	16	42.84 ± 10.92	p=0.002
	19	78.49 ± 21.26	ns
	22	98.82 ± 18.80	ns
	25	109.58 ± 16.59	ns
	28	137.55 ± 11.13	p=0.015
ZZ	16	30.46 ± 9.33	p=0.001
	19	65.58 ± 6.55	p=0.003
	22	87.19 ± 8.76	ns
	25	85.98 ± 8.41	ns
	28	101.10 ± 16.77	ns

3.4.3.3. I.t. Administration of Saline Versus i.t. Administration of Nor-BNI

Comparisons were also made at each level of intravesical treatment group. Figure 3.9 shows the same data as Figure 3.8 but broken out by intravesical treatment group (AA, AZ, ZA, or ZZ) rather than i.t. treatment. Panel A compares group AA given either i.t. saline or i.t. nor-BNI. Panels B, C, and D show the same comparison for groups AZ, ZA, and ZZ, respectively. Each intravesical treatment (AA, AZ, ZA, or ZZ) was analyzed separately at 1 and 4 mg/kg. After 1 mg/kg of U50,488H, the effects of i.t. treatment and time and the i.t. treatment x time interaction were not significant for any of the intravesical treatments. Therefore, data were not analyzed further.

After 4 mg/kg of U50,488H, there were significant effects of time at each level of intravesical treatment group (AA: [$F(4,60)=14.21$, $p<0.001$]; AZ: [$F(4,56)=22.04$, $p<0.001$]; ZA: [$F(4,48)=18.28$, $p<0.001$]; ZZ: [$F(4,44)=18.54$, $p<0.001$]. However, only group ZA showed a significant effect of i.t. treatment [$F(1,12)=10.68$, $p=0.007$]. Therefore, a t-test was conducted comparing VMRs in group ZA receiving intravesical nor-BNI with those in group ZA receiving i.t. saline. Group ZA given i.t. nor-BNI (93.46 ± 8.73) had significantly greater VMRs than those give i.t. saline (44.38 ± 5.55, $p<0.001$).

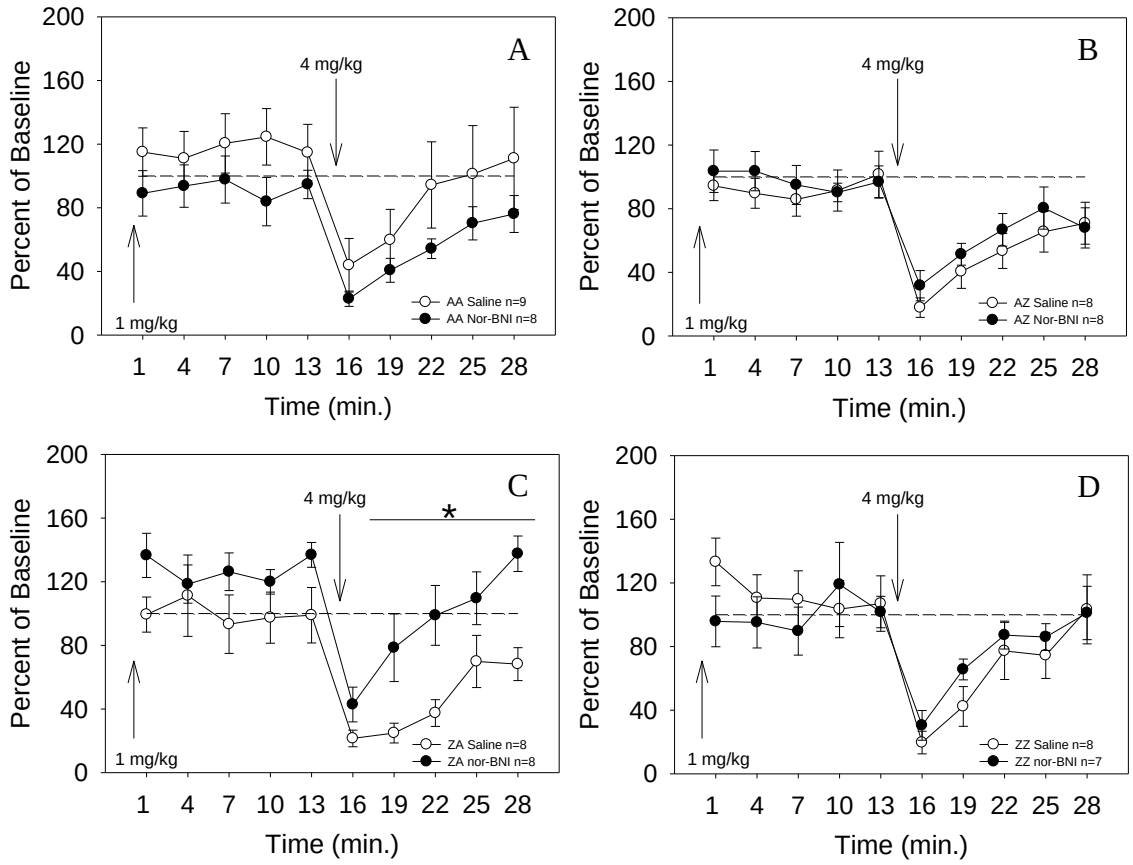

Figure 3.9. Responses to U50,488H after prior administration of i.t. nor-BNI or i.t. saline (n=7-9). Comparisons are made between animals given different i.t. treatments (i.t. saline or nor-BNI) at each level of intravesical treatment group (AA, AZ, ZA, or ZZ). Panel A compares group AA given i.t. saline with those given i.t. nor-BNI. Panels B, C, and D show the same comparison for groups AZ, ZA, and ZZ, respectively. Panels A, B, and D: After both 1 mg/kg and 4 mg/kg of U50,488H, the effect of i.t. treatment was not significant. Panel C: After 4 mg/kg of U50,488H, group ZA given i.t. nor-BNI had significantly greater responses than those give i.t. saline (*$p<0.001$).

3.4.3.4. Summary

Examination of data from animals given i.t. saline as a control vehicle (Panel A of Figure 3.8) revealed that VMRs to UBD after 1 mg/kg of U50,488H were markedly dissimilar to those seen in Experiment 1 (Figure 3.2). Comparing VMRs to 100 percent of baseline revealed that the facilitatory and inhibitory effects of 1 mg/kg of U50,488H and the inhibitory effects of 4 mg/kg of U50,488H were reduced in animals given i.t. saline compared to animals in Experiment 1. It is possible that some factor that differed between Experiments 1 and 3, such as i.t. catheterization, may have affected responses to U50,488H. Therefore, this experiment did not clearly address whether the effects of U50,488H on VMRs to UBD observed in Experiment 1 were due to activation of spinal κ-opioid receptors. However, some patterns did emerge.

There were 2 primary comparisons of interest in Experiment 3. First, VMRs of different intravesical treatment groups were compared to determine if intravesical treatments altered the effect of U50,488H on VMRs to UBD. Second, VMRs of different i.t. treatment groups were compared to determine if i.t. nor-BNI reduced the effects of U50,488H on VMRs to UBD. The only significant effects of intravesical treatment group or i.t. treatment were observed after 4 mg/kg U50,488H in group ZA. Comparing the different intravesical treatment groups revealed that VMRs of group ZA given nor-BNI were significantly greater than those of groups AA and AZ. Comparing the different i.t. treatment groups revealed that the VMRs of group ZA given i.t. nor-BNI were significantly greater than those of group ZA given i.t. saline. It is unclear why nor-BNI was only effective in this intravesical group.

3.4.4. Dynorphin Content after i.t. Catheterization

Animals receiving i.t. administration of saline did not respond to U50,488H in the same way as animals lacking i.t. catheters, making it difficult to determine whether nor-BNI was able to block the effects of U50,488H. In order to determine if i.t. catheterization altered the endogenous ligand component of the opioid system, dynorphin content in the thoracolumbar and lumbosacral spinal cord of naïve animals as well as animals receiving i.t. catheterization alone and i.t. catheterization coupled with saline administration was measured using ELISA. These data are shown in Figure 3.10. An ANOVA revealed that there were no significant differences between animals that were naïve, exposed to i.t. catheterization alone, or exposed i.t. catheterization coupled with saline administration. There was a significant effect of segment [$F(1,21)=101.86$, $p<0.001$], but no i.t. treatment x segment interaction. A two-sample t-test revealed that thoracolumbar segments (0.629 ± 0.018 ng/mg protein) contained a significantly greater amount of dynorphin than lumbosacral segments (0.354 ± 0.021 ng/mg protein, $p<0.001$). An overall greater content of dynorphin in the thoracolumbar segments compared to lumbosacral segments is in agreement with data from EIL-treated animals (Panel C of Figure 3.5). To summarize, there was a significant difference in dynorphin content between segments, but either i.t. catheterization alone or i.t. catheterization coupled with saline administration did not significantly alter thoracolumbar or lumbosacral dynorphin content.

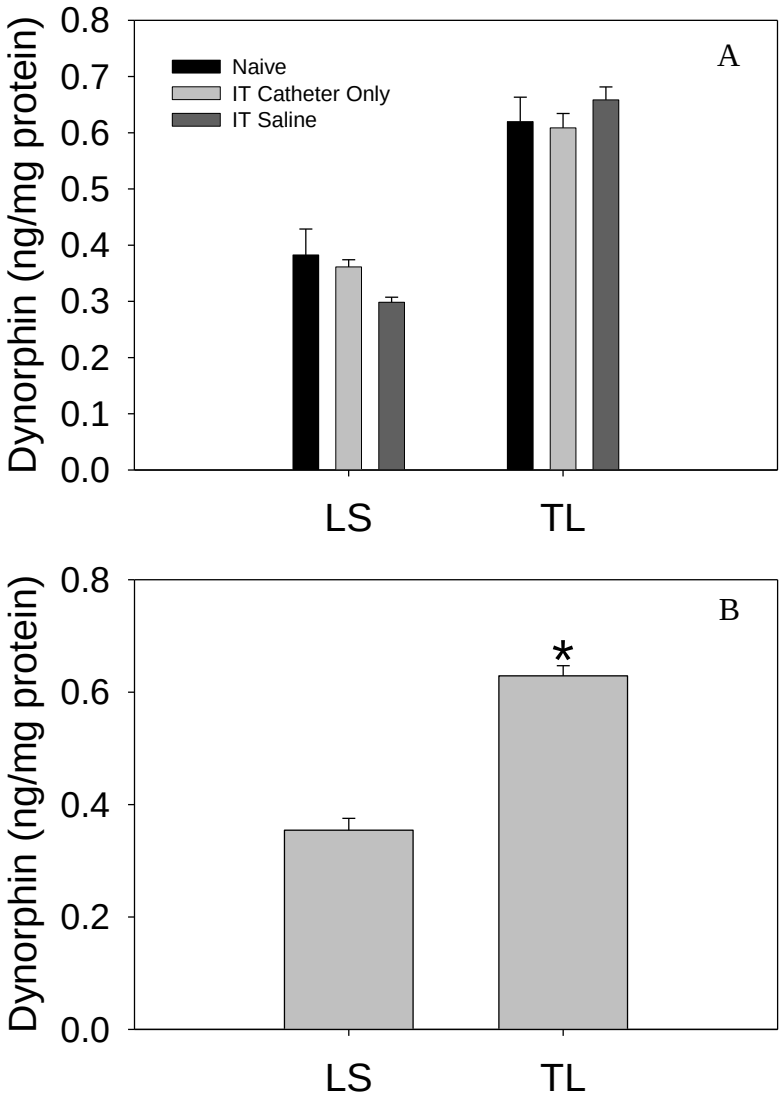

Figure 3.10. Spinal dynorphin content after intrathecal catheterization. Panel A: Lumbosacral and thoracolumbar dynorphin content for naïve animals, animals implanted with chronic i.t. catheters, and animals implanted with chronic i.t. catheters given saline (n=3-5/group). There were no significant differences in dynorphin content between naïve, i.t. catheter only, and i.t. saline animals in either the lumbosacral or thoracolumbar spinal cord. Panel B: Dynorphin content in the lumbosacral and thoracolumbar spinal cord collapsed across treatment. Dynorphin content in the thoracolumbar spinal cord was significantly greater than that in the lumbosacral spinal cord (*$p<0.001$).

CHAPTER 4

DISCUSSION

4.1. Purpose

Adult bladder inflammation recruits a reactive opioid inhibitory system that serves to suppress enhanced nociceptive input from the bladder (DeBerry et al., 2007). In some manner, EIL inflammation impairs the ability of this system to inhibit nociceptive input (DeBerry et al., 2007). The overarching goal of this thesis was to determine if this impairment of opioid inhibition involves changes in the κ-opioid system. Two ways in which the κ-opioid system may be altered are through changes in κ-opioid receptor function and changes in the concentration of endogenous ligand, dynorphin. These possibilities were examined in three experiments. The first measured changes in κ-opioid receptor function after adult and EIL inflammation through i.v. administration of U50,488H. The second examined changes in central (spinal cord) and peripheral (serum and bladder) content of dynorphin using ELISA. The third explored whether effects of U50,488H observed in Experiment 1 were due to a central site of action by administering i.t. nor-BNI 24 hours prior to testing.

4.2. Experiment 1: i.v. Administration of U50,488H in EIL-Treated Rats

κ-opioid receptor function was examined through systemic (i.v.) administration of U50,488H. Baseline VMRs to UBD were obtained prior to administration of U50,488H

and were not significantly altered by EIL or adult bladder inflammation. 1 mg/kg of U50,488H, a sub-maximal dose, inhibited VMRs of animals in all groups excluding group ZZ. 4 mg/kg of U50,488H produced robust inhibition of VMRs that decreased with time after drug administration.

The inhibition of visceral nociception observed in groups naïve, AA, AZ, and ZA at these doses of U50,488H is consistent with an existing literature that has examined the effects of systemic administration of U50,488H on distension of visceral tissues. Su et al. (2008) demonstrated inhibition of the VMR to UBD by i.v. administration of U50,488H with an ED50 of 1.34 mg/kg. In addition, Burton and Gebhart (1998) and Harada et al. (1995) found inhibition of the VMR to CRD by i.v. administration of U50,488H with ED50s of 1.8 to 2.6 mg/kg. Finally, Friese, Diop, Lambert, Riviere, & Dahl (1997) showed that i.v. administration of U50,488H inhibited cardiovascular responses to vaginal distension with an ED50 of 0.49 mg/kg.

However, EIL bladder inflammation followed by adult re-inflammation (group ZZ) dramatically altered the ability of U50,488H to suppress VMRs to UBD. In this treatment group, VMRs evoked by UBD were increased by 1 mg/kg U50,488H relative to baseline. Therefore, 1 mg/kg of U50,488H facilitated rather than inhibited VMRs to UBD. Similarly, the VMRs of group ZZ were less suppressed than those of the other groups following administration of 4 mg/kg of U50,488H, and this was the only group that had VMRs return to baseline levels after drug administration. These data indicate that 4 mg/kg was less able to inhibit VMRs to UBD in group ZZ. In conclusion, the current results as well as previous literature suggest that κ-opioid agonists normally inhibit visceral nociception. However, EIL and adult bladder inflammation reveal a facilitatory

effect of U50,488H that may normally be masked by the dominant inhibitory effect or activated when the inhibitory effect is lost. The inhibitory and facilitatory effects of U50,488H on VMRs to UBD could be mediated through several different mechanisms, including activation of κ-opioid receptors or alternative receptors at the periphery or spinal cord.

4.2.1. Mechanisms of U50,488H Inhibitory and Facilitatory Actions

4.2.1.1. Peripheral, Novel Site

The mechanism by which U50,488H produces visceral antinociception is controversial. U50,488H can act in the periphery to inhibit visceral nociception because U50,488H inhibits responses of decentralized primary afferents to UBD and CRD (Sengupta et al., 1996; Su et al., 1997 a,b). I.t. administration of U50,488H also fails to alter the VMR to CRD providing indirect support for a peripheral site of action (Danzebrink et al., 1995; Harada et al., 1995; for a review see Gebhart et al., 2000). However, attempts to reverse U50,488H-mediated inhibition with either selective κ-opioid receptor antagonists or a knockdown of the κ-opioid receptor suggest that U50,488H may act at a novel, peripheral opioid receptor. When Danzebrink et al. (1995) documented inhibition of VMRs to CRD by i.v. administration of U50,488H, they noted that the effects of 1 mg/kg U50,488H were only modestly reversed by i.v. administration of 1 mg/kg nor-BNI. They proposed that this may be due to an inadequate pretreatment time, but Sengupta et al. (1996) also found that neither a 20 mg/kg dose of nor-BNI administered 4 hours prior to testing nor two, 20 mg/kg doses of nor-BNI administered 24 and 48 hours prior to testing significantly reduced the inhibitory effects of i.a. administration of U50,488H on pelvic

nerve afferent responses to CRD. Su et al. (1997b) replicated this finding and also found that another κ-opioid receptor antagonist, DIPPA, was unable to reverse the inhibitory effects of i.a. administration of U50,488H on pelvic nerve afferent responses to CRD. Finally, Burton and Gebhart (1998) found that both nor-BNI and DIPPA were unable to reverse inhibition of the VMR to CRD produced by i.v. administration of a variety of κ-opioid agonists, including U50,488H. Su et al. (1997a) demonstrated similar results in the bladder. Two 20 mg/kg doses of nor-BNI administered 24 and 48 hours prior to testing did not significantly decrease inhibition of pelvic afferent firing during UBD produced by i.a. administration of U50,488H. As with selective κ-opioid receptor antagonists, i.t. administration of antisense oligodeoxynucleotide to the cloned κ-opioid receptor did not reverse U50,488H-induced inhibition of pelvic afferent responses to CRD (Joshi, Su, Porreca, & Gebhart, 2000).

In summary, inhibition of VMR and pelvic afferent responses to CRD or pelvic afferent responses to UBD by κ-opioid agonists was not generally affected by systemic administration of selective κ-opioid receptor antagonists or central administration of antisense oligonucleotide. The conclusion is that systemic administration of U50,488H is acting at a peripheral opioid receptor distinct from the cloned κ-opioid receptor, KOR1. It is still possible that U50,488H may act at least at a peripheral non-κ-opioid receptor because naloxone was able to attenuate inhibition of VMRs and pelvic afferent responses to CRD (Burton & Gebhart, 1998; Sengupta et al., 1996; Su et al., 1997b) and pelvic afferent responses to UBD (Su et al., 1997a). Therefore, i.v. administration of U50,488H may have inhibited VMRs to UBD in groups naïve, AA, AZ, and ZA in the current study by acting at either a peripheral, non-κ-opioid receptor or at a spinal/supraspinal site. Fur-

ther, peripheral receptors may have been disrupted by the treatment given to group ZZ, revealing an underlying facilitatory effect of U50,488H at another peripheral or central site. If U50,488H acts primarily at peripheral receptors, it would account for the failure of i.t. nor-BNI administration in Experiment 3 to influence the suppressive effects of 4 mg/kg of U50,488H in groups AA, AZ, and ZZ.

4.2.1.2. Spinal κ-opioid Receptors

While the previous section suggests that U50,488H can work at peripheral sites to modulate visceral nociception, it is possible that U50,488H may also act at the spinal level to inhibit the VMR to UBD. Activation of spinal κ-opioid receptors by U50,488H could provide one potential explanation for the hypersensitivity observed after administration of U50,488H in group ZZ. Knox and Dickenson (1987) and Sullivan and Dickenson (1991) demonstrated that lesser doses of U50,488H produced both facilitation and inhibition of spinal dorsal horn neuron responses to electrical C-fiber stimulation and noxious pinch, while greater doses consistently produced inhibition of spinal dorsal horn neuron responses to electrical C-fiber stimulation and formalin. If U50,488H acted centrally in the current study, then the i.v. administration of the 1 mg/kg dose may have been sufficient to produce a net inhibition of spinal dorsal horn neurons in groups naïve, AA, AZ, and ZA. Net inhibition would be reflected as a decrease in VMRs to UBD. However, both EIL and adult bladder inflammation may have shifted the dose-response curve for U50,488H to the left in group ZZ resulting in 1 mg/kg of U50,488H producing more facilitation than inhibition of spinal dorsal horn neurons analogous to the facilitatory ef-

fects observed by Knox and Dickenson (1987) with lesser doses of U50,488H. Net facilitation may be reflected as an increase in VMRs to UBD in group ZZ at the 1 mg/kg dose.

At a more molecular level, both the inhibitory and/or facilitatory effects of U50,488H may be mediated by actions at both κ-opioid receptor monomers and μ/κ heterodimers. Opioid receptors can form dimers or even oligomers, and oligomerization can alter the properties of each protomer (e.g. George et al., 2000; Waldhoer et al., 2005). Liu, von Gizycki, & Gintzler (2007) found that κ- as well as μ-opioid receptors are required for morphine antinociception in rats. As expected, administration of an antagonist selective for the μ-opioid receptor, β-funaltrexamine (β-FNA), reduced morphine-induced increases in tail flick latency to radiant heat. Surprisingly, nor-BNI was also able to the reverse effect of morphine. Morphine analgesia only required this κ-opioid receptor component in females, because nor-BNI did not have this effect in males (Liu et al., 2007). Concurrent activation of κ-opioid receptors by dynorphin was required for morphine analgesia in female rats because α-dynorphin antiserum also attenuated morphine antinociception (Liu et al., 2007). Liu et al. (2007) concluded that morphine was acting at a μ/κ heterodimer in female, but not male, rats to produce antinociception. In a subsequent study by Chakrabarti, Liu, and Gintzler (2010), the presence of a μ/κ heterodimer was confirmed using immunoprecipitation followed by visualization with Western blotting. The density of the 120 kDa μ/κ heterodimer was greater in female rats during proestrus than in diestrus (Chakrabarti et al., 2010). These data corresponded with the ability of nor-BNI to reverse morphine antinociception, i.e. morphine antinociception was only attenuated by nor-BNI in female rats during proestrus and not in diestrus (Chakrabarti et al., 2010). Proestrus is the stage of the estrous cycle during which circulating

concentrations of estrogen and progesterone peak (Smith, Freeman, & Neill, 1975). Therefore, it was proposed that formation of the μ/κ heterodimer may depend on these hormones. This hypothesis was supported by the finding that blocking estrogen or progesterone receptors with antagonists restored morphine antinociception in the presence of nor-BNI and reduced formation of the μ/κ heterodimer (Liu, Chakrabarti, Schnell, Wessendorf, & Gintzler, 2011).

Chakrabarti et al. (2010) proposed that the μ/κ heterodimer may mediate the antinociceptive effects of κ-opioids, while the κ-opioid receptor monomer may facilitate nociception. This could provide a mechanism by which both facilitation and inhibition of spinal dorsal horn neurons occurs with lower doses of U50,488H and only inhibition occurs at higher doses (Knox & Dickenson, 1987; Sullivan & Dickenson, 1991). If bladder nociception is mediated in this way, U50,488H could inhibit VMRs to UBD by acting at the κ-protomer of the μ/κ heterodimer. At the same time, the opposing facilitatory effects of U50,488H could occur through activation of a κ-opioid receptor monomer. Thus, U50,488H may act primarily at the μ/κ heterodimer to inhibit bladder sensitivity in groups naïve, AA, AZ, and ZA. In contrast, the ZZ treatment may down-regulate the μ/κ heterodimer, reducing the inhibitory effects of U50,488H and revealing underlying facilitatory effects of U50,488H acting at the κ-opioid receptor monomer.

In conclusion, it is possible that an interplay exists between spinal and peripheral inhibitory and facilitatory effects of i.v. administration of U50,488H on the VMR to UBD. Two sites that may mediate inhibition of the VMR to UBD are peripheral opioid receptors distinct from KOR1 and a spinal μ/κ heterodimer. Facilitation could be concurrently mediated through a spinal κ-opioid receptor monomer. Normally, the balance is

weighted toward inhibition. However, adult bladder inflammation tips this balance toward facilitation.

4.2.1.3. One Additional Consideration: Sodium Channels

As mentioned previously, inhibition of visceral nociception by systemic κ-opioid receptor agonists may be due to actions at peripheral opioid receptors distinct from κ-opioid receptors. However, a non-opioid mechanism for inhibition of responses to CRD and UBD by U50,488H and other arylacetamide κ-opioid receptor agonists has also been proposed. These agonists are known to block voltage-activated sodium channels in hippocampal CA3 neurons (Alzheimer & Bruggencate, 1990) and cardiac myocytes (Pugsley, Saint, Penz, & Walker, 1993; Pugsley, Saint, & Walker, 1994). U50,488H can occur as an enantiomer with κ-opioid receptor activity (-)-U50,488H, an enantiomer with no κ-opioid receptor activity (+)-U50,488H, or a racemic mixture (±)-U50,488H. (±)-U50,488H is the most commonly used isoform and was used in the current study, and the term U50,488H refers to this racemic form. All three enantiomers decreased voltage-activated sodium currents in colon sensory neurons in the DRG (Joshi, Lamb, Bielefeldt, & Gebhart, 2003; Su, Joshi, Kardos, & Gebhart, 2002). The effects of various stereoisomers on pelvic afferent responses to CRD were also examined. s.c. administration of all three forms of U50,488H, including (+)-U50,488H, inhibited pelvic afferent responses to CRD with similar ED50s (6.6-10.8 mg/kg; Su et al., 2002). As expected, inhibition by (+)-U50,488H (8 mg/kg) was non-opioid mediated because it was not reversed by naloxone. A final piece of evidence supporting a role for sodium channel blockade in the inhibition of pelvic afferent responses to CRD is that the sodium channel blocker mexiletine

inhibited pelvic afferent responses to CRD with a similar ED50 (9.1 mg/kg) to U50,488H stereoisomers.

It not clear whether sodium channel inhibition played a role in the inhibitory or facilitatory effects of U50,488H observed in the current study. The mechanisms which mediate inhibition of the VMR to CRD and UBD in intact animals may be very different from those that mediate inhibition of responses of dissociated pelvic afferents, the measure used to provide evidence for a role of sodium channel blockade in the inhibition of visceral nociception by U50,488H. For example, only (±)-U50,488H and (-)-U50,488H inhibited VMRs to CRD, and the ED50s required for this inhibition (4.7 mg/kg and 0.9 mg/kg, respectively) were lower than those required for inhibition of primary afferent activity (8.0 mg/kg and 6.6. mg/kg, respectively; Su et al., 2002). In addition, while 24-hour pretreatment with 10 mg/kg nor-BNI or 4 hour pretreatment with 0.5 mg/kg nor-BNI did not significantly reduce the inhibition produced by 10 mg/kg (±)-U50,488H, it was able to reduce the inhibition produced by 5 mg/kg (-)-U50,488H (Su et al., 2002). Therefore, at lower, potentially more selective doses of U50,488H, such as those used in the current study (1 mg/kg and 4 mg/kg), inhibition of visceral nocicieption may be at least partially mediated by κ-opioid receptors.

4.3. Experiment 2: Dynorphin Content in EIL-Treated Rats

4.3.1. Spinal Cord

In addition to altering κ-opioid receptor function, EIL and adult bladder inflammation altered spinal dynorphin content. In the lumbosacral spinal cord, EIL inflammation alone (group ZA) and EIL inflammation accompanied by adult re-inflammation

(group ZZ) significantly increased dynorphin content compared to all other groups. These increases were restricted to the lumbosacral segments because no such change was observed in the thoracolumbar cord. There is evidence that the lumbosacral spinal cord alone mediates acute visceral nociception, but the thoracolumbar spinal cord as well as the lumbosacral spinal cord can modulate visceral nociception under conditions of inflammation, (Ness & Gebhart, 1988b; Traub, 2000; Traub, Pechman, Iadarola, & Gebhart, 1992; Traub, Herdegen, & Gebhart, 1993; Traub & Murphy, 2002).

4.3.2. Bladder

Three different measures of bladder dynorphin content were used to provide different perspectives on how dynorphin input from the bladder was altered by EIL and adult bladder inflammation: total bladder content, bladder content normalized to bladder weight, and bladder content normalized to total protein. Each measure provided a unique perspective. Since total bladder content was directly proportional to bladder weight, it reflected the dynorphin supply of the entire bladder. Most importantly, because group AA served as the primary control comparison, total bladder content of dynorphin was significantly increased after adult bladder inflammation alone (group AZ), EIL inflammation alone (group ZA), and when EIL inflammation was followed by adult re-inflammation (group ZZ) compared to group AA. EIL inflammation alone produced the greatest increase in total bladder dynorphin, with group ZA having significantly greater total bladder dynorphin than groups AZ and ZZ.

Normalizing bladder content to bladder weight provided a measure of the amount of dynorphin per 1 mg section of the bladder. This measure was independent of bladder

weight. Bladder content per mg bladder weight was significantly increased in group ZA compared to groups naïve, AA, and ZZ. Since group ZZ had significantly heavier bladders than groups naïve, AA, and AZ, these data indicate that the increase in total bladder dynorphin content in group ZZ was primarily due to increased bladder weights. Group ZA was the only group displaying an increase in dynorphin concentration independent of bladder weight.

Finally, normalizing to bladder weight does not control for differences in the amount of protein extracted or the total protein concentration of each homogenate. Therefore, bladder dynorphin content was also normalized to total protein loaded. Using this measure, group ZA did not display an increase in bladder dynorphin, indicating that relatively higher total protein concentrations in group ZA compared to groups naïve, AA, and AZ may have contributed to increases in total bladder content and bladder content normalized to bladder weight. Bladder content of dynorphin, normalized to total protein, was significantly decreased in group ZZ compared to groups naïve and ZA. Group ZZ also displayed relatively higher total protein concentrations than groups naïve, AA, and AZ, possibly contributing to this decrease. However, higher protein concentrations do not completely account for decreased dynorphin content in group ZZ because group ZZ displayed decreases in total bladder content and bladder content normalized to bladder weight compared to group ZA without correcting for protein loaded.

It remains unclear which measure is most important for determining the magnitude of hypersensitivity after bladder inflammation. What is clear is that when a general measure of total bladder dynorphin is used, adult bladder inflammation alone (group AZ), EIL bladder inflammation alone (group ZA), and EIL inflammation followed by adult re-

inflammation (group ZZ) increased bladder dynorphin, and the greatest increase occurred after EIL bladder inflammation alone (group ZA).

4.3.3. Function

4.3.3.1. Spinal Cord

Adult re-inflammation (group ZZ) appeared to produce no additional increase or in dynorphin over and above that produced by EIL inflammation (group ZA). Adult bladder inflammation alone (group AZ) did not cause upregulation of dynorphin or dynorphin release because spinal dynorphin content was not significantly different from non-inflamed animals (group AA). These data are not consistent with studies in which arthritis and hindpaw inflammation increased dynorphin mRNA and peptide (Calza et al., 1998; Draisci & Iadarola 1989; Draisci et al., 1991; Hollt et al., 1987; Iadarola et al., 1988 a,b; MacArthur et al., 1999; Millan et al., 1985, 1986, 1987, 1988; Nahin et al., 1989; Nakamura, 1994; Noguchi et al., 1991; Przewlocka et al., 1992; Ruda et al., 1988; Weihe et al., 1989). In the present study, an increase may not have been observed in group AZ because of a delay in the synthesis of new dynorphin protein. For example, CFA-induced hindpaw inflammation does not increase spinal dynorphin until 3 days after induction of inflammation, while tactile and thermal hypersensitivity are evident by 6 hours after CFA injection. Similarly, adult bladder inflammation may upregulate prodynorphin expression and trigger synthesis of new dynorphin protein. However, the increase in dynorphin protein have may occurred at times greater than 24 hours after the treatment when tissue was collected and after the peak of inflammation and bladder hypersensitivity (Randich et al., 2006a). This could be tested by measuring dynorphin

levels at various times after these treatments in a parametric fashion, but this experiment was not performed in the current study.

4.3.3.2. Bladder

Changes in bladder content of dynorphin presumably reflect changes in the content of dynorphin in bladder primary afferents. Assuming primary afferents are the principal source of dynorphin, increases in bladder dynorphin content may indicate a potential for more release from peripheral and perhaps central terminals during noxious stimulation. If this assumption is made, then EIL or adult inflammation increase the total available pool of dynorphin in primary afferents because total bladder content of groups AZ, ZA, and ZZ was significantly increased relative to control group AA. Group ZA would have the greatest available pool, because it had the greatest total bladder content of dynorphin. When differences in bladder weight are controlled for, group ZA still has the greatest available pool because bladder content normalized to bladder weight was significantly increased in this group. Finally, when dynorphin content is normalized to total protein, group ZZ had less dynorphin content. The latter data indicate that bladder dynorphin content in group ZZ animals is less than that in group ZA regardless of the measure used. This may indicate that EIL inflammation is necessary to prime primary afferents to release dynorphin after adult re-inflammation.

This difference between groups ZA and ZZ in adult bladder dynorphin content may be viewed in two ways. One possibility is that dynorphin was released by adult inflammation in group ZZ. If dynorphin alters bladder sensitivity after release, then dynorphin may have more of an impact on sensitivity in group ZZ than any other group. If pe-

ripheral release of dynorphin inhibits bladder sensitivity, similar to the inhibition of VMRs to UBD produced by U50,488H in the current study in groups AA, AZ, and ZA as well as in non-inflamed adult rats in the study by Su et al. (2008d), then group ZZ should experience the least bladder sensitivity. However, this is not consistent with data from Randich et al. (2006b) indicating that this group actually displays the greatest degree of bladder hypersensitivity. Alternatively, dynorphin may be released in every group during UBD, and this release rather than the release after bladder inflammation per se may be important for bladder sensitivity. If the latter hypothesis is true, then dynorphin would have less of an effect in group ZZ than group ZA because there is less dynorphin available for release during UBD. This hypothesis is more consistent with previous data, because group ZZ would be less inhibited by dynorphin, which could contribute to bladder hypersensitivity. These possibilities could be tested by recording decentralized primary afferents during UBD in a set of similarly treated set of animals in the presence and absence of dynorphin antiserum. If dynorphin antiserum had no effect on primary afferent responses to UBD in groups AA, AZ, and ZA but altered responses to UBD in group ZZ, it would support the first possibility. If dynorphin antiserum altered responses to a greater extent in group ZA than group ZZ, it would support the second possibility.

4.4. Experiment 3: i.v. Administration of U50,488H and i.t. Administration of Nor-BNI in EIL-Treated Rats

One potential site of action of U50,488H is at spinal κ-opioid receptors. To determine whether the inhibitory and/or facilitatory effects of U50,488H observed in Experiment 1 were due to activation of spinal κ-opioid receptors, nor-BNI was administered

i.t. 24 hours prior to VMR testing. Baseline VMRs to UBD prior to U50,488H administration also were examined to determine the tonic effect of nor-BNI in each intravesical treatment group. There was a trend toward a decrease in baseline VMRs to UBD in group ZZ given nor-BNI compared to those given i.t. saline, and there was a trend toward increased baseline VMRs to UBD in group AA given i.t. nor-BNI compared to those given i.t. saline. However, neither intravesical nor i.t. treatment had a significant effect on baseline VMRs.

Percent of baseline VMRs to UBD following U50,488H administration were examined in each intravesical treatment group (group AA, AZ, ZA or ZZ). Compared to Experiment 1, the inhibitory potency of both 1 mg/kg and 4 mg/kg of U50,488H was reduced. In fact, 1 mg/kg U50,488H did not shift VMRs to significantly above or below baseline levels in any group. 4 mg/kg U50,488H suppressed VMRs, but not to the same extent as in Experiment 1. Examining the ability of nor-BNI to prevent suppression induced by 4 mg/kg U50,488H revealed that this suppression was only reduced by nor-BNI in group ZA. Therefore, no clean-cut data emerged from Experiment 3. One concern is that the i.t. catheter was a confounding variable in this experiment. I.t. catheterization may have reduced the ability of U50,488H to affect the κ-opioid system.

4.4.1. Dynorphin Content after i.t. Catheterization

I.t. catheterization by itself or i.t. catheterization coupled with administration of saline may have altered the effects of U50,488H in Experiment 3 relative to Experiment 1, in which animals did not receive i.t. catheters. To explore this possibility, changes in spinal dynorphin content after i.t. catheterization alone or i.t. catheterization combined

with i.t. saline administration were examined in this thesis. These studies showed that i.t. catheterization alone and i.t. catheterization coupled with i.t. saline administration did not alter dynorphin content in either the thoracolumbar or lumbosacral spinal cord. For this experiment, animals received adult intrathecal treatment at least 5 days after i.t. catheters were implanted. This was considered an ample amount of time to recover from i.t. catheter surgeries. Others have documented that i.t. catheterization may alter the κ-opioid system at 1 week after catheter implantation and beyond. For example, Millan et al. (1989) found that dynorphin content in the dorsal spinal cord of lumbar (L2-L6) segments was significantly increased 1 week after i.t. catheter implantation. This was associated with a decrease in the potency of U50,488H on tail withdrawal responses to noxious heat and pressure. In addition, Herman and Goldstein (1985) found that i.t. administration of dynorphin was more potent on the tail flick test 1 day after catheter implantation compared to 7 days or longer after catheter implantation. Similarly, Stevens and Yaksh (1986) and Prezewlocki et al. (1983) found that i.t. administration of dynorphin produced motor dysfunction at lower doses of dynorphin 1 and 4 days after catheter implantation compared to 7 days or longer.

The discrepancy between the current results and the increase in spinal dynorphin found by Millan et al. (1989) may be attributable to the fact that in the current study tissue was often collected more than 1 week after i.t. catheterization. This was necessary because animals were required to be in proestrus, and it often took several cycles before an animal was identified to be in this stage and able to be treated. Dynorphin content may have returned to normal levels when given the extended recovery time. Also, differences in methodology, such as sensitivity of assays used and segments analyzed, may

have contributed to the difference between the current results and those of previous studies.

4.5. Gonadal Hormone Influence on Bladder Sensitivity

Gonadal steroid hormones were not directly examined in this thesis. However, hormonal state is thought to have an important impact on bladder sensitivity as well as opioid antinociception. In addition, the present thesis has postulated an important role of the μ/κ heterodimer, which is thought to be regulated by gonadal hormones (Liu et al., 2007, 2011; Chakrabarti et al., 2010). Therefore, the following includes a very brief review of gonadal hormone influences on bladder sensitivity and the κ-opioid system since it may help guide future thinking and research in the area.

Sex differences in pain sensitivity in both humans and non-human animals are well recognized. For example, many pain conditions, including BPS/IC, fibromyalgia, and IBS, are more prevalent in women than men, and in general, women display lower pain thresholds than men (for a review, see Berkley, 1997). Gonadal hormones are thought to contribute to these differences. In addition, ovarian hormones lead to cyclic fluctuations in pain sensitivity during the menstrual cycle in women and estrous cycle in rats. For example, women with IC experience the greatest bladder hypersensitivity during the perimenstrual period when both progesterone and estradiol decrease (Powell-Boone et al., 2005). In non-inflamed rats, the greatest bladder hypersensitivity occurs during proestrus when progesterone and estrogen are highest (Ness et al., 2001). Adult bladder inflammation produces the greatest increase in bladder hypersensitivity during proestrus and metestrus (Ball et al., 2010) which are both phases during which progeste-

rone levels increase (Smith et al., 1975). Finally, Johnson and Berkley (2002) found that turpentine produces the greatest decrease in micturition thresholds during proestrus and estrus. Upon inspection of these data, no clear picture emerges when the influences of gonadal hormones are viewed in terms of absolute concentrations. However, IC patients experience the greatest bladder pain during a point of the menstrual cycle when gonadal hormones are decreasing, while rats experience the greatest bladder hypersensitivity during points of the estrous cycle when gonadal hormones peak. One possibility is that changes in hormone levels, rather than stable but elevated hormone levels, may be key for increasing bladder sensitivity. For example, acute but not chronic estrogen replacement increases VMRs to UBD in ovariectomized rats (Robbins, Mebane, Ball, Shaffer, & Ness, 2010). In addition, acute estrogen withdrawal by pellet explants increases VMRs to UBD (Robbins et al., 2010). In IC patients with cyclic symptom flares, a very limited, non placebo-controlled study found symptom improvement with leuprolide acetate and gonadal hormone supplementation with oral contraceptives (Lentz, Bavendam, Stenchever, Miller, & Smallridge, 2002). Robbins et al. (2010) proposed that dynamic changes in hormones may cause a mismatch between signaling by estrogen receptors of different subtypes with genomic or nongenomic effects acting at different sites on the nociceptive pathway, and that this mismatch may contribute to bladder sensitivity.

If this hypothesis is true then cyclic or abrupt fluctuations in hormonal levels should be reflected by fluctuations in the neural substrates underlying bladder sensitivity. With regard to the focus of this thesis, κ-opioid receptors, κ-opioid receptor immunoreactivity in the lumbosacral (L6-S1) spinal cord of female rats is greater in proestrus and estrus than in diestrus (Chang et al., 2000) and greater than in males (Harris et al., 2004).

Prodynorphin mRNA expression in the lumbar (L4-L5) spinal cord 24 hours after CFA-induced hindpaw inflammation is greater in females in diestrus and proestrus than females in estrus and males (Bradshaw, Miller, Ling, Malsnee, & Ruda, 2000). Formation of a μ/κ heterodimer is also greater in female rats in proestrus than females in diestrus and males (Chakrabarti et al., 2010). Therefore, κ-opioid receptor protein, dynorphin expression, and μ/κ heterodimer formation are all greatest around the time of the proestrus peak in estrogen and progesterone. Since bladder hypersensitivity is also greatest around proestrus (Ness et al., 2001), increases in dynorphin and the μ/κ heterodimer may occur as a compensatory response to increased nociception in normal rats. In contrast, the progressive increase in levels of estrogen and progesterone experienced during pregnancy results in an increase in dynorphin in the lumbar spinal cord (Medina, Dawson-Basoa, & Gintzler, 1993a; Medina, Want & Gintzler, 1993b). This increase in dynorphin is associated with antinociception in animal studies because dynorphin acts at κ-opioid receptors to increase jump thresholds to electric foot shock (Dawson-Basoa & Gintzler, 1996; Sander, Portoghese, & Gintzler, 1988). Also, these hormone levels are also associated with increased jump thresholds to electric foot shock produced by i.t. administration of U50,488H (Dawson-Basoa & Gintzler, 1996). Dynorphin remains increased during parturition, during which gonadal hormones rapidly decrease, complicating these results. Nonetheless, these data may indicate that the dynorphin-μ/κ heterodimer system is upregulated during times of increased estrogen and progesterone. When this increase in transient, such as during proestrus, inhibition by the dynorphin-μ/κ heterodimer system may be outweighed by pro-nociceptive processes, such as increases in pro-nociceptive peptides (Shaffer et al., 2011). In contrast, when this increase is sustained, such as during

pregnancy, these pro-nociceptive processes may not occur, revealing the inhibitory effects of the dynorphin-μ/κ heterodimer system.

4.6. Clinical Implications

From a clinical perspective, it is possible that an EIL insult to the bladder in humans may increase susceptibility for the development of BPS/IC. There is evidence showing that women with BPS/IC are more likely to recall recurrent UTI (Peters et al., 2009). Experiencing a second, adult UTI could possibly trigger symptom onset in these individuals by altering the κ-opioid system. In healthy women, κ-opioid receptor agonism may inhibit pain (Gear et al., 1996 a,b, 1999, 2003), and a dynorphin-κ-opioid receptor system has been associated with antinociception during pregnancy and parturition (Dawson-Basoa & Gintzler, 1996; Sander et al., 1998). Using a model of BPS/IC in this thesis, the pronociceptive effects of U50,488H suggest that κ-opioids lack antinociceptive efficacy and may worsen symptoms in individuals with chronic bladder pain. Fitting with these data, the only opioid agonist with an acceptable safety and efficacy profile in the treatment of bladder pain, tramadol, is a mixed μ-opioid agonist, serotonin releaser, and norepinephrine reuptake inhibitor, with no κ-opioid receptor activity (Agarwal, Yadav, Gupta, Singh, & Singh, 2008; Singh, Agarwal, Batra, Kishore, & Mandal, 2008). The mechanism for an impaired antinociceptive effect or relative hyperalgesia may be a downregulation of a κ-opioid inhibitory system which shifts the balance between antinociception and pro-nociception toward pro-nociception. A μ/κ heterodimer has been proposed to mediate κ-opioid inhibition (Chakrabarti et al., 2010). The formation and function of this heterodimer requires concurrent activation of ERα, ERβ, GPR30, and proge-

sterone receptors (PR; Liu et al., 2011). Women with IC display a trend toward decreased levels of estrogen compared to healthy controls (Powell-Boone et al., 2005). In rats, adult bladder inflammation significantly decreases estradiol levels across estrous phases and also produces a trend toward a decrease in progesterone (Shaffer et al., 2011). Therefore, one way to shift κ-opioid receptor function back toward net inhibition of pain transmission may be to supplement gonadal steroid hormones, which may upregulate this heterodimer. It would be important to maintain constant levels of estrogen and progesterone since dynamic changes in these hormones are associated with increased bladder sensitivity in both women and rats. A hormonal supplement that provides a constant level of estradiol and progestin for an extended period of time could potentially provide some symptom relief in BPS/IC patients through avoidance of pro-nociceptive processes and upregulation of the μ/κ heterodimer.

Finally, some theoretical possibilites for pharmaceutical treatment of BPS/IC unrelated to gonadal steroid hormones exist. For example, κ-opioid antinociception may be increased by blocking the pro-nociceptive κ-opioid receptor monomer. This could be accomplished by administration of an antagonist selective for the κ-opioid receptor monomer, if such antagonist did exist. Conversely, κ-opioid antinociception could be increased by administration of an agonist selective for the μ/κ heterodimer.

4.7. Conclusions

Normally, i.v. administration of U50,488H produces antinociception during UBD. However, when rats receive EIL bladder inflammation coupled with adult re-inflammation, U50,488H increases sensitivity to UBD. These data may reflect a change

in the κ-opioid system due to chronically increased levels of dynorphin in the lumbosacral spinal cord after EIL bladder inflammation. U50,488H may be acting at the spinal cord because i.t. administration of nor-BNI reversed inhibition of 4 mg/kg U50,488H in the group that received EIL bladder inflammation, but on the whole, data from the i.t. nor-BNI experiment were not conclusive. It was proposed that a μ/κ heterodimer may mediate κ-opioid antinociception. The function of this heterodimer may be altered by bladder inflammation, and this alteration may contribute to the results of the current study using a rat model of BPS/IC. In addition, the μ/κ heterodimer may be modulated by gonadal hormones. Therefore, one treatment option for chronic bladder pain in BPS/IC may be gonadal hormone therapy to upregulate κ-opioid antinociception.

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APPENDIX A

IACUC NOTICE OF APPROVAL

NOTICE OF RENEWAL

DATE: December 20, 2010

TO: ALAN RANDICH, Ph.D.
CH -201 1170
FAX: (205) 975-6110

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Neonatal Bladder Inflammation, Opioids, and Adult Bladder Pain
Sponsor: NIH
Animal Project Number: 110108016

As of January 29, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	B	90
Rats	C	150

Animal use must be renewed by January 28, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110108016 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.