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Spinal Cord Control of Ejaculatory Reflexes in Male Rats

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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SPINAL CORD CONTROL OF EJACULATORY REFLEXES IN MALE RATS

(Thesis format: Integrated Article)

by

Natalie Kozyrev

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Ejaculatory dysfunction impacts large numbers of men of all ages and around the world. In addition, a great majority of men with chronic spinal cord injury (SCI) experience ejaculatory dysfunction, which negatively impacts the quality of life of these individuals and their partners. SCI men emphasize the significance of regaining sexual function as their main goal. Currently, there is a marked absence of literature reporting the alterations to sexual function and ejaculation in particular in animal models of chronic SCI. In addition, there are many unanswered questions pertaining to the spinal cord control of ejaculation in healthy, intact men. It is known that ejaculation is controlled by a population of lumbar spinothalamic (LSt) cells in the lumbar spinal cord through their direct projections to preganglionic autonomic and motor neurons in the lumbosacral spinal cord. It is hypothesized that LSt cells control ejaculatory reflexes through the release of their neuropeptides galanin, cholecystokinin (CCK), gastrin-releasing peptide (GRP), and enkephalin onto receptors in autonomic and motor areas of the lumbosacral spinal cord. This hypothesis was tested in this thesis utilizing a paradigm in anesthetized and spinalized male rats, with stimulation of the sensory inputs via the dorsal penile nerve. Consistent with the hypothesis, mu and delta opioid receptor, galanin, CCK, and GRP receptor activation in LSt target areas in the lumbosacral spinal cord was demonstrated to be critical for ejaculatory reflexes. Next, the hypothesis that intrathecal infusions of the LSt neuropeptides can improve ejaculatory reflexes in male rats with chronic SCI was tested. Results indicated that intrathecal infusions of GRP and the mu opioid receptor agonist DAMGO improved ejaculatory reflexes in male rats with chronic contusion SCI. Finally, the hypothesis that the D3 receptor agonist 7-OH-DPAT will recover

ejaculatory function in male rats with chronic spinal cord injury was tested. Indeed, systemic infusions of 7-OH-DPAT greatly improved ejaculatory reflexes in SCI males. Together, the studies in this thesis further clarified the mechanisms involved in the spinal cord control of ejaculation in male rats and represent an initial but pivotal first step towards the recovery of ejaculatory function after chronic spinal cord injury.

Key Words: ejaculation, spinal ejaculation generator, chronic spinal cord injury, lumbosacral spinal cord, spinal cord contusion, galanin, cholecystokinin, enkephalin, gastrin-releasing peptide, dopamine D3 receptor.

Co-Authorship Statement

Chapter 2 entitled “Activation of gastrin-releasing peptide receptors in the lumbosacral spinal cord is required for ejaculation in male rats”, chapter 3 entitled “Activation of mu and delta opioid receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats”, chapter 4 entitled “Activation of galanin and cholecystokinin receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats”, chapter 5 entitled “Activation of mu opioid and GRP receptors in the lumbosacral spinal cord can improve ejaculatory reflexes in male rats with chronic contusion spinal cord injury” and chapter 6 entitled “Systemic infusions of 7-OH-DPAT rescued ejaculatory reflexes in male rats with chronic contusion spinal cord injury” were written by Natalie Kozyrev with inputs by Dr. Lique M. Coolen. Experimental procedures and data analysis were performed by Natalie Kozyrev and intellectual input was provided by Lique M. Coolen and Michael N. Lehman.

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List of abbreviations

ABC, avidin-biotin-horseradish peroxidase complex

BCM, muscle of the bulbocavernosus

BNSTpm, posteromedial bed nucleus of the stria terminalis

CAN, central autonomic nucleus

CCK, cholecystokinin

DMSO, dimethyl sulfoxide

DOR, delta opioid receptor

DPN, dorsal penile nerve

DRG, dorsal root ganglion

EMG, electromyography

ERK, extracellular-regulated protein kinase

GRP, gastrin-releasing peptide

GIRK, G-protein-coupled inwardly-rectifying K+

IML, intermediolateral cell column

LSt, lumbar spinothalamic

MAP, mitogen-activated protein

MEK, mitogen-activated protein extracellular signal-regulated kinase

MOR, mu opioid receptor

MPOA, medial preoptic area

NPGi, nucleus of the paragigantocellularis

PB, phosphate buffer

PBS, phosphate buffered saline

pERK, phosphorylated MAP kinase

PVN, paraventricular nucleus of the hypothalamus

SCI, spinal cord injury

SNB, sacral nucleus of the bulbocavernosus

SPN, sacral parasympathetic nucleus

SV, seminal vesicle

SVP, seminal vesicle pressure

Chapter 1: Introduction

Spinal cord control of ejaculatory reflexes in male rats: Effects of spinal cord injury

1.1 Introduction

Male sexual dysfunction, including erectile and ejaculatory dysfunction, commonly occurs in adult men of all ages and around the world. While the mechanisms of erectile dysfunction are well-known and various effective treatment options are currently available, the mechanisms underlying the control of ejaculation remain largely unknown. Premature ejaculation is one of the most frequently reported ejaculatory dysfunctions and at present there are no effective treatment options available (Vale, 1999). Ejaculation is also severely altered in men with chronic spinal cord injury (SCI) (Brackett et al., 1998). However, among paraplegic men, the recovery of normal sexual function is the highest priority and is even ranked above recovery of locomotor function (Anderson, 2004). The current review will summarize literature describing the central control of ejaculation in humans and animal models. In addition, the effects of chronic SCI on ejaculation are reviewed. Finally, potential treatments geared toward the recovery of normal sexual function in men suffering from premature ejaculation and in men with chronic SCI will be reviewed.

1.2 Ejaculation

Male sexual behavior is highly complex and consists of several components including the pursuit and investigation of the female, mounts and intromissions finally culminating in ejaculation, a process that is closely associated with orgasm in men (Meisel R, 1994) and reward in male rats (Hull et al., 2002). Ejaculation is physiological process that refers to the expulsion of seminal

contents from the urethral meatus (Marberger, 1974; McKenna, 1999) and is composed of two separate components, emission and expulsion. Emission refers to the secretion of seminal contents from the accessory sex glands, including the vas deferens, seminal vesicles and prostate (Wang et al., 1991) and the closure of the bladder neck and external uretheral sphincter to avert retrograde ejaculation (Newman et al., 1982). Expulsion refers to the coordinated, rhythmic contractions of the striated perineal muscles and the bulbocavernosus muscle (BCM) in particular, as well as the smooth muscles of the urethra, which forcefully expel semen from the urethral meatus (Breedlove and Arnold, 1980, 1981; Breedlove et al., 1983).

1.3 The Spinal Ejaculation Generator

It has been known for some time from studies of chronic SCI patients and animal models that ejaculation is a reflex and the central mechanisms that regulate this reflex are localized to the lumbosacral spinal cord (Comarr, 1970; Comarr and Gunderson, 1975; McKenna, 1997; McKenna, 1999). Previous studies have shown that vibratory penile stimulation is sufficient to trigger ejaculatory reflexes in men with complete spinal cord injuries above the 10^{th} thoracic spinal segment (Brackett et al., 1998; Brackett, 1999; Sonksen and Ohl, 2002). Similarly, stimulation of the dorsal penile nerve (DPN), the sensory branch of the pudendal nerve, triggers ejaculatory reflexes in male rats with complete spinal cord transection (Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012). The fact that ejaculatory reflexes persist in spinalized men and male rats (Comarr, 1970; Comarr and Gunderson, 1975; Pescatori et al., 1993; Brackett et al., 1998; McKenna, 1999) despite the complete absence of functional reciprocal connections

with supraspinal regions suggests that ejaculation is regulated by a control center in the lumbosacral spinal cord. This control center is known as the spinal pattern generator (McKenna et al., 1991; Carro-Juarez and Rodriguez-Manzo, 2007), spinal pacemaker (Sachs and Garinello, 1979), or spinal ejaculation generator (Marberger, 1974; Marson and McKenna, 1994) and it controls ejaculation in two main ways. First, it coordinates sympathetic, parasympathetic and motor outflow to trigger the two components of ejaculation: emission and expulsion. Secondly, the spinal ejaculation generator integrates this autonomic and motor outflow with sensory inputs necessary to induce ejaculation which are conveyed during sexual activity (Coolen et al., 2004; Allard et al., 2005; Coolen, 2005; Young et al., 2009).

1.3.1 LSt Cells Form an Integral Component of the Spinal Ejaculation Generator

About a decade ago, substantial progress was made in identifying a population of interneurons in the lumbosacral spinal cord that form a critical component of the spinal ejaculation generator (Truitt and Coolen, 2002). These interneurons are located in lamina 10 around the central canal and the medial portion of lamina 7 in lumbar levels (L3 – L4) of the spinal cord and have direct projections to the parvocellular subparafascicular thalamic nucleus (SPFp) (Ju et al., 1987; Coolen et al., 2003; Truitt et al., 2003). Moreover, these lumbar spinothalamic (LSt) cells, named according to their anatomical position in the spinal cord and thalamic projections, are sexually-dimorphic and show neural activation, visualized with Fos, specifically following ejaculation in male but not in female rats (Truitt and Coolen, 2002; Coolen et al., 2003; Truitt et al., 2003). In addition, LSt cells co-express several neuropeptides including galanin, cholecystokinin (CCK), enkephalin and gastrin-releasing peptide (GRP) (Newton, 1993; Nicholas et al., 1999; Phan and

Newton, 1999; Newton and Phan, 2006; Sakamoto et al., 2008; Kozyrev et al., 2012) and project their axons to autonomic preganglionic neurons (Xu et al., 2006) and pudendal motoneurons (Newton, 1993; Newton and Phan, 2006) in the thoracolumbar and lumbosacral spinal cord. Galanin, a marker of LSt cells, is expressed in axons projecting to preganglionic sympathetic and parasympathetic neurons as well as pudendal motor neurons (Newton, 1993; Truitt and Coolen, 2002). Neural activation of LSt cells, based on Fos and galanin co-expression, is specifically related to the onset of ejaculation (Truitt et al., 2003; Coolen et al., 2004). Targeted lesions of the LSt cells with intraspinal injections of a neurotoxin SSP-Saporin abolished ejaculation in copulating male rats but spared all other components of male rat sexual behavior including pursuit and investigation of the female, mounts and intromissions (Truitt and Coolen, 2002). Furthermore, targeted lesions of the LSt cells with the same neurotoxin prevented rhythmic contractions of the BCM following DPN, urethral or pharmacological stimulation with the dopamine D3 receptor agonist 7-OH-DPAT, in anesthetized and spinalized male rats (Staudt, 2011). In addition, in male rats with LSt lesions, galanin-immunoreactive fibers normally projecting to autonomic nuclei in the lumbosacral spinal cord were absent, confirming that LSt cells are required for these functional projections (Truitt and Coolen, 2002). Together these studies demonstrated that LSt cells are essential for ejaculation in male rats and that LSt cells regulate ejaculation through intraspinal connections to preganglionic autonomic and motor neurons in the lumbosacral spinal cord (Truitt and Coolen, 2002; Truitt et al., 2003; Staudt, 2011). Thus, LSt cells are a population of interneurons in the lumbar spinal cord which comprise a pivotal component of the spinal ejaculation generator and transform sensory inputs during sexual activity into motor and secretory outputs (McKenna, 1999).

1.3.2 Spinal Inputs to the Ejaculation Generator

During sexual activity, the DPN, the sensory branch of the pudendal nerve, plays a central role in the transmission of sensory inputs to the spinal ejaculation generator required to trigger ejaculation (Marberger, 1974; Hull et al., 2002). Indeed, electrical stimulation of the DPN triggers rhythmic contractions of the BCM similar to those observed in copulating male rats (Pescatori et al., 1993). Furthermore, both DPN stimulation in anesthetized and spinalized male rats and ejaculation in mating males triggers phosphorylated ERK (pERK) and phosphorylated NMDA receptor subunit 1 (pNR1) in LSt cells (Staudt et al., 2010, 2011). Moreover, injections of antagonists for ERK and NMDA receptors blocked DPN stimulation-induced BCM bursting in male rats (Staudt et al., 2010, 2011). Pudendal nerve terminals are found in close proximity to the LSt cells in the lumbosacral spinal cord and are mainly localized to medial dorsal horn and dorsal gray commissure (DGC) (McKenna and Nadelhaft, 1986; Ueyama et al., 1987). Presumably, these sensory signals are relayed to the LSt cells through intraspinal connections between the LSt cells and the dorsal horn and DGC. However, precisely how this transfer occurs is currently unknown as there are no known direct projections from the dorsal horn and DGC to the LSt cells. These sensory inputs are conveyed by the DPN during sexual activity prior to ejaculation and activate the LSt cells to induce ejaculation and may include visceral sensory, somatosensory, proprioceptive or noxious inputs (Carro-Juarez and Rodriguez-Manzo, 2005). Some sensory inputs related to the summation of sexual activity may also be conveyed by the hypogastric and pelvic nerves (McKenna, 2001; Coolen et al., 2004; Coolen, 2005). Pelvic nerve afferents terminate in the vicinity of pudendal nerve afferent terminals in the lumbosacral spinal cord, at around segments L6 – S1 (Nadelhaft and Booth, 1984). Furthermore, the pelvic nerve

conveys sensory stimuli associated with mating to supraspinal regions including the central tegmental field and lesions of the pelvic nerve dramatically reduce neural activation of the central tegmental field induced by mating (Wersinger et al., 1993). Unlike the pudendal and pelvic nerves, the hypogastric nerve terminates in the dorsal horn of the thoracolumbar spinal cord, in segments T13 – L2 in male rats (De Groat W, 1990). Nevertheless, electrical stimulation of the intermesenteric nerve which contains fibers of the hypogastric nerve triggered seminal plug expulsion in anesthetized rats (Bernabe et al., 2007). In men, the penis is innervated by a large number of sensory fibers which include mainly small, unmyelinated Aδ and C fibers terminating in free nerve endings (Halata and Munger, 1986). These sensory fibers of the penis can detect temperature, pain and mechanical stimuli including touch, pressure and stretch (Kitchell et al., 1982). In addition, the penis exclusively contains a genital end bulb which is a type of encapsulated nerve ending that may regulate sensory responses to stimulation of the glans (Coolen et al., 2004). Furthermore, the sensitivity of penile receptors is greatly enhanced by penile erection (Johnson, 1988). In summary, penile receptors convey sensory information regarding temperature, touch, stretch, pressure and pain via the DPN as well as the pelvic and hypogastric nerves to the lower thoracic, lumbar and sacral spinal cord. These sensory inputs ultimately converge in the spinal ejaculation generator and trigger the activation of LSt cells, which in turn triggers ejaculation.

1.3.3 Outputs of the Spinal Ejaculation Generator

During the emission phase of ejaculation, autonomic nuclei in the thoracolumbar and lumbosacral spinal cord supply sympathetic and parasympathetic innervation to the pelvic

viscera including the seminal vesicles, ductus deferens and prostate(Giuliano and Clement, 2005). The intermediolateral cell column (IML) and dorsal central autonomic nucleus (DCN) located in the thoracolumbar spinal cord in spinal segments (T13 – L2) contain sympathetic preganglionic neurons that send sympathetic ouflow mainly through the hypogastric and pelvic nerves to the visceral organs (Hancock and Peveto, 1979a; Nadelhaft and McKenna, 1987; Baron and Janig, 1991; Giuliano et al., 1997). Among the two populations of sympathetic preganglionic neurons, it is those of the DCN which send projections to the prevertebral ganglia and are implicated in the control of the visceral organs while IML neurons are thought to be involved mainly in the control of cardiovascular function (Janig and McLachlan, 1987). Parasympathetic preganglionic neurons are located in the sacral parasympathetic nucleus (SPN) in spinal segments (S2-S5) which innervate the pelvic organs including the prostate (Orr and Marson, 1998), urethra (Vizzard et al., 1995) and penis (Marson and Carson 3rd, 1999). In summary, the emission phase of ejaculation is under the control of both the sympathetic and parasympathetic preganglionic cell populations as are many of the physiological events leading up to emission, including contractions of the ductus deferens (Kolbeck and Steers, 1992; Kihara and de Groat, 1997) and the discharge of prostatic contents into the urethra (Wang et al., 1991). The expulsion phase of ejaculation is under the control of pudendal motoneurons in the lumbosacral spinal cord (Coolen et al., 2004). These pudendal motoneurons are localized to the spinal nucleus of the bulbocavernosus (SNB) as well as the dorsolateral (DL) nucleus in male rats (Schroder, 1980; McKenna and Nadelhaft, 1986; Ueyama et al., 1987), and to Onuf's nucleus in men (Schroder, 1985; Thor et al., 1989). Motoneurons in these nuclei innervate the bulbospongiosus and ischiocavernosus striated perineal muscles and the external urethral and anal sphincters. Coordinated contractions of these muscles and sphincters trigger the ejection of semen from the

urethral meatus (Bohlen et al., 1980; Gerstenberg et al., 1990; Holmes et al., 1991; Holmes and Sachs, 1991). Notably, the striated perineal muscle contractions occur concurrently with the pleasurable orgasmic sensations experienced at ejaculation (Gerstenberg et al., 1990).

1.4 Supraspinal Sites Regulating Ejaculation

Several supraspinal regions in the brain and brainstem provide descending modulation of the spinal ejaculation generator. These supraspinal regions exert excitatory and inhibitory influences over the spinal ejaculation generator to regulate ejaculation.

1.4.1 The Medial Preoptic Area (MPOA)

One such region is the medial preoptic area (MPOA) of the hypothalamus (Pehek et al., 1989; Markowski et al., 1994; Hull et al., 1997; Hull et al., 2004), a central area in regulating male sexual behavior (Hull et al., 2004). The MPOA exerts its excitatory effects on ejaculation through dopamine D2 (Hull et al., 1989; Hull et al., 1992) and D3 receptors (Kitrey et al., 2007) and electrical stimulation of the MPOA triggers contractions of the striated penile muscles in anesthetized male rats (Sato and Christ, 2000). Moreover, the activation of D3 receptors has been recently shown to be critical for ejaculation in male rats (Clement et al., 2007; Kitrey et al., 2007; Clement et al., 2009b). Since there are no known direct projections from the MPOA to the lumbosacral spinal cord, it is likely that the MPOA exerts its effects on ejaculation through

connections with other brain regions involved in the control of ejaculation, including the nucleus paragigantocellularis (NPGi)(Marson et al., 1992; Marson and McKenna, 1992; Yells et al., 1992; Murphy et al., 1999) and the paraventricular nucleus of the hypothalamus (PVN) (Marson and McKenna, 1994).

1.4.2 Nucleus of the paragigantocellularis (NPGi)

Neurons of the NPGi in the ventral medulla are well-known for their inhibitory effects of ejaculation in male rats (Marson et al., 1992; Marson and McKenna, 1992; Yells et al., 1992). It is currently suggested that this descending inhibition is serotonergic in nature and is mediated by 5-hydroxytryptamine (5-HT)(Marson and McKenna, 1992). Bilateral lesions of the NPGi enhanced sexual performance in sexually-naïve male rats, by reducing the frequency of mounts, intromission and latency to ejaculation. In addition, males with such lesions demonstrated increased latency to copulate to exhaustion and dramatically augmented numbers of ejaculations prior to the onset of sexual exhaustion (Yells et al., 1992). Furthermore, bilateral lesions of the NPGi markedly reduced the latency to the onset of ex copula reflexes (Marson et al., 1992).

In addition, 50% of anesthetized male rats with intact spinal cords displayed the urethrogenital reflex which can normally be elicited only in animals with completely transected spinal cords, following the removal of descending inhibition (Marson et al., 1992). NPGi neurons have direct projections to the ventral horn in the vicinity of pudendal motor neurons in the lumbosacral spinal cord (Marson and McKenna, 1992). Moreover, intrathecal infusions of 5-HT prevented the rhythmic contractions of the striated perineal muscles and ejaculation induced by stimulation

of the urethra while infusions of the 5-HT antagonist abolished this inhibitory effect (Marson and McKenna, 1992). This finding supports the notion that 5-HT exerts descending inhibitory influences on ejaculation. However, conflicting evidence exists for the inhibitory effects of serotonin on ejaculation. It has been demonstrated that the serotonin 1A receptor agonist 8-OH-DPAT inhibits emissions elicited in the ex copula paradigm (Lee et al., 1990). In contrast, other studies demonstrated enhanced effects of 8-OH-DPAT on ejaculation in copulating animals (Haensel et al., 1991; Fernandez-Guasti et al., 1992; Coolen et al., 1996; Coolen et al., 1997b). In addition, another study found a facilitative effect of 8-OH-DPAT on urethral stimulation-induced rhythmic bursting patterns of the BCM, indicative of the expulsion phase of ejaculation (Carro-Juarez and Rodriguez-Manzo, 2001). One proposed explanation for the discrepancies is that the two components of ejaculation: emission and expulsion can occur independently of one another (Staudt, 2011). Indeed, the seminal vesicles are not required for the contractions of striated perineal muscles in copulating male rats (Cukierski et al., 1991). Similarly, the emission of seminal fluids into the urethra or urethral stimulation is not required for the expulsion reflex in male rats (Holmes and Sachs, 1991). Therefore, the inhibitory effects of 8-OH-DPAT on emission can exist alongside enhanced effects of expulsion in mating animals. In summary, descending projections of the NPGi exert inhibitory influences on ejaculatory reflexes in the ex copula paradigm and on ejaculatory behavior in copulating male rats by acting on motor neurons in the ventral horn of the lumbosacral spinal cord. Moreover, bilateral lesions of the NPGi facilitated ejaculatory behavior in mating animals and ejaculatory reflexes in anesthetized rats, providing further evidence of the inhibitory effects of NPGi on ejaculation. However, due to conflicting results, it is currently unclear whether this inhibition of ejaculation is mediated by serotoninergic mechanisms or whether other neurotransmitters may be involved.

1.4.3 The paraventricular nucleus of the hypothalamus (PVN)

The PVN is another brain region which provides descending excitatory modulation of ejaculation. Axons of PVN neurons terminate in the lumbosacral spinal cord and release the neurotransmitter oxytocin (Wagner and Clemens, 1991, 1993). Specifically, PVN neurons project to the pudendal motor neurons in the lumbar spinal cord around segments L5 – L6 in the region of the SNB (Wagner and Clemens, 1991). Moreover, neurophysin (the product of oxytocin and vasopressin) containing fibers and putative terminals were observed in close contact to SNB motor neurons(Wagner and Clemens, 1993). Lesions of the PVN resulted in an absence of neurophysin-containing fibers in the pudendal motor neurons, indicating that these fibers originated in the PVN (Wagner and Clemens, 1993). Additional support for the role of the PVN comes from a recent report that oxytocin receptors in the brain regulate ejaculation triggered by infusions of the D3 agonist 7-OH-DPAT (Clement et al., 2008). Specifically, i.c.v. infusions of an oxytocin antagonist resulted in a dose-dependent blockade of sexual responses elicited by 7-OH-DPAT, including changes in seminal vesicle pressure (SVP), intracavernosal pressure (ICP) and rhythmic bursting of the BCM (Clement et al., 2008). In addition, intrathecal administration of the oxytocin antagonist to the L6 lumbar segment significantly diminished ejaculation and the duration of BCM bursting but had no effect on ICP responses (Clement et al., 2008). From these results, the authors concluded that while brain oxytocin receptors mediate ejaculatory responses triggered by infusions of 7-OH-DPAT, spinal oxytocin receptors only control the occurrence of ejaculation. These findings provide further support to the idea that oxytocin is released from axon terminals of the PVN onto oxytocin receptors expressed on

pudendal motor neurons in the lumbosacral spinal cord to modulate ejaculation in male rats (Clement et al., 2008). Furthermore, the levels of oxytocin in blood plasma are substantially enhanced in male rats (Stoneham et al., 1985), rabbits (Todd and Lightman, 1986) and men (Murphy et al., 1987) following ejaculation. PVN lesions do not prevent ejaculation but reduce the amount of seminal contents ejected in copulating male rats suggesting that the PVN is critical for the emission component of ejaculation (Ackerman et al., 1997).

1.5 Supraspinal network displaying neural activation specifically with ejaculation

It has been demonstrated that certain regions of the posterior thalamus, MPOA, bed nucleus of the stria terminals (BNST) and medial amygdala (MEA) express Fos, a marker of neural activation, following various components of sexual behavior in male rats (Coolen et al., 2004). In addition, certain subdivisions of these regions express Fos specifically following ejaculation and not following other components of male rat sexual behavior including intromissions and mounts (Coolen et al., 2004). These regions include subdivisions of the posterior thalamus, BNST and MEA. In particular, these include the medial subregion of the parvocellular subparafascicular thalamic nucleus (SPFp), the lateral zone of the posterior medial MEA, the posterior medial BNST and the posterodorsal preoptic nucleus (PDPn) (Baum and Everitt, 1992; Coolen et al., 1996, 1997a). Although all of these regions show neural activation with ejaculation, most of these, in particular the posterior medial MEA and PDPn, are not critical for the display of ejaculatory behavior (Coolen et al., 2004). In contrast, lesions of the SPFp, including the zona incerta abolished ejaculation in male rats (Maillard and Edwards, 1991). However, when lesions were restricted to the SPFp, ejaculatory behavior was not affected (Heeb and Yahr, 2000). These

results indicate that the SPFp is involved in the relay of sensory signals associated with ejaculation but is not involved in inducing ejaculation in male rats (Coolen et al., 2004).

1.6 LSt-SPFp pathway

It has long been unknown precisely how sensory signals associated with ejaculation are conveyed to supraspinal regions. Several years ago, a candidate pathway was identified which transmits sensory information associated with ejaculation from the sexual organs to the thalamus. Specifically, the DPN transmits sensory signals from the periphery to the LSt cells and from there these signals are relayed to the medial SPFp in the thalamus by way of the spinothalamic pathway (Ju et al., 1987; Coolen et al., 2003). The LSt – medial SPFp pathway is involved in the transmission and processing of sensory information associated with ejaculation (Truitt et al., 2003). In support of this hypothesis, the SPFp and the LSt cells demonstrate neural activation specifically following ejaculation (Truitt et al., 2003). Neural activation, visualized with Fos expression, is not observed following other aspects of male sexual behavior including mounts and intromissions. Moreover, several neuropeptides are expressed in the LSt – medial SPFp pathway including galanin, CCK and enkephalin (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003). Of these neuropeptides, galanin infusions in the medial SFPp have been shown to suppress sexual behavior in male rats, indicating that endogenous galanin is released in the medial SFPp following ejaculation and acts to suppress subsequent mating for a short duration during the post-ejaculatory interval or for a longer duration once sexual satiety is reached (Coolen et al., 2004).

1.7 Summary: Spinal cord control of ejaculation

In summary, ejaculation is controlled by the LSt cells in the lumbosacral spinal cord which form a critical component of the spinal ejaculation generator. Sensory signals from the periphery are conveyed by the DPN to activate the LSt cells and trigger ejaculation. In turn, the LSt cells convert these sensory signals into secretory or motor outputs to induce the emission and expulsion components of ejaculation. Outputs of the LSt cells include sympathetic and parasympathetic preganglionic neurons in the IML, CAN and SPN respectively as well as motor neurons in the SNB. Both sympathetic and parasympathetic preganglionic neurons are involved in initiating the emission component of ejaculation by triggering the secretion of seminal fluids into the prostatic urethra, closure of the bladder neck and contractions of the seminal vesicles, prostate and vas deferens. Expulsion is controlled by motor neurons which induce contractions of the striated perineal muscle thereby eliciting the ejection of seminal contents from the urethral meatus. In addition, supraspinal regions in the brain and brainstem provide excitatory and inhibitory descending modulation of ejaculation. Excitatory input is provided by the MPOA and PVN while inhibitory modulation originates in the NPGi in the ventral medulla. Finally, sensory signals associated with ejaculation are conveyed from the LSt cells to the medial SPFp in the posterior thalamus through the spinothalamic pathway. Neural activation of the SPFp specifically following ejaculation or infusions of galanin inhibit sexual behavior in male rats. In addition, the medial SPFp has a number of connections with other brain regions including the BNST, MPOA and MEA that are involved in male rat sexual behavior and that demonstrate neural activation

specifically with ejaculation. Therefore, the medial SPFp likely exerts inhibition of mating through its connections with these brain regions.

1.8 Ejaculation is severely impaired following chronic spinal cord injury

Traumatic spinal cord injury (SCI) radically alters erectile and ejaculatory function in men. In particular, SCI is associated with severely disrupted ejaculation. Of men with SCI, 0% to 55% (with a median of 15%) can achieve ejaculation with a partner during sexual intercourse or during masturbation (Biering-Sorensen and Sonksen, 2001). Moreover, the majority of SCI men can only ejaculate with the aid of intense penile vibratory stimulation or electroejaculation and both of these procedures are effective first-line treatment of ejaculatory disorders in SCI men (Soler and Previnaire, 2011). Complete transection of the spinal cord results in a loss of sensation and voluntary muscle control below the level of the lesion in the spinal cord. Even though SCI men can ejaculate through intense vibratory stimulation and electroejaculation, the ejaculation is sometimes retrograde, suggesting that ejaculatory dysfunction in SCI men may be due to the lack of coordination between sympathetic, parasympathetic and motor neurons (Soler and Previnaire, 2011).

It is known that fewer than 10% of men with complete SCI are able to ejaculate with sexual intercourse or genital stimulation (Bors, 1960; Comarr, 1970). Preliminary studies of men with SCI determined that vibratory penile stimulation is an effective means to trigger ejaculation in patients with spinal cord lesions above the 12^{th} thoracic segment (Brindley, 1984). More recently, similar studies of men with SCI found that an intact spinal cord between the 10^{th}

thoracic and 2nd lumbar segments (T10 – L2) as well as an intact sacral spinal cord is necessary to elicit ejaculation (Soler et al., 2008). Therefore, the preservation of intact preganglionic autonomic and motor neurons is necessary in order to integrate the autonomic and somatic outflow required to trigger ejaculation with sensory inputs conveyed by the DPN during sexual intercourse or genital stimulation. In addition, these regions of the spinal cord must be intact to coordinate the autonomic and motor outflow to trigger the two phases of ejaculation: emission and expulsion.

Among available treatments for ejaculatory dysfunction following SCI, midodrine appears to have promising results. Midodrine, an alpha1-adrenergic agonist, is predominantly used as a remedy for neurogenic orthostatic hypotension (Soler et al., 2008), is a vasoconstrictor of venous capacitance vessels as well as arterial resistance and has been shown to be effective in the treatment of anejaculation in men with SCI (Staerman et al., 2001; Soler et al., 2007; Courtois et al., 2008). In addition to enhancing ejaculation and orgasmic sensations in men after SCI, midodrine use also has a low reported incidence of autonomic dysreflexia, a dangerous condition which frequently accompanies ejaculation and orgasm in SCI patients (Sheel et al., 2005; Courtois et al., 2008). The dramatic increase in blood pressure and heart rate immediately prior to ejaculation typically return to normal within minutes following ejaculation in healthy able-bodied men (Littler et al., 1974; Nemec et al., 1976; Kruger et al., 1998). However, in men with SCI, these considerable increases in blood pressure and heart rate do not return to baseline levels for several hours, and sometimes for days following ejaculation (Sheel et al., 2005). Autonomic dysreflexia is a cardiovascular deficit associated with SCI and occurs due to a dysregulation of the sympathetic nervous system following SCI (Sheel et al., 2005). Autonomic dysreflexia is particularly common in SCI patients with lesions of the cervical or upper thoracic

spinal cord, at or above T6 and involves a sudden, extreme rise in blood pressure, headache, perspiration, bradycardia and anxiety (Karlsson, 1999; Teasell et al., 2000).

Intense penile vibratory stimulation is considerably more effective than sexual intercourse in eliciting ejaculation in SCI with 65% of SCI men reaching ejaculation by these means compared to 5 – 15 % achieving ejaculation through sexual intercourse (Soler et al., 2007, 2008). During penile vibratory stimulation, there is a strong activation of the autonomic nervous system in response to intense stimulation of the DPN (Soler et al., 2007, 2008). Moreover, vibratory stimulation can be used as a home treatment to enhance bladder capacity and alleviate spasticity in SCI men (Biering-Sorensen et al., 2005). In addition, penile vibratory stimulation can be utilized to facilitate ejaculatory reflexes in SCI men and with repeated use can improve ejaculatory function and responsiveness during sexual intercourse (Elliott, 2002; Soler et al., 2008). Patients with upper motor neuron lesions (above T10) are often able to ejaculate and orgasm when penile vibratory stimulation is combined with midodrine (Soler et al., 2007, 2008). Indeed, penile vibratory stimulation in conjunction with midodrine elicited ejaculation in 84% of men with complete SCI and of these, 59% were able to reach orgasm as well (Soler et al., 2008). However, vibratory stimulation is ineffective in SCI men with injuries to the T11-S4 spinal segments, since this method depends on intact preganglionic autonomic neurons in the lumbosacral spinal cord (Brackett et al., 2010). Penile vibratory stimulation triggers ejaculation through intense stimulation of the DPN and subsequent powerful activation of the autonomic nervous system. Because the spinal cord must be intact between segments T11-S4 for vibratory stimulation to be effective, this suggests the presence of a spinal ejaculation generator in humans, which is localized to the lumbosacral spinal cord. While the existence of the spinal ejaculation generator is well-established in male rats, it is not known whether this generator also exists in

men and whether it is located in the lumbosacral spinal cord. However, studies in SCI men indicate that if a spinal ejaculation generator indeed exists in men, it is localized to the lumbosacral spinal cord, to coordinate the autonomic and motor outflow required for ejaculation and to integrate this outflow with sensory signals from the sexual organs during sexual activity.

Electroejaculation is utilized as a treatment for ejaculatory dysfunction in SCI men in whom penile vibratory stimulation is ineffective in eliciting ejaculation or who have spinal cord lesions caudal to T11 which disrupt the ejaculatory reflex arc (Brackett et al., 2010). The electroejaculation protocol involves inserting an electrical probe into the rectum such that the probe is adjacent to the rectal wall in close proximity to the seminal vesicles and prostate gland (Soler and Previnaire, 2011). Next, electrical stimulation of the prostate and seminal vesicles is administered in waves, with increasing voltage as the procedure progresses until ejaculation ensues. One disadvantage of electroejaculation is the considerable discomfort experienced by patients undergoing the procedure. These patients tend to have some residual sensation following the SCI and an invasive procedure like electroejaculation can produce painful sensations requiring the administration of spinal anesthetics (Halstead et al., 1987; Bennett et al., 1988; Biering-Sorensen and Sonksen, 2001). Because electroejaculation is a procedure which is invasive, requires the use of medical equipment and may require the use of spinal anesthesia, it cannot be used as a home treatment for anejaculation in men with SCI. Another disadvantage of electroejaculation is that it often results in retrograde ejaculation with very low antegrade sperm motility and motile sperm counts compared to a procedure such as penile vibratory stimulation (Halstead et al., 1987; Bennett et al., 1988; Ohl et al., 1996; Ohl et al., 1997). Therefore, penile vibratory stimulation is a more suitable procedure for sperm retrieval for reproductive purposes compared to electroejaculation in SCI men (Halstead et al., 1987; Bennett et al., 1988; Ohl et al.,

1996; Ohl et al., 1997). One significant advantage of electroejaculation is that it has a success rate of nearly 100% in triggering ejaculation in men with SCI that do not respond to genital stimulation or penile vibratory stimulation. Moreover, the effectiveness of electroejaculation does not depend on the level of spinal cord lesion or on an intact ejaculatory reflex arc (T11-S4) (Halstead et al., 1987; Bennett et al., 1988).

1.9 Summary: Effects of spinal cord injury on ejaculation

In summary, ejaculation is severely impaired and a greater amount and intensity of stimulation may be necessary to trigger ejaculation in men with SCI (Elliott, 2002). One possibility is that ejaculatory function is dramatically diminished following chronic SCI due to the loss of supraspinal influences on the ejaculatory reflex arc in the lower thoracic, lumbar and sacral spinal cord (Soler and Previnaire, 2011). Intense penile vibratory stimulation is a promising treatment of ejaculatory dysfunction in SCI men (Sonksen et al., 1994; Ohl et al., 1997; Brackett et al., 1998) which induces intense vibratory stimulation of the DPN and as a result triggers powerful activation of autonomic nuclei in the lumbosacral spinal cord (Soler et al., 2007). It is not clear whether penile vibratory stimulation also triggers activation of motor neurons in Onuf's nucleus, in the sacral spinal cord. In male rats, stimulation of the DPN triggers rhythmic bursting of the perineal muscles, including the BCM, which are under the control of motor neurons in the SNB in the lumbosacral spinal cord (Pescatori et al., 1993; McKenna, 1999; Staudt et al., 2010; Kozyrev et al., 2012; Kozyrev, 2013c, 2013b). Moreover, rhythmic bursting of the BCM is required for the expulsion phase of ejaculation indicating that vibratory

stimulation likely triggers the activation of autonomic as well as motor neurons in the lumbosacral spinal cord, to induce the emission and expulsion components of ejaculation in SCI men. The DPN is required for ejaculation induced by penile vibratory stimulaiton since local anesthesia of the DPN prevents ejaculation in SCI men (Wieder et al., 2000). Penile vibratory stimulation in combination with midodrine is even more effective and can restore ejaculation and orgasmic sensations in up to 80% of SCI men (Soler et al., 2007, 2008). In addition, SCI men who do not respond to vibratory stimulation alone are more likely to ejaculate and experience orgasmic sensations when this type of stimulation is supplemented with midodrine (Soler et al., 2007, 2008). Interestingly, repeated sessions of penile vibratory stimulation can reduce the threshold necessary to trigger ejaculation in SCI men (Elliott, 2002) suggesting that recurrent DPN stimulation can improve the conductance of sensory signals required to activate autonomic and motor nuclei in the lumbosacral spinal cord and induce ejaculation. Therefore, repeated sessions of penile vibratory stimulation can be useful in treating ejaculatory dysfunction and may be easily administered at home (Elliott, 2002).

1.10 Animal models of chronic spinal cord injury

Animal models, by imitating the human experience, can be used to study the effects of SCI on ejaculation. Currently, there are three validated models of human SCI that are frequently utilized in animal studies. The first model, complete spinal transection, results in the complete denervation of the spinal cord from supraspinal regions and is a model of complete spinal cord injury (Wrathall, 1992; Jacob et al., 2001; Young, 2002; Gris et al., 2003; Jacob et al., 2003; Onifer et al., 2007). The second model is the clip compression model in which an aneurysm clip is positioned around the spinal cord but the dura is left undisturbed. Next, the clip is tightly

clasped around the cord for one minute to induce trauma to the spinal cord. The last method, contusion injury of the spinal cord, is performed using a spinal cord impactor, such as the Infinite Horizon IH0400 Impactor (200 kdyne, Precision Systems and Instrumentation). The clip compression model and the contusion injury models are considered to be models of partial SCI and are validated models of SCI in humans (Wrathall, 1992; Young, 2002; Onifer et al., 2007).

1.11 Thesis rationale and objectives

Given that men with SCI emphasize the importance of regaining sexual function as their primary goal (Anderson, 2004), it is astounding to observe the absence of literature describing the changes to sexual function and ejaculation in particular in animal models of chronic SCI. In addition, many more questions remain regarding the spinal cord control of ejaculation in intact, healthy men. Although some progress has been made in the spinal cord research of ejaculatory reflexes, the literature is still lacking and treatment options for ejaculatory dysfunction are unsatisfactory. However, recent advances in science have generated insight into the spinal cord control of ejaculation. It is currently known that ejaculation is under the control of LSt cells in the lumbosacral spinal cord (Truitt and Coolen, 2002; Truitt et al., 2003; Staudt, 2011). Targeted lesions of the LSt cells abolish ejaculation and ejaculatory reflexes via their direct projections to preganglionic sympathetic neurons in the IML and CAN and to preganglioninc parasympathetic neurons in the SPN as well as motor neurons in the SNB (Truitt and Coolen, 2002; Staudt, 2011). My overall hypothesis is that LSt cells control ejaculatory reflexes via release of their neuropeptides galanin, CCK, GRP, and enkephalin in the IML, CAN, SPN, and pudendal

motorneurons. In these LSt target areas, neuropeptide receptor activation is therefore critical in triggering ejaculation. This hypothesis is tested in Chapters 2, 3, and 4 utilizing the DPN stimulation model described above in anesthetized, spinalized male rats. In Chapter 5, I will determine the effects of chronic spinal injury using a contusion paradigm on ejaculatory reflexes. Moreover, I will next test the hypothesis that administration of the LSt neuropeptides can alleviate the detrimental effects of chronic spinal cord injury in male rats. Finally, in Chapter 6, I will test the hypothesis that D3 receptor agonist will improve ejaculatory function in male rats with chronic spinal cord injury. The D3 receptor agonist 7-OH-DPAT has been demonstrated to facilitate ejaculation in spinal intact and acutely spinalized rats (Ahlenius and Larsson, 1995; Clement et al., 2007), but the effect of this agonist on impaired ejaculatory function in rats with spinal cord injury has not been determined.

Together, the studies in this thesis further elucidate the mechanisms by which the spinal ejaculation generator controls ejaculatory function in male rats. Moreover, these studies will form a first, but critical step towards an improvement of ejaculatory dysfunction following chronic spinal cord injury.

Chapter 2: Activation of gastrin-releasing peptide receptors in the lumbosacral spinal cord is required for ejaculation in male rats[1]

[1] Research article published in J Sex Med: 9(5) 1303 – 1318

2.1 Introduction

Male sexual behavior is a complex and rewarding behavior that ultimately results in ejaculation (Hull et al., 2002). Ejaculation is defined as the expulsion of seminal fluid from the urethral meatus (Marberger, 1974; McKenna, 1999) and comprises two phases: emission and expulsion. Emission refers to the secretion of seminal fluids from the accessory sex glands, contraction of the ductus deferens, and closure of the bladder neck and external urethral sphincter. Expulsion refers to the forceful ejection of semen which is produced by the rhythmic contractions of the striated perineal and smooth muscles of the urethra, and the bulbocavernosus muscle (BCM) in particular (Bohlen et al., 1980; Gerstenberg et al., 1990; Holmes et al., 1991; Holmes and Sachs, 1991; Pescatori et al., 1993).

It is well known that ejaculation is a reflex and that the central components that regulate the ejaculatory reflex are located in the lumbosacral spinal cord (Griffith et al., 1973; Pescatori et al., 1993; McKenna, 1999) (Figure 1). Indeed, ejaculatory reflexes remain intact in animals and men with complete spinal cord transection (Griffith et al., 1973; Pescatori et al., 1993; McKenna, 1999). Thus, ejaculation is mediated by a spinal control center, referred to as a spinal ejaculation generator (Marberger, 1974; McKenna, 1999; Carro-Juarez and Rodriguez-Manzo, 2007), spinal pattern generator (McKenna et al., 1991), or spinal pacemaker (Sachs and Garinello, 1979). This spinal ejaculation generator is suggested to coordinate sympathetic, parasympathetic, and motor outflow to induce the emission and expulsion phases of ejaculation. In addition, the spinal ejaculation generator is hypothesized to integrate this autonomic and motor outflow with sensory inputs associated with the summation of sexual activity, thereby triggering ejaculation.

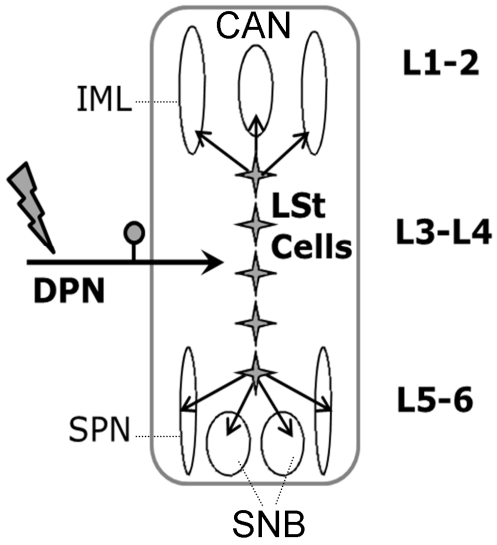

Figure 1 Schematic diagram of the spinal ejaculation generator

Lumbar spinothalamic (LSt) cells receive sensory inputs related to the summation of sexual activity via the dorsal nerve of the penis (DPN), triggering activation of the LSt cells. LSt cells send axonal projections to preganglionic sympathetic neurons in the central autonomic nucleus (CAN) and intermediolateral cell column (IML), to preganglionic parasympathetic neurons in the sacral parasympathetic nucleus (SPN), and to pudendal motorneurons in the sacral nucleus of the bulbocavernosus (SNB). It is hypothesized that the neuropeptides expressed in the axon termimals of the LSt cells trigger ejaculatory reflexes by acting in the LSt target areas.

The spinal ejaculation generator is composed of a population of interneurons in the central gray of lumbar levels L3-4 that play a pivotal role in the control of ejaculation, and are hypothesized to convert sensory signals into motor or secretory outputs (Truitt et al., 2003). This population of interneurons is located in lamina 10 and the medial portion of lamina 7 of lumbar

segments 3 and 4 (L3-4), contain the neuropeptides galanin, cholecystokinin, gastrin releasing peptide, and enkephalin (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008), and have projections to a nucleus in the thalamus: the parvocellular subparafascicular thalamic nucleus (Ju et al., 1987; Coolen et al., 2003; Truitt et al., 2003). Based on their anatomical location in the spinal cord and thalamic projections, these particular cells are referred to as lumbar spinothalamic (LSt) cells (Truitt and Coolen, 2002).

Moreover, LSt cells have additional characteristics that are consistent with their role as a critical component of the spinal ejaculation generator. Mating-related sensory inputs induce activation of LSt cells, thereby triggering ejaculation (Sachs and Garinello, 1979; Pescatori et al., 1993; McKenna, 2001; Coolen et al., 2004). These sensory inputs are relayed via the sensory branch of the pudendal nerve, or dorsal penile nerve (DPN) (Staudt et al., 2010, 2011). Mating or stimulation of the DPN, which reliably triggers ejaculatory reflexes in anesthetized and spinalized male rats, induces expression of markers of neural activation cFos (Truitt et al., 2003) and phosphorylated MAP kinase (pERK) (Staudt et al., 2010) in LSt cells. Moreover, inhibition of MAP kinase kinase (MEK) completely abolished ejaculation induced by DPN stimulation (Staudt et al., 2010) . Therefore, DPN fibers transmit sensory inputs to LSt cells and in turn trigger ejaculation via activation of the MAP kinase pathway.

LSt cells are ideally located to coordinate outflow necessary for ejaculation. LSt cells have axonal projections to target regions of the spinal cord implicated in ejaculatory control (Newton, 1993; Allard et al., 2005; Xu et al., 2006). Specifically, LSt cells project to preganglionic sympathetic neurons in the intermediolateral cell column (IML) and central autonomic nucleus (CAN) of the thoracolumbar spinal cord and to the preganglionic parasympathetic neurons in the sacral parasympathetic nucleus (SPN) of the lumbosacral spinal

cord (McKenna, 1997; Allard et al., 2005) and motor neurons in the spinal nucleus of the bulbocavernosus (SNB) (Newton, 1993; Sakamoto et al., 2008). LSt cells and their axonal projections contain several neuropeptides, including galanin, cholecystokinin, enkephalin and gastrin releasing peptide (GRP) (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008). Thus, we hypothesize that these neuropeptides act in the LSt target areas to mediate ejaculatory reflexes. In support of this hypothesis, GRP, a member of the family of bombesin-like peptides (McDonald et al., 1979; Battey and Wada, 1991), has been implicated in male erectile and ejaculatory function in spinally-intact males utilizing the *ex-copula* reflex paradigm (Sakamoto et al., 2008). However, in order to study the role of GRP in ejaculatory reflexes, it is necessary to eliminate supraspinal influences on the spinal ejaculation generator.

Therefore, the purpose of the present study was to test the role of GRP in the control of ejaculation using a physiologically-relevant model of ejaculation involving electrical stimulation of the DPN in anesthetized, spinalized male rats. Stimulation of the DPN reliably triggers ejaculation in all mammals, including rats (Pescatori et al., 1993), monkeys (Herbert, 1973) and humans (Wieder et al., 2000). Moreover, in anesthetized and spinalized male rats, DPN stimulation elicits rhythmic contractions of the BCM similar to those observed in a mating animal during ejaculation (Coolen et al., 2004; Staudt et al., 2010), and reflexive penile erections are evident (Pescatori et al., 1993; Rampin et al., 1997b). The aim of the present study was twofold. First, it was confirmed that GRP is expressed in LSt cells and their axon terminals in autonomic and motor areas in the lumbosacral spinal cord. Next, it was tested whether GRP receptor activation is required to trigger the emission and expulsion phases of ejaculation and whether GRP is sufficient to trigger ejaculatory reflexes in the absence of DPN stimulation.

2.2 Materials and Methods

2.2.1 *Animals*

Adult male Sprague Dawley rats (4-5 months) were obtained from Charles River (Sherbrook, Quebec, Canada and Wilmington, MA, USA) and were housed in pairs in standard housing cages on a 12-hour light/dark cycle with lights off at 10 a.m. Food and water were available *ad libitum*. All procedures were approved by the University Committee on Use and Care of Animals at Western University and the University of Michigan and conform to the guidelines outlined by the National Institutes of Health.

2.2.2 Experimental designs

2.2.2.1 Intrathecal infusion of GRP receptor antagonist RC-3095

Male rats were anesthetized with urethane (1.5 g/kg, i.p.), a laminectomy was performed between the 6^{th} and the 8^{th} thoracic spinal segments and the spinal cord was completely transected. Next, the dorsal penile nerve (DPN) and the muscle of the bulbocavernosus (BCM) were exposed and the surrounding connective tissue was removed. In preparation for electromyographic (EMG) recordings, silver recording electrodes, connected to the PowerLab/7SP Data Acquisition System (ADInstruments, Inc., Colorado Springs, CO) were

inserted bilaterally into the BCM and a ground electrode was placed into the muscle of the right thigh. A bipolar stimulating electrode connected to the SD9 Square Pulse Stimulator (Grass Technologies, West Warwick, RI) was positioned directly on top of the DPN. Initial stimulation of the DPN was performed in order to verify the completeness of the spinal cord transection, indicated by a rhythmic bursting pattern of the BCM (Figure 2). Electrical stimulation of the DPN comprised square wave pulses of 1 ms duration, 4 V at 60 Hz for 10 seconds. These stimulation parameters have previously been demonstrated to trigger rhythmic bursting of the BCM indicative of the expulsion phase of ejaculation in 100% of control animals (Staudt et al., 2010, 2011). Experiments started 2 hours following transection of the spinal cord to allow for potential acute effects of spinal cord transection to subside. A second laminectomy was performed at spinal level T10 in preparation for intrathecal administration of neuropeptides. A small incision was made in the dura mater at the site of the second laminectomy and a polyethylene catheter (Caly-Adams PE-10, Parsippany, NJ, USA) was inserted into the subarachnoid space at lumbar levels, to deliver drugs presumably to the entire lumbosacral spinal cord. First, 10 µl/min of saline was infused and BCM EMG activity was recorded for 25 minutes. Following these 25 minutes, the DPN was stimulated twice with 5 minute intervals between stimulation, at 30Hz and 60Hz in a counter balanced manner (testing trial 1; control trial). BCM activity was recorded for 90 seconds, the time period for a typical BCM bursting activity (Lodder and Zeilmaker, 1976). One hour later, the procedure was repeated in the same animals, which now received a 10 µl infusion (over 1 minute) of either 0.9% saline (N = 10) or one of two doses of the GRP receptor antagonist RC-3095, 20 nmol (N = 9) and 10 nmol (N = 6) (Sigma-Aldrich Canada Ltd. (Oakville, Ontario)). BCM activity was recorded for 25 minutes, after which the

DPN was stimulated at 30Hz and 60 Hz as described above in a counterbalanced manner, and the BCM EMG activity was recorded (testing trial 2; drug trial).

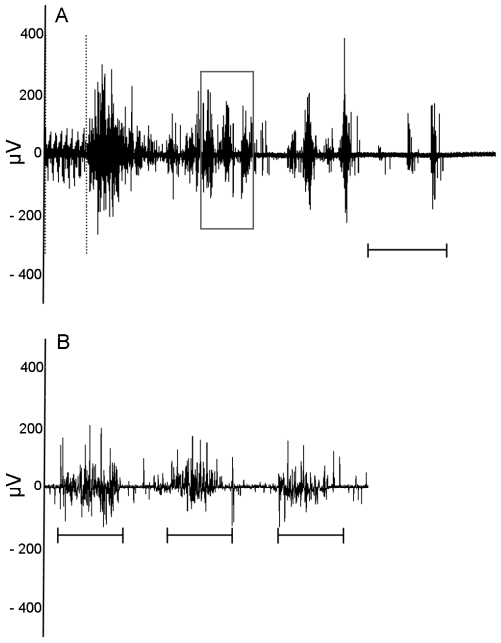

Figure 2 Example of characteristic BCM EMG induced by DPN stimulation.

Dashed lines in the EMG trace in (**A**) represent the period of DPN stimulation followed by 90 seconds of EMG recording showing BCM events (activity above baseline) and clusters of events, i.e. BCM bursts. Time is presented on horizontal scale (Scale bar in **A** represents 20 seconds) and activity amplitude (in µV) on vertical scale. The four bursts indicated by the box (16 seconds of recording) are shown on an enlarged time scale in (**B**) to clearly show the clusters of BCM events within bursts and the return to baseline activity between bursts.

2.2.2.2 Intrathecal infusion of GRP^{20-29} agonist

A separate group of male rats was anesthetized, spinalized, and prepared as described above. Males received intrathecal 10 µl infusions of 0.9 % saline (N=11) and BCM EMG was recorded for 10 minutes following infusion. Then, the DPN was stimulated at 5 HZ, 10 HZ, 30 HZ and 60 HZ in a randomized and counterbalanced manner and BCM EMG was recorded for a period of 90s following stimulation (testing trial 1; control trial). One hour after the last DPN stimulation, the procedure was repeated in the same group of animals, which now received 10 µl intrathecal infusions of either 0.9% saline (N=11) or one of two doses of GRP agonist (GRP^{20-29} 0.2 nmol, n=7; 0.5 nmol, n=7 (Phoenix Pharmaceuticals Inc. USA, Burlingame, CA)) and BCM EMG was recorded for 10 minutes after infusion of vehicle or drug. Next, the DPN was stimulated at 5, 10, 30 and 60 HZ in a counterbalanced order as described above (testing trial 2; drug trial).

2.2.3 BCM EMG analysis

BCM EMG recordings were analyzed for the 25 or 10 minutes following the infusion of the antagonist or agonist respectively, and for 90 seconds following each DPN stimulation. The numbers of events and bursts, as well as latencies to first event were analyzed. The analysis of numbers of events was performed using LabChart 7 (AD Instruments), and all events that were above baseline activity were recorded (Staudt et al., 2011). In addition, numbers of bursts were analyzed by two independent observers blinded to experimental treatment groups. Bursts were defined as a group of 10 or more events, without return to baseline activity between events. These criteria were based on a detailed analysis of the BCM bursting activity in a subgroup of control animals (n = 15). The average number of events per burst, number of bursts and time intervals between successive bursts during a period of 90 seconds following a 30 or 60 Hz DPN

stimulation were calculated, There was an average of 48.56 (ranging from 10 – 253) or 62.57 (ranging from 10 – 315) BCM events per burst after 30 and 60 Hz DPN stimulation respectively. Furthermore, there was a mean of 7.4 bursts (ranging between 3 – 12 bursts) or 8.07 bursts (ranging between 4 – 12 bursts) following 30 or 60 Hz resp. Analysis of time (s) between bursts revealed that there was an average of 2.54 seconds between successive bursts (ranging from 0.2 - 7 seconds).

Effects of antagonist treatments on numbers of events or bursts were compared both within animals (between the two testing trials (control and drug trials) and within the same drug treatment group) and between groups (within each of the two testing trials, and between drug treatment groups), separately for the 30 or 60 Hz stimulations, using two-way repeated ANOVA (factors: Drug treatment and Testing trial) and Holm-Sidak post hoc tests, Effects of agonist treatments on numbers of events or bursts were compared within animals (between the five stimulations (following infusion without stimulation, 5, 10, 30, 60 Hz)) and between treatment groups (within each stimulation setting), separately for each of the two testing trials, using a two-way repeated ANOVA (factors: Drug treatment and Stimulation) and Holm-Sidak post hoc tests, A 95% confidence level was used for all tests.

2.2.4 Immunohistochemistry experiments:

2.2.4.1 GRP expression in LSt cells

In order to verify that LSt cells and their axon terminals in autonomic and motor areas express GRP, 8 male rats were perfused transcardially with 10 mL 0.9 % saline followed by 500 mL of

4% paraformaldehyde in phosphate buffer (0.1 PB). Spinal cords were removed and postfixed for 1 hour in the same fixative and subsequently placed in cryoprotective solution (20 % sucrose in 0.1 PB with 0.01 % sodium azide) until further processing for immunohistochemistical detection of GRP and galanin, a marker for LSt cells (Truitt and Coolen, 2002; Truitt et al., 2003; Coolen et al., 2004; Staudt et al., 2010, 2011).

2.2.4.2 DPN stimulation-induced pERK after RC-3095

To verify that GRP antagonist did not prevent activation of LSt cells, expression of phosphorylation of ERK (pERK) in LSt cells was examined following stimulation of the DPN (Staudt et al., 2010). Male rats (n = 6) were anesthetized and spinalized as described above. Two hours after spinalization, the DPN and BCM were exposed and vehicle (saline, n = 3) or drug (RC-3095; Sigma-Alderich Inc.; 20 nmol; n = 3) were infused in a volume of 10 µl intrathecally as described above. 25 Minutes later, the DPN was stimulated at 30 Hz and BCM EMG activity was recorded for 90 seconds. Five minutes following DPN stimulation, animals were perfused transcardially with 5 mL 0.9 % saline solution and 500 mL of 4% paraformaldehyde in 0.1M PB. Spinal cords were removed and postfixed for 1 hour in the same fixative and subsequently placed in cryoprotective solution (20 % sucrose in 0.1 PB with 0.01 % sodium azide) until further processing for immunohistochemical detection of pERK and galanin. The stimulation parameters and time of perfusion were previously shown to be optimal for visualization of pERK expression in LSt cells (Staudt et al., 2010). Moreover, it was previously shown that spinalization and exposure of DPN and BCM without electrical stimulation of the DPN did not induce pERK expression in LSt cells (Staudt et al., 2010), hence negative control groups were not included in this experiment.

2.2.5 *Immunohistochemistry*

Spinal cords were sectioned using a freezing microtome (Thermo Fisher Scientific, Walldorf, Germany) into 12 parallel series of 35 um coronal sections in cryoprotectant solution (30 % sucrose, 30 % ethylene glycol in 0.1 M PB with 0.01 % sodium azide) and stored at -20 C until further processing. Free floating sections containing lower thoracic, lumbar, and sacral spinal levels were thoroughly rinsed in 0.1 M saline buffered sodium phosphate (PBS) between incubations and blocked with 1% H_2O_2 for 10 minutes prior to incubation. All antibody incubations were performed in incubation solution containing 0.1% bovine serum albumin and 0.4% Triton X-100 (BP151-500, Thermo Fisher Scientific, Ottawa, Ontario) in PBS at room temperature with gentle agitation.

Galanin/GRP Dual Fluorescence: One series of sections for four animals (2 spinal intact and 2 spinalized) were dual stained for galanin and GRP. Sections were incubated overnight with rabbit anti-galanin (1:60,000; T-4334; Bachem, Torrance, CA) and with biotinylated goat anti-rabbit for one hour (1:500; Vector Laboratories, Burlingame, CA, USA), avidin horseradish peroxidase complex (ABC-elite, 1:1000 in PBS; 1 hour; Vector Laboratories), biotinylated tyramine (BT; 1:250 in PBS containing 1uL/ml of 3% H_2O_2 for 10; NEL700 /700A; PerkinElmer Life Sciences, Boston, MA), and Alexa 488-conjugated streptavidin (1:100 in PBS, 30 minutes (Jackson ImmunoResearch Laboratories, West Grove, PA). Next, sections were incubated with rabbit anti-GRP (1:2,000; Phoenix (H-027-13)) and Alexa 555-conjugated goat anti-rabbit for 30 minutes in PBS (1:100; Jackoson ImmunoResearch Laboratories, West Grove, PA) and mounted

on plus charged slides, coverslipped with gelvatol and stored in the dark at 4°C. One series of sections of 4 animals (2 spinal intact and 2 spinalized) was stained using this same procedure, but were first processed for GRP immunoreactivity (-ir; using rabbit-anti-GRP; 1:40,000) and subsequently for galanin (rabbit anti-galanin; 1:3,000).

Galanin/pERK Dual Fluorescence: One series of sections were processed using the same protocol as described above using rabbit anti-pERK (1:1,000; overnight; Cell Signalling # 26L) and rabbit anti-galanin (1:3,000).

For all procedures, the omission of primary antibodies resulted in a complete loss of signal at specific wavelengths and served as a control manipulation. Moreover, all primary antibodies have been previously characterized (Truitt et al., 2003; Staudt et al., 2010, 2011) .

2.2.6 Data Analysis:

2.2.6.1 Analysis for Galanin/GRP co-localization

Sections of the lumbosacral spinal cord containing LSt cells were analyzed for expression of GRP using a Leica DM5000B (Leica Microsystems, Wetzlar, Germany). In all 8 animals included in the analysis, all galanin-ir and CGRP-ir cells were analyzed for co-expression of GRP-ir or galanin-ir respectively. The percentages of galanin-ir co-expressing GRP and vice versa were determined for each animal and a group mean was calculated. In addition, the co-localization of galanin and GRP was analyzed in LSt axons in the intermediolateral cell column

(IML), central autonomic nucleus (CAN), sacral parasympathetic nucleus (SPN), and spinal nucleus of the bulbocavernosus (SNB) using a Zeiss laser-scanning confocal microscope system (Zeiss LSM510; Carl Zeiss MicroImaging, Inc., Thornwood, NY). Alexa 555-fluorescence was imaged using a 567 nm emission filter and a HeNe laser and Alexa 488 was imaged using a 505 nm filter and Argon laser. Images were generated by projecting several 1 µm optical sections collected at 1 µm intervals through areas of interest in the z-axis using a 63x water-immersion objective.

2.2.6.2 pERK Expression in LSt cells

pERK expression in LSt cells was analyzed on a DM5000B Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Specifically, all lumbar cells expressing galanin-ir were analyzed for expression of pERK. Data are expressed as the mean percentages of LSt cells that express pERK for each animal. The group means were calculated and compared between animals that received an infusion of RC-3095 (20nmol) and saline-treated controls, using Student t tests with 95% confidence levels.

2.2.6.3 Pressure recording in seminal vesicles

Based on findings from the above described experiments, an additional experiment was conducted to analyze pressure in the seminal vesicles (SV), which is considered an indicator of the emission phase (Clement et al., 2008). Spinal cords were transected, and BCM and DPN were exposed as described above. In addition, the right SV was exposed by coeliotomy. SV pressure (SVP) was measured using a pressure catheter (ADInstruments, model number: SPR-

671(1.4F, Single, Straight, 15cm, Ny) attached to a catheter interface cable (ADInst, model number: AEC-IOD) and connected to a Bridge AMP (ADInst). The pressure catheter was inserted into the lumen of the SV and secured prior to recording. SVP and BCM EMG (as described above) were simultaneously recorded and analyzed using LabChart 7 (ADInst). There were three groups of animals (N=3 per group). The first group of control animals received DPN stimulation at 5, 10, 30 and 60 Hz 10 minutes after intrathecal saline infusion (control Trial 1) and identical treatment in trial 2 (control Trial 2). The second group of animals received intrathecal saline and DPN stimulation at 30 and 60 Hz 25 minutes later (control Trial 1), and subsequent intrathecal RC-3095 (20 nmol; Trial 2; drug trial) again followed by DPN stimulation at 30 and 60 Hz 25 minutes after each infusion. The third group of animals received intrathecal saline and DPN stimulation at 5,10, 30, and 60 Hz 10 minutes later (Trial 1; control trial) and subsequent GRP agonist (GRP^{20-29}; Trial 2; drug trial) followed by DPN stimulation at 5, 10, 30, and 60 Hz 10 minutes after each infusion.

Analysis: BCM EMG was analyzed as described above and numbers of bursts were compared within animals and between trails 1 and 2 using t-tests or the Mann-Whitney –Wilcoxon test. SVP was analyzed using LabChart7 (ADInst). SVP increased simultaneously with BCM EMG bursts and the numbers of times that increased SVP occurred were counted and compared within animals between trials 1 and 2 using t-tests with confidence levels of 95%.

2.3 Results

2.3.1 GRP expression in LSt cells and axons

GRP was co-expressed by all LSt cells analyzed, based on analysis of 119 galanin-IR cells in eight animals (Figure 3). Moreover, 100% GRP-IR neurons in the lumbosacral spinal cord co-expressed galanin-IR, suggesting that GRP is exclusively expressed by LSt cells. There was no effect of spinal transection on co-expression of galanin and GRP-ir. In addition, qualitative analysis showed that virtually all galanin-IR axons in IML, CAN, SPN, and SNB co-expressed GRP (Figure 3). Hence, GRP may be released in autonomic and motor areas to mediate ejaculation However, a few GRP-IR fibers that did not co-express galanin-IR were detected dorsal to the SPN (Figure 3J-L), raising the possibility that the SPN also receives a minor GRP input from cells other than LSt neurons and outside the lumbosacral spinal cord.

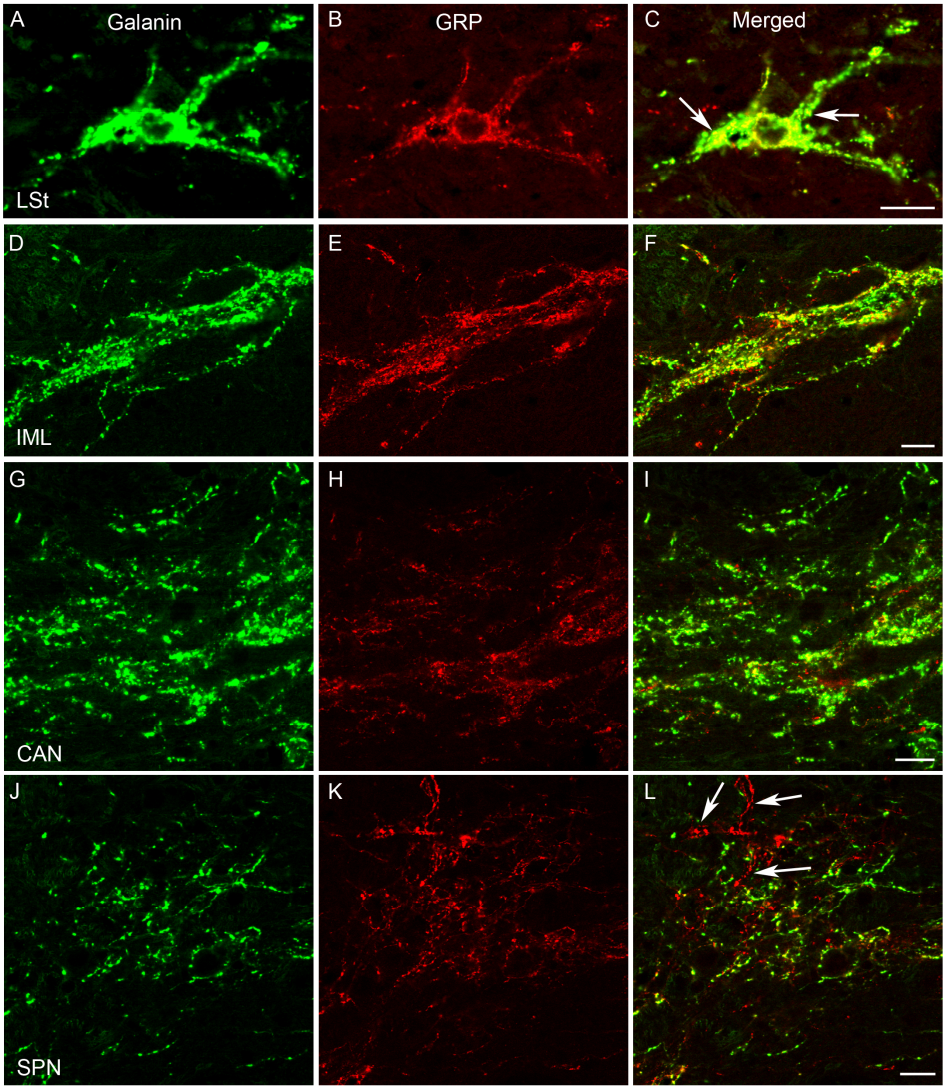

Figure 3 The co-expression of galanin-ir and GRP-ir in LSt cells, IML, CAN and SPN of the male rat

Confocal images (1 µm optical sections) illustrating the co-expression of galanin-ir **(A)** and GRP-ir **(B)** in LSt cells **(A-C)**, IML **(D-F)**, CAN **(G-I)**, and SPN **(J-L)** in a spinal intact male rat. Galanin-ir was immunoprocessed using TSA amplification, while GRP was immunoprocessed using a conjugated secondary antibody. Arrows in **(C)** indicate putative close contacts of galanin/GRP-ir axon terminals with LSt cells. Arrows in L indicate GRP-ir fibers dorsal to the SPN that do not co-express galanin-ir. Scale bars indicate 20 µm.

2.3.2 GRP receptor antagonist blocked ejaculatory reflexes

GRP receptor antagonist RC-3095 significantly reduced DPN stimulation-induced ejaculatory reflexes at both 30 and 60 Hz stimulation frequencies as evidenced by the reduced numbers of BCM events and bursts (Figure 4C-F). There were main effects of testing trial on the numbers of BCM events for both 30 ($F(1, 43)$ 13.760; $p=0.002$; Figure 4C) and 60 Hz stimulation frequencies ($F(1, 41)$ 12.727; $p=0.003$; Figure 4D). Post hoc analyses revealed that animals treated with either dose of RC-3095 during the second, drug trial (trial 2; Figure 4B) had significantly decreased BCM events in response to 30 and 60 Hz DPN stimulation compared to their DPN stimulation-induced BCM events following saline treatment in their first control trial (trial 1; 60 Hz: $p=0.008$ (10nmol); $p=0.040$ (20nmol); 30 Hz: $p=0.022$ (10nmol); $p=0.029$ (20nmol); Figure 4C-D and Figure 5C-D). There were no significant differences between the two groups treated with the lower and higher doses of RC-3095. Saline treated animals did not differ in their BCM events during trial 2 compared to trial 1 (30 Hz: $p=0.130$; 60 Hz: $p=0.280$, Figure 4C-D and Figure 5A-B), demonstrating that the reduction in BCM events was not an effect of repeated testing, but rather of RC-3095 treatment. Similarly, there were main effects of testing trial on the numbers of BCM bursts for both 30 Hz ($F(1,48) = 56.209$; $p < 0.001$; Figure 4E) and 60 Hz ($F(1,48) = 41.878$; $p < 0.001$; Figure 4F) stimulation frequencies. Males treated with either dose of RC-3095 during trial 2 (drug trial) had significantly lower numbers of BCM bursts compared to trial 1 (control trial) with saline treatment (30Hz: $p < 0.001$ (10 nmol), 60 Hz: $p < 0.001$ (20nmol). Once again, there was no effect of the dosage of the antagonist and of repeated testing, as saline-treated animals did not differ in the numbers of BCM bursts during the second compared to the first testing trial (Figure 4E-F).

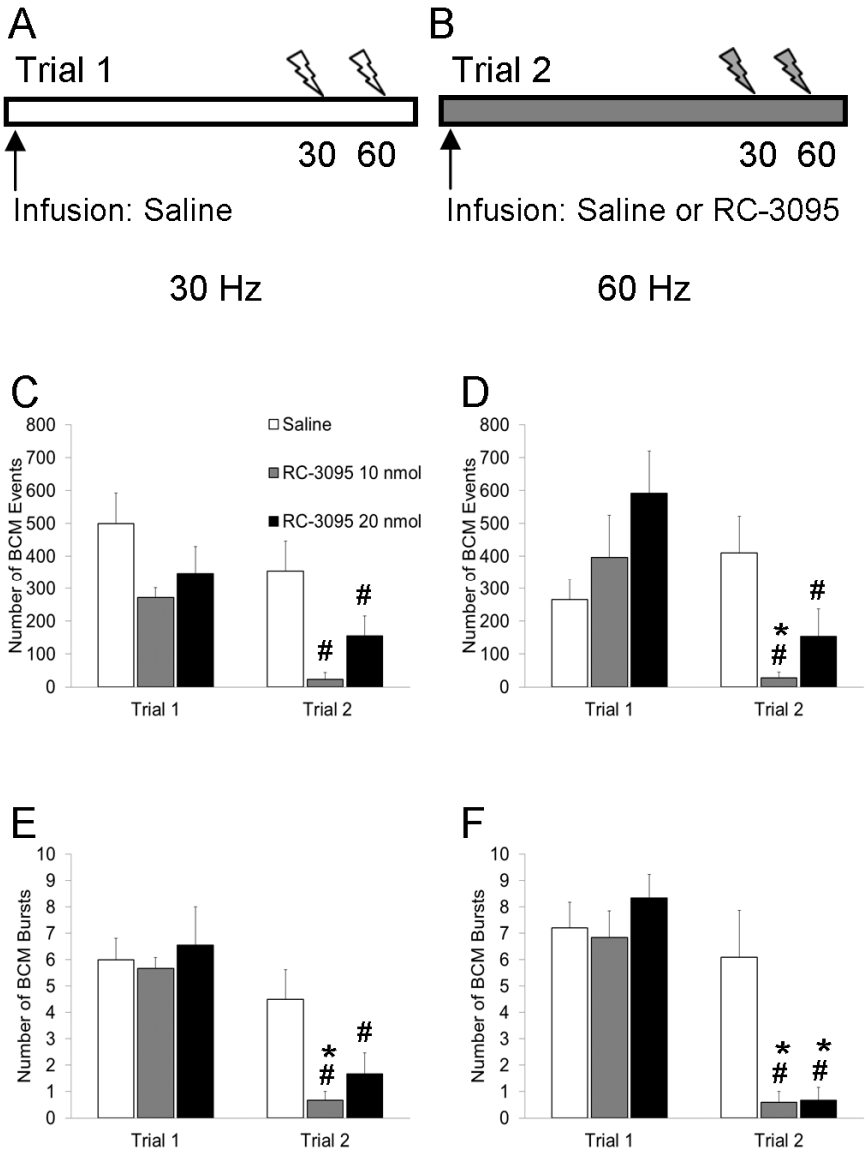
A
Trial 1
30 60
Infusion: Saline
B
Trial 2
30 60
Infusion: Saline or RC-3095
30 Hz
60 Hz
C
D
Saline
RC-3095 10 nmol
RC-3095 20 nmol
Number of BCM Events
Trial 1
Trial 2
E
F
Number of BCM Bursts

Figure 4 Effects of GRP receptor antagonist RC-3095 infusion on the numbers of BCM events and bursts in male rats

Quantitative analysis of BCM events and bursts following infusions with GRP receptor antagonist RC-3095 During Trial 1 **(A**; control trial**)**, all groups received saline infusions (arrow) and 30 and 60 Hz DPN stimulation (bolt arrows; in counterbalanced manner). During Trial 2 **(B**; drug trial**)**, the same groups received saline or RC-3095 (10 or 20 nmol) infusions (arrow) followed by 30 and 60 Hz DPN stimulation (bolt arrows; in counterbalanced manner). Numbers of BCM events are shown in **(C)** (30 Hz) and **(D)** (60 Hz); while numbers of BCM bursts are shown in **(E)** (30 Hz) and **(F)** (60 Hz). # denotes significant differences from Trial 1 within the same treatment group. * indicates significant differences from controls within the same testing trial.

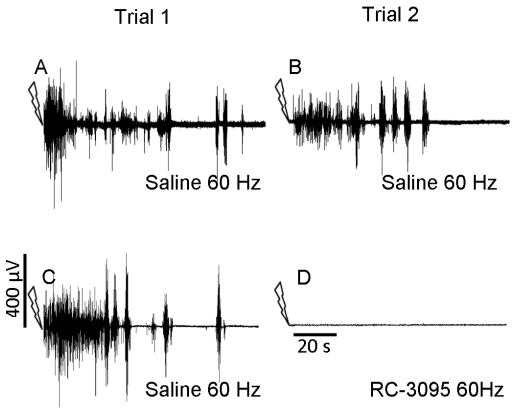

Figure 5 Infusions of RC-3095 prevent DPN stimulation-induced bursting of the BCM in male rats.

Representative EMG recording traces depicting BCM activity induced by 60Hz DPN stimulation (indicated by arrow) following intrathecal infusions of saline or GRP antagonist RC-3095. DPN stimulation induced characteristic BCM bursting following infusion of saline in trial 1 **(A, C)** and again following saline in trial 2 **(B; same animal as A)**. In contrast, infusion of the GRP antagonist RC-3095 (20nmol) in trail 2 completely prevented BCM activity **(D; same animal as C)**. Each trace represents 90 seconds of BCM activity.

A significant interaction between drug treatment and testing trial was detected for numbers of BCM events at the 60 Hz stimulation ($F(2,48) = 6.941$; $P = 0.005$). Post hoc analyses demonstrated that during trial 2 (drug trial), animals treated with 10 nmol RC-3095 had significantly fewer numbers of BCM events induced by 60 Hz stimulation compared to saline controls ($p=0.004$; Figure 4D). A trend towards significance was observed in animals treated with the higher dose of RC-3095 (20nmol) and received 60 Hz DPN stimulation ($P = 0.06$; Figure 4D). Interactions between drug treatment and testing trial were also detected for numbers of BCM bursts at 30 Hz ($F(2,48) = 5.250$; $P = 0.014$) and 60 Hz ($F(2,48) = 6.941$; $P = 0.005$) stimulations. Specifically, during trial 2 (drug trial), animals treated with the lower dose of RC-3095 had significantly lower numbers of BCM bursts compared to saline controls for both 30 Hz ($P = 0.01$; Figure 4E) and 60 Hz ($P < 0.001$; Figure 4F) stimulations and animals treated with the higher dose had fewer BCM bursts compared to saline controls following 60 Hz stimulation ($P = 0.003$; Figure 4F). There were no significant differences between the two groups treated with the lower and higher doses of RC-3095. Finally, there were no differences in numbers of

BCM events nor bursts between groups during the first control trial, when all groups were treated with saline (Figure 4C-F), demonstrating that group differences during the second testing trial were caused by drug treatments, rather than differences in BCM bursting responses.

2.3.3 *RC-3095 did not affect DPN stimulation-Induced pERK in LSt cells*

In order to verify that GRP antagonist RC-3095 blocks BCM bursting via actions in LSt target areas rather than acting directly on LSt cells, DPN stimulation-induced neural activation of LSt cells was examined. In both antagonist treated and control males, DPN stimulation induced pERK in 97 ± 0.03% of LSt cells, similar to the activation reported previously in control males (Staudt et al., 2010). Thus, GRP receptor antagonist treatment did not affect the activation of LSt cells in response to DPN stimulation (Figure 6).

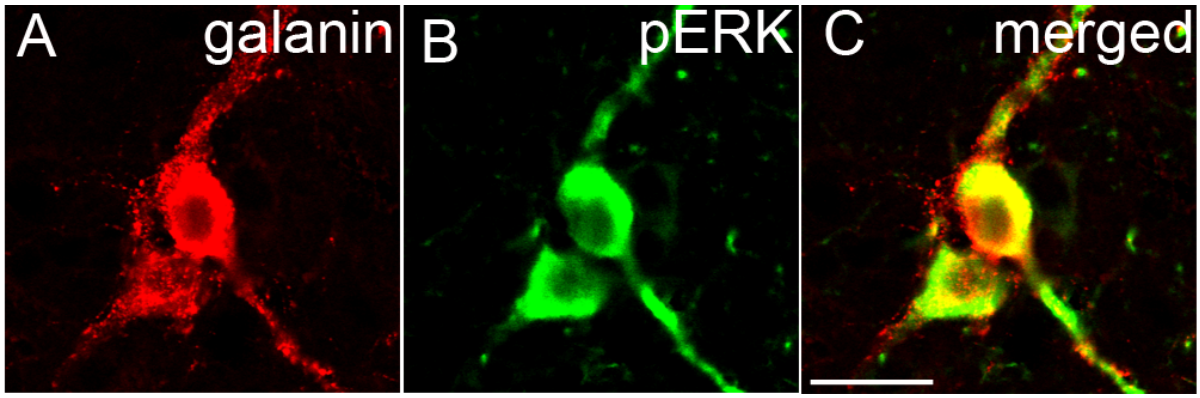

Figure 6 Expression of pERK in LSt cells induced by DPN stimulation

Confocal images (1 µm optical sections) demonstrating pERK expression **(B)** in LSt cells **(A)** induced by DPN-stimulation. The pattern of pERK labeling is representative of all antagonist- and control-treated animals. Scale bar indicates 20 µm.

2.3.4 *GRP^{20-29} agonist facilitated BCM activity*

During the first testing trial (control trial), there was an overall effect of DPN stimulation parameters on the number of BCM events ($F(4, 121)= 28.878$; $P < 0.001$) and bursts ($F(4,121) = 54.775$; $P < 0.001$). Post hoc tests showed that following 30Hz DPN stimulation, all treatment groups had significantly increased numbers of BCM events (compared to infusion, 5Hz, or 10Hz, $P < 0.001$; Figure 7C), and bursts (compared to infusion, 5Hz, or 10Hz, $P < 0.001$; Figure 7E). DPN stimulation at 60 Hz also increased numbers of BCM events and bursts (compared to infusion, 5Hz, or 10Hz, $P < 0.001$; Figure 7C, 7E). These data confirm that 30 and 60 Hz stimulations reliably trigger BCM bursting while 5 and 10 Hz represent sub-threshold levels of stimulation. Moreover, saline infusions alone (without DPN stimulation) did not trigger any BCM activity (Figure 7C, 7E, 8A). Finally, during control trial 1, there were no differences between treatment groups in the numbers of BCM events nor bursts (Figure 7C,7E), demonstrating that there were no initial group differences in BCM bursting responses and that repeated DPN stimulations in the randomized order did not affect BCM responses to subsequent stimulations.

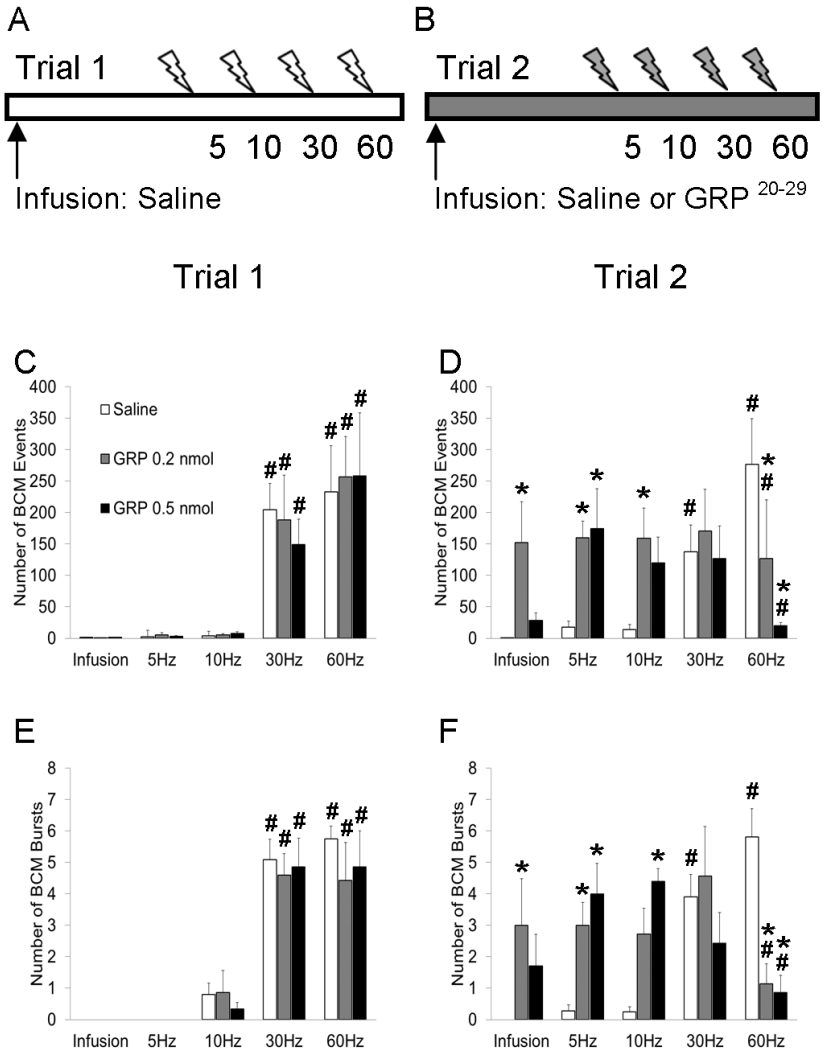
A
Trial 1
5 10 30 60
Infusion: Saline
B
Trial 2
5 10 30 60
Infusion: Saline or GRP 20-29
Trial 1
Trial 2
C
D
Saline
GRP 0.2 nmol
GRP 0.5 nmol
Number of BCM Events
Infusion
5Hz
10Hz
30Hz
60Hz
E
F
Number of BCM Bursts

Figure 7 Effects of GRP infusions on the numbers of BCM events and bursts in male rats.

Quantitative analysis of BCM events and bursts following infusions with GRP^{20-29}. During Trial 1 **(A**; control trial**)** all groups received intrathecal saline (arrow) followed by four DPN stimulations (bolt arrows) at 5-60 Hz (in counterbalanced manner). During Trial 2 **(B**; drug trial**)**, all groups received intrathecal GRP^{20-29} (10 or 20 nmol; arrow) again followed by four DPN stimulations at 5-60 Hz (bolt arrows; in counterbalanced manner). Numbers of events are shown in **(C)** (Trial 1) and **(D)** (Trial 2), while numbers of bursts are shown in **(E)** (Trial 1) and **(F)** (Trial 2). # denotes significant differences from BCM activity following infusion, 5 Hz, and 10 Hz within the same treatment group and trial, * indicates significant differences from the saline control group within the same DPN stimulation and trial.

During the second testing trial (drug trial), there were overall effects of DPN stimulations on BCM bursts ($F(4,120) = 2.795$, $P = 0.031$)) and there were significant interactions between stimulations and drug treatments for events (($F(8, 119) = 5.048$; $P < 0.001$) and bursts ($F(8,120) = 8.053$; $P < 0.001$). First, the GRP^{20-29} receptor agonist significantly triggered BCM activity in the absence of DPN stimulation in a subset of animals, (43% of 0.2 nmol and 43% of 0.5 nmol; Figure 7D, 7F and Figure 8F). Latencies to BCM activity were 149 ± 13.66 (0.2 nmol) and 148 ± 5.69 (0.5 nmol) seconds post-infusion. Comparisons of numbers of BCM events and bursts showed a significant increase in BCM activity in animals treated with the low dose of GRP^{20-29} (0.2 nmol) compared to saline controls (events: $P = 0.015$; bursts: $P = 0.008$) and a trend toward significance compared to animals treated with the high dose of GRP^{20-29} (0.5 nmol; events: P=0.07; Figures 7D, 7F). These results indicate that in a portion of the animals, the low dose of GRP^{20-29} was sufficient for triggering ejaculatory reflexes in the absence of sensory stimulation.

Furthermore, intrathecal infusions of GRP^{20-29} facilitated ejaculatory reflexes in response to subthreshold levels of DPN stimulation (5-10 Hz) as evidenced by the increased numbers of events and bursts (Figures 7D, 7F, 8D). Post hoc analyses revealed significantly increased BCM events in response to DPN stimulation in animals treated with low dose of GRP^{20-29} (0.2 nmol) at 5 Hz ($P = 0.022$) and 10 Hz ($P = 0.042$) compared to saline-treated control animals (Figure 7D). Similarly, animals treated with the high dose of GRP^{20-29} (0.5 nmol) demonstrated significantly increased BCM events in response to 5 Hz DPN stimulation ($P = 0.012$; Figure 7D). Animals treated with the low dose of GRP^{20-29} (0.2 nmol) also had significantly more BCM bursts in response to 5 Hz DPN stimulation ($P = 0.016$) and a trend to significance at 10 Hz ($P = 0.041$) compared to saline-treated animals (Figure 7F). Similarly, animals treated with the high dose of GRP^{20-29} (0.5 nmol) demonstrated significantly increased BCM bursts after 5Hz ($P = 0.001$) and 10 Hz ($P = 0.012$) DPN stimulation compared to saline controls (Figure 7F).

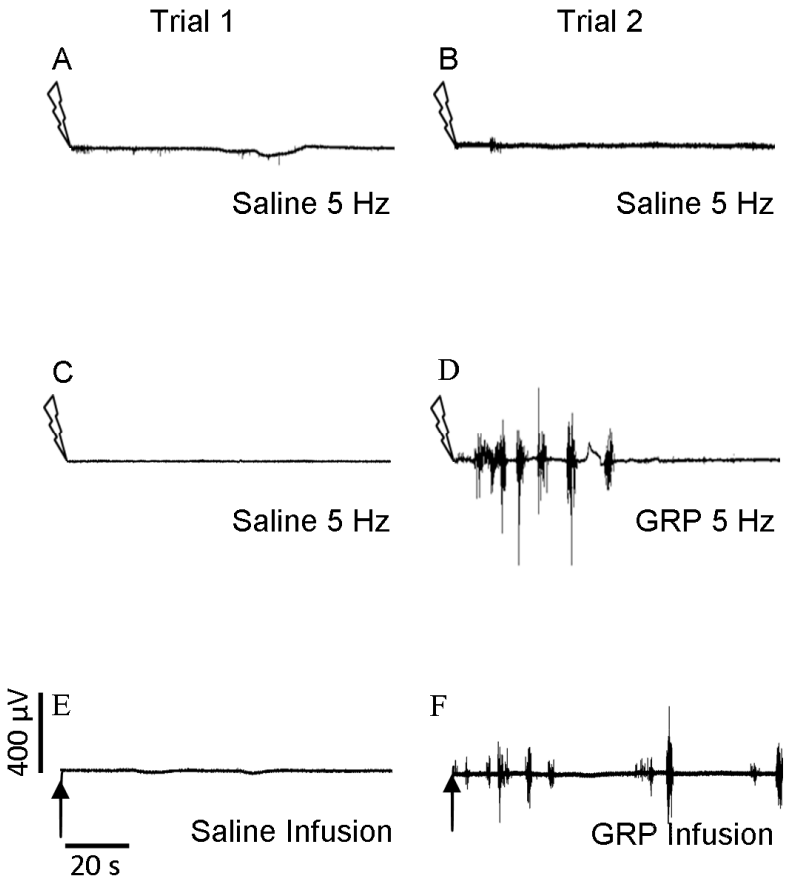

Figure 8 Effects of GRP infusions on BCM activity in male rats.

Representative EMG recording traces showing BCM activity induced by subthreshold stimulation of the DPN (5Hz; indicated by bolt arrows) following intrathecal infusion of saline **(A-C)** or GRP^{20-29} **(D)**. Subthreshold DPN stimulation did not induce BCM activity following intrathecal infusions of saline in trial 1 **(A, C)**, or following saline in trail 2 **(B; same animal as A)**. In contrast, subthreshold DPN stimulation following intrathecal infusion of GRP^{20-29} (0.2 nmol) did induce BCM activity **(D; same animal as C)**. Infusion of GRP^{20-29} (0.2 nmol) **(F)** but

not saline **(E; same animal as F)** also induced BCM activity without DPN stimulation. Each trace represents 90 seconds of BCM activity.

GRP^{20-29} treatment did not affect BCM activity at 30 Hz stimulation. But, unexpectedly, GRP agonist treatment significantly decreased BCM activity in response to high levels of DPN stimulation (60 Hz) at both the lower dose (events: p= 0.016; bursts: $P < 0.001$) and the higher dose (events: $P < 0.001$; bursts: $P < 0.001$) of GRP^{20-29} compared to saline-treated control animals (Figure 7D-F). Finally, saline treatment did not affect increased BCM events and bursts following 30 Hz (Events; infusion: $P = 0.007$, 5Hz: p=0.017, 10Hz: $P = 0.037$; Bursts; infusion: $P < 0.001$, 5Hz: $P < 0.001$, 10Hz: $P = 0.001$) or 60 Hz (Events; infusion, 5Hz, 10Hz: $P < 0.001$, 30Hz: $P = 0.006$; Bursts; infusion, 5Hz, 10Hz: $P < 0.001$) DPN stimulation, confirming that there was no effect of repeated DPN stimulations (Figure 7D-F).

2.3.5 *GRP mediates SVP Increases following DPN stimulation*

The findings described above confirm a role for GRP in the expulsion phase of ejaculatory reflex. Next, we determined the role of GRP for increases in SVP, indicative of the emission phase. Since effects of DPN stimulation on SVP have not yet been documented, saline-treated controls were analyzed. DPN stimulation triggered SVP increases that were concurrent with BCM EMG bursts in saline treated animals (Figure 9). There were no significant differences in the numbers of SVP increases following DPN stimulation between trial 1 and trial 2 in animals treated with saline (at 5, 10, 30, or 60 Hz; Table 1), showing lack of effect of repeated DPN stimulation. Similarly, and in concordance to the results described above, there were no

significant differences in the numbers of BCM bursts induced by DPN stimulation between trial 1 and trial 2 in saline treated animals (Table 1). The frequency of SVP increases was closely coupled to the numbers of BCM bursts. Each SVP increase was accompanied by a BCM burst, although a number of DPN-stimulation induced BCM bursts occurred in the absence of SVP increases, in animals that received 30 or 60 Hz DPN stimulation. In the absence of BCM activity following subthreshold levels of DPN stimulation (Trial 1: control trial; saline infusion, 5 Hz, and 10 Hz) there was a similar absence of SVP increases (Table 1).

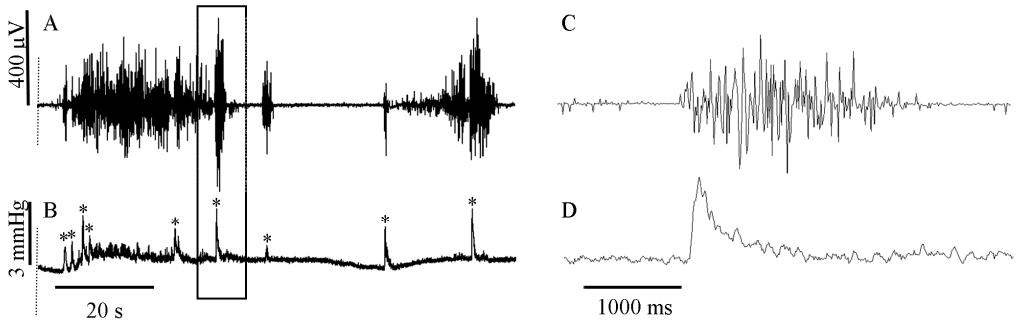

Figure 9 SVP and concurrent BCM activity induced by DPN stimulation

A representative example of a simultaneous recording of BCM activity and SVP (90 seconds duration) after 60 Hz DPN stimulation 10 minutes following intrathecal saline infusion. The dotted lines in A and B indicate the end of DPN stimulation. Stars in (B) indicate SVP increases. A fragment of the trace indicated in the box area in A and B is enlarged in C and D to clearly show a BCM burst (C) coupled with a single increase in SVP (D) over 4 seconds of recording

Table 1 Numbers of SVP increases and BCM bursts following infusions of saline, RC-3095 and GRP in male rats.

Numbers of seminal vesicle pressure (SVP) increases and BCM bursts in animals treated with saline-saline (n=3), saline-RC3095 (20nmol; n =3) or saline-GRP (0.2nmol; n = 3) in trial 1-trial 2 respectively, and with DPN stimulation at 0, 5, 30, or 60 Hz frequencies. # indicates significant difference from trial 1 within the same treatment group and stimulation parameter.

Saline-saline (n = 3)	**Trial 1: Numbers of SVP**	**Trial 2: Numbers of SVP**	**Trial 1: Numbers of BCM bursts**	**Trial 2: Numbers of BCM bursts**
Infusion	0	0	0	0
5 Hz	0	0	0	0
10 Hz	0	0	0	0
30 Hz	3.33 ± 1.43	2.33 ± 0.94	8 ± 1.08	8.33 ± 0.62
60 Hz	4 ± 0.71	2.33 ± 1.03	8.33 ± 0.24	9.33 ± 0.62
Saline-RC3095 20nmol (n = 3)	**Trial 1: Numbers of SVP**	**Trial 2: Numbers of SVP**	**Trial 1: Numbers of BCM bursts**	**Trial 2: Numbers of BCM bursts**
30 Hz	4.66 ± 1.86	0 #	6.66 ± 0.47	0 #
60 Hz	3.66 ± 2.03	0 (P =0.052)	8 ± 0.71	0 #
Saline-GRP 0.2nmol (n = 3)	**Trial 1: Numbers of SVP**	**Trial 2: Numbers of SVP**	**Trial 1: Numbers of BCM bursts**	**Trial 2: Numbers of BCM bursts**
Infusion	0	3.66 ± 1.89	0	5 ± 2.86
5 Hz	0	1.33 ± 0.24 #	0	4.33 ± 0.62 #
10 Hz	0	2 ± 0.41 #	0	4 ± 0.71 #
30 Hz	1 ± 0.41	3.33 ± 0.47 #	7.33 ± 0.62	6.66 ± 0.62
60 Hz	4.33 ± 2.09	2 ± 0.41	8 ± 0.41	6.33 ± 0.62

GRP appears to be critically involved in the regulation of both BCM EMG activity and SVP increases (Table 1, Figure 10). Indeed, RC-3095 (20 nmol) treatment completely blocked both BCM EMG bursts (confirming results described above) and SVP increases in response to 30 Hz (BCM: $P < 0.001$; SVP: $p = 0.03$) and 60 Hz (BCM: $p = 0.001$; SVP: $P = 0.052$) DPN stimulation (Table 1). Moreover, in 2/3 animals, GRP^{20-29} triggered BCM bursts and SVP increases in the absence of DPN stimulation (Table 1, Figure 10). In addition, GRP^{20-29} facilitated BCM bursts and SVP increases in response to subthreshold levels: 5 Hz (BCM: $p = 0.004$; SVP: $P = 0.005$) and 10 Hz (BCM: $P = 0.005$; SVP: $P = 0.013$) of DPN stimulation (Table 1).

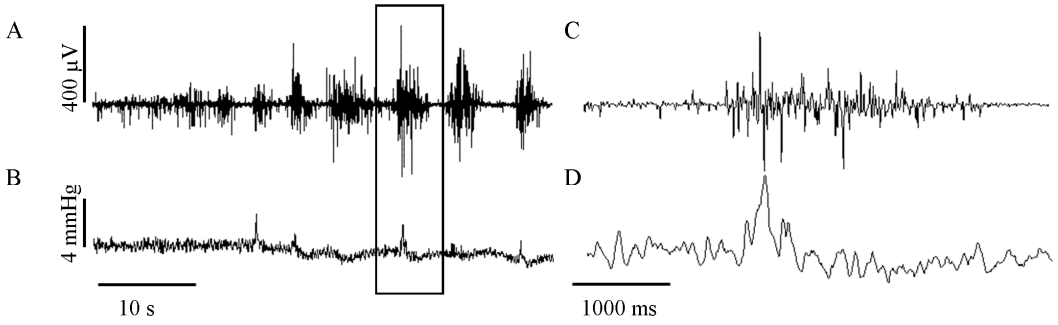

Figure 10 BCM activity and concurrent increases in SVP triggered by infusions of GRP in a male rat

Simultaneous recording of BCM activity (A) and SVP (B) in one animal (45 seconds of EMG recording) 145 seconds after an intrathecal infusion of GRP^{20-29} (0.2nmol). The traces in A and B are magnified to show one BCM burst (C) occurring simultaneously with an increase in SVP (D).

2.4 Discussion

The findings of this set of studies support the hypothesis that GRP plays a critical role in the control of ejaculation by acting in autonomic and motor areas targeted by LSt axons. GRP was found to be present in all LSt cells and LSt axon projections within the lumbosacral spinal cord. As predicted, intrathecal administration of GRP receptor antagonist RC-3095 severely disrupted both the emission and expulsion phases of ejaculation in response to sensory stimulation, reflected in the reduced numbers of SVP increases and BCM activity in response to DPN stimulation in RC-3095-treated male rats. In contrast, intrathecal infusion of GRP^{20-29} facilitated both the emission and expulsion phases of ejaculation, as evidenced by the surge in the numbers of SVP increases and BCM bursts respectively in male rats treated with GRP^{20-29}. In particular, GRP^{20-29} was most effective in facilitating ejaculation after sub-threshold sensory stimulation (5-10 Hz), signifying the presence of a cumulative effect of GRP receptor activation by intrathecal infusion of GRP and the release of endogenous GRP or other neuropeptides in response to stimulation of the DPN.

GRP is a bombesin-like peptide that is widely distributed in the central nervous system of mammals (Marberger, 1974; Roth et al., 1982; McKenna, 1999) and is involved in many physiological processes including the suppression of feeding behavior (Merali et al., 1993) circadian rhythms (Drouyer et al., 2010; Francl et al., 2010; Gamble et al., 2011) and itching behavior (Sun and Chen, 2007). In the lumbosacral spinal cord, GRP was shown here to be exclusively expressed by LSt cells, as 100% of LSt neurons, identified by immunoreactivity for galanin, co-expressed GRP and vice versa. Moreover, virtually all galanin-IR LSt axons in the autonomic and motor areas co-expressed GRP. This finding is in slight contrast with a previous

report of a sub-population of GRP-IR neurons that do not co-express galanin dorsal to the central canal in the lumbar spinal cord (Sakamoto et al., 2008). The discrepancy is possible due to differences in the sensitivity of immunohistochemical detections: in the present study, two different immunohistochemistry protocols were performed in which either one of the neuropeptide signals were amplified for optimal sensitivity of peptide detection. Results from both protocols were identical and complete co-localization of galanin- and GRP-ir was found in soma and axon terminals. Moreover, the present study utilized spinal cord tissues from both spinal intact and spinal transected male rats, thereby correcting for the possibility of supraspinal influences on galanin or GRP expression. A few GRP-IR fibers that did not co-express galanin-IR were observed in the region of and dorsal to the SPN, suggesting that the SPN receives inputs from GRP neurons outside of the spinal ejaculation generator and the lumbosacral spinal cord. Sources of GRP indeed exist outside of the spinal ejaculatory network (Roth et al., 1982). The SPN is known for its role in erectile function (Rampin et al., 1997a; Tang et al., 1998; Giuliano and Rampin, 2004) and emission (Allard et al., 2005; Johnson, 2006) and destruction of sacral autonomic centers abolishes reflex erections (Everaert et al., 2010) but a specific role for SPN in the expulsion phase of ejaculation has not been documented. Therefore, it is likely that GRP axons located dorsally to the SPN are predominantly involved in erectile function and emission, but not expulsion (Morgan et al., 1981; Rampin et al., 1997b; Tang et al., 1998). Indeed, one of the sources of GRP outside of the spinal ejaculation generator is the paraventricular nucleus of the hypothalamus (Roth et al., 1982), an area well known to be involved in erectile function (Chen et al., 1997; Argiolas and Melis, 2005) and to project to the lumbosacral spinal cord (Shen et al., 1990; Tang et al., 1998). It is currently unknown if GRP in the PVN or its projections mediate erection, and this possibility can be tested in future studies.

The expression of GRP in LST cells and projections is consistent with the proposed role of GRP in control of ejaculation via action in autonomic and motor areas in the lumbosacral spinal cord (see Figure 3), Moreover, intrathecal injections of GRP receptor antagonist RC-3095 blocked BCM bursting in response to DPN stimulation. It is assumed that RC-3095 acted primarily in the areas of the spinal cord that are located adjacent to the pia-arachnoid space, including all LSt target areas. Indeed, GRP receptor expression has been reported in the SPN and SNB of the lumbosacral spinal cord (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008), however its expression in other LSt target regions, including the CAN and IML requires further investigation. Another possibility is that GRP or GRP antagonist treatment acts directly on the LSt cells. Galanin- and GRP-ir axon terminals arising from LSt cells that make putative contacts with LSt cells have previously been observed (see Figure 5; (Truitt et al., 2003)) and thus GRP may be released directly onto LSt cells to control ejaculation. However, RC-3095 treatment did not decrease neural activity of LSt cells, indicated by pERK expression, substantiating that RC-3095 likely blocks the ejaculatory reflex by acting on GRP receptors downstream of the LSt cells, rather than directly disrupting activity of LSt cells.

In a portion of the animals [33-43%], GRP^{20-29} was sufficient in triggering ejaculatory reflexes in the absence of DPN stimulation. It is possible that the differences in the ability of GRP to induce ejaculatory reflexes between groups of rats are due to differences in receptor densities for neuropeptides or receptors expressed in the spinal ejaculation generator, including GRP. In addition, GRP^{20-29} was not sufficient to trigger ejaculatory reflexes in all the animals indicating that a higher dose of GRP agonist may have been necessary to trigger BCM bursting and SVP increases in some animals. Alternatively, the lack of responses to GRP^{20-29} in all animals suggests that other neuropeptides expressed in LSt cells and potentially released in LSt target

areas may act in concert with GRP to control ejaculation. These neuropeptides include galanin (Newton, 1993; Kozyrev, 2013b), cholecystokinin (Ju et al., 1987; Kozyrev, 2013b) and enkephalin (Nicholas et al., 1999; Kozyrev, 2013c). An unexpected finding was that the higher dosages of GRP^{20-29} caused significant suppression of BCM activity induced by the highest stimulation parameters. A similar finding is that stimulation of the DPN at higher frequencies (such as 120 Hz) does not trigger ejaculatory reflexes (unpublished personal observations). Hence, such stimulation by agonists and high frequency may represent a super physiological manipulation that leads to dysfunction or possible muscle fatigue.

An advantage of the current study of the role of GRP in ejaculation is the use of the DPN stimulation paradigm which utilizes anesthetized and spinalized male rats and elicits rhythmic contractions of the BCM and concurrent SVP increases, akin to those observed in freely-moving animals during mating (Beyer et al., 1982; Holmes and Sachs, 1991; Staudt et al., 2010; Normandin and Murphy, 2011; Staudt et al., 2011). Thus, complete transection of the spinal cord permits the study of ejaculation as a reflex response in the absence of supraspinal influences (Sachs and Garinello, 1980; Chung et al., 1988; Monaghan and Breedlove, 1992). The DPN is the primary afferent that transmits sensory information related to the summation of mating to the spinal ejaculation generator and triggers ejaculation (Hart, 1968; McKenna and Nadelhaft, 1986). Indeed, bilateral transection of the DPN blocks ejaculation in mating rats (Lodder and Zeilmaker, 1976). Although the pathways whereby sensory information is transmitted from the DPN to the LSt cells are currently unknown, it has been shown that DPN nerve fibers terminate in the lower lumbar and upper sacral levels of the spinal cord in the general vicinity of the LSt cells, dorsal horn and dorsal grey commissure (DCG) (McKenna and Nadelhaft, 1986; Ueyama et al., 1987; Yells et al., 1992; Rampin et al., 1997b). Furthermore, it has previously been shown that DPN

stimulation activates LSt cells in a similar manner as ejaculatory behavior in freely moving animals (Staudt et al., 2010; Staudt, 2011). The mechanism for LSt cell activation is through NMDA receptors (Staudt et al., 2011) and map kinsase signaling (Staudt et al., 2010). Moreover, this activation is required as pharmacological blockade of either of these components results in the blockade of DPN stimulation-induced ejaculatory reflexes in male rats (Staudt et al., 2010; Staudt, 2011). Therefore, the activation of LSt cells is essential for the transmission of sensory information from the DPN to the outputs of the spinal cord ejaculation generator (Staudt, 2011). A previous study demonstrated effects of GRP treatment on erectile and ejaculatory reflexes using an ex copula paradigm in spinal intact rats (Sakamoto et al., 2008). However, in this paradigm, it is not possible to eliminate actions of GRP on the supraspinal areas that control ejaculation from the spinal ejaculation generator, including the nucleus of the paragigantocellularis (nPGi) (Marson and McKenna, 1992; Yells et al., 1992; Normandin and Murphy, 2011), the medial preoptic area (MPOA) (Marson, 2004) and the paraventricular nucleus of the hypothalamus (Marson and McKenna, 1990, 1994; Murphy and Hoffman, 2001; Normandin and Murphy, 2011). Moreover, emissions or ejaculations as observed in the *ex-copula* paradigm are not dependent on the spinal ejaculation generator (Staudt, 2011) as lesions of the LSt cells did not block sexual reflexes using this paradigm. While, in contrast, intact LSt cells were pivotal for ejaculatory reflexes as induced in three accepted paradigms for ejaculation (Staudt, 2011), including DPN or urethrogenital stimulation, and pharmacological activation of D3 receptors in spinal transected anesthetized male rats (Staudt, 2011) as well as for ejaculatory behavior in freely mating male rats (Truitt and Coolen, 2002).

In conclusion, these data confirm that GRP receptor activation in the lumbosacral spinal cord is required for sensory stimulation-induced ejaculation reflex in anesthetized, spinalized male rats.

Furthermore, GRP appears to act by activating receptors in the outputs of the LSt cells. However, GRP receptor expression in the target regions of the LSt cells remains to be elucidated. In addition, the roles of other neuropeptides expressed in the LSt axons, including enkephalin, galanin and cholecystokinin in the control of ejaculatory behavior and reflexes remain to be investigated.

Chapter 3: Activation of mu and delta opioid receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats

3.1 Introduction

Ejaculation is a complex physiological phenomenon that is highly rewarding and culminates in the ejection of seminal contents from the urethral meatus (Marberger, 1974; McKenna, 1999). Ejaculation involves two phases, emission and expulsion. During the emission phase, seminal fluids are secreted from the accessory sex glands, including the prostate, seminal vesicles and vas deferens and the bladder neck and external urethral sphincter shut to avert retrograde ejaculation. During the expulsion phase, rhythmic contractions of the striated perineal muscles, and the bulbocavernosus muscle (BCM) in particular, forcefully expel semen from the urethral meatus (Bohlen et al., 1980; Gerstenberg et al., 1990; Holmes et al., 1991; Holmes and Sachs, 1991). Ejaculation is a reflex controlled by a spinal ejaculation generator in the lumbosacral spinal cord (Pescatori et al., 1993; McKenna, 1999). The spinal ejaculation generator (Marberger, 1974; McKenna, 1999; Carro-Juarez and Rodriguez-Manzo, 2007) is thought to control ejaculation by closely coordinating sympathetic, parasympathetic, and motor components to initiate emission and expulsion (Coolen et al., 2004; Allard et al., 2005; Coolen, 2005; Staudt et al., 2010; Kozyrev et al., 2012). In addition, it is postulated that the spinal ejaculation generator triggers ejaculation by integrating sensory inputs conveyed by the dorsal penile nerve (DPN), the sensory branch of the pudendal nerve, during copulation with autonomic and motor outflow. The spinal ejaculation generator consists of a set of interneurons in lamina 10 and the medial portion of lamina 7 of lumbar segments 3 and 4 (L3-4) that are integral in the control of ejaculation. These lumbar interneurons are referred to as lumbar spinothalamic (LSt) cells due to their anatomical position in the lumbar spinal cord and projections to the subparafascicular parvocellular nucleus

of the thalamus (Ju et al., 1987; McKenna, 1997; Coolen et al., 2003; Truitt et al., 2003). One proposed role of LSt cells is to transform sensory signals associated with the summation of sexual activity into motor or secretory outputs (Truitt and Coolen, 2002). LSt cells display neural activation specifically with ejaculation but not with other components of sexual behavior following copulation or electrical stimulation of the DPN in male rats (Sachs and Garinello, 1979; Pescatori et al., 1993; McKenna, 1997; Coolen et al., 2004). Markers of neural activation in LSt cells with ejaculation include cFos (Truitt et al., 2003) and phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK) (Staudt et al., 2010) and ejaculation is triggered through activation of the mitogen activated protein (MAP) kinase pathway (Staudt et al., 2010) following mating in intact animals or DPN stimulation in anesthetized and spinalized male rats.

Furthermore, LSt cells have axonal projections to LSt target regions (Newton, 1993; Allard et al., 2005; Xu et al., 2006) including preganglionic sympathetic, parasympathetic and motor neurons in the thoracolumbar and lumbosacral spinal cord (Newton, 1993; McKenna, 1997; Sakamoto et al., 2008). LSt cells express several neuropeptides including galanin, cholecystokinin, gastrin releasing peptide (GRP), and enkephalin (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008). Thus, we hypothesize that these neuropeptides act in the LSt target areas to modulate ejaculatory reflexes. Indeed, our laboratory has recently revealed a critical role for GRP in the control of ejaculation in male rats (Kozyrev et al., 2012). Intrathecal infusions of GRP antagonist RC-3095 completely abolished ejaculatory reflexes while intrathecal infusion of GRP agonist GRP^{2029} triggered ejaculatory reflexes in 43-66 % of animals and facilitated ejaculatory reflexes in response to subthreshold frequencies (5-10 Hz) of DPN stimulation in 100 % of male rats (Kozyrev et al., 2012). In the current study we test if the opioid peptide enkephalin is critical for regulation of ejaculation.

A recent study has also identified a critical role for opioids in the control of ejaculatory reflexes in male rats (Carro-Juarez and Rodriguez-Manzo, 2009). Specifically, it was reported that intravenous injections of the opioid agonist morphine blocked ejaculatory reflexes in male rats and that this effect was reversed by systemic pretreatment with the opioid antagonist naloxone (Carro-Juarez and Rodriguez-Manzo, 2009). Furthermore, intravenous naloxone induced the genital ejaculatory motor pattern (Carro-Juarez and Rodriguez-Manzo, 2009). From these results, the authors concluded that endogenous opioids regulate the activity of the spinal ejaculation generator by exerting inhibitory effects. However, the non-specific nature of the opioid agonist and antagonist used in this study as well as the intravenous mode of drug delivery constitute significant confounds in the interpretation of the results. Enkephalin, which is expressed by LSt cells and axons (Nicholas et al., 1999), is an endogenous ligand for both mu (MOR) and delta opioid receptors (DOR) (Cesselin, 1995), and can thus trigger ejaculation by acting on MOR and/or DOR in LSt target regions. Hence, the purpose of the present study was to investigate the individual contributions of MOR and DOR on ejaculatory reflexes induced by electrical stimulation of the DPN in anesthetized and spinalized male rats. Stimulation of the DPN in anesthetized and spinalized animals is a physiologically relevant model (Giuliano et al., 2010), reliably inducing ejaculation in all mammals, including rodents (Pescatori et al., 1993), primates (Herbert, 1973), and men (Wieder et al., 2000). First, it was tested whether enkephalin is required for ejaculatory reflexes in male rats using intrathecal delivery of MOR and DOR antagonists during simultaneous recordings of seminal vesicle (SV) pressure, a marker of emission, and rhythmic contractions of the BCM, a marker of expulsion in anesthetized and spinalized male rats. Next, it was tested whether enkephalin is sufficient to trigger ejaculation in male rats in the absence of DPN stimulation or following subthreshold levels of DPN stimulation

(5-10 Hz) that do not trigger ejaculatory reflexes in male rats in control conditions (Kozyrev et al., 2012) and intrathecal infusions of specific MOR and DOR agonists in the lumbosacral spinal cord during recordings of SV pressure and BCM contractions.

3.2 Materials and Methods

3.2.1 *Animals*

Adult male Sprague Dawley rats (225-250 grams) were acquired from Charles River (Wilmington, MA, USA) and pair-housed in standard housing cages on a 12-hour light/dark cycle with lights off at 9 pm. Food and water were available ad libitum. All procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and conformed to the guidelines outlined by the National Institutes of Health.

3.2.2 Experimental Designs

3.2.2.1 *Surgical procedures*

Male rats were anesthetized with urethane (1.5 g/kg, i.p.) and a laminectomy was carried out between the sixth and the eighth thoracic spinal segments. A complete transaction of the spinal cord was performed at the seventh thoracic spinal segment. Subsequently, animals were prepared

for electromyographic (EMG) recordings of bulbocavernosus muscle (BCM) bursting and seminal vesicle pressure (SVP); indicators of expulsion and emission components of ejaculations respectively (Pescatori et al., 1993; Clement et al., 2008). The BCM and the dorsal penile nerve (DPN) were surgically exposed and the surrounding connective tissue was removed. Silver recording electrodes, connected to the PowerLab/7SP Data Acquisition System (AD Instruments, Inc., Colorado Springs, CO, USA) were placed bilaterally into the BCM and a ground electrode was inserted into the muscle of the right thigh. In a subset of animals, the right seminal vesicle was exposed by coeliotomy and a pressure catheter (AD Instruments Inc., model number: SPR-671(1.4 F, Single, Straight, 15 cm, Ny) connected to a catheter interface cable (AD Instruments Inc., model number: AEC-IOD) and attached to a Bridge AMP (AD Instruments Inc.), was gently guided into the lumen of the SV and fixed in place. Finally, for stimulation of the DPN, a bipolar stimulating electrode connected to the SD9 Square Pulse Stimulator (Grass Technologies, West Warwick, RI, USA) was arranged directly above the DPN. 2-4 Hours prior to any of the experiments, the DPN was stimulated (4 V at 60 Hz square wave pulses of 1 ms duration, for total of 10 seconds) in order to verify the completeness of the spinal cord transection. These established stimulation parameters consistently trigger rhythmic bursting of the BCM, indicative of the expulsion phase of ejaculation, in all animals (Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012).

3.2.2.2 *Intrathecal infusion of opioid receptor antagonists*

Pharmacological experiments began at least two hours following transection of the spinal cord in order to let the potential acute effects of spinal cord transection to diminish. A minor incision was performed in the dura mater at the site of the laminectomy and a polyethylene catheter (Caly-Adams PE-10, Parsippany, NJ, USA) was guided into the subarachnoid space until the open end reached the mid-lumbar levels, such that saline or drug infused through the catheter would bathe the entire lumbosacral spinal cord. First, 10 µL of saline was delivered (at 10 µl/minute rate) and BCM EMG activity was recorded for 25 minutes. Next, the DPN was stimulated at 30 Hz and 60 Hz (4 V, square wave pulses of 1 ms duration, for total of 10 seconds) in a counterbalanced manner, with 5-minutes between the stimulations (testing trial 1; control trial). BCM EMG and SVP were recorded. An hour later, the procedure was performed again in the same animals, which were now administered a 10 µL infusion of opioid receptor antagonists, after which the DPN was stimulated at 30 Hz and 60 Hz in a counterbalanced manner, and BCM EMG activity and SVP were recorded (testing trial 2; drug trial). We previously established that repeated stimulations and infusions of saline did not affect BCM EMG bursting in response to or without DPN stimulation (Kozyrev et al., 2012), therefore all animals received drug treatment in testing trial 2 (drug trial). In experiment 1, one of two doses of the mu opioid receptor antagonist H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) 0.3 nmol (N = 7) or 3 nmol (N = 8) were infused and BCM EMG was recorded. In experiment 2, the most effective dose of CTOP was administered, 3 nmol, and BCM EMG and SVP were recorded simultaneously (N = 6). In experiment 3, one of three doses of the delta opioid receptor antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP), [Bachem Americas, Inc. Torrance, CA, USA], 0.4 nmol (N = 6), 4 nmol (N = 7), or 40 nmol (N = 6) were infused and BCM EMG and SVP were recorded.

3.2.2.3 Intrathecal infusion of opioid receptor agonists

Four separate groups of male rats was anesthetized, spinalized, and prepared as described above. In testing trial 1: control trial, all male rats received 10 µL intrathecal infusions of 0.9% saline (N = 30) and BCM EMG and SVP were recorded for 10 minutes after infusion. Next, the DPN was stimulated at 5 HZ, 10 HZ, 30 HZ and 60 HZ in a randomized and counterbalanced order, and BCM EMG and SVP were recorded for the duration of 90 seconds following stimulation (testing trial 1; control trial). We previously determined that repeated stimulation using these parameters did not affect BCM EMG (Kozyrev et al., 2012). One hour following the final DPN stimulation, the procedure was repeated in the same groups of animals, which now received 10 µL intrathecal infusions of one of two doses of mu opioid receptor agonist (D-Ala2, N-Me-Phe4, glycinol5) – Enkephalin [DAMGO 0.1 nmol, N = 7; 10 nmol, N = 8; (Bachem Americas, Inc. Torrance, CA, USA)] or one of two doses of the delta opioid receptor agonist [DAla2] Deltorphin II, 1.3 nmol, N = 8; 13 nmol, N = 8; [American Peptide Co, Sunnyvale, CA, USA] and BCM EMG and SVP were recorded for 10 minutes after infusion. Finally, the DPN was stimulated at 5, 10, 30, and 60 HZ in a counterbalanced manner as described above and previously (testing trial 2; drug trial; (Kozyrev et al., 2012)).

3.2.3 *BCM EMG and SVP Analysis*

BCM EMG and SVP recordings were analyzed using methods we previously described (Kozyrev et al., 2012). Briefly, data were analyzed for the 25 or 10 minutes following the infusion of the antagonist or agonist, respectively. In addition, EMG and SVP were analyzed for 90 seconds following each DPN stimulation, which is the time span of a characteristic DPN-stimulation induced ejaculatory reflex in control male rats (Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012). The numbers of events, bursts, and SVP increases were analyzed (Kozyrev et al., 2012) using LabChart 7.35 (AD Instruments Inc.). An event is defined as an increase in EMG above baseline; a burst is a cluster of EMG events (10 or more events) without return to baseline; and an increase in SVP is counted each time it occurs concurrently with BCM EMG activity. These definitions were previously determined (Kozyrev et al., 2012). For the antagonist experiments, the effects of antagonist treatments on the numbers of events, bursts and SVP increases were compared both within animals (within the same drug dose, between testing trial 1: control trial and testing trial 2: drug trial) and between groups (between different doses within testing trial 1 or 2: drug trial), separately for the 30 and 60 Hz stimulations, using two-way repeated Anova (factors: Testing trial and Drug dose) and Holm-Sidak post hoc tests. For the agonist experiments, numbers of events, bursts and SVP increases were compared within animals (between testing trial 1: control trial and testing trial 2: drug trial) and between treatment groups (between different doses within testing trial 1 or 2: drug trial), separately for each stimulation setting (following infusion without stimulation, 5, 10, 30, 60 Hz), using a two-way repeated Anova (factors: Testing trial and Drug Dose) and Holm-Sidak post hoc tests. A 95% confidence level was used for all tests.

3.2.4 Immunohistochemistry Experiments

3.2.4.1 *DPN Stimulation-induced pERK after intrathecal opioid receptor antagonist treatment*

To verify that mu opioid receptor antagonist CTOP and delta opioid receptor antagonist TIPP did not prevent the activation of LSt cells, expression of phosphorylation of ERK (pERK) in LSt cells was examined following stimulation of the DPN (Staudt et al., 2010; Kozyrev et al., 2012). Male rats (N = 9) were anesthetized and spinalized as described above. Two hours after spinalization, the DPN and BCM were exposed and vehicle (saline, N = 4) or drug (CTOP 3 nmol; N =3, TIPP 40 nmol; N = 2, Bachem Americas, Inc. Torrance, CA, USA) were infused in a volume of 10 µL intrathecally as described above. Following a 25-minute infusion, the DPN was stimulated at 30 Hz and BCM EMG activity and SVP were recorded for 90 seconds. Five minutes following DPN stimulation, animals were perfused transcardially with 5 mL 0.9% saline solution and 500 mL of 4% paraformaldehyde in 0.1 M PB. Spinal cords were removed and postfixed for 1 hour in the same fixative and then transferred into a cryoprotective solution (20% sucrose in 0.1 PB with 0.01% sodium azide) until further processing for immunohistochemical visualization of pERK and galanin. The stimulation parameters and time of perfusion were previously shown to be optimal for detection of pERK expression in LSt cells (Staudt et al., 2010; Kozyrev et al., 2012). Furthermore, previous experiments have shown that spinalization and the surgical exposure of the DPN and the BCM in the absence of electrical stimulation of the DPN do not induce pERK expression in LSt cells (Staudt et al., 2010), hence negative control groups were not included in this experiment.

3.2.5 Immunohistochemistry: Galanin/pERK dual fluorescence

Spinal cords were cut using a freezing microtome (Thermo Fisher Scientific, Walldorf, Germany) into 12 parallel series of 35 um coronal sections in cryoprotectant solution (30% sucrose, 30% ethylene glycol in 0.1 M PB with 0.01% sodium azide) and stored at -20°C until further processing. Free floating sections of the lower thoracic, lumbar, and sacral spinal levels were thoroughly rinsed in 0.1 M saline buffered sodium phosphate (PBS) between incubations and blocked with 1% H_2O_2 for 10 minutes before incubation. All antibody incubations were performed in incubation solution containing 0.1% bovine serum albumin and 0.4% Triton X-100 (BP151-500, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) in PBS at room temperature with gentle agitation. Spinal cord sections were incubated overnight with rabbit anti-pERK (1:1,000; overnight; Cell Signaling #26 L) and with biotinylated goat anti-rabbit for one hour (1:500; Vector Laboratories, Burlingame, CA, USA), avidin horseradish peroxidase complex (ABC-elite, 1:1,000 in PBS; 1 hour;Vector Laboratories), biotinylated tyramine (BT; 1:250 in PBS containing 1 uL/mL of 3% H_2O_2 for 10; NEL700/700A; PerkinElmer Life Sciences, Boston, MA, USA), and Alexa 488-conjugated streptavidin (1:100 in PBS, 30 minutes (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Next, sections were incubated with rabbit anti-galanin (1:3,000; overnight, T-4334; Bachem, Torrance, CA, USA) and Alexa 555-conjugated goat anti-rabbit for 30 minutes in PBS (1:100; Jackson Immuno-Research Laboratories) and mounted on plus charged slides, cover-slipped with gelvatol and stored in the dark at 4°C. For all immunohistochemistry procedures, the omission of primary antibodies

resulted in a complete loss of signal at specific wavelengths and all primary antibodies have been previously validated (Truitt et al., 2003; Staudt et al., 2010, 2011; Kozyrev et al., 2012)

3.2.6 Data Analysis

3.2.6.1 *pERK Expression in LSt Cells*

pERK expression in LSt cells was analyzed on a DM5000B Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Specifically, all lumbar cells expressing galanin-ir were analyzed for expression of pERK, as cytopplasmic galanin-ir is exclusively expressed in LSt cells. Data are expressed as the mean percentages of LSt cells that display pERK for each animal. The group means were calculated and compared between animals that received an infusion of CTOP (3 nmol) or TIPP (40 nmol) and saline-treated controls, using Student t-tests with 95% confidence levels.

3.3 Results

3.3.1 *Mu Opioid Receptor Antagonist CTOP Suppressed Ejaculatory Reflexes*

Mu opioid receptor antagonist CTOP significantly decreased DPN stimulation-induced ejaculatory reflexes at both 30 and 60 Hz stimulation frequencies as reflected in the reduced numbers of BCM events, bursts, and SVP increases. There were main effects of testing trial on

the numbers of BCM events for both 30 Hz ($F(1, 29) = 88.436$; $P < 0.001$; Figure 11A) and 60 Hz stimulation frequencies ($F(1, 29) = 62.013$; $P < 0.001$; Figure 11A). Post hoc analyses revealed that animals treated with either dose of CTOP during the second, drug trial had significantly decreased BCM events in response to 30 or 60 Hz DPN stimulation compared to their DPN stimulation induced BCM events following saline treatment in their first control trial (trial 1; 60 Hz: P <0.001 (0.3 nmol); P = 0.009 (3 nmol); 30 Hz: P = <0.001 (0.3 nmol); P <0.001 (3 nmol); Figure 11A). There were no significant differences between the two groups treated with the lower and higher doses of CTOP for each stimulation frequency.

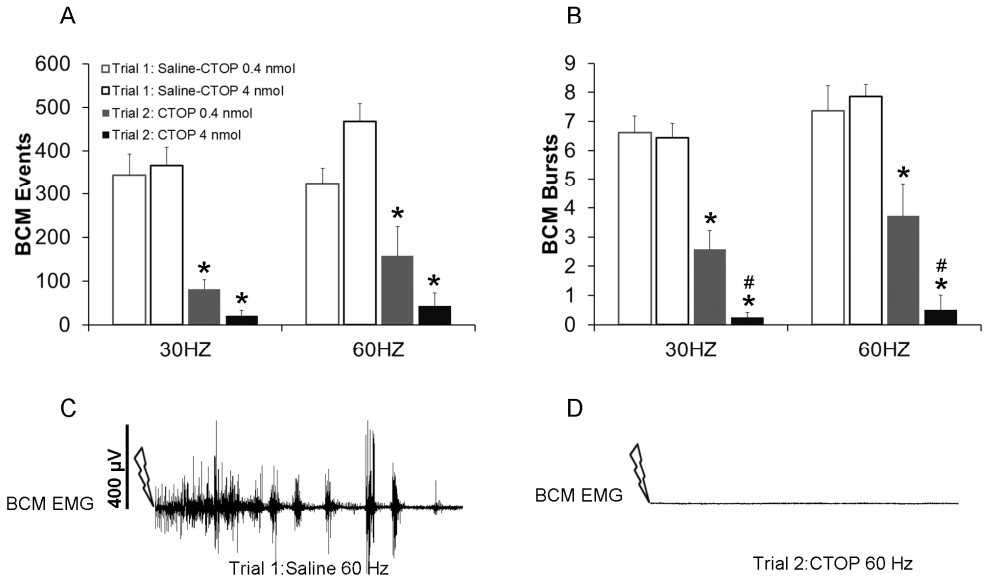

Figure 11 Effects of CTOP infusions on the numbers of BCM events and bursts induced by DPN stimulation in male rats.

Quantitative analysis of BCM events (A) and bursts (B) in response to 30 and 60 Hz DPN stimulations following infusions of saline in trial 1 (control trial; white bars) or one of two doses of CTOP (0.4 or 4 nmol) in trial 2 (drug trial; filled bars). Data are presented as Mean SEM. * denotes significant differences from trial 1 within the same treatment group, while # indicates significant differences between treatment groups within the same testing trial. Representative EMG traces (90 seconds duration) following 60 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (**C**: control trial) and CTOP (**D**: same animals as in **C**).

Similarly, there were main effects of testing trial on the numbers of BCM bursts for both 30 Hz ($F(1, 29) = 108.732$; $P < 0.001$; Figure 11B) and 60 Hz ($F(1, 29) = 123.431$; $P < 0.001$; Figure 11B) stimulation frequencies. In addition, there were significant interactions between testing trial and drug dosage for both stimulation frequencies (30Hz: $F(1.29)=11.83$; $P = 0.023$; 60 Hz: $F(1,29) = 23.87$; $P = 0.016$). CTOP during trial 2 (drug trial) significantly reduced BCM bursts compared to trial 1 (control trial) with saline treatment (30 Hz: $P < 0.001$ [0.3 nmol], 30 Hz: $P < 0.001$ [3 nmol], 60 Hz: $P < 0.001$ [0.3 nmol] 60 Hz: $P < 0.001$ [3 nmol]; Figure 11B, C, D). There was an effect of dose on the numbers of BCM bursts in testing trial 2: drug trial. Specifically, there were significantly fewer BCM bursts following the higher dose compared to the lower dose of CTOP (30 Hz: $P = 0.003$ [3 nmol]; Figure 11B, 60 Hz: $P = 0.006$ [3 nmol]; Figure 11B). Finally, during the first trial, there were no significant differences between groups in the numbers of BCM events or bursts.

In a separate group of males, CTOP (3 nmol) also severely impaired the emission component of ejaculation as indicated by significantly decreased numbers of SVP increases (Figure 12). There were main effects of testing trial on the numbers of SVP increases ($F(1, 23) = 120.143$; $P < 0.001$; Figure 12C, D, E), as well as BCM events ($F(1, 23) = 513.688$; $P < 0.001$; Figure 12A, D, E), and BCM bursts ($F(1, 23) = 118.433$; $P < 0.001$; Figure 12B, D, E). Post hoc analyses revealed that CTOP (3 nmol) in trial 2 (drug trial), male rats displayed significantly fewer numbers of SVP increases, BCM events and bursts compared to control trial 1, for both 30 Hz (events: $P < 0.001$; bursts: $P < 0.001$; SVP: $P < 0.001$; Figure 12A-E) and 60 Hz (events: $P < 0.001$; bursts: $P < 0.001$; SVP: $P < 0.001$; Figure 12A-E). Overall, these results confirm the hypothesis that intrathecal CTOP suppresses both the emission and expulsion components of ejaculation and that activation of mu opioid receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats.

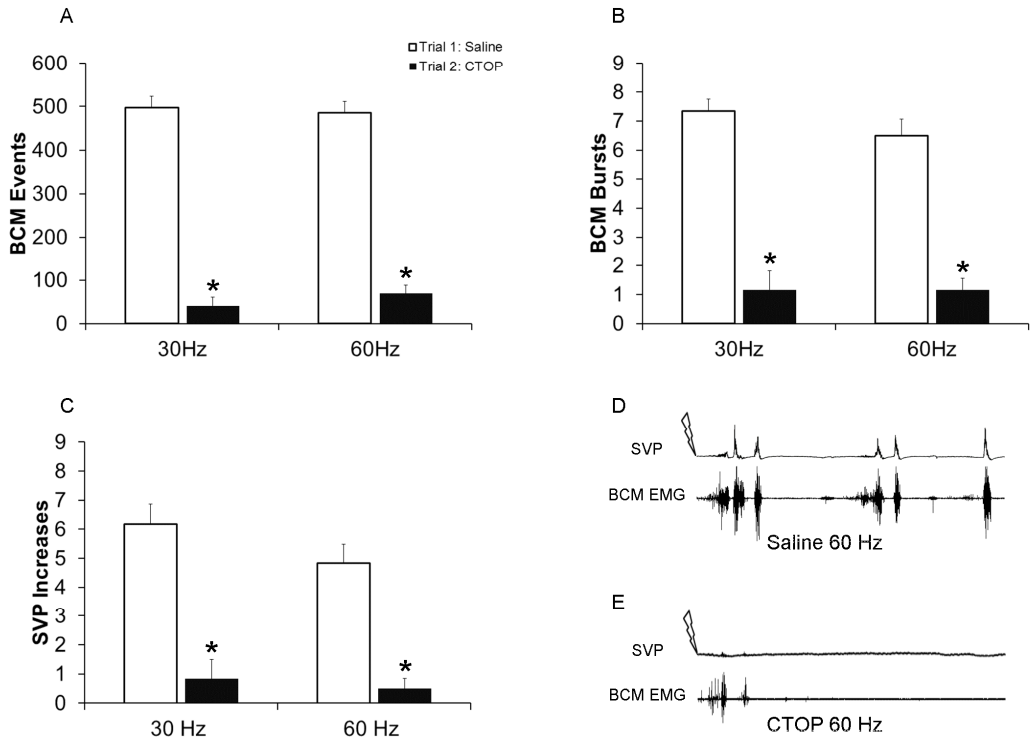

Figure 12 Effects of CTOP on the numbers of BCM bursts and SVP increases induced by DPN stimulation in male rats.

Quantitative analyses of BCM events **(A)** bursts, **(B)** and SVP increases **(C)** in response to 30 and 60 Hz DPN stimulation following infusions of saline in trial 1 (control trial; white bars) or CTOP (4 nmol) in trial 2 (drug trial; filled bars). Data are presented as Mean SEM. * denotes significant differences from trial 1 (control trial). EMG and concurrent SVP traces of 90 seconds duration following 60 Hz DPN stimulation (arrow) after an intrathecal infusion of saline **(D control trial)** and CTOP **(E same animal as in D)**.

3.3.2 *Delta Opioid Receptor Antagonist TIPP Suppressed Ejaculatory Reflexes*

Delta opioid receptor antagonist TIPP significantly decreased DPN stimulation-induced ejaculatory reflexes at both 30 and 60 Hz stimulation frequencies as reflected in the reduced numbers of BCM events, BCM bursts, and SVP increases. There were main effects of testing trial on the numbers of BCM events for both 30 Hz ($F(1,37) = 20.131$; $P < 0.001$; Figure 13A) and 60 Hz stimulation frequencies ($F(1, 37) = 26.666$; $P < 0.001$; Figure 13A), main effect of drug dosage for 60 Hz ($F(2,37)=6.18$; $P = 0.01$) and significant interactions between testing trial and drug dosage for both 30 and 60 Hz (30 Hz: ($F(2,37) = 5.861$; $P = 0.012$; 60 Hz ($F(2,37) = 4.349$; $P = 0.031$). Animals treated with the higher (4 nmol) and highest (40 nmol) but not the lower (0.4 nmol) dose of TIPP during the second, drug trial had significantly decreased BCM events in response to 30 Hz ($P = 0.012$: 4 nmol; $P = <0.001$**:** 40 nmol; Figure 13A) and 60 Hz (60 Hz: $P = 0.014$, 4 nmol*;* $P <0.001$, 40 nmol; Figure 13A) stimulation frequencies compared to saline treatment in trial 1 (control trial). In addition, there was an effect of dosage, as animals treated with the highest dose of TIPP (40 nmol) in the second trial (drug trial) displayed significantly fewer BCM events compared to those treated with the lower dose of TIPP (0.4 nmol) for both the 30 Hz ($P = 0.004$**,** 40 nmol; Figure 13A) and 60 Hz ($P < 0.001$**,** 40 nmol; Figure 13A) stimulation frequencies and compared to animals treated with the middle dose of TIPP (4 nmol) in response to the 60 Hz ($P = 0.003$**,** 4 nmol; Figure 13A) stimulation (with a trend for the 30 Hz ($P = 0.075$**,** 4 nmol; Figure 13A)).

Similarly, numbers of BCM bursts were reduced for both 30 Hz ($F(1, 37) = 23.209$; $P < 0.001$; Figure 13B) and 60 Hz ($F(1, 37) = 21.298$; $P < 0.001$; Figure 13B) stimulation frequencies. Males treated with the middle (4 nmol) and the highest (40 nmol) but not the lower

(0.4 nmol) dose of TIPP during trial 2 (drug trial) had significantly fewer BCM bursts compared to trial 1 (control trial) with saline treatment (30 Hz: $P = 0.004$ [4 nmol]; Figure 13B, 30 Hz: $P < 0.001$ [40 nmol]; Figure 13B, 60 Hz: $P = 0.018$ [4 nmol]; Figure 13B, 60 Hz: $P < 0.001$ [40 nmol]; Figure 13B, D, E).

Finally, TIPP reduced SVP increases and there were main effects of testing trial for both 30 Hz ($F(1, 37) = 19.041$; $P < 0.001$; Figure 13C) and 60 Hz ($F(1, 37) = 9.059$; $P = 0.008$; Figure 13C) stimulation frequencies. Males treated with the highest (40 nmol) but not the middle (4 nmol) or lower (0.4 nmol) doses of TIPP during trial 2 (drug trial) had significantly fewer SVP increases compared to trial 1 (control trial) with saline treatment (30 Hz: $P < 0.001$ [40 nmol], 60 Hz: $P = 0.002$ [40 nmol]; Figure 13C, D, E).

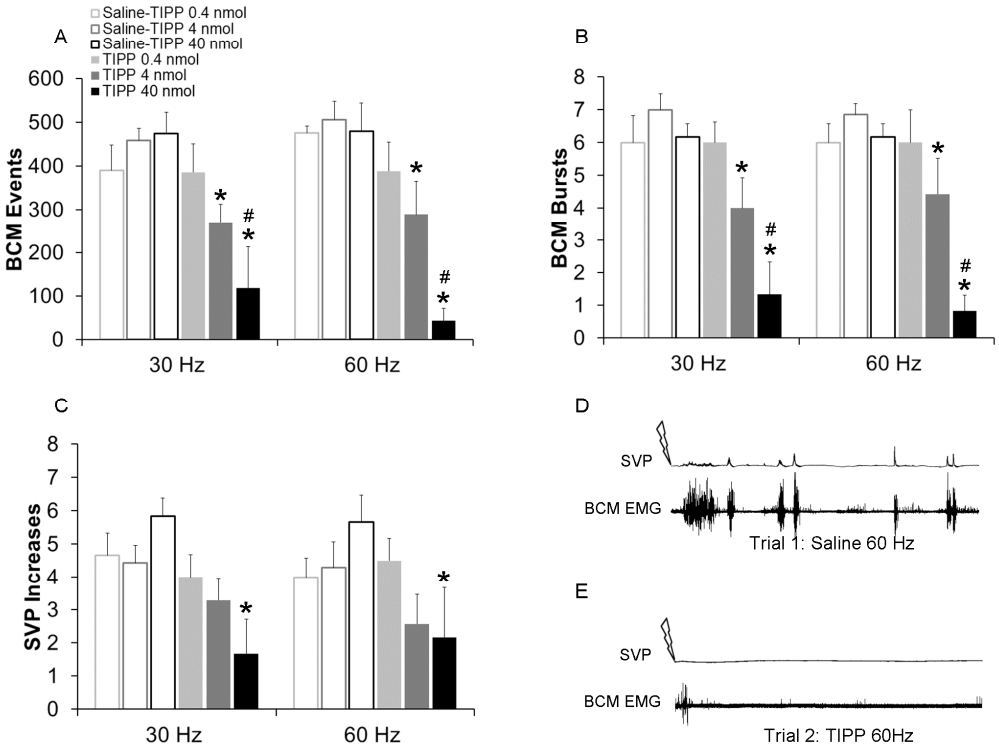
A
Saline-TIPP 0.4 nmol
Saline-TIPP 4 nmol
Saline-TIPP 40 nmol
TIPP 0.4 nmol
TIPP 4 nmol
TIPP 40 nmol
BCM Events
600
500
400
300
200
100
0
30 Hz
60 Hz
B
BCM Bursts
8
7
6
5
4
3
2
1
0
30 Hz
60 Hz
C
SVP Increases
8
7
6
5
4
3
2
1
0
30 Hz
60 Hz
D
SVP
BCM EMG
Trial 1: Saline 60 Hz
E
SVP
BCM EMG
Trial 2: TIPP 60Hz

Figure 13 Effects of TIPP infusions on the numbers of BCM events, bursts and SVP increases triggered by DPN stimulation in male rats

Quantitative analyses of BCM events (**A**), bursts (**B**) and SVP increases (**C**) in response to 30 and 60 Hz DPN stimulation following infusions of saline in trial 1 (control trial; white bars) or one of three doses of TIPP (0.4, 4 or 40 nmol) in trial 2 (drug trial; filled bars). Data are presented as Mean SEM. * denotes significant differences from trial 1 within the same treatment group, while # indicates significant differences between treatment groups within the same testing trial. EMG traces of 90 seconds duration following 60 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (**D**: control trial) and TIPP (**E**: same animal as in **C**).

There were no significant differences for any of the parameters between the two groups of animals during trial 1 (control trial) when both groups received saline, indicating that group differences in trial 2 were caused by drug treatment.

3.3.3 *CTOP and TIPP did not affect DPN Stimulation-Induced pERK in LSt Cells*

In order to test that mu opioid receptor antagonist CTOP and delta opioid receptor antagonist TIPP suppressed BCM bursting and SV pressure via actions in LSt target areas rather than by preventing activation of LSt cells, DPN stimulation-induced activation of LSt cells was examined. In control treated males, DPN stimulation induced pERK in 95.4 ± 2.7 % of LSt cells, as reported previously (Staudt et al., 2010, 2011; Kozyrev et al., 2012). Neither CTOP nor TIPP infusions prior to DPN stimulation affected LSt activation (CTOP: 96.5 ± 3.5 % (Figure 14); TIPP: 100 ± 0 % of LSt cells).

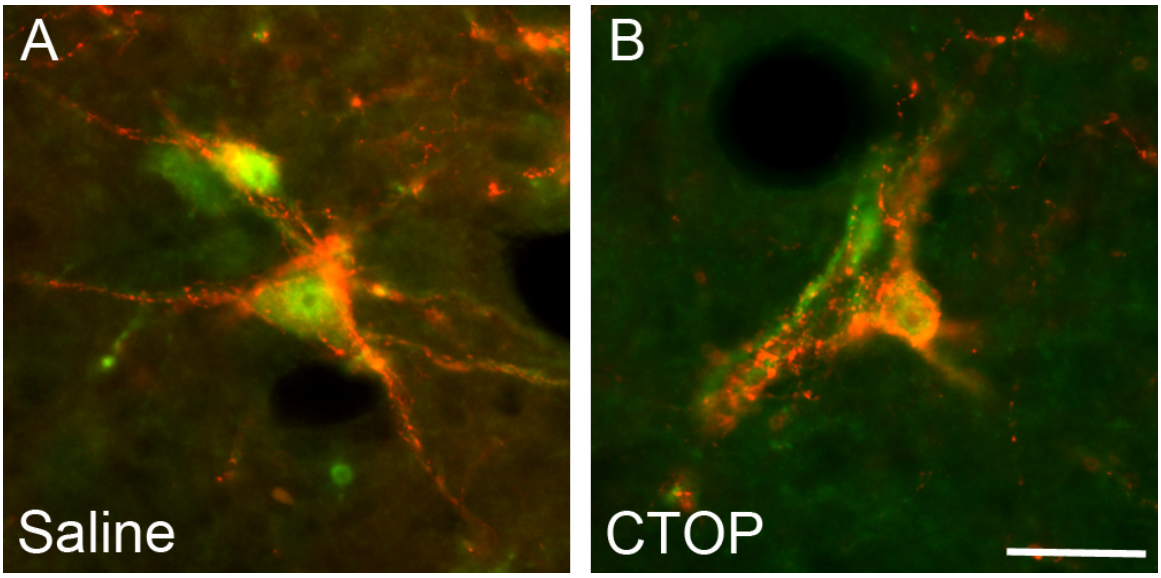

Figure 14 CTOP infusions did not prevent the activation of LSt cells induced by DPN stimulation in male rats.

Confocal images (1 µm optical sections) demonstating pERK expression in LSt cells induced by DPN stimulation following infusions of saline **(A)** and the mu opioid receptor antagonist CTOP **(B)**. The pattern of pERK labeling is characteristic of all CTOP and saline-treated animals. Scale bar indicates 20 µm.

3.3.4 *Mu Opioid Receptor Agonist DAMGO Triggered and Facilitated Ejaculatory Reflex*

The mu opioid receptor agonist DAMGO triggered BCM events, bursts and SVP increases in the absence of DPN stimulation. In addition, DAMGO dose-dependently facilitated BCM events, bursts and SVP increases induced by subthreshold levels of DPN stimulation (5-10 Hz). For the numbers of BCM events, there were main effects of testing trial for all stimulation frequencies (Infusion (0 Hz: ($F(1, 29) = 6.400$; $P = 0.025$); 5 Hz: ($F(1, 29) = 11.407$; $P = 0.005$); 10 Hz: ($F(1, 29) = 41.312$; $P < 0.001$); 30 Hz ($F(1, 29) = 42.443$; $P < 0.001$); and 60 Hz ($F(1, 29)$

= 73.256; $P < 0.001$); Figure 15A). Moreover, there were effects of drug dosage (5 Hz: (F(1, 29) = 8.637; $P = 0.012$), 10 Hz: (F(1, 29) = 29.005; $P < 0.001$) and 30 Hz (F(1, 29) = 4.763; $P = 0.048$) DAMGO-induced BCM events in the absence of DPN stimulation, but only following the higher dose (10 nmol) and in 100% of males (Infusion: $P = 0.009$ compared to saline in trail 1; $P = 0.024$ compared to lower dosage in trial 2; Figure 15A). DAMGO treatment also induced BCM events following subthreshold DPN stimulation; but this effect was only observed with the lower dosage (0.1 nmol) and not the higher dose (10 nmol) (5-10 Hz: $P < 0.001$; compared to saline treatment in trial 1; 5-10 Hz: $P < 0.001$ compared to higher dosage in trial 2; Figure 15A). In contrast, DAMGO reduced numbers of BCM events following 30 and 60 Hz DPN stimulation frequencies, which reliably triggered BCM activity following saline in trial 1 (Figure 15A; 30Hz: $P < 0.001$, 10 nmol compared to saline in trial 1 and compared to lower dosage in trial 2; 60 Hz: $P < 0.001$, 10 nmol; $P < 0.001$, 0.1 nmol; compared to saline in trial 1).

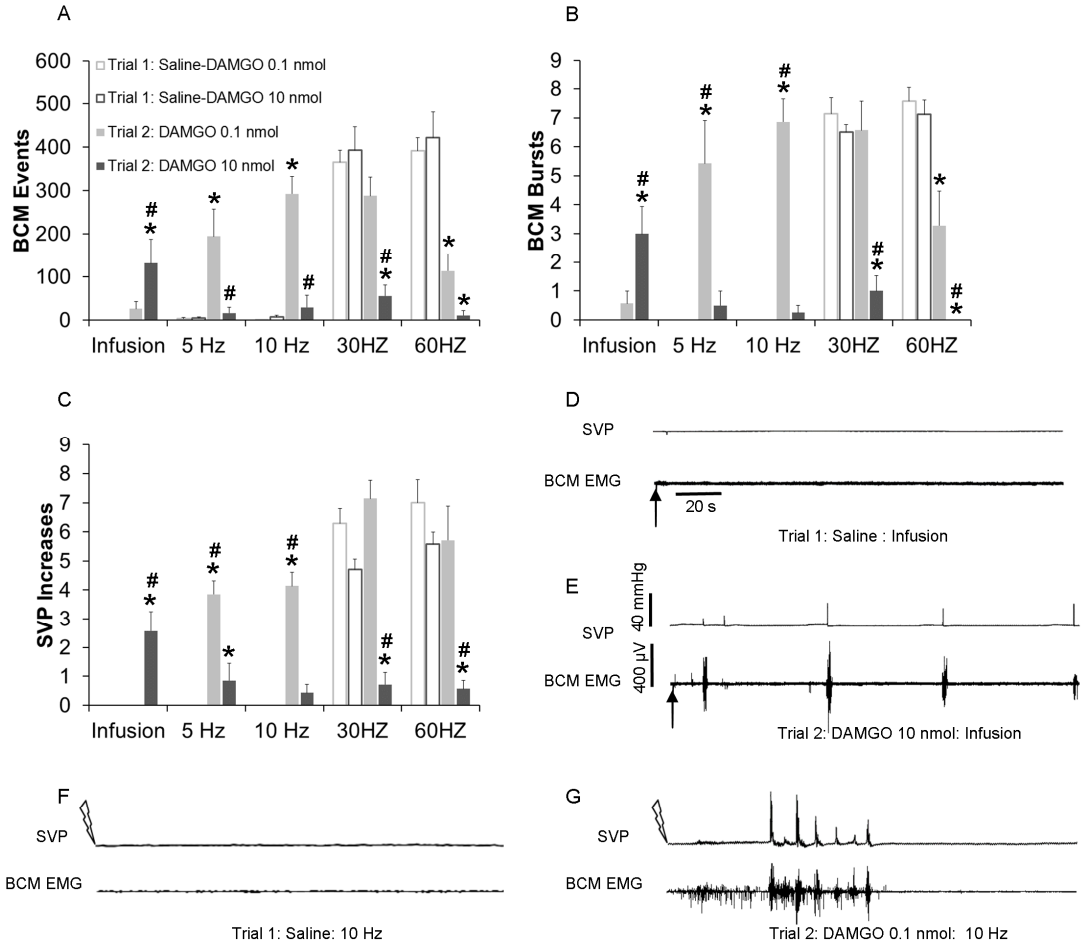

Figure 15 Effects of DAMGO infusions on the numbers of BCM events, bursts and SVP increases in male rats.

Quantitative analyses of BCM events **(A),** bursts **(B)** and SVP increases **(C)** in response to infusion, 5 Hz, 10 Hz, 30 Hz and 60 Hz DPN stimulation following intrathecal infusions of saline in trial 1 (control trial; white bars) or one of two doses of DAMGO (0.1 or 10 nmol) in trial 2 (drug trial; filled bars). Data are presented as Mean SEM. Note absence of white bars for infusions, 5, and 10 Hz, as these stimulation frequencies do not trigger BCM events, bursts, nor SVP increases in control conditions. * denotes significant differences from trial 1 within the

same treatment group, while # indicates significant differences between treatment groups within the same testing trial. Representative EMG traces (180 seconds duration) following an intrathecal infusion of saline **(D: control trial)** and DAMGO **(E: 10 nmol: Same animals as in D)** in the absence of DPN stimulation. EMG and concurrent SVP traces (90 seconds duration) following 10 Hz DPN stimulation (arrow) after an intrathecal infusion of saline **(F: control trial)** and DAMGO **(G: 0.1 nmol: same animal as in F)**.

The effects of DAMGO on BCM bursts were nearly identical to those described above for BCM events with main effects of testing trial and drug dosage for all stimulation frequencies except 30 Hz (Figure 15B; Testing trial: Infusion, 0 Hz: ($F(1, 29) = 10.726$; $P = 0.006$; 5 Hz: ($F(1, 29) = 16.136$; $P = 0.001$; 10 Hz: ($F(1, 29) = 80.755$; $P < 0.001$; and 60 Hz ($F(1, 29) = 78.692$; $P < 0.001$); Drug dosage: and main effects of drug dosages: Infusion: $F(1, 29) = 4.960$; $P = 0.044$; 5 Hz: ($F(1, 29) = 11.152$; $P = 0.005$; 10 Hz: ($F(1, 29) = 69.792$; $P < 0.001$; and 60 Hz ($F(1, 29) = 8.074$; $P = 0.014$). Post hoc tests showed that DAMGO induced BCM bursts in the absence of DPN stimulation in males treated with the higher dose (10 nmol) but not the lower dose (0.1 nmol; Infusion: $P = 0.001$; compared to saline in trial 1; $P = 0.001$ compared to lower dosage in trail 2). DAMGO also facilitated BCM bursts following subthreshold DPN stimulations, but only with the lower (0.1 nmol) and not the higher (10 nmol) dose (5 Hz: $P < 0.001$; 10 Hz $P < 0.001$; compared to saline in trial 1 or higher dose in trial 2). DAMGO reduced numbers of BCM bursts following 60 Hz DPN stimulation (lower dose (0.1 nmol): $P < 0.001$ compared to saline in trial 1; higher dose (10 nmol): $P < 0.001$ compared to saline in trial 1 and $P = 0.014$ compared to lower dose in trial 2).

Finally, effects of DAMGO on SVP increases mirrored those on BCM activity (Figure 15C). There were main effects of testing trial (Infusion (0 Hz: ($F(1, 27) = 8.168$; $P = 0.014$; 5 Hz: ($F(1, 27) = 28.106$; $P < 0.001$; 10 Hz: ($F(1, 27) = 63.157$; $P < 0.001$; 30 Hz ($F(1, 27) = 6.866$; $P = 0.022$; and 60 Hz ($F(1, 27) = 16.471$; $P < 0.002$; Figure 15C) and of drug dosage (Infusion: ($F(1, 27) = 8.168$; $P = 0.014$; 5 Hz: ($F(1, 27) = 5.807$; $P = 0.033$; 10 Hz: ($F(1, 27) = 31.383$; $P < 0.001$; 30 Hz:($F(1, 27) = 18.103$; $P = 0.001$; and 60 Hz:($F(1, 27) = 9.874$; $P = 0.008$). DAMGO infusions alone, in the absence of DPN stimulation, caused increases in SVP in males treated with the higher dose (10 nmol) but not the lower dose (0.1 nmol) (Infusion: $P < 0.001$; compared to saline in trial 1 or to the lower dose in trial 2). Furthermore, DAMGO increased SVP following subthreshold DPN stimulation (5 Hz: $P < 0.001$, 0.1 nmol; $P = 0.047$, 10 nmol ; 10 Hz: $P < 0.001$; 0.1 nmol) compared to saline treatment in trial 1. The lower dosage of DAMGO was significantly more effective in causing SVP increases than the higher dosage (5 Hz: $P < 0.001$; 10 Hz: $P < 0.001$). Lastly, the higher dose of DAMGO (10 nmol) decreased SVP increases induced by threshold DPN stimulation (30 Hz ($P < 0.001$) and 60 Hz ($P < 0.001$) compared to saline in trial 1 or to the lower dose in trial 2).

During trail 1, there were no significant differences in BCM events, bursts, or SVP increases between groups, indicating that the differences observed in trial 2 were due to the effects of DAMGO.

3.3.5 *Delta Opioid Receptor Agonist Deltorphin Facilitated Ejaculatory reflexes*

In contrast to the effects of DAMGO, Deltorphin did not induce BCM activity or SVP increases in the absence of DPN stimulation (Figure 16). However, deltorphin enhanced the

emission and expulsion components of the ejaculatory reflex following subthreshold DPN stimulation (Figure 16). There were main effects of testing trial on BCM events (5 Hz: ($F(1, 31) = 33.286$; $P < 0.001$; 10 Hz: ($F(1, 31) = 56.543$; $P < 0.001$; 30 Hz ($F(1, 31) = 6.059$; $P = 0.027$; 60 Hz ($F(1, 31) = 5.710$; $P < 0.031$; Figure 16A), BCM bursts (5 Hz: ($F(1, 31) = 121.00$; $P < 0.001$; 10 Hz: ($F(1, 31) = 108.432$; $P < 0.001$; 60 Hz ($F(1, 31) = 14.608$; $P < 0.002$; Figure 16B), and SVP increases (5 Hz: ($F(1, 31) = 52.417$; $P < 0.001$; 10 Hz: ($F(1, 31) = 54.000$; $P < 0.001$; and 60 Hz ($F(1, 31) = 8.590$; $P = 0.011$; Figure 16C), and a significant interaction between testing trial and drug dosage on BCM bursts for 60 Hz ($F(1, 31) = 5.020$; $P = 0.042$) stimulation. Deltorphin II (1.3 nmol or 13 nmol) significantly increased numbers of BCM events, burst and SVP increases following 5 Hz (events: 1.3 nmol: $P < 0.001$; 13 nmol: $P = 0.006$; bursts: 1.3 nmol: $P < 0.001$; 13 nmol: $P < 0.001$; SVP: 1.3 nmol: $P < 0.001$; 13 nmol: $P < 0.001$; Figure 16 A-E) and 10 Hz (events: 1.3 nmol: $P < 0.001$; 13 nmol: $P < 0.001$; bursts: 1.3 nmol: $P < 0.001$; 13 nmol: $P < 0.001$; SVP: 1.3 nmol: $P < 0.001$; 13 nmol: $P < 0.001$; Figure 16 C) stimulation frequencies. Deltorphin did also slightly increase BCM events following 30 Hz, but only after the lower dosage, and without effects on BCM bursts or SVP increases and 60 Hz stimulation frequencies ($P = 0.012$). Finally, the higher dose of deltorphin (13 nmol) reduced BCM activity and SVP increases following 60 Hz DPN stimulation (events: $P = 0.011$; bursts: $P < 0.001$; SVP increases: $P = 0.011$) compared to saline treatment in trial 1. As noted for each of the experiments presented above, there were again no differences in BCM or SVP parameters between groups during the saline control trial 1.

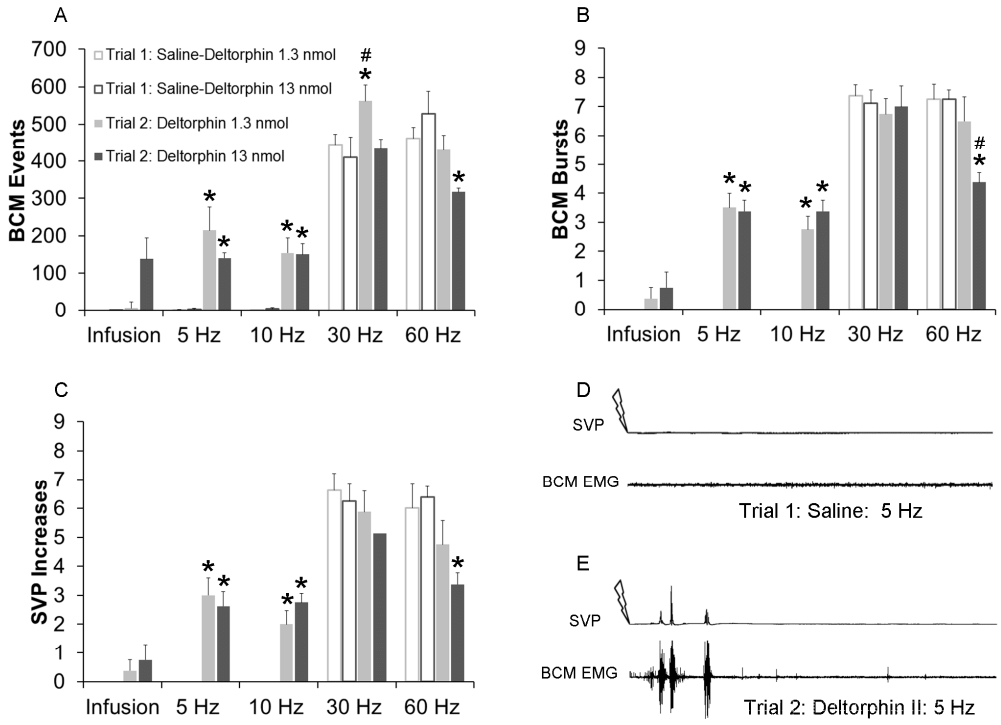

Figure 16 Effects of Deltorphin II on the numbers of BCM events, bursts and SVP increases in male rats.

Quantitative analyses of BCM events **(A)** bursts **(B)** and SVP increases **(C)** following infusion, 5 Hz, 10 Hz, 30 Hz and 60 Hz DPN stimulation after intrathecal infusions of saline in trial 1 (control trial; white bar) or deltorphin II (1.3 or 13 nmol) in trial 2 (drug trial; filled bars). Data are presented as Mean SEM. Note absence of white bars for infusions, 5, and 10 Hz, as these stimulation frequencies do not trigger BCM events, bursts, nor SVP increases in control conditions.* denotes significant differences from trial 1 (control trial), while # indicates significant differences from lower dosage within the same testing trial. EMG and SVP traces (90 seconds duration) following 5 Hz DPN stimulation (arrow) after an intrathecal infusion of saline **(D: control trial)** and deltorphin II **(E: same animal as in D)**.

3.4 Discussion

The results of these experiments support the hypothesis that enkephalin plays a critical role in the control of ejaculation by acting on mu and delta opioid receptors in LSt target areas in the lumbosacral spinal cord. Antagonist studies showed that activation of either MOR or DOR is critical for the emission and expulsion phases of ejaculation in response to sensory stimulation in male rats. In addition, activation of either MOR or DOR by agonist administration at lower dosages facilitated both the emission and expulsion phases of ejaculation following sub-threshold sensory stimulation. Finally, stimulation of MOR, but not DOR was sufficient to trigger ejaculatory reflexes in the absence of sensory stimulation in a large subset (75 %) of male rats.

Together, these findings demonstrate that endogenous activation of MOR or DOR plays a facilitative role in the control of ejaculation. However, infusions of higher dosages receptor agonists for either the MOR or DOR disrupted ejaculation. This finding is in conformity with previous studies which showed that systemic and intravenous morphine exerts inhibitory effects on ejaculatory behavior (Myers and Baum, 1979; Szechtman et al., 1981; Wiesenfeld-Hallin and Sodersten, 1984) and reflexes in male rats (Myers and Baum, 1979; Carro-Juarez and Rodriguez-Manzo, 2009). The effects of morphine have been proposed to be partially mediated via actions on peripheral tissues (Agmo et al., 1992). The current findings demonstrate that the inhibitory effects of high doses of opioid receptor agonists are mediated via central actions within the lumbosacral spinal cord. Noteworthy, our previous studies showed similar inhibitory effects of higher doses of GRP agonists on ejaculatory reflexes, while lower doses of GRP agonists triggered or facilitated ejaculation. Therefore, it is possible that high doses of GRP or opioid

receptor ligands cause mechanisms of desensitization of the G-protein coupled receptors at the LST target sites (Wess, 1997); thereby preventing the ability of DPN stimulation to trigger further action of endogenous ligands on receptor activation. It is also possible that infusion of the higher dose of mu and delta receptor agonists prevented DPN stimulation-induced ejaculatory reflexes by acting on the processing of the sensory inputs relayed via the DPN. The DPN is comprised of A δ and C-fibers (Johnson and Halata, 1991) but the specific contributions of A δ and C-fibers to ejaculation have not been investigated. Intrathecal infusions of opioid agonists inhibit A δ and C-fibers (Yeomans and Proudfit, 1996) raising the possibility that the disruption of ejaculatory reflexes to threshold sensory stimulation after the higher dose but not the lower dose of opioid agonists is mediated by inhibition of A δ and C-fibers in the dorsal horn of the lumbosacral spinal cord. In addition, A δ and C-fiber neurons have cell bodies in the dorsal root ganglia and express mu and delta opioid receptors (Ikoma et al., 2007) indicating that presynaptic inhibition of DPN synaptic transmission may have contributed to disruption of ejaculation. Finally, the primary excitatory neurotransmitter released from A δ and C-fibers is glutamate (Russell, 2007) which activates NMDA receptors in LSt cells to trigger ejaculatory reflexes in male rats (Staudt et al., 2011). Opioids in turn, can inhibit the release of glutamate (Russell, 2007) thereby blocking the activation of LSt cells in the lumbosacral spinal cord.

One of the main findings of the current study was that infusion of the higher dose of DAMGO (10 nmol) was sufficient to trigger ejaculatory reflexes in the absence of sensory stimulation in a large proportion of male rats (75 %). Mu and delta opioid receptors are inhibitory G-protein coupled receptors (Russell, 2007); therefore, it is likely that the mechanism of action whereby endogenous opioids trigger or facilitate ejaculatory reflexes is by means of inhibition of inhibitory neurons in LSt target regions. However, at this time, the localization of

mu or delta opioid receptor in LSt target areas is unknown. Future studies identifying the exact pre- or postsynaptic localization of these receptors are critical to elucidate the mechanisms by which opioids influence ejaculatory function. For example, opioids can exert analgesia through inhibition of inhibitory GABA (γ – aminobuteric acid) neurons (Basbaum and Fields, 1984). Opioids inhibit GABA-mediated synaptic transmission by reducing the likelihood of presynaptic neurotransmitter release (Capogna et al., 1993; Vaughan et al., 1997) and this effect is mediated by voltage-dependent potassium conductance (Vaughan et al., 1997). Mu but not delta opioid receptors are specifically coupled to this potassium conductance (Vaughan et al., 1997) and the subsequent inhibition of GABAergic synaptic transmission; therefore we speculate that this may explain that intrathecal infusions of DAMGO, a selective MOR agonist, but not deltorphin, a selective DOR agonist, triggered ejaculatory reflexes in the absence of DPN stimulation. However, localization of presynaptic MOR on LSt axons has not been confirmed.

A second main finding of the current study was intrathecal infusions of mu or delta opioid antagonists CTOP or TIPP, severely disrupted ejaculatory reflex induced by DPN stimulation that reliably triggered ejaculation under control conditions. Conversely, facilitation of ejaculatory reflexes by infusions of mu or delta receptor agonists DAMGO or deltorphin, was observed after DPN stimulation at sub-threshold parameters that do not trigger ejaculation in control animals. Moreover, antagonist infusions did not disrupt activation of LSt cells, indicating that opioid antagonists acted on neurons downstream from LSt cells, rather than on inputs to LSt cells. These data support our hypothesis that endogenous opioids are primarily acting in LSt target areas to regulate ejaculatory reflexes. As mentioned above, a critical next step is detailed localization of the expression of mu and delta receptors within these LSt target areas. The findings that both mu and delta receptors are involved in mediating ejaculation, indicates that

enkephalin may act on both receptors. Indeed, studies have shown that opioid receptors may form heterodimers (Gomes et al., 2000; Pfeiffer et al., 2003). In addition, activation of delta receptors influences subsequent activation of mu receptors. Indeed, mu and delta opioid receptors demonstrate a high degree of sequence homology and a common opioid receptor binding site within the helical transmembrane core has been proposed to account for the ligand-directed signaling or biased agonism which occurs at opioid receptors (Pradhan et al., 2012).

In addition to forming complex interaction between mu and delta receptors, opioid receptors also form complex interaction with other GPCR. We have previously shown that GRP regulates ejaculatory reflexes, showing findings similar to those described here. Thus, it is possible that a complicated interplay exists between the actions of co-expressed neuropeptides, including enkephalin and GRP (Kozyrev et al., 2012), in LSt cells and axons, such that co-release of multiple neuropeptides may be required to induce ejaculation, but inhibition of each one alone may disrupt ejaculation.

In conclusion, these data support the hypothesis that activation of mu and delta opioid receptors in the lumbosacral spinal cord is required for sensory stimulation-induced emission and expulsion in anesthetized and spinalized male rats. The endogenous opioid ligand enkephalin may act through the activation of both mu and delta receptors in the outputs of the LSt cells. However, precise localization of mu and delta opioid receptor expression in the spinal ejaculation generator is currently unknown and needs to be elucidated in future studies. Furthermore, the roles of other neuropeptides expressed in the LSt cells and axons, including galanin and cholecystokinin, in the control of ejaculation remain an open question. Finally, these data indicate that mu and delta opioid antagonists may be useful for the treatment of ejaculatory

dysfunction, specifically to delay the onset of ejaculation in men afflicted with premature ejaculation.

Chapter 4: Activation of galanin and cholecystokinin receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats

4.1 Introduction

Ejaculation refers to the forceful discharge of seminal contents from the urethral meatus (Marberger, 1974; McKenna, 1999) and consists of two phases: emission and expulsion. Emission refers to the secretion of seminal fluids from the prostate, seminal vesicles and vas deferens and these processes are under the control of parasympathetic preganglionic neurons in the sacral parasympathetic nucleus (SPN) in the upper sacral spinal cord (Hancock and Peveto, 1979b; Nadelhaft and Booth, 1984; Coolen et al., 2004). In addition, emission involves the contraction of the ductus deferes and closure of the bladder neck, in order to prevent retrograde ejaculation, and these processes are under the control of sympathetic preganglionic neurons in the intermediolateral cell column (IML) and dorsal central autonomic nucleus (DCN) in the thoracolumbar spinal cord (Hancock and Peveto, 1979a; Nadelhaft and McKenna, 1987; Baron and Janig, 1991; Coolen et al., 2004). The expulsion component is characterized by rhythmic contractions of the striated perineal muscles, particularly the muscle of the bulbocavernosus (BCM), accompanied by the forceful expulsion of semen from the urethral meatus and is under sympathetic and motor control (Bohlen et al., 1980; Gerstenberg et al., 1990; Holmes et al., 1991; Holmes and Sachs, 1991; Coolen et al., 2004). Motorneurons that control the rhythmic contraction of the BCM are located in the spinal nucleus of the bulbocavernosus (SNB) in the lumbosacral spinal cord in male rats (Schroder, 1980; McKenna and Nadelhaft, 1986; Ueyama et al., 1987). It well established that ejaculation is a spinal reflex and the essential components that control this reflex are localized to the lumbosacral spinal cord (Pescatori et al., 1993; McKenna, 1999). This spinal control center is referred to as the spinal ejaculation generator (Marberger, 1974; McKenna, 1999; Truitt and Coolen, 2002; Truitt et al., 2003; Staudt et al., 2010; Staudt,

2011; Staudt et al., 2011; Kozyrev et al., 2012; Kozyrev, 2013c) and contols ejaculation through a complex coordination of the sympathetic, parasympathetic, and motor components required to facilitate emission and expulsion (Coolen et al., 2004; Coolen, 2005; Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012; Kozyrev, 2013c) with sensory inputs during sexual activity. The latter are primarily relayed via the dorsal penile nerve (DPN) (Coolen et al., 2004; Coolen, 2005; Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012; Kozyrev, 2013c) . The spinal ejaculation generator is composed of lumbar spinothalamic (LSt) cells, in lamina 10 and the medial portion of lamina 7 of lumbar segments 3 and 4, (L3-4). LSt cells are essential for ejaculation (McKenna, 1999; Truitt and Coolen, 2002) which they trigger via projections to DCN, IML, SPN, and SNB (Newton, 1993; McKenna, 1997; Truitt and Coolen, 2002; Coolen et al., 2004; Allard et al., 2005; Sakamoto et al., 2008; Sun et al., 2009; Young et al., 2009; Kozyrev et al., 2012; Kozyrev, 2013c). The LSt cells express the neuropeptides galanin, cholecystokin (CCK), enkephalin and gastrin-releasing peptide (GRP) (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Sakamoto et al., 2008). It is hypothesized that LSt cells transform sensory signals conveyed by the DPN associated with the summation of coitus into motor or secretory outputs to trigger emission and expulsion (Truitt and Coolen, 2002). There is abundant evidence to support the notion that LSt cells are an integral component of the spinal ejaculation generator and are essential for ejaculation. Firstly, it has been shown that LSt cells express cFos (Truitt et al., 2003) and phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK) (Staudt et al., 2010), markers of neural activation, exclusively with ejaculation but not with other components of male rat sexual behavior (Truitt et al., 2003). Secondly, acute transection of the spinal cord at the mid-thoracic spinal segments followed by electrical stimulation of the DPN triggers ejaculatory reflexes in male rats (Staudt et al., 2010; Staudt, 2011; Staudt et al., 2011; Kozyrev et

al., 2012; Kozyrev, 2013c). Thirdly, LSt cells display neural activation, specifically pERK (Staudt et al., 2010) and phosphorylated NMDA receptor subunit 1 (pNR1) (Staudt et al., 2011) following stimulation of the DPN, akin to mating animals. This neural activation is critical for ejaculation in male rats since blocking ERK and NMDA receptor activation with specific antagonists resulted in the absence of a coordinated pattern of BCM bursting, representative of ejaculation, after electrical stimulation of the DPN (Staudt et al., 2010, 2011). Similarly, targeted ablation of LSt cells completely abolished ejaculation but did not affect mounts or intromissions in male rats (Truitt and Coolen, 2002).

LSt cells co-express several neuropeptides including gastrin releasing peptide (GRP), enkephalin, galanin and cholecystokin (CCK) (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008; Kozyrev et al., 2012). Therefore we hypothesized that these neuropeptides are released from LSt cell axon terminals onto receptors of preganglionic sympathetic (IML and CAN), parasympathetic (SPN) and motor (SNB) neurons to modulate ejaculatory reflexes. Indeed, our laboratory has recently revealed a critical role for the GRP and the mu and delta opioid receptors in the control of ejaculation in male rats (Kozyrev et al., 2012; Kozyrev, 2013c). It was discovered that intrathecal infusions of the GRP antagonist RC-3095, mu and delta opioid receptor antagonists CTOP and TIPP, respectively, severely disrupted ejaculatory reflexes in male rats. Conversely, intrathecal infusion of GRP agonist GRP^{2029} or the opioid receptor agonist DAMGO triggered ejaculatory reflexes in 43-66 % and 75 % of male rats respectively. In addition, intrathecal infusion of GRP^{2029}, the delta receptor agonist deltorphin II or DAMGO facilitated ejaculatory reflexes following subthreshold levels of DPN stimulation in 100 % of male rats (Kozyrev et al., 2012; Kozyrev, 2013c).

The purpose of the current study was to test the respective roles of galanin and CCK in the control of ejaculatory reflexes in male rats. It was hypothesized that LSt cells release galanin and CCK from their axon terminals onto their target regions, is critical for triggering the emission and expulsion components of ejaculation. The current study aimed to investigate the individual contributions of galanin and cholecystokin receptor activation in the control of emission and expulsion reflexes induced by electrical stimulation of the DPN in anesthetized and spinalized male rats, and simultaneously recordings of seminal vesicle (SV) pressure and concurrent rhythmic contractions of the BCM, markers of emission and expulsion respectively.

4.2 Materials and Methods

4.2.1 *Animals*

For these experiments, adult male Sprague Dawley rats (225-250 grams) were obtained from Charles River (Wilmington, MA, USA) and housed in pairs in standard housing cages on a reverse 12-hour light/dark cycle with lights off at 9 a.m. Food and water were available ad libitum. All procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and conformed to the guidelines outlined by the National Institutes of Health.

4.2.2 Experimental Designs

4.2.2.1 Intrathecal infusions of Galanin or Cholecystokinin antagonists

Procedures were identical to our previously described studies(Brumovsky et al., 2006). Male rats were deeply anesthetized with urethane (1.5 g/kg, i.p.), a laminectomy was performed between the sixth and the eighth thoracic spinal segments and a complete transaction of the spinal cord was performed at the same spinal level. Next, the bulbocavernosus muscle (BCM) and the dorsal penile nerve (DPN) were surgically exposed and the surrounding connective tissue was removed. Subsequently, silver recording electrodes, attached to the PowerLab/7SP Data Acquisition System (AD Instruments, Inc., Colorado Springs, CO, USA) were inserted bilaterally into the BCM and a ground electrode was placed into the muscle of the right thigh in preparation for electromyographic (EMG) recordings. Additionally, the right seminal vesicle was exposed by coeliotomy for the purpose of measuring and recording seminal vesicle (SV) pressure, an marker of the emission component of ejaculation (Clement et al., 2008; Kozyrev et al., 2012; Kozyrev, 2013c). SV pressure (SVP) was measured with a pressure catheter (AD Instruments Inc., model number: SPR-671(1.4 F, Single, Straight, 15 cm, Ny) attached to a catheter interface cable (AD Instruments Inc., model number: AEC-IOD) and connected to a Bridge AMP (AD Instruments Inc.). The pressure catheter was carefully inserted into the lumen of the SV and secured in place prior to recording. In preparation for stimulation of the DPN, a bipolar stimulating electrode connected to the SD9 Square Pulse Stimulator (Grass Technologies, West Warwick, RI, USA) was positioned directly above the DPN. Initial stimulation of the DPN was performed immediately after spinal cord transection was in order to verify that the spinal cord transection is

complete, as evidenced by rhythmic bursting of the BCM and simultaneous SVP increases. Initial electrical stimulation of the DPN comprised square wave pulses of 1 ms duration, 4 V at 60 Hz for 10 seconds. These established DPN stimulation parameters reliably trigger rhythmic bursting of the BCM, corresponding to the expulsion phase of ejaculation, in all saline-treated control animals (Staudt et al., 2010; Staudt, 2011; Staudt et al., 2011; Kozyrev et al., 2012; Kozyrev, 2013c). Pharmacological experiments began two hours after spinal cord transection in order to allow the acute effects of spinal cord transection to subside. Next, a small incision was made in the dura mater at the site of the laminectomy and a polyethylene catheter (Caly-Adams PE-10, Parsippany, NJ, USA) was carefully inserted into the subarachnoid space until the open end reached the 3rd lumbar-4th lumbar (L3-L4) spinal segments.

In each animal, in trial 1, 10 µL of 0.9% sterile saline (or 0.9% sterile saline solution mixed with a small amount of dimethyl sulfoxide (DMSO) for animals subsequently treated with galantide) was infused through the polyethylene catheter to bath the entire lumbosacral spinal cord and BCM EMG activity was recorded for 25 minutes. Following the saline infusion, the DPN was stimulated at 30 Hz and 60 Hz in a counterbalanced order, with 5-minute rest periods between the stimulations (testing trial 1; control trial). Recordings of BCM EMG and SVP spanned a period of 90 seconds, corresponding to the duration of a representative ejaculatory reflex triggered by DPN stimulation in male rats (Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012; Kozyrev, 2013c). The entire procedure was repeated one hour later in the same animals, which now received a 10 µL infusion of one of two doses of the galanin antagonist galantide dissolved in a solution of 0.9 % sterile saline mixed with a small amount of DMSO (1 nmol, N = 8; 10 nmol, N = 8; and N=6 in a second experiment [Phoenix Pharmaceuticals Inc. USA, Burlingame, CA, USA]), or one of two doses of the cholecystokinin (CCK) antagonist

proglumide (71 nmol, N = 6; 714 nmol, N = 6 [Sigma-Aldrich Corp. St. Louis, MO, USA]). After drug infusion, BCM EMG and SVP were recorded for 25 minutes, followed by stimulation of the DPN at 30 Hz and 60 Hz in a counterbalanced order, and concurrent recordings of BCM EMG and SVP (testing trial 2; drug trial). Our previous experiments have demonstrated that saline infusions or repeated DPN stimulations do not affect parameters of ejaculatory reflex (Kozyrev et al., 2012; Kozyrev, 2013c).

4.2.2.2 Intrathecal infusions of galanin or cholecystokinin

Three separate groups of male rats were anesthetized, spinalized, and prepared as described above. In testing trial 1: control trial, male rats received 10 μL intrathecal infusions of 0.9% saline (N = 20) or 0.9% saline mixed with DMSO (N = 8; CCK group) and BCM EMG and SVP were recorded for 10 minutes after infusion. Next, the DPN was stimulated at 5 HZ, 10 HZ, 30 HZ and 60 HZ in a randomized and counterbalanced order, and BCM EMG and SVP were recorded for the duration of 90 seconds following stimulation (testing trial 1; control trial). One hour following the final DPN stimulation, the procedure was repeated in the same groups of animals, which now received 10 μL intrathecal infusions of one of two doses of galanin (0.16 nmol, N = 6 and N = 7 in a second experiment; 0.32 nmol, N = 7 (Bachem Americas, Inc. Torrance, CA, USA)] or CCK dissolved in 0.9% sterile saline and DMSO solution [CCK (26-33) (sulfated), 4.35 nmol; N = 8] (Phoenix Pharmaceuticals Inc. USA, Burlingame, CA, USA) and BCM EMG and SVP were recorded for 10 minutes after infusion. Following each drug infusion, the DPN was stimulated at 5, 10, 30, and 60 HZ in a counterbalanced order as described

previously (testing trial 2 and 3; drug trials) (Kozyrev et al., 2012). Our previous studies have demonstrated that saline infusions or repeated DPN stimulations do not affect parameters of ejaculatory reflex (Kozyrev et al., 2012; Kozyrev, 2013c).

4.2.3 *BCM EMG and SVP Analysis*

Analyses were performed as described in or previous studies (Staudt et al., 2010; Kozyrev et al., 2012; Kozyrev, 2013c). Analysis of BCM EMG and SVP recordings spanned 25 minutes or 10 minutes after infusion of the antagonist or agonist, respectively, and 90 seconds after every DPN stimulation. The numbers of BCM events, bursts, and SVP increases were analyzed. The analysis of numbers of BCM events was carried out using LabChart 7.35 (AD Instruments Inc.), and all events that were above baseline activity were included in the analysis (Staudt et al., 2010; Staudt, 2011; Staudt et al., 2011; Kozyrev et al., 2012; Kozyrev, 2013c). Additionally, the numbers of bursts and SVP increases were analyzed. The antagonist experiments were analyzed such that the effects of antagonist treatments on the numbers of events, bursts and SVP increases were compared within animals (within the same drug dose, between testing trial 1: control trial and testing trial 2: drug trial) and between groups (between different doses within testing trial 1: control trial and testing trial 2: drug trial), for the 30 and 60 Hz stimulations separately, using two-way repeated anova (factors: Testing trial and Drug dose) and Holm-Sidak post hoc tests. The galanin infusion experiments were analyzed such that the effects of neuropeptide treatment on the numbers of events, bursts and SVP increases were compared within animals (within the same drug dose, between testing trial 1: control trial and testing trial 2: drug trial) and between

treatment groups (separately for each stimulation setting: Infusion, 5 Hz, 10 Hz, 30 Hz, and 60 Hz), and separately for each of the two testing trials, using a two-way repeated anova (factors: Testing trial and DPN stimulation) and Holm-Sidak post hoc tests. A 95% confidence level was utilized for all tests. The cholecystokinin experiment was analyzed using a two-way repeated anova and the effects of CCK treatment on the numbers of events, bursts and SVP increases were compared within testing trials (with the saline and drug trials) and between stimulation settings (Infusion, 5 Hz, 10 Hz, 30 Hz, and 60 Hz) as well as between the two testing trials within each stimulation setting using a two-way repeated anova (factors: Testing trial and DPN stimulation) and Holm-Sidak post hoc tests.

4.2.4 Immunohistochemistry Experiments

4.2.4.1 *DPN Stimulation-induced pERK after intrathecal opioid receptor antagonist treatment*

To confirm that the galanin antagonist galantide and the CCK antagonist proglumide disrupted ejaculatory reflexes by acting on LSt target regions and not by preventing the activation of LSt cells, the expression of pERK in LSt cells was examined following intrathecal infusions of galantide or proglumide and stimulation of the DPN (Staudt et al., 2010; Kozyrev et al., 2012; Kozyrev, 2013c). Male rats (N = 9) were anesthetized and spinalized as described above. Two hours after spinalization, the DPN and BCM were exposed and galantide (10 nmol, N =4) or proglumide (714 nmol, N = 2) were infused in a volume of 10 µL intrathecally as described previously. Following a 25-minute infusion, the DPN was stimulated at 30 Hz and BCM EMG

and SVP were recorded for 90 seconds. Five minutes following DPN stimulation, animals were perfused transcardially with 5 mL 0.9% saline solution and 500 mL of 4% paraformaldehyde in 0.1 M PB. Spinal cords were removed and postfixed for 1 hour in the same fixative and then transferred into a cryoprotective solution (20% sucrose in 0.1 PB with 0.01% sodium azide) until further processing for immunohistochemical visualization of pERK and galanin. The stimulation parameters and time of perfusion were previously shown to be optimal for detection of pERK expression in LSt cells (Staudt et al., 2010; Kozyrev et al., 2012; Kozyrev, 2013c). In addition, it was previously shown that intrathecal infusions of saline do not prevent DPN stimulation induced pERK experession in LSt cells (Kozyrev et al., 2012; Kozyrev, 2013c). Furthermore, previous experiments have shown that spinal cord transection and the surgical exposure of the DPN and the BCM without electrical stimulation of the DPN does not lead to pERK expression in LSt cells (Staudt et al., 2010), hence negative control groups were not included in this experiment.

4.2.5 Immunohistochemistry: Galanin/pERK dual fluorescence

Spinal cords were sliced with a freezing microtome (Thermo Fisher Scientific, Walldorf, Germany) into 12 parallel series of 35 um coronal sections, placed into a cryoprotectant solution (30% sucrose, 30% ethylene glycol in 0.1 M PB with 0.01% sodium azide) and stored at -20°C until further processing. Free floating sections of the lower thoracic, lumbar, and sacral spinal cord were thoroughly rinsed in 0.1 M saline buffered sodium phosphate (PBS) between incubations and blocked with 1% H2O2 for 10 minutes prior to incubation. All antibody incubations were performed in incubation solution containing 0.1% bovine serum albumin and

0.4% Triton X-100 (BP151-500, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) in PBS at room temperature with gentle agitation. Spinal cord sections were incubated overnight with rabbit anti-pERK (1:1,000; overnight; Cell Signaling #26 L) and with biotinylated goat anti-rabbit for one hour (1:500; Vector Laboratories, Burlingame, CA, USA), avidin horseradish peroxidase complex (ABC-elite, 1:1,000 in PBS; 1 hour;Vector Laboratories), biotinylated tyramine (BT; 1:250 in PBS containing 1 uL/mL of 3% H2O2 for 10; NEL700/700A; PerkinElmer Life Sciences, Boston, MA, USA), and Alexa 488-conjugated streptavidin (1:100 in PBS, 30 minutes (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Next, sections were incubated with rabbit anti-galanin (1:3,000; overnight, T-4334; Bachem, Torrance, CA, USA) and Alexa 555-conjugated goat anti-rabbit for 30 minutes in PBS (1:100; Jackson Immuno-Research Laboratories) and mounted on plus charged slides, cover-slipped with gelvatol and stored in the dark at 4°C. For all immunohistochemistry procedures, the omission of primary antibodies resulted in a complete loss of signal at specific wavelengths and all primary antibodies have been previously characterized (Truitt et al., 2003; Staudt et al., 2010, 2011; Kozyrev et al., 2012; Kozyrev, 2013c).

4.2.6 Data Analysis

4.2.6.1 *pERK Expression in LSt Cells*

pERK expression in LSt cells was analyzed using a DM5000B Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Essentially, all neurons in the lumbar spinal cord

expressing galanin-ir were analyzed for expression of pERK and percentages of galanin-ir cells co-expressing pERK were calculated for each animal. Differences between groups were analyzed using ANOVA and Holms-Sidak post hoc tests.

4.3 Results

4.3.1 *Galantide Suppressed Ejaculatory Reflexes*

The galanin antagonist galantide significantly suppressed DPN stimulation-induced ejaculatory reflexes at both 30 and 60 Hz stimulation frequencies as evidenced in the reduced numbers of BCM events, and bursts. There were main effects of testing trial on the numbers of BCM events for both 30 Hz ($F(1, 29) = 50.905$; $P < 0.001$; Figure 16A) and 60 Hz stimulation frequencies ($F(1, 29) = 30.213$; $P < 0.001$; Figure 17A). Post hoc analyses revealed that animals treated with either dose of galantide during the second trial (drug trial) had significantly decreased BCM events in response to 30 and 60 Hz DPN stimulations compared to saline treatment in their first control trial (trial 1; 30 Hz: $P = 0.003$ (1 nmol); $P < 0.001$ (10 nmol); 60 Hz: $P = 0.008$ (1 nmol); $P < 0.001$ (10 nmol); Figure 17A, C, D). In addition, animals treated with the higher dose of galantide during trial 2 displayed significantly fewer BCM events in response to 60 Hz (trial 1; $P = 0.022$ [10 nmol]) DPN stimulation frequency compared to saline treatment in trial 1 (Figure 17 A, C, D). None of these effects were attributable to pre-existing group differences, as in control trial 1, when all animals received saline there were no significant differences in the numbers of

BCM events in response to either 30 or 60 Hz DPN stimulations between groups (control trial; Figure 17 A, C).

The effects of galantide on BCM bursts paralleled the effects on BCM events. There were main effects of testing trial on the numbers of BCM bursts for both 30 Hz ($F(1, 29) = 100.213$; $P < 0.001$) and 60 Hz ($F(1, 29) = 218.400$; $P < 0.001$) stimulation frequencies (Figure 17 B). Specifically, male rats treated with either dose of galantide during trial 2 (drug trial) demonstrated significantly fewer BCM bursts compared to trial 1 (control trial) with saline treatment (trial 1; 30 Hz: $P < 0.001$ [1 nmol]; $P < 0.001$ [10 nmol]; 60 Hz: $P < 0.001$ [1 nmol]; $P < 0.001$ [10 nmol]; Figure 17 B, C, D). In addition, there were significant interactions between factors of testing trial and drug dosage on the number of BCM bursts for both 30 Hz ($F(1, 29) = 4.916$; $P = 0.045$) and 60 Hz ($F(1, 29) = 6.067$; $P = 0.029$) stimulation frequencies. Post hoc analyses demonstrated that male rats treated with the higher dose of galantide during trial 2 (drug trial) had significantly fewer BCM bursts in response to 60 Hz (trial 2: $P = 0.041$ [10 nmol]) DPN stimulation frequency compared to trial 1 (Figure 17 B, C, D). There were no significant differences in the numbers of BCM bursts after 30Hz or 60 Hz DPN stimulation within testing trial 1, control trial, indicating that differences in dosage in trial 2 (drug trial) were due to the effects of galantide (Figure 17 B, C).

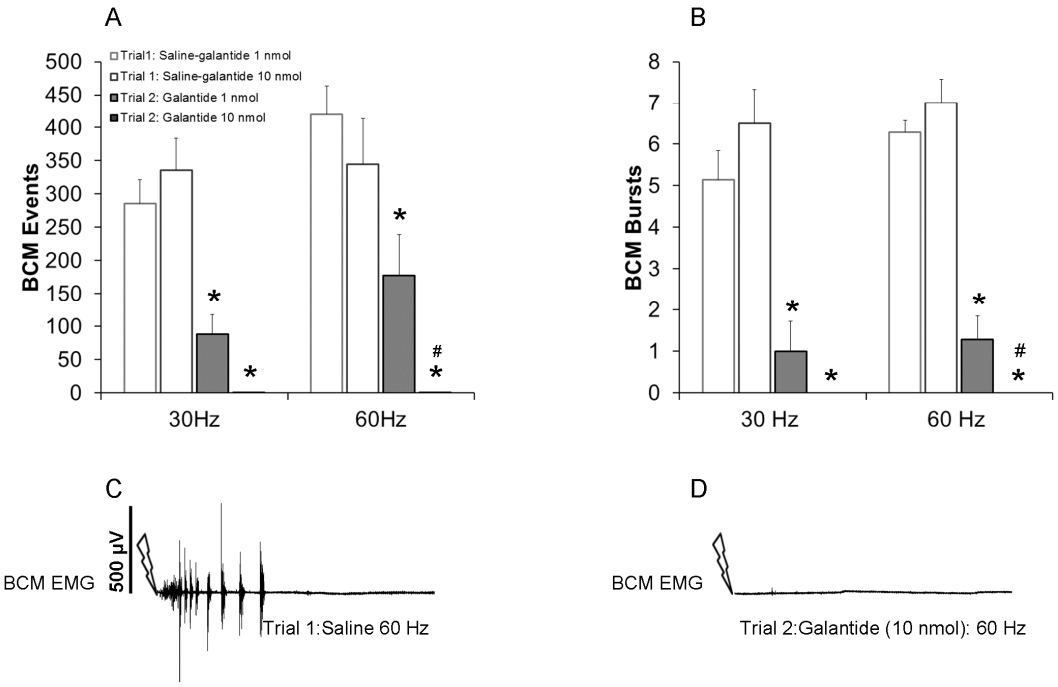

Figure 17 Effects of galantide on numbers of BCM events and bursts in male rats.

Quantitative analyses and accompanying EMG traces of BCM events and bursts following intrathecal infusions of galantide. Quantitative analysis of BCM events (**A**) and bursts (**B**) in response to 30 and 60 Hz DPN stimulation following infusions of saline in trial 1 (control trial) or one of two doses of galantide (1 or 10 nmol) in trial 2 (drug trial). EMG traces of 90 seconds duration following 60 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (**C**: control trial) and galantide (**D**: same animals as in **C**). * denotes significant differences from trial 1 within the same treatment group, while # indicates significant differences between treatment groups within the same testing trial.

The effects of galantide on SVP increases, a marker of the emission component of ejaculation, was tested in a separate group of animals in which BCM EMG and SVP were recorded simultaneously following intrathecal infusion of the higher dose of galantide (10 nmol) (N = 6). The effects on BCM events and bursts were completely replicated, and moreover were paralleled by significant decreases in numbers of SVP increases. There were main effects of testing trial on the numbers of BCM events ($F(1, 23) = 30.227$; $P = 0.003$), BCM bursts ($F(1, 23) = 53.382$; $P < 0.001$) and SVP increases ($F(1, 23) = 110.401$; $P < 0.001$). Post hoc analyses revealed that male rats treated with galantide in trial 2 showed significantly fewer numbers of BCM events, bursts and SVP increases for both 30 Hz (events: $P = 0.004$; bursts: $P < 0.001$ (Figure 18A); SVP: $P < 0.001$; Figure 18B) and 60 Hz (events: $P = 0.001$; bursts: $P < 0.001$(Figure 18A); SVP: $P < 0.001$; Figure 18B, C, D) stimulation frequencies compared to saline treatment in trial 1. Overall, these results confirm that intrathecal galantide suppresses both the emission and expulsion components of ejaculation, indicating that activation of galanin receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats.

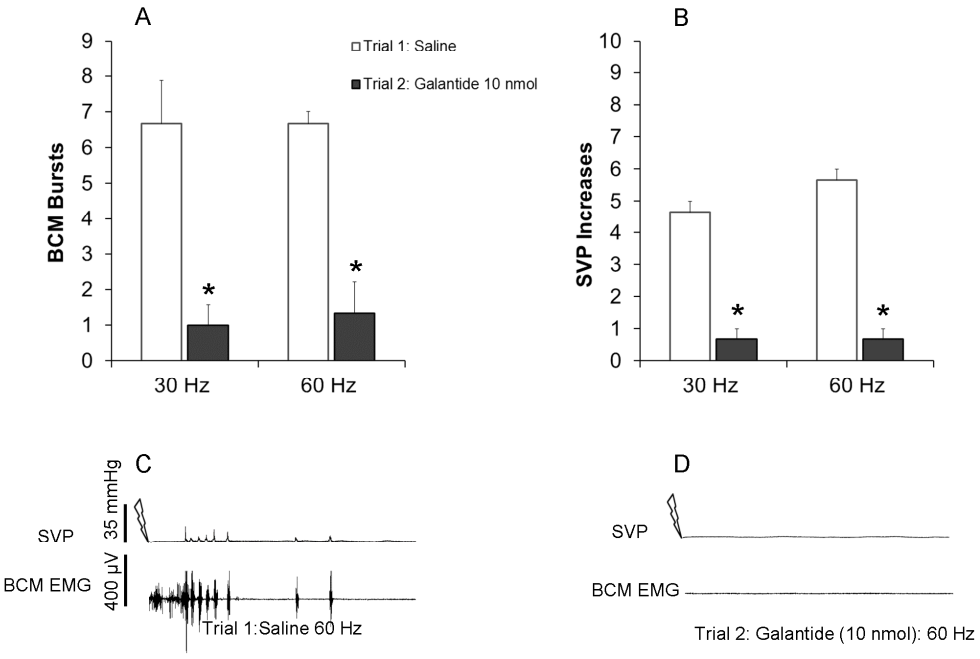

Figure 18 Effects of galantide on the numbers of BCM bursts and SVP increases in male rats.

Quantitative analyses and accompanying EMG traces of BCM bursts and SVP increases following intrathecal infusions of galantide. Quantitative analysis of BCM bursts (**A**) and SVP increases (**B**) in response to 30 and 60 Hz DPN stimulation following infusions of saline in trial 1 (control trial) or galantide (10 nmol) in trial 2 (drug trial). EMG and concurrent SVP traces of 90 seconds duration following 60 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (**C**: control trial) and galantide (**D**: same animals as in **C**). * denotes significant differences from trial 1 (control trial).

4.3.2 *Proglumide Dose-Dependently Suppresses Ejaculatory Reflexes*

The CCK antagonist proglumide dose-dependently disrupted DPN stimulation-induced ejaculatory reflexes in response to 30 and 60 Hz stimulation frequencies as reflected in the reduced numbers of BCM events, bursts, and SVP increases. There were main effects of testing trial on the numbers of BCM events for both 30 Hz ($F(1, 23) = 23.289$; $P < 0.001$) and 60 Hz ($F(1, 23) = 5.446$; $P = 0.042$) stimulation frequencies. Post hoc analyses revealed that animals treated with the higher but not the lower dose of proglumide during the second trial (drug trial) had significantly decreased BCM events in response to 30 and 60 Hz DPN stimulation compared to saline treatment in the first control trial (30 Hz: $P < 0.001$ (714 nmol); 60 Hz: $P = 0.020$ (714 nmol); Figure 19A).

There was also a main effect of drug dosage on the numbers of BCM events following 60 Hz DPN stimulation ($F(1, 23) = 5.025$; $P = 0.049$) and a significant interaction between factors of drug dosage and testing trial on the numbers of BCM events following 30 Hz DPN stimulation ($F(1, 23) = 9.846$; $P = 0.011$). Post hoc analyses revealed that male rats treated with the higher dose of proglumide (714 nmol) during trial 2 (drug trial) displayed significantly fewer BCM events following 30 Hz ($P = 0.015$) and 60 Hz DPN stimulation frequencies ($P = 0.018$) compared to animals that received the lower dose of proglumide (71 nmol); Figure 19A. There were no significant differences in the numbers of BCM events in response to 30 or 60 Hz DPN stimulation frequencies between the groups during trial 1 (control trial) when both groups of animals received infusions of saline (Figure 19A).

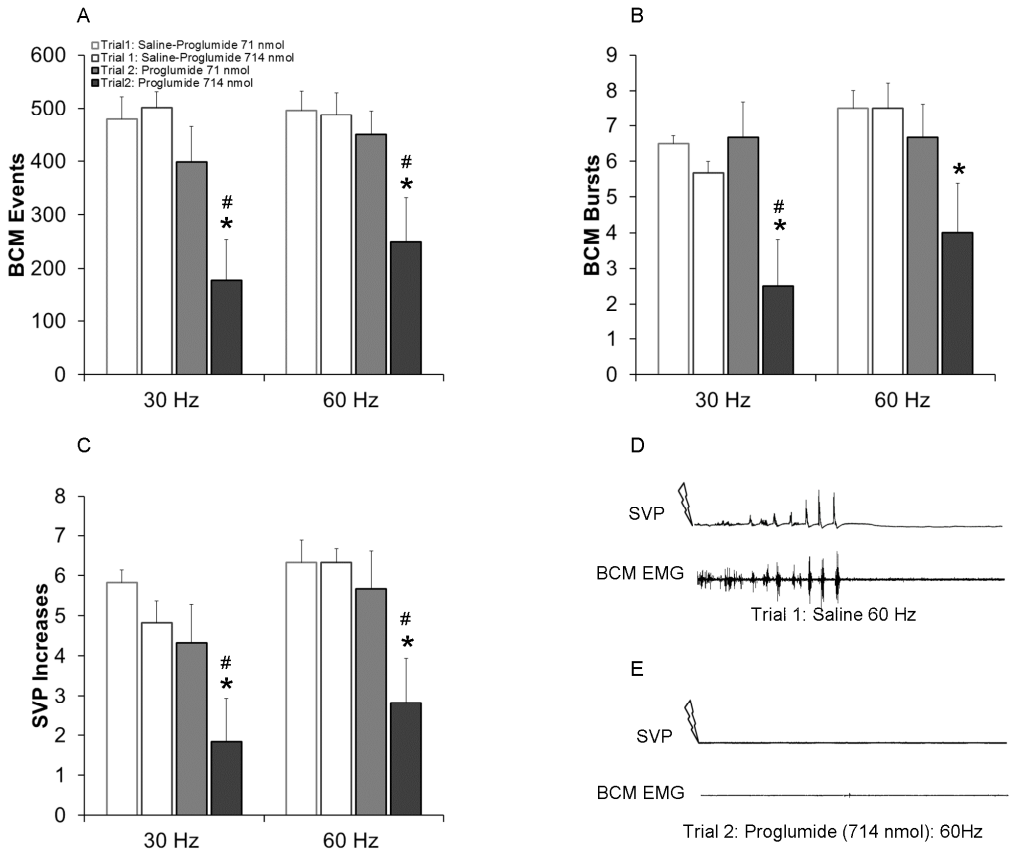

Figure 19 Effects of proglumide on the numbers of BCM events, bursts and SVP increases in male rats.

Quantitative analyses and accompanying EMG traces of BCM events, bursts and SVP increases following intrathecal infusions of proglumide. Quantitative analysis of BCM events (A), bursts (B) and SVP increases (C) in response to 30 and 60 Hz DPN stimulation following infusions of saline in trial 1 (control trial) or one of two doses of proglumide (71 or 714 nmol) in trial 2 (drug trial). EMG traces of 90 seconds duration following 60 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (D: control trial) and galantide (E: same animals as in C). * denotes significant differences from trial 1 within the same treatment group, while # indicates significant differences between treatment groups within the same testing trial.

Similarly, there were main effects of testing trial on the numbers of BCM bursts for 60 Hz ($F(1, 23) = 6.680$; $P = 0.027$) stimulation frequency, and for drug dosage on the numbers of BCM bursts for 30 Hz ($F(1, 23) = 8.007$; $P = 0.018$) DPN stimulation frequency. Post hoc analyses revealed that male rats that received infusions of the higher dose (714 nmol) of proglumide during testing trial 2 (drug trial) displayed significantly fewer BCM bursts following 30 Hz ($P = 0.022$; Figure 19B) and 60 Hz ($P = 0.014$; Figure 19B, D, E) DPN stimulation frequencies. Furthermore, male rats treated with the higher dose (714 nmol) of proglumide showed a significant reduction in the numbers of BCM bursts following 30 Hz ($P = 0.003$) with a trend towards significance following 60 Hz ($P = 0.062$) DPN stimulation frequency compared to male rats treated with the lower dose of proglumide (71 nmol; Figure 19B). There were no significant differences between the groups in the numbers of BCM bursts following 30 or 60 Hz DPN stimulations during trial 1 (control trial; Figure 19B).

In addition, intrathecal infusions of proglumide dose-dependently suppressed SVP increases. There were main effects of testing trial on the numbers of SVP increases following 30 Hz ($F(1, 23) = 10.565$; $P = 0.009$) and 60 Hz ($F(1, 23) = 6.288$; $P = 0.031$) stimulation frequencies. Post hoc analyses demonstrated that the higher dose (714 nmol) of proglumide significantly reduced numbers of SVP increases following 30 Hz ($P = 0.012$; 714 nmol; Figure 19C) and 60 Hz ($P = 0.014$; 714 nmol; Figure 19C) stimulation frequencies. Furthermore, there was an effect of dosage during trial 2 (drug trial) whereby animals receiving the higher dose of proglumide (714 nmol) displayed significantly fewer SVP increases in response to 30 Hz ($P = 0.039$; Figure 19C) and 60 Hz ($P = 0.021$; Figure 19C, D, E) stimulation frequencies. Finally, there were no differences between groups during trial 1 (control trial; Figure 19C).

4.3.3 *Galantide and Proglumide Did Not Prevent DPN Stimulation-Induced pERK in LSt Cells*

In order to test that the galanin antagonist galantide and the CCK antagonist proglumide suppressed BCM bursting and SV pressure by acting in LSt target areas instead of acting directly on LSt cells, DPN stimulation-induced neural activation of LSt cells was examined following intrathecal infusions of each antagonist. It has previously been shown that in control treated animals, DPN stimulation induced pERK in 97 ± 0.03 % of LSt cells (Kozyrev et al., 2012). Similarly, intrathecal infusions of galantide and proglumide did not prevent DPN stimulation-induced activation of LSt cells. Indeed, DPN stimulation induced pERK in 94.05 ± 0.03% and 87.93 ± 0.07 % of LSt cells in galantide (10 nmol) and proglumide (714 nmol) treated male rats, respectively. These results indicate that galantide and proglumide are primarily acting in LSt target regions to suppress the emission and expulsion components of ejaculation, and not directly on LSt cells.

4.3.4 *Galanin Facilitated BCM Events, Bursts and SVPs*

In control trial 1, it was confirmed that 30 and 60 Hz DPN stimulation triggered BCM events and burst, while saline infusion or DPN stimulation at 5 and 10 Hz did not trigger BCM activity. In Trial 2, galanin facilitated BCM events, bursts and SVP increases. There were main effects of testing trial on the numbers of BCM events for Infusion: ($F(1, 25) = 17.937$; $P = 0.001$); 5 Hz: ($F(1, 25) = 17.853$; $P = 0.001$; 10 Hz: ($F(1, 31) = 54.000$; $P < 0.001$) stimulation

frequencies. Post hoc tests demonstrated that infusions of the lower dose of galanin, in the absence of DPN stimulation, during trial 2 (drug trial) increased number of BCM events (0.15 nmol $P = 0.003$; 0.3 nmol; P=0.051) compared to trial 1 (control trial; Figure 20A). Moreover, galanin facilitated BCM events after sub threshold DPN stimulation in male rats treated with either dose of galanin demonstrated significantly greater numbers of BCM events following 10 Hz (0.15 nmol: $P < 0.001$; 0.3 nmol: $P < 0.001$; Figure 20A) DPN stimulation. There was an effect of dosage for the 5 Hz stimulation frequency during trial 2 (drug trial). Specifically, animals treated with the higher dose of galanin demonstrated significantly greater numbers of BCM events following 5 Hz DPN stimulation compared to animals treated with the lower dose of galanin (0.3 nmol: $P = 0.014$; Figure 20A).

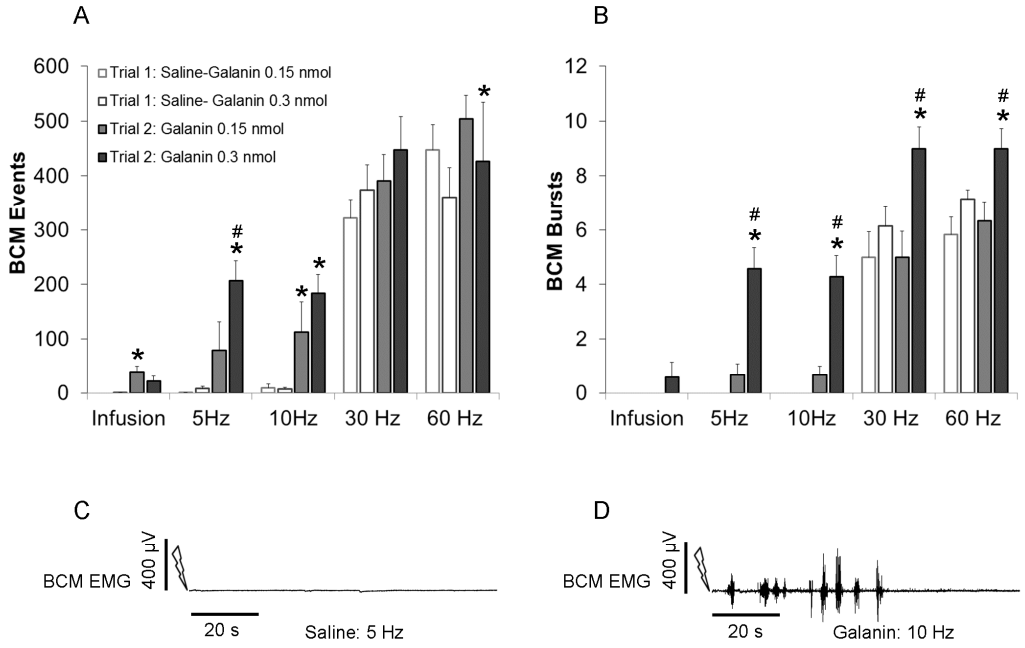

Figure 20 Effects of galanin on numbers of BCM events and bursts in male rats

Quantitative analyses and accompanying EMG traces of BCM events and bursts following intrathecal infusions of galanin. Quantitative analysis of BCM events (**A**) and bursts (**B**) in response to infusion and DPN stimulation at 5 Hz, 10 Hz, 30 Hz and 60 Hz (counterbalanced) following infusions of saline in trial 1 (control trial) or one of two doses of galanin (0.15 or 0.3 nmol) in trial 2 (drug trial). EMG traces of 90 seconds duration following 10 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (**C**: control trial) and galanin (**D**: same animals as in C). * denotes significant differences from trial 1 within the same treatment group, while # indicates significant differences between treatment groups within the same testing trial.

The effects of galanin on BCM bursts were similar to the effects on BCM events, except that galanin infusions in the absence of DPN stimulation did not trigger BCM bursting activity. However, galanin did facilitate BCM bursts induced by DPN stimulation. There were main effects of testing trial on the numbers of BCM bursts for 5 Hz: ($F(1, 25) = 31.406$; $P < 0.001$ and 10 Hz: ($F(1, 25) = 30.305$; $P < 0.001$) stimulation frequencies. There were also main effects of drug dosage for 5 Hz ($F(1, 25) = 17.453$; $P = 0.002$; 10 Hz: ($F(1, 25) = 16.183$; $P = 0.002$) and 60 Hz ($F(1, 25) = 9.739$; $P = 0.010$) stimulation frequencies. and significant interactions between the factors for 5 Hz ($F(1, 25) = 17.453$; $P = 0.002$; 10 Hz: ($F(1, 25) = 16.183$; $P = 0.002$ and 30 Hz ($F(2, 25) = 6.119$; $P = 0.018$). Post hoc tests revealed that the higher dose (0.3 nmol) of galanin during trial 2 (drug trial) significantly increased numbers of BCM bursts following 5 Hz ($P < 0.001$), 10 Hz ($P < 0.001$), 30 Hz ($P < 0.001$), and 60 Hz ($P=0.049$) stimulation frequencies compared to trial 1 (control trial) (Figure 20B, C, D). Post hoc analyses demonstrated that male rats treated with the higher dose of galanin in trial 2 (drug trial: 0.3 nmol) had significantly more

BCM bursts compared to the lower dose of agalnin (0.15 nmol) (5 Hz: $P < 0.001$; 10 Hz: $P < 0.001$; 30 Hz: $P = 0.004$; and 60 Hz: $P = 0.006$; Figure 20B).

Effects of galanin (0.3 nmol) in SVP increases were tested in a separate group of male rats (N = 7) with simulatenous recordings of SVP and BCM EMG. In this group of animals, intrathecal infusions of galanin (0.3 nmol) facilitated both the emission and expulsion components of ejaculation as evidenced in the increased numbers of BCM events, bursts and SVP increases following subthreshold levels (5-10 Hz) of DPN stimulation. First, there were main effects of stimulation frequency on the numbers of BCM events ($F(4, 69) = 36.451$; $P < 0.001$), bursts ($F(4, 69) = 37.889$; $P < 0.001$) and SVP increases ($F(4, 69) = 18.603$; $P < 0.001$). In saline-treated males, in trial 1 (saline trial), only 30 and 60 Hz DPN stimulation frequencies increased NCM events, bursts, and SVP increases as expected (all $P < 0.001$ compared to saline infusion) (Figure 21A, B). There were also significant interactions between main factors of testing trial and stimulation frequency on the numbers of BCM events ($F(4, 69) = 11.633$; $P < 0.001$), bursts ($F(4, 69) = 10.774$; $P < 0.001$) and SVP increases ($F(4, 69) = 8.779$; $P < 0.001$).

Post hoc analyses demonstrated that male rats treated with galanin in trial 2 had significantly greater numbers of BCM events, bursts and SVP following 5 Hz (events: $P < 0.001$; bursts: $P < 0.001$; Figure 21A, C, D; SVP: $P < 0.001$; Figure 21B, C, D) and 10 Hz (events: $P = 0.008$; bursts: $P = 0.003$; Figure 21A; SVP: $P = 0.003$; Figure 21B) DPN stimulation frequencies compared to control trial 1. Overall, these results indicate that intrathecal galanin facilitates but does not trigger the emission and expulsion components of ejaculation. However, contrary to the first galanin experiment where it was found that intrathecal galanin facilitated numbers of BCM events and bursts not only in response to subthreshold (5-10 Hz) but also following threshold (30-60 Hz) levels of DPN stimulation, in this second experiment,

threshold levels of DPN stimulation (60 Hz) significantly decreased the numbers of BCM bursts and SVP increases following galanin infusions in trial 2 (bursts: $P = 0.041$; Figure 21A; SVP: $P = 0.036$; Figure 21B).

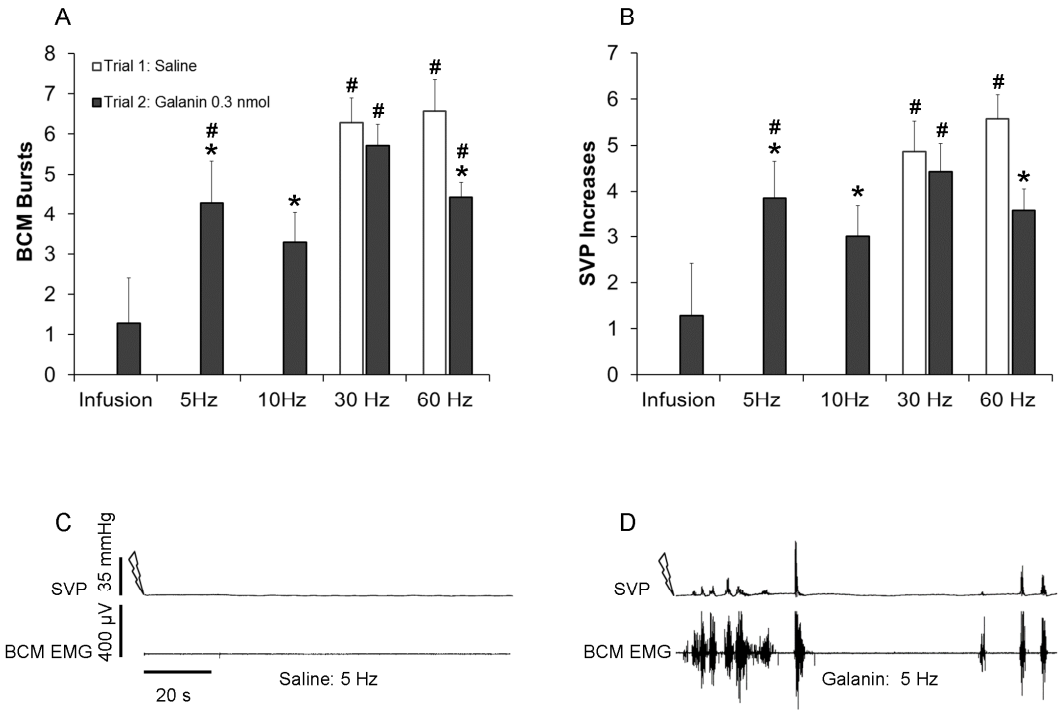

Figure 21 Effects of galanin on numbers of BCM bursts and SVP increases in male rats.

Quantitative analyses and accompanying EMG traces of BCM bursts and SVP increases following intrathecal infusions of galanin. Quantitative analysis of BCM bursts (A) and SVP increases(B) in response to infusion, 5 Hz, 10 Hz, 30 Hz and 60 Hz DPN stimulation (counterbalanced) following intrathecal infusions of saline in trial 1 (control trial) or galanin (0.3 nmol) in trial 2 (drug trial). EMG traces of 90 seconds duration following 10 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (C: control trial) and galanin (D: same

animals as in C). * denotes significant differences from trial 1 (control trial), while # indicates significant differences from infusion, 5Hz and 10 Hz within the same testing trial.

4.3.5 *Cholecystokinin (CCK) Facilitated BCM Events, Bursts and SVPs*

CCK (26-33) facilitated both the emission and expulsion components of ejaculation as evidenced by the increased numbers of BCM events, bursts and SVP increases following subthreshold levels of DPN stimulation. There were main effects of stimulation frequency on the numbers of BCM events ($F(4, 59) = 51.540$; $P < 0.001$), bursts ($F(4, 59) = 56.316$; $P < 0.001$) and SVP increases ($F(4,59) = 38.292$; $P < 0.001$). As expected, during trial 1 (saline trial) 30 and 60 Hz DPN stimulation increased numbers of BCM events, bursts and SVP increases compared to saline infusion, 5 or 10 Hz stimulation (all $P < 0.001$; Figure 22A-B). . There were also significant interactions between main factors of testing trial and stimulation frequency on the numbers of BCM events ($F(4, 59) = 4.897$; $P = 0.006$) and bursts ($F(4, 59) = 5.493$; $P = 0.004$). Post hoc analyses revealed that following CCK in trial 2 there were significantly greater numbers of BCM events, bursts and SVP increases following 10 Hz (events: $P = 0.040$; Figure 22A; bursts: $P = 0.005$; Figure 22B; SVP: $P = 0.013$; Figure 22C) DPN stimulation in trial 2 (drug trial) compared to trial 1 (saline trial). CCK did not significantly affect SVP increased following 5 Hz DPN stimulation during trial 2 compared to trial 1, even though BCM bursts and events were increased ($P = 0,05$ and 0.001; Figure 22B). Taken together, the results indicate that intrathecal infusions of CCK facilitate both the emission and expulsion components of ejaculatory reflexes following subthreshold levels of DPN stimulation (10 Hz) in male rats.

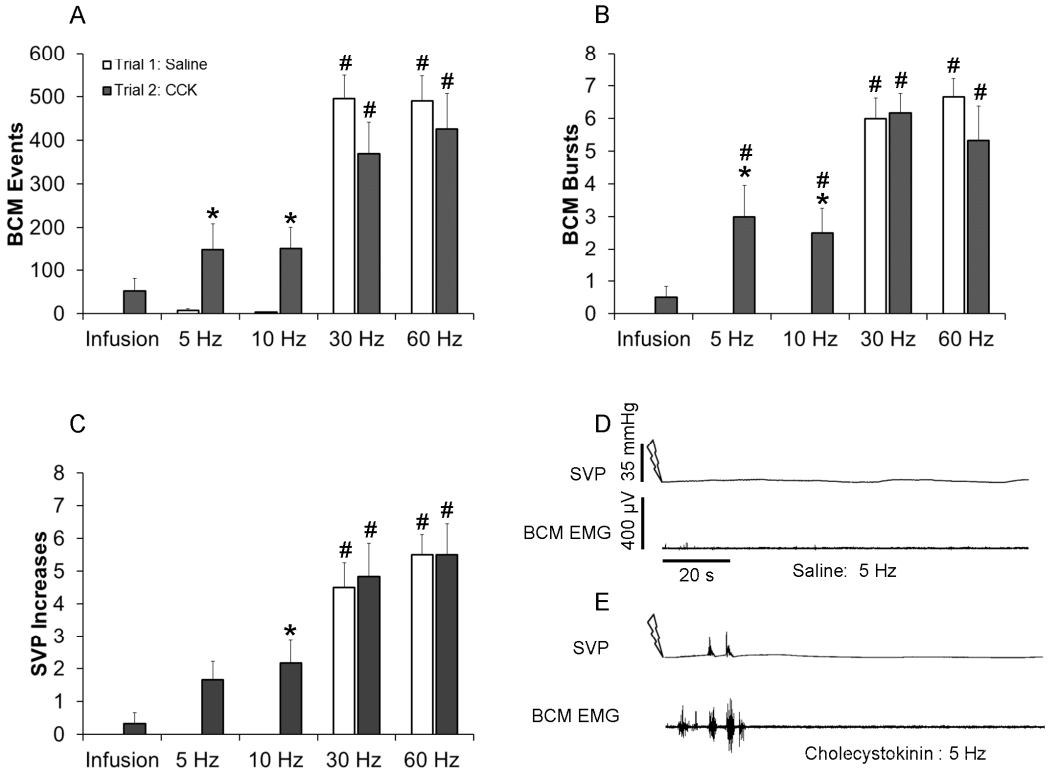

Figure 22 Effects of CCK on the numbers of BCM events, bursts and SVP increases in male rats.

Quantitative analyses and accompanying EMG traces of BCM events, bursts and SVP increases following intrathecal infusions of CCK. Quantitative analysis of BCM events (A), bursts (B) and SVP increases (C) in response to infusion, 5 Hz, 10 Hz, 30 Hz and 60 Hz DPN stimulation (counterbalanced) following intrathecal infusions of saline in trial 1 (control trial) or CCK (4.35 nmol) in trial 2 (drug trial). EMG traces of 90 seconds duration following 5 Hz DPN stimulation (arrow) after an intrathecal infusion of saline (D: control trial) and CCK (E: same animals as in D). * denotes significant differences from trial 1 (control trial), while # indicates significant differences from infusion, 5 Hz and 10 Hz within the same testing trial.

4.4 Discussion

These data support the hypothesis that galanin and CCK receptor activation in LSt target areas is critical for ejaculatory reflexes in male rats by acting on galanin and CCK receptors in LSt target areas in the lumbosacral spinal cord. As predicted, intrathecal infusions of galanin or CCK receptor antagonists, galantide and proglumide respectively, prevented both the emission and expulsion phases of ejaculation in response to sensory stimulation of the DPN in male rats. Conversely, galanin and CCK agonists facilitated ejaculatory reflexes following sub- threshold sensory stimulation (5–10 Hz) in 100% of male rats. In contrast, galanin or CCK infusions in the absence of DPN stimulation were not sufficient to trigger ejaculatory reflexes. This finding is in contrast with our previous observations that agonists for GRP or mu opioid receptors do trigger ejaculatory reflexes (Kozyrev et al., 2012; Kozyrev, 2013c). It is therefore possible that galanin and CCK act synergistically with the endogenous release of neuropeptides from LSt axon terminals, including galanin (Newton, 1992a, 1993), CCK (Phan and Newton, 1999), GRP (Sakamoto et al., 2008; Kozyrev et al., 2012) and enkephalin (Nicholas et al., 1999; Kozyrev, 2013c), upon sensory stimulation of the DPN.

Even though the group data presented in the results shows a lack of significant induction by galanin or CCK of ejaculation in the absence of sensory stimulation, intrathecal infusions of galanin and CCK triggered ejaculatory reflexes in 15 % (3/20) and 16 % (1/6) of male rats respectively. Perhaps the ability of intrathecal infusions of galanin or CCK to trigger ejaculatory reflexes in the absence of sensory stimulation in a minority of male rats may be explained by the presence of individual differences in galanin and CCK receptor densities in the lumbosacral spinal cord. It is possible that male rat ‘responders’ in the current study have a lower threshold to

ejaculation and are more likely to exhibit the characteristic aspects of 'premature ejaculation'. Indeed, a previous study observed inherent differences in the spinal command of ejaculation between rats classified as 'rapid ejaculators' as compared to 'sluggish ejaculators' and 'normal ejaculators' based on their ejaculation frequency in a set of mating tests (Borgdorff et al., 2009). Specifically, BCM contractions following microstimulation of the spinal ejaculation generator, corresponding to the expulsion phase of ejaculation, were significantly accelerated in 'rapid ejaculators' compared with 'sluggish ejaculators' and 'normal ejaculators'(Borgdorff et al., 2009). Overall, the agonist experiments indicate that activation of galanin and CCK receptors in the lumbosacral spinal cord facilitates ejaculatory reflexes following subthreshold levels of DPN stimulation (5-10 Hz) but is not sufficient to trigger ejaculatory reflexes in the majority of male rats in the absence of sensory stimulation of the DPN.

LSt cells and axons co-express galanin and CCK and the expression of both neuropeptides has been shown to be sexually dimorphic (Newton, 1992a, 1993; Phan and Newton, 1999). Specifically, male rats have a significantly greater number of both galanin and CCK-ir neurons and greater optical densities for both galanin and CCK than female rats and males with a testicular feminization mutation (Tfm) that lack functional androgen receptors (Newton and Phan, 2006). Therefore, androgens and functional androgen receptors regulate the expression of galanin and CCK in LSt cells. In addition, the expression of GRP, another neuropeptide expressed in LSt cells and axons, has been shown to be androgen-dependent since castration significantly reduced the intensity of GRP-ir fibers in the lumbar spinal cord in male rats and this reduction was prevented by androgen replacement (Sakamoto et al., 2008).

Galanin is a 29/30 amino acid long neuropeptide encoded by the GAL gene (Evans et al., 1993) that is widely distributed in the brain, spinal cord and gastrointestinal tract of mammals

(Kask et al., 1997; Hokfelt et al., 1999) and is involved in a variety of physiological functions including feeding, nociception, cognition, regulation of blood pressure and mood (Mechenthaler, 2008) . Galanin-ir is expressed in dorsal root ganglion cells (DRG) (Hokfelt et al., 1993; Kask et al., 1997; Landry et al., 2005), but in the lumbosacral spinal cord, is expressed exclusively in LSt cells (Truitt and Coolen, 2002; Truitt et al., 2003; Staudt, 2011). Furthermore, galanin-ir axon terminals exclusively deriving from LSt cells are in close proximity to cell bodies and proximal dendrites of autonomic preganglionic and motor neurons (Newton, 1992b; Ohmachi et al., 1996). A predominately inhibitory, hyperpolarizing neuropeptide (Ito, 2009), galanin mediates its effects through its receptors: GALR1, GALR2 and GALR3. Galanin receptors are inhibitory G-protein-coupled receptors that are associated with G-protein coupled inwardly-rectifying K+ (GIRK) channels (Kask et al., 1997; Wang et al., 1998) and GALR1 receptors have the highest densities postsynaptically compared to the other two receptor types (Brumovsky et al., 2006; Landry et al., 2006). In the lumbosacral spinal cord, GalR1 receptors have been observed in dorsal and ventral horns, lateral spinal nuclei (Brumovsky et al., 2006) and numerous GALR1 positive mRNA neurons were detected in lamina X, presumably in the vicinity of the LSt cells (Brumovsky et al., 2006). GALR2 mRNA expression is considerably diminished compared to GALR1 mRNA expression and is confined primarily to the dorsal horn (Brumovsky et al., 2006). However, the most intensely labeled neurons are observed in the ventral horn, likely on motorneurons, and a few GALR2 positive mRNA neurons are detected in the sympathetic and parasympathetic lateral cell columns, in the locations of the IML and SPN (Brumovsky et al., 2006). In light of these findings, it is likely that galanin facilitates emission and expulsion by acting on GALR1 and GALR2 receptors in LSt target regions, specifically on autonomic preganglionic neurons in the lateral cell columns and on motoneurons in the ventral horns of the

lumbosacral spinal cord in male rats. As previously mentioned, GALR1 receptors have been also observed in lamina X, in the region of LSt cells. However, it remains to be tested whether these receptors are expressed on LSt cells or other types of neurons in the lumbosacral spinal cord. If GALR1 receptors are indeed expressed on LSt cells, this suggests the possibility of autoreceptor interactions or reciprocal connections. However, intrathecal pretreatment with galantide in the current study did not prevent the activation of LSt cells following DPN stimulation suggesting that autoreceptors are not the primary targets of galanin released from the axon terminals of LSt cells. Alternatively, galantide did not diffuse deeply into the spinal cord to the location of the LSt cells.

Similarly to galanin, CCK is one of the first peptide hormones to be isolated from the gastrointestinal tract and is widely distributed in the nervous system of mammals (Vanderhaeghen et al., 1975). CCK is involved in many physiological processes including digestion, satiety, and is known to induce anxiety and drug tolerance to opioids (Bradwejn, 1993; Greenough et al., 1998; Fukazawa et al., 2007). The actions of CCK are mediated by two classes of receptors: CCKA and CCKB subtypes (Oz et al., 2007) which belong to a family of G-protein-coupled receptors (Oz et al., 2007). As galanin, CCK is expressed in DRG cells and involved in the maintenance of neuropathic pain following nerve injury (Brewer et al., 2003; Kim et al., 2009) reflected in substantial increases in CCK mRNA following injuries to the CNS (Xu et al., 1993; Brewer et al., 2003). In the spinal cord, CCK is expressed in LSt cells and LSt axons. CCK receptor expression has been described in motor neurons (Cortes et al., 1990; Schiffmann et al., 1991; Truitt et al., 2003). In addition, CCK depolarized neurons in lamina X in the lumbar spinal cord (Phelan and Newton, 2000), the precise location of LSt cells. Bath application of CCK has also been shown to depolarize isolated, hemisected lumbar spinal cord

ventral horn neurons and motorneurons in neonatal rats (Suzue et al., 1981) and this depolarizing action was mediated by CCKB type receptors (Oz et al., 2007). Activation of postsynaptic CCKB receptors can enhance the excitability of motoneurons and other types of neurons in ventral spinal cord of rats (Oz et al., 2007). This may also be the mechanism by which CCK facilitated BCM bursting.

CCK is an antagonist of endogenous opioids in the brain and spinal cord (Cesselin, 1995; Wiesenfeld-Hallin et al., 1999). It appears that CCK mediates its noxious effects through CCKB type receptors. The systemic administration of the CCKB receptor antagonist but not opioids successfully reduced mechanical allodynia in an ischemic model of SCI (Xu et al., 1994). The tendency of CCK to antagonize opioids may explain why the effect of CCK infusions on ejaculatory reflexes following subthreshold sensory stimulation (5-10 Hz) was somewhat dampened compared to galanin infusions in this study (Figures 4 – 6). LSt cells are hypothesized to release their co-expressed neuropeptides, including enkephalin, onto neurons in their target regions, thereby triggering ejaculation. Indeed, our laboratory recently found that intrathecal infusions of the mu opioid receptor agonist DAMGO consistently triggered ejaculatory reflexes in the absence of sensory stimulation in 75 % of male rats (Kozyrev, 2013c). Thus, activation of mu opioid receptors in the lumbosacral spinal cord was sufficient in a large majority of animals to trigger ejaculatory reflexes in male rats. Infusions of CCK in the current study may have suppressed the facilitative effects of opioids on ejaculatory reflexes in male rats by antagonizing endogenous opioids released from the LSt cells onto target regions following stimulation of the DPN. Although data from the two neuropeptide experiments were not directly compared, it is evident that the numbers of events, bursts and SVP increases following infusions of galanin (Figures 20 – 21) are somewhat higher compared to the numbers of events, bursts and SVP

increases following infusions of CCK (Figure 22). Furthermore, infusions of galanin produced a coordinated bursting pattern of the BCM and a robust increase in the numbers of BCM bursts and concurrent SVP increases (Figure 20D and 21D) while infusions of CCK resulted in a modest increase in BCM bursts and simultaneous SVP increases but not a full coordinated bursting pattern characteristic of ejaculation (Figure 22E).

In the first set of experiments, the higher dose of galanin (0.3 nmol; Figure 20) facilitated ejaculatory reflexes not only in response to subthreshold levels of DPN stimulation (5-10 Hz) but also following threshold stimulation frequencies (30-60 Hz). However, in the subsequent group of male rats, intrathecal infusion of the same dose of galanin (0.3 nmol; Figure 21) significantly decreased numbers of BCM events, bursts and SVP increases following 60 Hz DPN stimulation. In these animals, galanin (0.3 nmol) still facilitated numbers of BCM events, bursts and SVP increases in response to subthreshold levels of DPN stimulation (5-10 Hz). However, the discrepancies in ejaculatory responses between the two groups of male rats suggest that galanin consistently facilitates ejaculatory reflexes to subthreshold (5-10 Hz) but not to threshold (30-60 Hz) levels of DPN stimulation.

Since the activation of galanin or CCK receptors was not sufficient to trigger ejaculatory reflexes in the majority of male rats tested, the activation of galanin or CCK receptors may be required in combination with activation of other receptors in LSt target areas in order to trigger ejaculation. One candidate is the GRP receptor. Indeed, it was recently shown by our laboratory that activation of GRP receptors is required to trigger ejaculation and that intrathecal infusions of GRP triggered ejaculatory reflexes in up to 66% of male rats (Kozyrev et al., 2012). Other candidates include the mu and delta opioid receptors, whose ligand enkephalin is co-localized in LSt cells and axons (Nicholas et al., 1999) along with galanin (Newton, 1993; Newton and Phan,

2006), CCK (Newton, 1993; Newton and Phan, 2006) and GRP (Sakamoto et al., 2008; Kozyrev et al., 2012).

Infusions of galanin significantly reduced the numbers of BCM events, bursts and SVP increases following threshold DPN stimulation (60 Hz). This finding is in agreement with previous reports of intrathecal infusions of GRP, DAMGO and deltorphin II that have all significantly reduced the numbers of BCM events, bursts and SVP increases following 60 Hz DPN stimulation (Kozyrev et al., 2012; Kozyrev, 2013c). This consistent suppression of ejaculatory reflexes to threshold DPN stimulation (60 Hz) may be due to the effects of intrathecal infusions of neuropeptides combined with the endogenous release of opioids following DPN stimulation that cumulatively act to inhibit or desensitize the G-protein coupled receptors in autonomic and motor regions involved in ejaculation and suppress the transmission of sensory inputs to the LSt cells which are required to trigger ejaculation.

In conclusion, these data support the hypothesis that activation of galanin and CCK receptors in the lumbosacral spinal cord is required for sensory stimulation-induced emission and expulsion in anesthetized and spinalized male rats. Galanin and CCK likely act on receptors in LSt cell target regions, including motoneurons in the SNB, and autonomic preganglioninc neurons in the IML, CAN and SPN in the lumbosacral spinal cord. Overall, these data suggest that the co-release of neuropeptides from LSt cell axon terminals including galanin, CCK, enkephalin and GRP, triggers ejaculatory reflexes in male rats. Conversely, the release of only one of these neuropeptides is not sufficient to trigger ejaculatory reflexes in all animals in the absence of added sensory stimulation. Finally, these data suggest that galanin and CCK antagonists may be promising pharmacological agents in the treatment of ejaculatory disorders, particularly for the purpose of delaying the onset of emission and expulsion in men suffering

from premature ejaculation.

Chapter 5: Activation of mu opioid and GRP receptors in the lumbosacral spinal cord can improve ejaculatory reflexes in male rats with chronic contusion spinal cord injury

5.1 Introduction

Ejaculatory function is severely impaired in men with spinal cord injuries (SCI) (Brackett et al., 1998). A great majority of these patients are men with 85% between the ages of 18 - 45 years (Ohl et al., 1989). Of these SCI men, just 12 – 15 % are able to ejaculate but only with the aid of intense vibratory stimulation or electro-ejaculation (Elliott, 2006). Among paraplegic men, regaining sexual function is of the utmost importance, and is even ranked higher than regaining locomotor function (Anderson, 2004). However, the mechanisms whereby chronic SCI so dramatically disrupts ejaculation in men with SCI are currently unknown.

Male sexual behavior is a complex and highly rewarding behavior which includes several components including the pursuit and investigation of a female, mounts and intromissions, finally culminating in ejaculation (Coolen et al., 2004; Allard et al., 2005; Coolen, 2005) In men, ejaculation occurs concurrently with the pleasurable experience of orgasm (Bohlen et al., 1980; Elliott, 2002). Similarly, male rats associate ejaculation with reward (Tenk et al., 2009) Therefore, ejaculation is an exceptionally rewarding and complex physiological process which terminates in the expulsion of seminal contents from the urethral meatus (Giuliano and Clement, 2005; Clement P, 2011) and comprises two phases: emission and expulsion. The emission phase involves the secretion of seminal fluids from the prostate, seminal vesicles and vas deferens and closure of the bladder neck and external urethral sphincter. The expulsion phase comprises rhythmic contractions of the striated perineal muscles, particularly the bulbocavernosus muscle (BCM), to forcefully expel seminal contents from the urethral meatus (Bohlen et al., 1980;

Gerstenberg et al., 1990; Holmes et al., 1991; Holmes and Sachs, 1991). Ejaculation is a reflex under the control of the spinal ejaculation generator, located in the lumbosacral spinal cord (Pescatori et al., 1993; McKenna, 1999; Coolen et al., 2004; Allard et al., 2005; Coolen, 2005). The spinal ejaculation generator is comprised of interneurons in lamina 10 and the medial portion of lamina 7 of lumbar segments 3 and 4 (L3-4). These lumbar interneurons are known as lumbar spinothalamic (LSt) cells because of their anatomical location in the lumbar spinal cord and direct projections to the subparafascicular parvocellular thalamic nucleus (Coolen et al., 2003; Truitt et al., 2003; Giuliano and Clement, 2005; Clement P, 2011). Moreover, they are pivotal in the control of ejaculatory behavior (Truitt et al., 2003) and reflexes (Staudt, 2011) in male rats. The spinal ejaculation generator is thought to regulate ejaculation by coordinating sympathetic, parasympathetic, and motor ouflow required to stimulate emission and expulsion (Coolen et al., 2004; Coolen, 2005) and by integrating the sensory inputs, conveyed by the dorsal penile nerve (DPN) during sexual activity, with autonomic and motor ouflow to produce ejaculation (Coolen et al., 2004; Allard et al., 2005; Coolen, 2005; Giuliano and Clement, 2005; Clement P, 2011). The LSt cells, which form an integral component of the spinal ejaculation generator, are hypothesized to transform sensory signals associated with copulation into motor or secretory outputs (Truitt and Coolen, 2002). In support of this notion, LSt cells are activated specifically with ejaculation but not with other components of male sexual behavior (Truitt et al., 2003). In addition, LSt cells express neural activation, including cFos (Truitt et al., 2003) and phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK) (Staudt et al., 2010) following ejaculation triggered by mating or electrical stimulation of the dorsal penile nerve (DPN) in male rats (Sachs and Garinello, 1979; Pescatori et al., 1993; McKenna, 1997; Coolen et al., 2004). Moreover, activation of the MAP kinase pathway is required for ejaculation

following mating in intact animals or DPN stimulation in anesthetized and spinalized male rats (Staudt et al., 2010). Finally, LSt cells control ejaculation via their axon projections to numerous target regions involved in ejaculation (Newton, 1993; Allard et al., 2005; Xu et al., 2006) including preganglionic sympathetic, parasympathetic and motor neurons in the thoracolumbar and lumbosacral spinal cord (Newton, 1993; McKenna, 1997; Sakamoto et al., 2008). LSt cells and axons co-express various neuropeptides including enkephalin, galanin, cholecystokinin and gastrin releasing peptide (GRP) (Ju et al., 1987; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008). Thus, it was predicted that these neuropeptides modulate ejaculatory reflexes by acting in the LSt target areas. Indeed, our laboratory utilized a physiological paradigm to test that hypothesis, using stimulation of the DPN in spinalized, anestitized male rats. This DPN stimulation model is a physiologically relevant model of ejaculation (Clement P, 2011) which utilizes anesthetized and spinalized animals and consistently triggers ejaculatory reflexes in male rats (Pescatori et al., 1993), primates (Herbert, 1973), and men (Wieder et al., 2000). It has been demonstrated that antagonism of each of the neuropeptides receptors in LSt target areas abolish ejaculatory reflexes (Kozyrev et al., 2012; Kozyrev, 2013c, 2013b). Moreover, infusions of agonists for the receptors these neuropeptides triggered ejaculatory reflexes in the absence of sensory stimulation (Kozyrev et al., 2012; Kozyrev, 2013c, 2013b) or facilitated ejaculation following subthreshold sensory stimulation incapable of triggering ejaculation in control conditions.

The purpose of the present study was to test whether intrathecal infusions of receptor agonists for the neuropeptides enkephalin, galanin, and GRP, individually and in combination, can restore ejaculatory reflexes in a chronic model of contusion SCI in anesthetized and spinalized male rats. Specifically, it was tested whether infusion of each agonist individually or

infusions of a cocktail of these agonists is sufficient to trigger ejaculatory reflexes in male rats in the absence of sensory stimulation or followed by electrical stimulation of the DPN. Parameters of emission and expulsion were analyzed during neuropeptide infusions and following DPN stimulation.

5.2 Materials and Methods

5.2.1 *Animals*

Adult male Sprague Dawley rats (225-250 grams) were acquired from Charles River (Wilmington, MA, USA) and pair housed in standard housing cages on a reverse 12-hour light/dark cycle with lights off at 9 a.m. Food and water were available ad libitum. All procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and conformed to the guidelines outlined by the National Institutes of Health.

5.2.2 *Spinal Cord Surgeries*

Male rats were randomly assigned to either the spinal cord injury (SCI) group (N = 16) or the sham group (N = 17) for these experiments. All surgical procedures were performed under ketamine (87 mg/ml) / xylazine (13 mg/ml) anesthesia (1 ml/kg i.p. injection). Backs were

shaved and thoroughly cleaned with betadine, iodine and 70 % alcohol solution. Lacrilube opthalamic ointment (Allergen, Irevine, CA) was applied to the eyes in order to avoid drying. In both SCI and sham animals, a laminectomy was performed between the 6^{th} and 7^{th} thoracic spinal segments (T6-T7) and was made large enough in order to ensure accessibility of the spinal cord during the impact injury. Only the SCI group of male rats was subjected to the contusion spinal cord injury, performed with the IH-400 impactor (Precision Systems and Instrumentation, LLC, Lexington, KY), positioned 3 mm above the cord and a force of 200-216 kdyn was applied directly to the intact dura of the exposed spinal cord at the T6/7 segments. After the contusion, the layers of muscle and skin were sutured separately, the muscle was sutured with absorbable sutures and the skin was closed using wound clips. Subcutaneous injections of the analgesic ketoprofen (5 mg/ml/kg s.c.), and the antibiotic Baytril (5 mg/ml/kg s.c.), were administered immediately following the surgery and for up to three days thereafter in order to alleviate pain and prevent infection. The sham animals were injected with ketoprofen and Baytril in the same manner and for the same period of time as the SCI animals. Bladders of SCI males were expressed twice daily until bladder function returned (typically after 7-10 days).

5.2.3 *Locomotor testing*

Locomotor testing was conducted three times beginning one week following the contusion/sham surgeries and each week thereafter in order to assess locomotor recovery following spinal cord contusion. Locomotor activity was measured during the first half of the light phase, for 15 minutes, in a large area and using 16 × 16 photobeam array (Med Associates

Inc, Vermont). Locomotor activity was expressed as the time animals spent ambulatory in the horizontal position, or rearing in a vertical position. Locomotor activity was analyzed using a two-way repeated anova (factors: SCI and time) and Holm-Sidak post-hoc tests to compare locomotor activity two, three and four weeks following spinal cord contusion (or sham) surgery within each group of animals and between sham and SCI animals, separately for ambulatory and vertical time.

5.3 Experimental Designs

5.3.1 *Intrathecal infusions of neuropeptides*

Four to five weeks following the spinal cord contusion surgeries, ejaculatory reflexes were measured as described before (Kozyrev et al., 2012; Kozyrev, 2013b, 2013a). Male rats were deeply anesthetized with urethane (1.5 g/kg, i.p.), backs were shaved and a laminectomy was made between the seventh and the eighth thoracic spinal segments, immediately caudal to the injury site, and a complete transaction of the spinal cord was performed at the same spinal level. Next, animals were positioned supine and the skin, fat, connective tissues and ligaments surrounding the reproductive organs were removed to expose the muscle of the bulbocavernosus (BCM) and the dorsal penile nerve (DPN). Then, silver recording electrodes, attached to the PowerLab/7SP Data Acquisition System (AD Instruments, Inc., Colorado Springs, CO, USA) were bilaterally implanted into the BCM and a ground electrode was inserted into the muscle of the right thigh prior to the start of electromyographic (EMG) recordings. In addition, a small

incision was made in the abdomen and the right seminal vesicle was located for the purpose of measuring and recording seminal vesicle (SV) pressure, a marker of the emission phase of ejaculation (Clement et al., 2008; Kozyrev et al., 2012; Kozyrev, 2013c, 2013b). SV pressure (SVP) was measured with a pressure catheter (AD Instruments Inc., model number: SPR-671(1.4 F, Single, Straight, 15 cm, Ny) connected to a catheter interface cable (AD Instruments Inc., model number: AEC-IOD) and attached to a Bridge AMP (AD Instruments Inc.). The pressure catheter was carefully guided into the lumen of the SV and fixed in place before recording commenced. Lastly, a bipolar stimulating electrode connected to the SD9 Square Pulse Stimulator (Grass Technologies, West Warwick, RI, USA) was arranged directly on top of the DPN, in preparation for stimulation. Immediately following spinal cord transection, the DPN was stimulated in order to verify the completeness of the spinal transection, reflected in the release of supraspinal inhibition and the corresponding rhythmic bursting of the BCM and concurrent increases in SVP. The initial electrical stimulation of the DPN was composed of square wave pulses of 1 ms duration, 4 V at 60 Hz for 10 seconds. These DPN stimulation parameters have been well established to reliably trigger increases in SVP and concurrent rhythmic bursting of the BCM, corresponding to the emission and expulsion components of ejaculation, in all saline-treated control animals (Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012; Kozyrev, 2013c, 2013b).

Pharmacological experiments were conducted as described previously (Kozyrev et al., 2012; Kozyrev, 2013a, 2013b) and began two hours after spinal cord transection in order to allow the acute effects of spinal cord transection to subside. In preparation for neuropeptide infusions, a small opening was made in the dura mater in the location of the laminectomy and a polyethylene catheter (Caly-Adams PE-10, Parsippany, NJ, USA) was gently guided into the

subarachnoid space until the open end reached the 3rd lumbar-4th lumbar (L3-L4) spinal segments.

In the first testing trial (saline trial; Figure 23A), 10 µL of 0.9 % saline was intrathecally infused with a polyethylene catheter to bathe the entire lumbosacral spinal cord. The catheter was left in place for 10 minutes after injection in order to let the contents diffuse in the lumbosacral spinal cord and SVP and BCM EMG activity were recorded for the entire duration. After the saline infusion, the DPN was stimulated at 5, 10, 30 Hz and 60 Hz in a counterbalanced manner, with 5-minute intervals between the stimulations. We have previously established that saline infusions and repeated stimulations do not affect ejaculatory reflexes (Kozyrev et al., 2012) . Moreover, we established that 5 and 10 Hz DPN stimulations in control conditions do not trigger ejaculation, while 30 and 60 Hz DPN stimulations under control conditions reliably trigger ejaculatory reflexes in all control animals. SVP and BCM EMG were recorded for at least 90 seconds following nerve stimulation, corresponding to the duration of a characteristic ejaculatory reflex triggered by DPN stimulation in male rats (Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012; Kozyrev, 2013b, 2013c). One hour later, this procedure was repeated twice in the same animals, but using neuropeptide receptor agonists (Trials 2 and 3). Half the animals received a 10 µL infusion of galanin (0.3 nmol [Bachem Americas, Inc. Torrence, CA, USA]; Figure 23B) and a 10 µL infusion of the delta opioid receptor agonist [DAla2] Deltorphin II (13 nmol ; [American Peptide Co, Sunnyvale CA, USA]; N (SCI) = 5; N (sham) = 6; Figure 23B: Trial 3. The other half of the animals received 10 µL infusion of gastrin releasing peptide (GRP $^{20\text{-}29}$; 0.2 nmol; [Phoenix Pharmaceuticals Inc. Burlingame, CA, USA]; Figure 23B: Trial 2) and a 10 µL infusion of the mu opioid receptor agonist (D-Ala2, N-Me-Phe4, glycinol5) – Enkephalin (DAMGO; [Bachem Americas, Inc. Torrence, CA, USA]; 1 nmol; N (SCI) = 11, N

(sham) = 11; Figure 23). Each pair of drugs was delivered in a counterbalanced order of infusion and there was a rest period of 2-3 hours between neuropeptide infusions in order to allow the effects of the previous neuropeptide on ejaculatory reflexes to subside. Recordings of SVP and BCM EMG were obtained during and following drug infusion for a total period of 10 minutes. Next, the DPN was stimulated at 5, 10, 30 and 60 Hz in a counterbalanced manner, and concurrent recordings of SVP and BCM EMG were obtained for 90 seconds following DPN stimulation (Testing trial 2 and 3; drug trials).

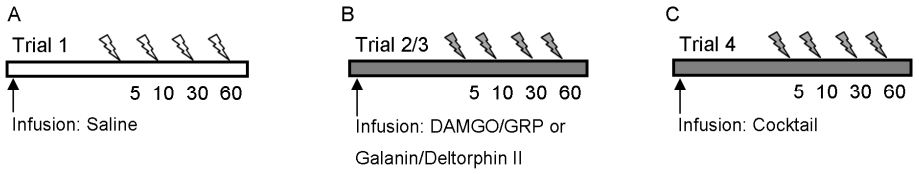

Figure 23 Timeline of experimental manipulations.

In trial 1, all male rats received intrathecal infusions of saline (A; Arrow) followed by intrathecal infusions of DAMGO (1 nmol) and GRP (0.2 nmol) or galanin (0.3 nmol) and deltrophin II (13 nmol) in trials 2 and 3 (B: Arrow [in a counterbalanced order]). Finally in trial 4, three hours following infusion of the last neuropeptide, male rats received intrathecal infusions of a cocktail of neuropeptides (C: Arrow). Following each infusion, BCM EMG and SVP activity was recorded for 15 minutes. Next, the DPN was stimulated at 5, 10, 30 and 60 Hz in a counterbalanced manner (A – C) and SVP and BCM EMG activity was recorded for 90 seconds following each DPN stimulation.

5.3.2 *Intrathecal infusions of a cocktail of neuropeptides*

Three hours following the last neuropeptide infusion, male rats (N (SCI) = 11; N (sham) = 7) received 10 µL intrathecal infusion of a cocktail of neuropeptides composed of galanin (0.3 nmol), deltorphin (13 nmol,), DAMGO (1 nmol) and GRP (0.2 nmol) and recordings of SVP and BCM EMG were obtained for 15 minutes (Figure 23C: Trial 4). After the infusion, the DPN was stimulated at 5, 10, 30, and 60 Hz in a counterbalanced manner with five minute intervals between stimulations.

5.3.3 *BCM EMG and SVP Analysis*

SVP and BCM EMG recordings were analyzed for 10 minutes following infusion of each neuropeptide, and for 90 seconds each time the DPN was stimulated. The numbers of SVP increases and concurrent BCM events and bursts were analyzed. Analysis of the numbers of BCM events was performed with LabChart 7.35 (AD Instruments Inc.) software and all events that were above baseline activity were included in the analysis (Kozyrev et al., 2012; Kozyrev, 2013b, 2013a). The numbers of SVP increases and BCM bursts were analyzed as previously described (Kozyrev et al., 2012; Kozyrev, 2013b, 2013a). Briefly, 8 or more consecutive BCM events above baseline were considered a ‘burst’ and all concurrent increases in SVP above baseline were included in the analysis.

5.3.4 *Statistical analyses*

Statistical analyses of the neuropeptide experiments involved comparing the effects of each neuropeptide on the numbers of SVP increases, BCM events and bursts within animals (within the same drug, between testing trial 1: control trial and testing trial 2 and 3: drug trials) and between SCI and sham groups of animals (separately for each stimulation setting: Infusion, 5 Hz, 10 Hz, 30 Hz, and 60 Hz), and separately for each of the three testing trials, using a two-way repeated anova (factors: drug and SCI) and Holm-Sidak post hoc tests. A 95% confidence level was utilized for all tests. In a separate analysis, the effects of the order of drug delivery were tested for Trials 2 and 3. One way ANOVA was conducted to determine differences in the effects of each drug between the half the animals receiving the drug in the second and in the third trial. Only if no differences were detected were all animals included in the analysis. Similarly, the cocktail infusion experiment was analyzed by comparing the effects of the cocktail on the numbers of SVP increases, BCM events and bursts within animals (between different stimulation settings: Infusion, 5 Hz, 10 Hz, 30 Hz and 60 Hz) as well as between sham and SCI male rats using a two-way repeated anova (factors: DPN stimulations and SCI) and Holm-Sidak post hoc tests.

5.4 Results

5.4.1 *Locomotor activity was impaired in SCI male rats*

There was no evidence of recovery in either ambulatory or vertical time in the SCI group of male rats. Moreover, SCI males did not differ in activity at 4 weeks compared to 2 weeks post-injury. In addition, both ambulatory (horizontal) and vertical time were significantly reduced in SCI male rats compared to sham male rats following surgery (contusion or sham spinal cord trauma). There were main effects of SCI on ambulatory time ($F(1, 68) = 5.360$; $P = 0.031$; Figure 24A) and vertical time ($F(1, 68) = 11.537$; $P = 0.003$; Figure 24B). Post hoc analyses demonstrated that sham males spent significantly more ambulatory time exploring their chamber compared to SCI males three weeks following surgery ($P = 0.007$; Figure 24A). In addition, sham males spent significantly greater vertical time in the chamber compared to SCI males two weeks ($P = 0.004$), 3 weeks ($P = 0.002$) and four weeks ($P = 0.010$) following surgery (Figure 24B).

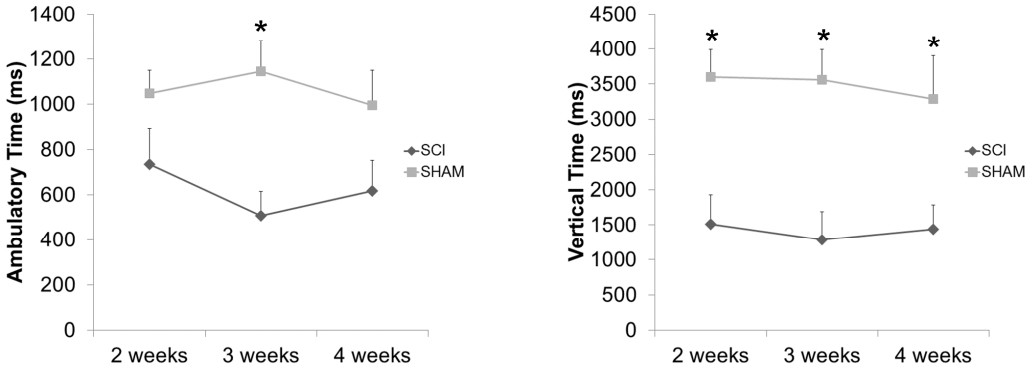

Figure 24 Locomotor activity in sham and SCI male rats 2, 3 and 4 weeks after surgery.

Locomotor acitivity expressed as the mean combined time (ms) sham and SCI male rats spent in ambulatory (A) and vertical (B) locomotion during three 15-minute trials, two, three and four weeks following spinal cord contusion injury (or sham surgery).

5.4.2 *Experiment 1: Ejaculatory reflexes were absent in SCI males*

In the first experiment, ejaculatory reflexes were examined in sham and SCI males following saline (control Trial 1), and subsequently following GRP or DAMGO (Trials 2 and 3). First, under control saline conditions, in trial 1, SCI male rats demonstrated severely disrupted BCM events, bursts, and SVP increases following 30 and 60 HZ DPN stimulation, parameters that reliable trigger ejaculation in control animals (Figures 25D, 25E, 25D, 26E, 27D, 27E). For both stimulation parameters, there were main effects of SCI on BCM events ($F(1,59) = 31.25$; $P < 0.001$ and $F(1,59) = 33.53$; $P < 0.001$), BCM bursts ($F(1,59) = 48.94$; $P < 0.001$ and $F(1,59) = 83.55$; $P < 0.001$), and SVP increases ($F(1,59) = 39.38$; $P < 0.001$ and $F(1,59) = 72.98$; $P < 0.001$). Specifically, whereas sham animals (following saline infusions), display increased BCM events, bursts and SVP increases following either 30 or 60 Hz (all $P < 0.001$); SCI animals display no BCM activity nor SVP changes at all and were significantly different from sham controls in all parameters (all $P < 0.001$). Hence, SCI severely disrupted the ability of DPN stimulation to trigger ejaculatory reflexes.

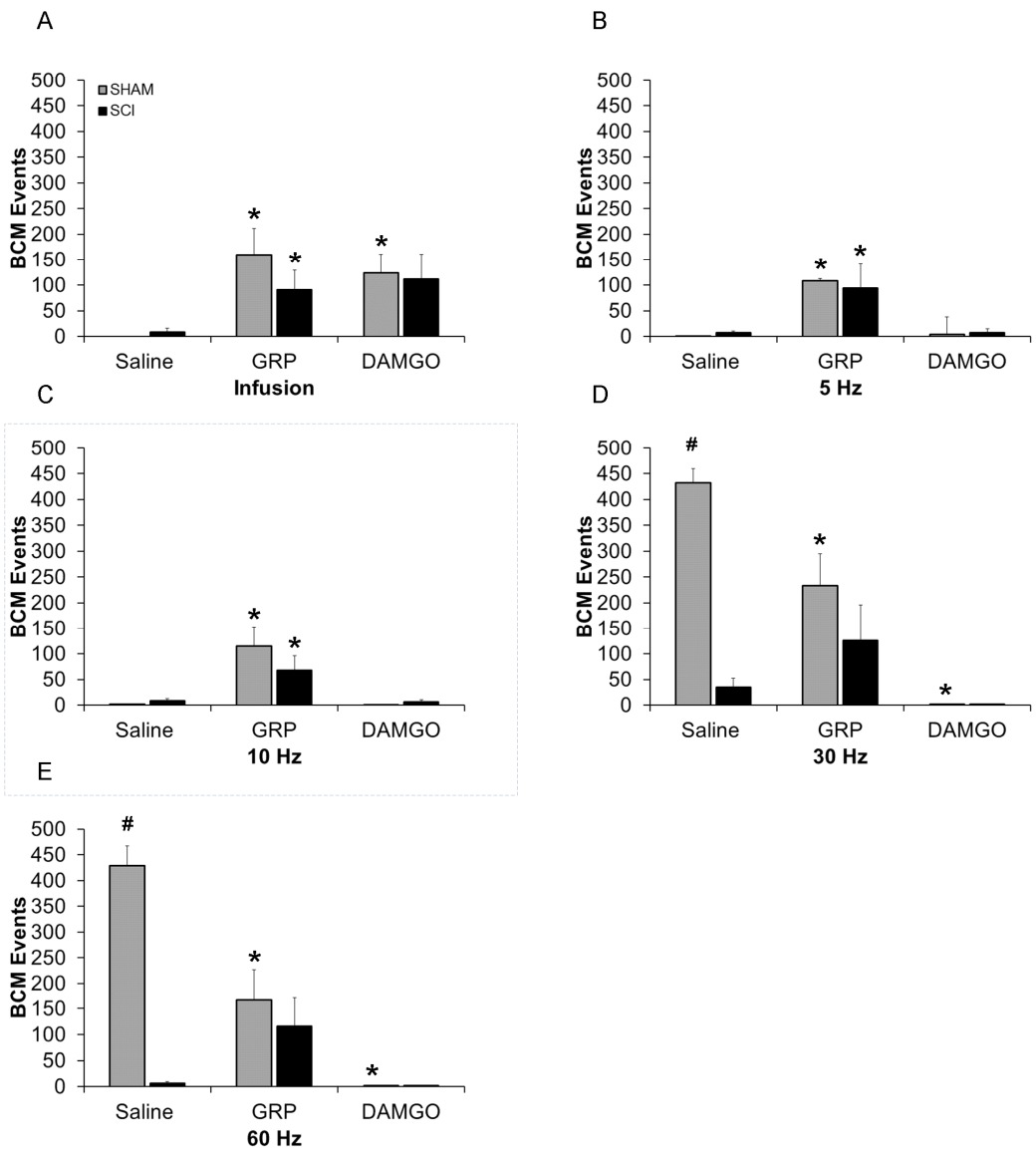
A
SHAM
SCI
BCM Events
500
450
400
350
300
250
200
150
100
50
0
Saline
GRP
DAMGO
Infusion
B
5 Hz
C
10 Hz
D
30 Hz
E
60 Hz
*
#

Figure 25 Effects of DAMGO and GRP infusions on the numbers of BCM events in sham and SCI male rats.

Quantitative analysis of BCM events following intrathecal infusions of saline (A: trial 1[control trial]) and DAMGO and GRP (**A**: trials 2 and 3 [drug trials]), followed by 5 Hz **(B)**, 10 Hz **(C)**, 30 Hz **(D)**, and 60 Hz **(E)** DPN stimulations (in a counterbalanced order) in sham and SCI male rats. * denotes significant differences from saline (trial 1: control trial) within sham or SCI; # denotes significant differences between treatment groups (sham vs. SCI) within the same testing trial.

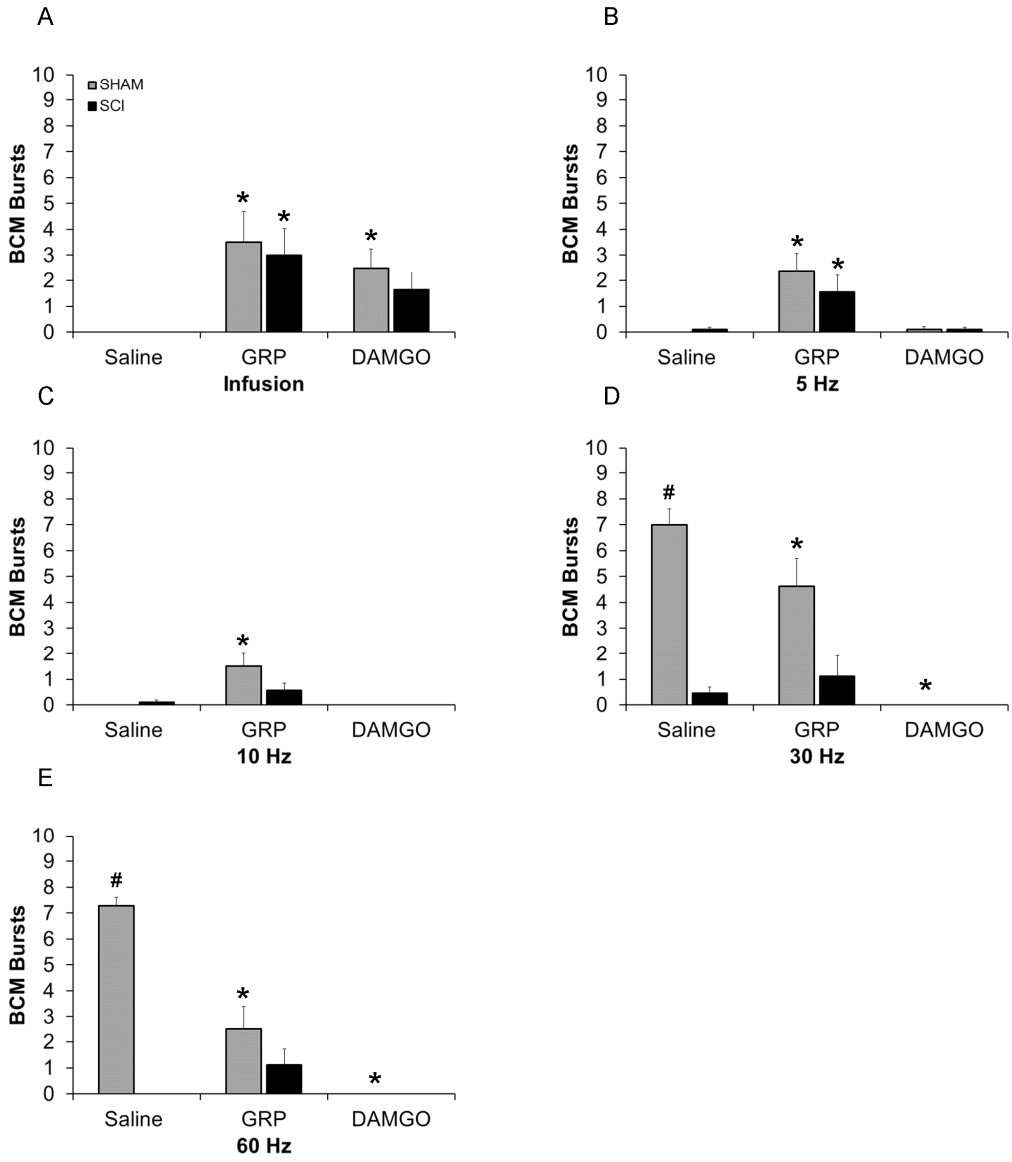
A
SHAM
SCI
BCM Bursts
Saline
GRP
DAMGO
Infusion
B
5 Hz
C
10 Hz
D
30 Hz
E
60 Hz

Figure 26 Effects of GRP and DAMGO infusions on the numbers of BCM bursts in sham and SCI male rats.

Quantitative analysis of BCM bursts following intrathecal infusions of saline (A: trial 1[control trial]) and DAMGO and GRP (**A**: trials 2 and 3 [drug trials]), followed by 5 Hz **(B)**, 10 Hz **(C)**, 30 Hz **(D)**, and 60 Hz **(E)** DPN stimulations (in a counterbalanced order) in sham and SCI male rats. * denotes significant differences from saline (trial 1: control trial) within the same treatment group. # denotes significant differences between treatment groups (sham vs. SCI) within the same testing trial.

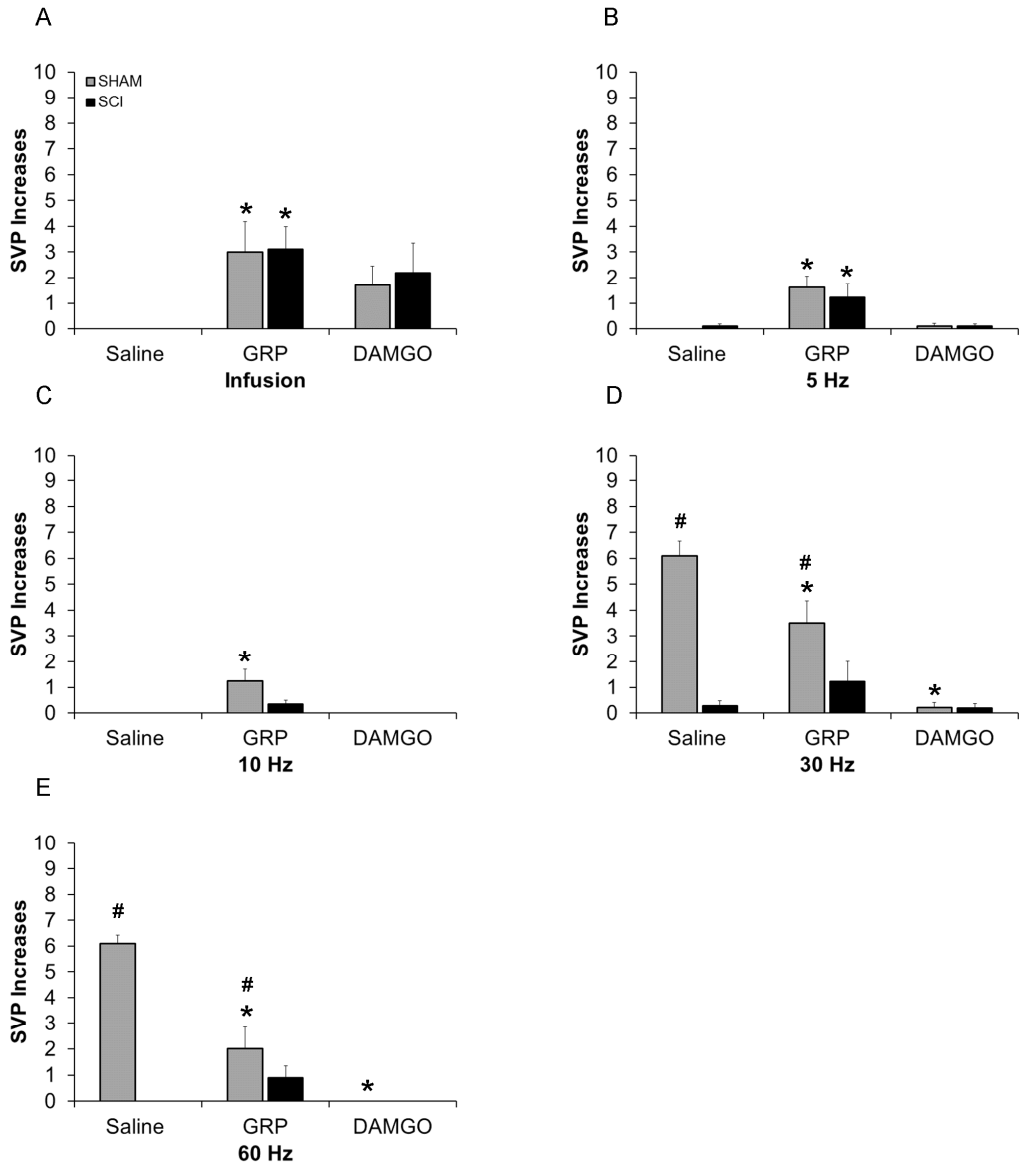
A
SHAM
SCI
SVP Increases
Saline
GRP
DAMGO
Infusion
B
5 Hz
C
10 Hz
D
30 Hz
E
60 Hz

Figure 27 Effects of GRP and DAMGO infusions on the numbers of SVP increases in sham and SCI male rats.

Quantitative analysis of SVP increases following intrathecal infusions of saline (A: trial 1[control trial]) and DAMGO and GRP (**A**: trials 2 and 3 [drug trials]), followed by 5 Hz **(B)**, 10 Hz **(C)**, 30 Hz **(D)**, and 60 Hz **(E)** DPN stimulations (in a counterbalanced order) in sham and SCI male rats. * denotes significant differences from saline (trial 1: control trial) within the same treatment group. # denotes significant differences between treatment groups (sham vs. SCI) within the same testing trial.

5.4.3 *Infusions of GRP and DAMGO in sham and SCI male rats*

Following the saline trial (Trial 1), animals received GRP and DAMGO infusions in counter balanced order during Trials 2 and 3. However, preliminary data showed that DAMGO infusions prevented the facilitatory effects of the subsequent GRP infusions, although this effect was not significant because of the small numbers of animals tested. In contrast, GRP infusions did not affect the facilitatory effects of subsequent DAMGO infusions. Therefore, data for GRP in Trial 3 were not included in the analysis, and analysis was based on effects of GRP in Trial 2 and DAMGO in Trails 2 and 3.

There were main effects of drug treatment (GRP and DAMGO in Trials 2 and 3) on all three parameters of ejaculatory reflex: numbers of BCM events (infusion: ($F(2,59) = 11.578$; $P < 0.001$; Figure 25A); 5 Hz: ($F(2, 59) = 11.637$; $P < 0.001$; Figure 25B); 10 Hz: ($F(2, 59) = 14.755$; $P < 0.001$; Figure 25C); 30 Hz: ($F(2, 59) = 22.937$; $P < 0.001$; Figure 25D); 60 Hz: ($F(2, 59) =$

20.186; $P < 0.001$; Figure 25E), BCM bursts (infusion: ($F(2, 59) = 12.987$; $P < 0.001$; Figure 26A), 5 Hz: ($F(2, 59) = 17.113$; $P < 0.001$; Figure 26B), 10 Hz: ($F(2, 59) = 13.326$; $P < 0.001$; Figure 26C), 30 Hz: ($F(2, 59) = 26.569$; $P < 0.001$; Figure 26D), 60 Hz: ($F(2, 59) = 42.239$; $P < 0.001$; Figure 26E), and SVP increases (infusion: ($F(2, 59) = 9.086$; $P < 0.001$; Figure 27A), 5 Hz: ($F(2, 59) = 15.422$; $P < 0.001$; Figure 27B), 10 Hz: ($F(2, 59) = 13.295$; $P < 0.001$; Figure 27C), 30 Hz: ($F(2, 59) = 19.958$; $P < 0.001$; Figure 27D), 60 Hz ($F(2, 59) = 35.523$; $P < 0.001$; Figure 27E). In addition, there were main effects of SCI on the numbers of SVP increases, BCM events and bursts for 30 and 60 Hz (30 HZ: SVP increases: ($F(1, 59) = 39.381$; $P < 0.001$); BCM events: ($F(1, 59) = 31.248$; $P < 0.001$); BCM bursts: ($F(1, 59) = 48.942$; $P < 0.001$; 60 Hz: SVP increases: ($F(1, 59) = 72.984$; $P < 0.001$); BCM events: ($F(1, 59) = 33.530$; $P < 0.001$); BCM bursts: $F(1, 59) = 83.552$; $P < 0.001$), and on numbers of SVP increases for 10 Hz ($F(1, 59) = 5.681$; $P = 0.026$) . Furthermore, there were significant interactions between SCI and drug treatment on the numbers of SVP increases, BCM events and bursts for 30 and 60 Hz (30 Hz: SVP increases: ($F(2, 59) = 18.420$; $P < 0.001$); BCM events ($F(2, 59) = 17.185$; $P < 0.001$); BCM bursts: ($F(2, 59) = 19.344$; $P < 0.001$; and 60 Hz: SVP increases: ($F(2, 59) = 40.377$; $P < 0.001$); (BCM events: ($F(2, 59) = 23.978$; $P < 0.001$); BCM bursts: ($F(2, 59) = 47.729$; $P < 0.001$), and on the numbers of SVP increases and BCM bursts for 10 Hz (SVP increases: ($F(2, 59) = 4.491$; $P = 0.019$; Figure 27C); BCM bursts: $F(2, 59) = 3.413$; $P = 0.045$; Figure 26C).

5.4.4 *Infusions of GRP triggered and facilitated ejaculatory reflexes in male SCI rats*

Intrathecal infusions of the gastrin-releasing peptide (GRP) agonist GRP^{2029} improved ejaculatory reflexes in male rats with SCI, following drug infusion alone and in response to subthreshold (5 – 10 Hz) levels of DPN stimulation (Figures 25 – 27, 28C, 28D, 29A-29D). First, in sham control males, GRP infusions in trial 2, in the absence of DPN stimulation triggered ejaculatory reflexes and increased numbers BCM events ($P = 0.001$), bursts ($P < 0.001$), and SVP ($P = 0.001$) compared to saline infusions in trial 1, replicating our previous findings (Kozyrev et al., 2012). Moreover, GRP infusions facilitated ejaculatory reflexes following 5 and 10 Hz DPN stimulation, evidenced by significant increases in numbers of BCM events ($P = 0.002$ and $P < 0.001$), BCM bursts ($P < 0.001$ both), and SVP increases ($P < 0.001$ both), and again confirming our previous findings (Kozyrev et al., 2012). Moreover, in SCI males, GRP infusions alone, and in conjunction with subthreshold DPN stimulation triggered and facilitated ejaculatory reflexes. In SCI males, GRP infusions increased numbers of BCM events ($P = 0.006$), BCM bursts ($P = 0.002$), and SVP increases ($P = 0.003$) compared to saline in trial 1. Also, in SCI males GRP increased these parameters following 5 Hz (BCM events: $P = 0.005$; BCM bursts: $P = 0.004$; SVP increases: $P = 0.004$) and 10 Hz (BCM events: $P = 0.014$) compared to saline infusions and DPN stimulation. Furthermore, ejaculatory reflexes in SCI males treated with GRP were identical to those of sham males.

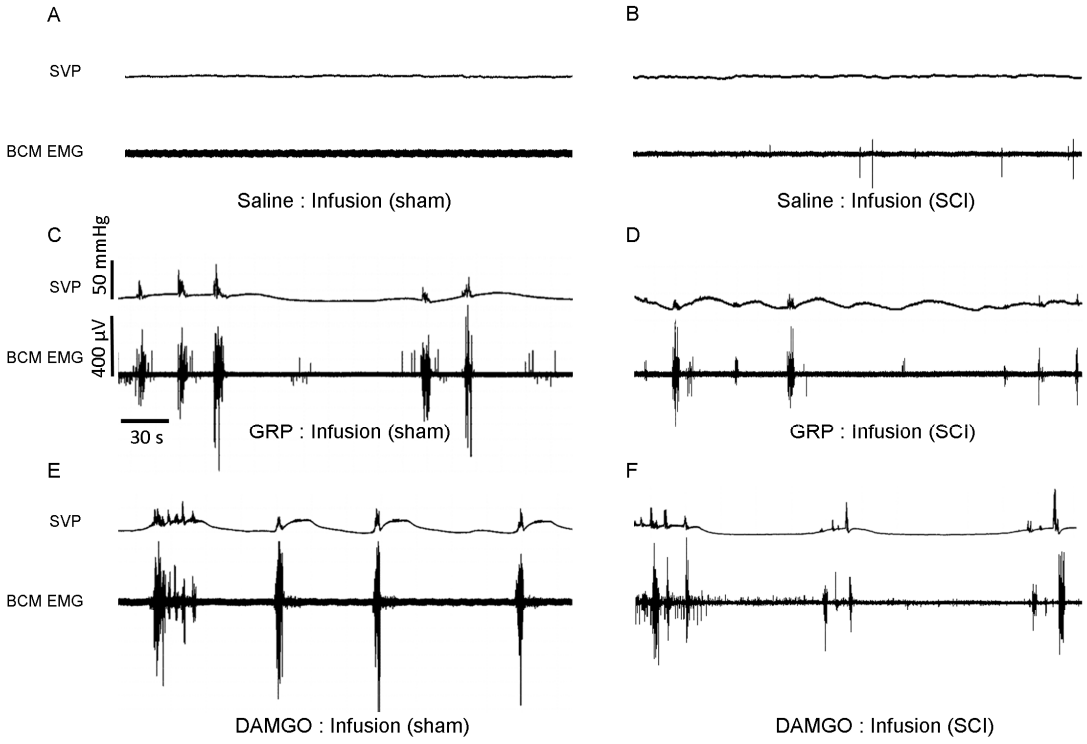

Figure 28 Representative BCM EMG and SVP recording traces showing SVP increases and concurrent BCM activity induced by intrathecal infusions of GRP and DAMGO in sham and SCI male rats.

Infusions of saline did not trigger SVP increases or BCM activity in sham **(A)** or SCI **(B)** male rats. In contrast, intrathecal infusions of GRP **(C: Sham, D: SCI)** and DAMGO **(E: Sham [same animal as in A], F: SCI [same animal as in B])** induced SVP increases and simultaneous BCM bursts in the absence of DPN stimulation in a subset of sham and SCI male rats. Each trace represents 200 seconds of recordings.

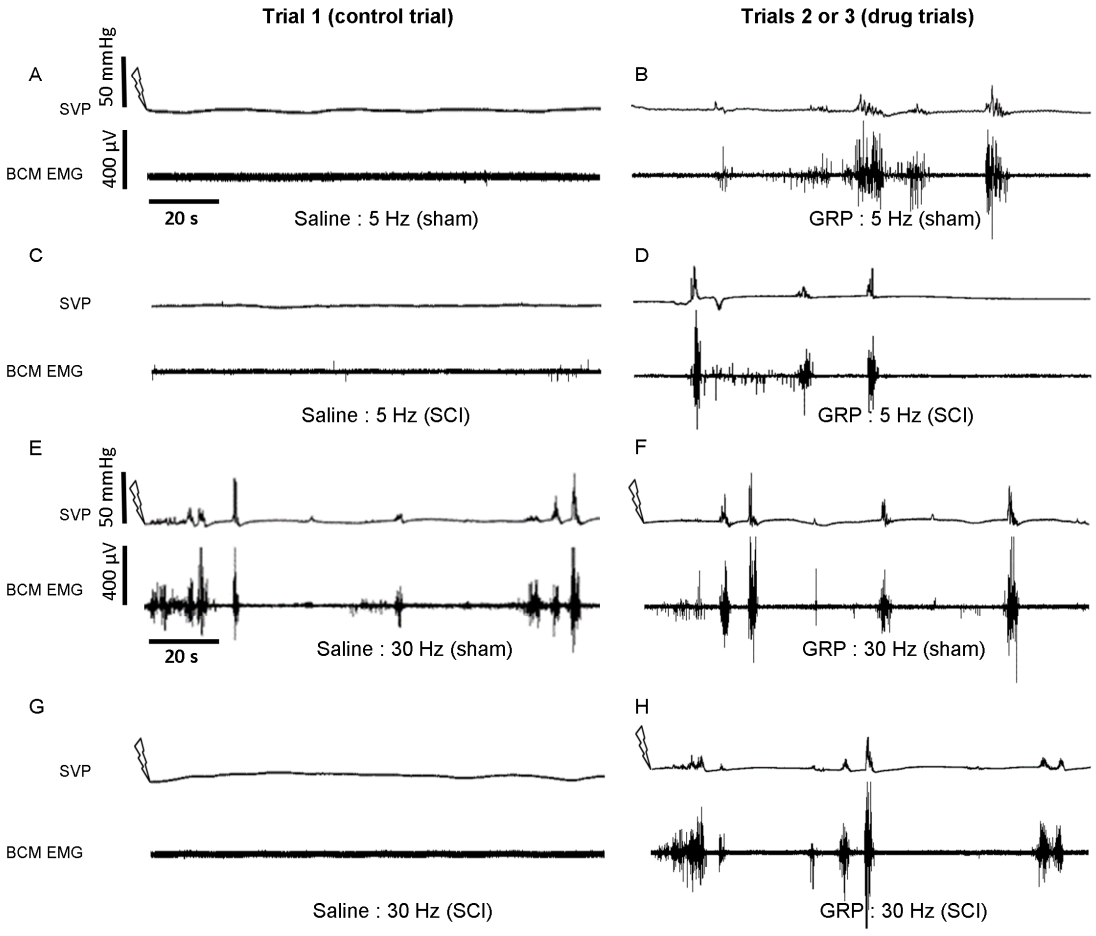
Trial 1 (control trial)
Trials 2 or 3 (drug trials)
A
B
SVP
50 mmHg
BCM EMG
400 µV
20 s
Saline : 5 Hz (sham)
GRP : 5 Hz (sham)
C
D
SVP
BCM EMG
Saline : 5 Hz (SCI)
GRP : 5 Hz (SCI)
E
F
SVP
50 mmHg
BCM EMG
400 µV
20 s
Saline : 30 Hz (sham)
GRP : 30 Hz (sham)
G
H
SVP
BCM EMG
Saline : 30 Hz (SCI)
GRP : 30 Hz (SCI)

Figure 29 Representative BCM EMG and SVP recording traces depicting SVP and concurrent BCM EMG activity triggered by subthreshold (5 Hz) and threshold (30 Hz) levels of DPN stimulation following infusions of saline and GRP in sham and SCI male rats

Representative BCM EMG and SVP recording traces depicting SVP and concurrent BCM EMG activity triggered by subthreshold (5 Hz) and threshold (30 Hz) levels of DPN stimulation in sham and SCI male rats. Subthreshold DPN stimulation (5 Hz) following saline infusions did not induce BCM EMG or SVP activity in sham **(A)** or SCI **(C)** male rats. Conversely, subthreshold DPN stimulation (5 Hz) following infusions of GRP triggered emission (SVP) and explusion (EMG) reflexes in both sham **(B [same animal as in A])** and SCI **(D [same animal as in C])** male rats. Next, threshold DPN stimulation (30 Hz) triggered BCM EMG and SVP activity in sham **(E)** but not in SCI **(G)** males. Intrathecal infusions of GRP followed by threshold DPN stimulation (30 Hz) triggered BCM EMG and SVP increases in sham males **(F [same animal as in E])** and rescued ejaculatory reflexes in SCI males **(H [same animal as in G]**).

Finally, in sham males, numbers of BCM events, bursts and SVP increases were significantly lower compared to those following 30 or 60 Hz stimulation after saline treatment in Trail 1 (all $P < 0.001$), but still appeared increased compared to subthreshold or no stimulation (statistical comparisons were not conducted). Hence, although GRP inhibited ejaculatory reflexes, they were still triggered by DPN stimulation. However, SCI males were even more affected by these inhibitory effects of GRP, and numbers of BCM bursts and SVP increases were significantly lower compared to sham males under the same GRP and DPN stimulation conditions (BCM bursts: 30 Hz: $P < 0.001$and 60 Hz: $P = 0.036$; SVP increases: 30 Hz $P = 0.01$).

5.4.5 *Infusions of DAMGO triggered ejaculatory reflexes in male SCI rats*

In sham animals, DAMGO (1 nmol) triggered ejaculatory reflexes (BCM events: $P = 0.001$; BCM bursts: $P < 0.001$; SVP increases: $P = 0.011$). Moreover, DAMGO infusions triggered ejaculatory reflexes in SCI males, even though this only reached statistical significance for SVP increases ($P = 0.028$), with strong trends for BCM events ($P = 0.053$) and bursts ($P = 0.07$). There were no differences between sham and SCI males in these parameters. In sham males, DAMGO did not facilitate ejaculatory reflexes following sub threshold DPN stimulation. Moreover, DAMGO completely ablated the ability of 30 and 60 Hz DPN stimulation to trigger ejaculation. These findings replicate our previous findings (Kozyrev, 2013b: Chapter 3) and indicate that once DAMGO is infused to stimulate ejaculation, subsequent ejaculatory reflexes can no longer be triggered by DPN stimulation. DAMGO infusions in SCI animals had the same lack of effect on DPN stimulation-induced ejaculatory reflexes.

5.4.6 *Experiment 2: SCI disrupts ejaculatory reflexes, but deltorphin or galanin do not improve function.*

First, the effects of SCI observed in the first experiment, were again observed in this second experiment. Whereas, 30 and 60 Hz DPN stimulation following saline infusions (control Trial 1) reliably triggered ejaculatory reflexes in sham males; SCI males showed no ejaculatory reflexes under any conditions (Figures 30, 31, 32).

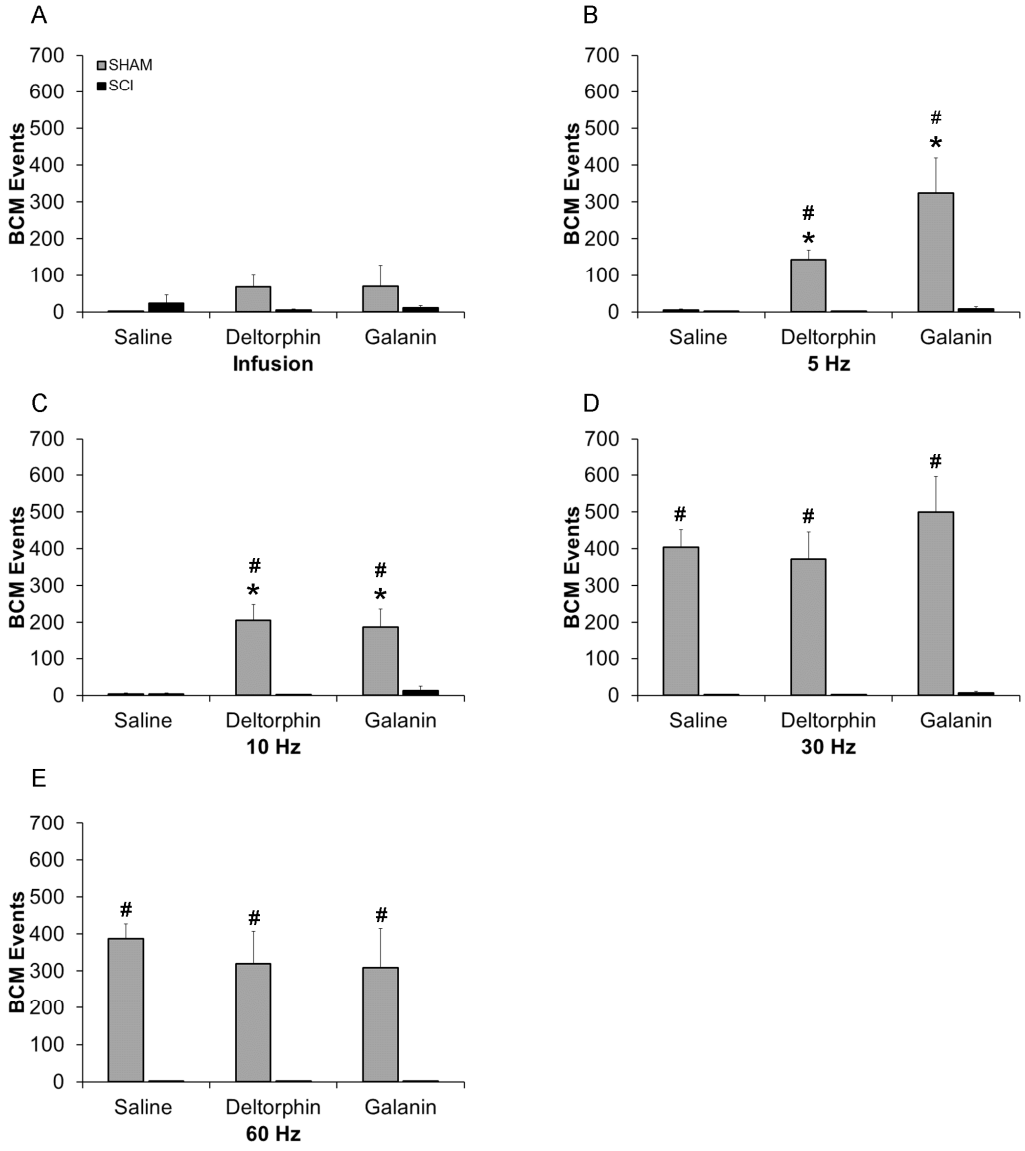
A
SHAM
SCI
BCM Events
700
600
500
400
300
200
100
0
Saline
Deltorphin
Galanin
Infusion
B
5 Hz
C
10 Hz
D
30 Hz
E
60 Hz
#
*

Figure 30 Effects of galanin and deltorphin II infusions on the numbers of BCM events in sham and SCI male rats.

Quantitative analysis of BCM events following intrathecal infusions of saline (**A**: trial 1[control trial]) and galanin and deltorphin II (**A**: trials 2 and 3 [drug trials]), followed by 5 Hz **(B)**, 10 Hz **(C)**, 30 Hz **(D)**, and 60 Hz **(E)** DPN stimulations (in a counterbalanced order) in sham and SCI male rats. * denotes significant differences from saline (trial 1: control trial) within the same treatment group. # denotes significant differences between treatment groups (sham vs. SCI) within the same testing trial.

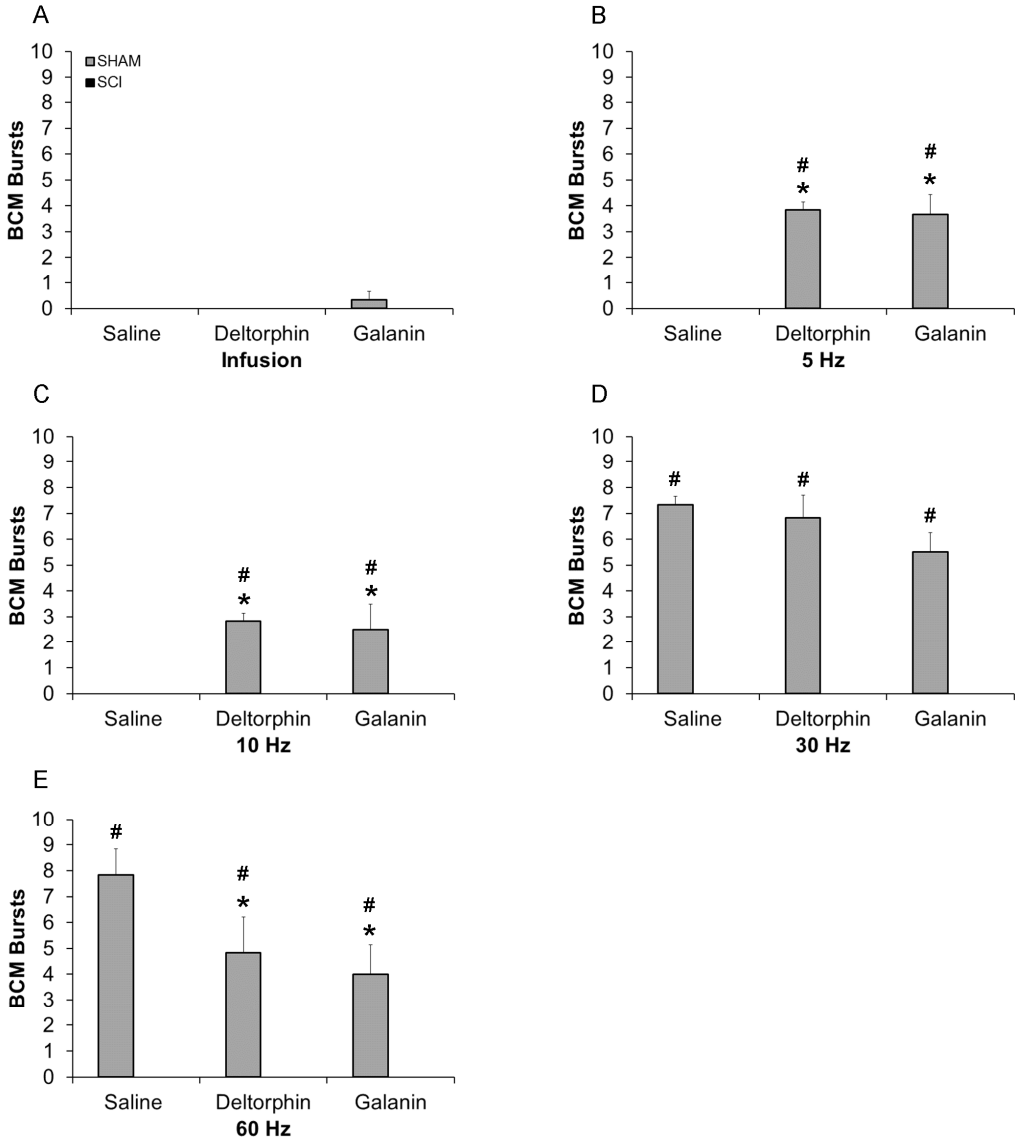
A
SHAM
SCI
BCM Bursts
Saline
Deltorphin
Galanin
Infusion
B
5 Hz
C
10 Hz
D
30 Hz
E
60 Hz

Figure 31 Effects of galanin and deltorphin infusions on the numbers of BCM bursts in sham and SCI male rats

Quantitative analysis of BCM bursts following intrathecal infusions of saline (A: trial 1[control trial]) and galanin and deltorphin II (**A**: trials 2 and 3 [drug trials]), followed by 5 Hz **(B)**, 10 Hz **(C)**, 30 Hz **(D)**, and 60 Hz **(E)** DPN stimulations (in a counterbalanced order) in sham and SCI male rats. * denotes significant differences from saline (trial 1: control trial) within the same treatment group. # denotes significant differences between treatment groups (sham vs. SCI) within the same testing trial.

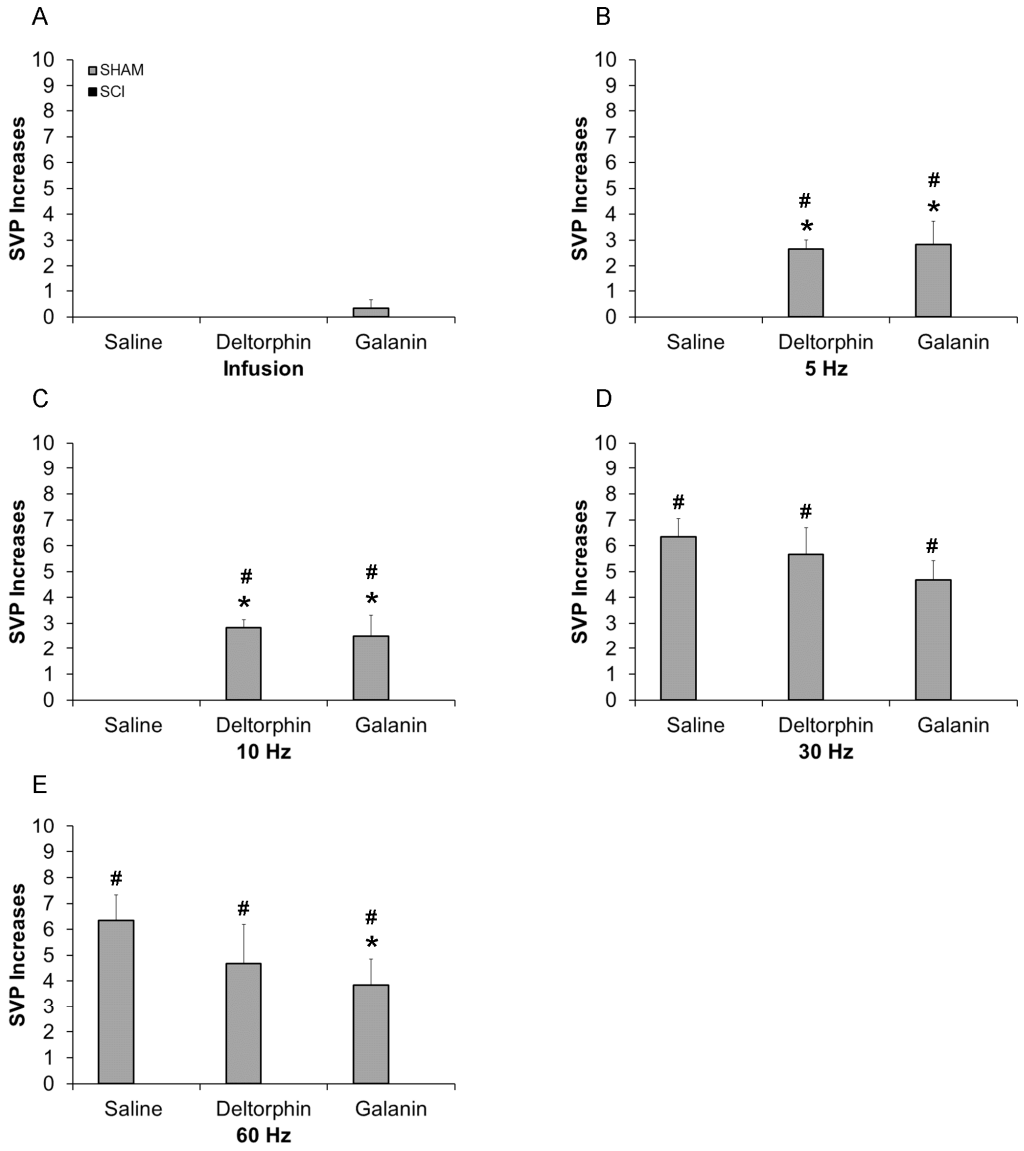
A
SHAM
SCI
SVP Increases
Saline
Deltorphin
Galanin
Infusion
B
5 Hz
C
10 Hz
D
30 Hz
E
60 Hz

Figure 32 Effects of galanin and deltorphin infusions on the numbers of SVP increases in sham and SCI male rats

Quantitative analysis of SVP increases following intrathecal infusions of saline (**A**: trial 1[control trial]) and galanin and deltorphin II (**A**: trials 2 and 3 [drug trials]), followed by 5 Hz **(B)**, 10 Hz **(C)**, 30 Hz **(D)**, and 60 Hz **(E)** DPN stimulations (in a counterbalanced order) in sham and SCI male rats. * denotes significant differences from saline (trial 1: control trial) within the same treatment group & denotes significant differences between treatment groups (sham vs. SCI) within the same testing trial.

There were main effects of SCI on the numbers of BCM events and bursts, and SVP increases for all stimulation parameters (Infusions: Events: ($F(1,32) = 12.685$; $P = 0.006$; 5 Hz BCM events: $F(1, 32) = 12.685$; $P = 0.006$; BCM bursts: $F(1, 32) = 54.144$; $P < 0.001$; SVP increases: $F(1, 32) = 47.903$; $P < 0.001$; 10 Hz: BCM events: $F(1, 32) = 38.976$; $P < 0.001$; BCM bursts: $F(1, 32) = 36.113$; $P < 0.001$; SVP increases: $F(1, 32) = 36.113$; $P < 0.001$; Figure 32C; 30 Hz: BCM events: $F(1, 32) = 41.255$; $P < 0.001$; BCM bursts: $F(1, 32) = 229.685$; $P < 0.001$; SVP increases: $F(1, 32) = 95.582$; $P < 0.001$; and 60 Hz BCM events: $F(1, 32) = 31.001$; $P < 0.001$; BCM bursts: $F(1, 32) = 29.730$; $P < 0.001$; SVP increases: $F(1, 32) = 20.089$; $P = 0.002$). There were main effects of drug on the numbers of BCM events, bursts and SVP increases for 5, 10, and 60 Hz DPN stimulation frequencies (5 Hz: BCM events: $F(2, 32) = 7.942$; $P = 0.003$; BCM bursts: $F(2, 32) = 19.753$; $P < 0.001$; SVP increases: $F(2, 32) = 5.395$; $P = 0.015$; 10 Hz: BCM events: $F(2, 32) = 5.984$; $P = 0.010$; BCM bursts: $F(2, 32) = 4.547$; $P = 0.025$; SVP increases: $F(2, 32) = 7.409$; $P = 0.004$; 60 Hz: BCM bursts: $F(2, 32) = 4.054$; $P = 0.035$). Additionally, there were significant interactions between main factors of SCI and drug on the

numbers of SVP increases, BCM events and bursts for 5, 10, and 60 Hz (5 Hz: BCM events: $F(2, 32) = 7.942$; $P = 0.003$; BCM bursts: $F(2, 32) = 19.753$; $P < 0.001$; SVP increases: $F(2, 32) = 5.395$; $P = 0.015$; 10 Hz: BCM events: $F(2, 32) = 5.671$; $P = 0.012$; BCM bursts: $F(2, 32) = 4.547$; $P = 0.025$; SVP increases: $F(2, 32) = 7.409$; $P = 0.004$; 60 Hz: BCM bursts: $F(2, 32) = 4.054$; $P = 0.035$).

Post hoc tests showed that infusion of galanin and deltorphin facilitated ejaculatory reflexes in 100 % of sham males but did not affect ejaculatory reflexes in SCI males (Figures 30B, 30C, 31B, 31C, 32B, 32C, and 33A-33F). In sham males, both galanin and deltorphin facilitated ejaculatory reflexes following subthreshold DPN stimulation, evidenced by significantly greater numbers of BCM events and bursts, and SVP increases (all $P < 0.001$), confirming our previous findings (Kozyrev, 2013b) (Chapter 4). Also, as we reported previously, galanin and deltorphin infusions had a slight dampening effect on ejaculatory reflexes induced by 30 (galanin: BCM bursts: $P = 0.024$) or 60 Hz (galanin: BCM bursts: $P < 0.001$ and SVP increases: $P = 0.009$; deltorphin: BCM bursts: $P = 0.006$) DPN stimulation in sham males, compared to saline conditions in Trial 1. In contrast, galanin and deltorphin had no effect on ejaculatory reflexes in SCI males (Figure 30, 31, 32, 33, 34).

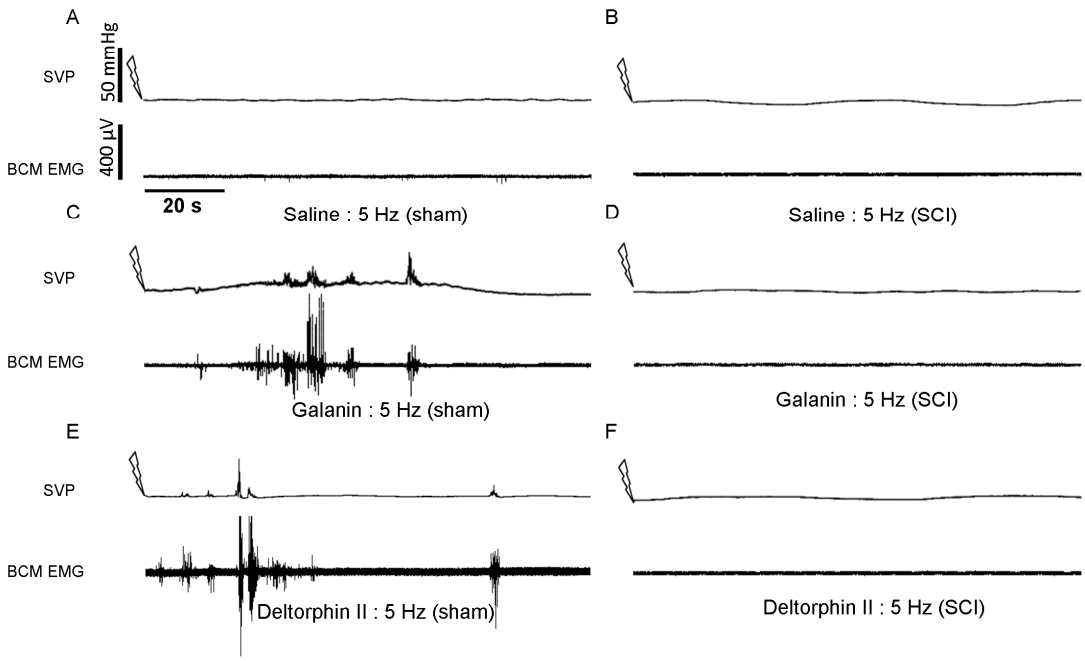

Figure 33 Representative BCM EMG and SVP recording traces depicting SVP and concurrent BCM EMG activity triggered by subthreshold (5 Hz) DPN stimulation following infusions of saline, galanin and deltorphin in sham and SCI male rats.

Subthreshold DPN stimulation (5 Hz) following saline infusions did not induce BCM EMG or SVP activity in sham **(A)** or SCI **(B)** male rats. Next, subthreshold DPN stimulation (5 Hz) following infusions of galanin and deltrophin II triggered emission (SVP) and explusion (EMG) reflexes in sham (**C** [galanin]; **E** [deltorphin II]) but not in SCI (**D** [galanin]; F [deltorphin II]) male rats. Each trace shown represents 90 seconds of recordings.

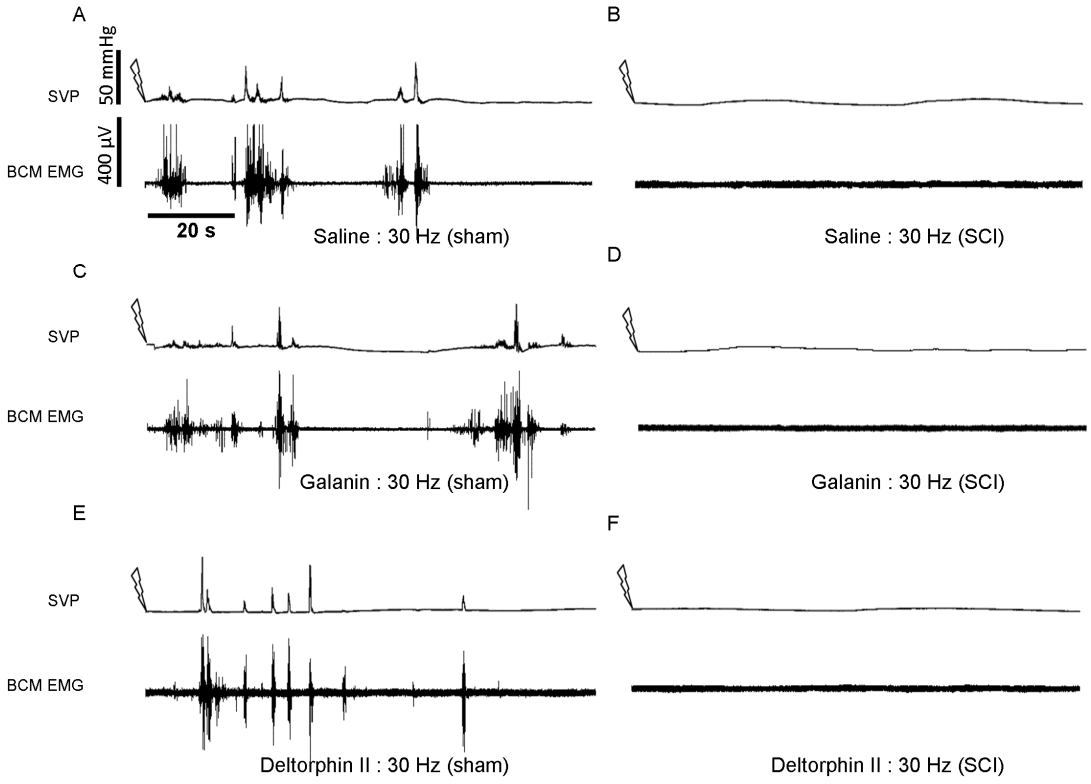

Figure 34 Representative BCM EMG and SVP recording traces depicting SVP and concurrent BCM EMG activity induced by threshold (30 Hz) DPN stimulation following infusions of saline, galanin and deltorphin in sham and SCI male rats.

30 Hz DPN stimulations following saline infusions induced BCM EMG and SVP activity in sham **(A)** but not in SCI **(B)** male rats. Next, 30 Hz DPN stimulation following infusions of galanin and deltrophin II triggered SVP increases and concurrent BCM bursts in sham (**C** [galanin]; **E** [deltorphin II]) but not in SCI (**D** [galanin]; **F** [deltorphin II]) male rats. Each trace shown represents 90 seconds of recordings.

5.4.7 *Experiment 3: Cocktail infusions triggered ejaculatory reflexes in a subset of SCI males*

In the final intrathecal infusion experiment, all sham and SCI animals received intrathecal infusions of a cocktail of all four receptor agonists (10 µl of 0.3 nmol galanin, 1 nmol DAMGO, 13 nmol deltrophin II and 0.2 nmol GRP). There were main effects of DPN stimulation parameters on the numbers of BCM events ($F(4, 89) = 4.862$; $P = 0.002$) and bursts ($F(4, 89) = 2.930$; $P = 0.027$), but only a trend on SVP increases ($P = 0.074$). Post hoc tests showed that in sham males, cocktail infusions did not trigger or facilitate ejaculatory reflexes. However, in contrast, in SCI males, cocktail infusions did trigger ejaculatory reflexes in the absence of DPN stimulation; but cocktail infusions did not facilitate ejaculatory reflexes following DPN stimulation. In SCI males infused with the cocktail, numbers of BCM events and bursts were higher compared to all DPN stimulation frequencies (compared to 5 Hz: BCM events: $P < 0.001$; BCM bursts: $P = 0.002$; 10 Hz: BCM events: $P < 0.001$; BCM bursts: $P = 0.008$; 30 Hz: BCM events: $P < 0.001$; BCM bursts: $P = 0.01$; 60 Hz: BCM events: $P < 0.001$; BCM bursts: $P = 0.003$; Figure 35).

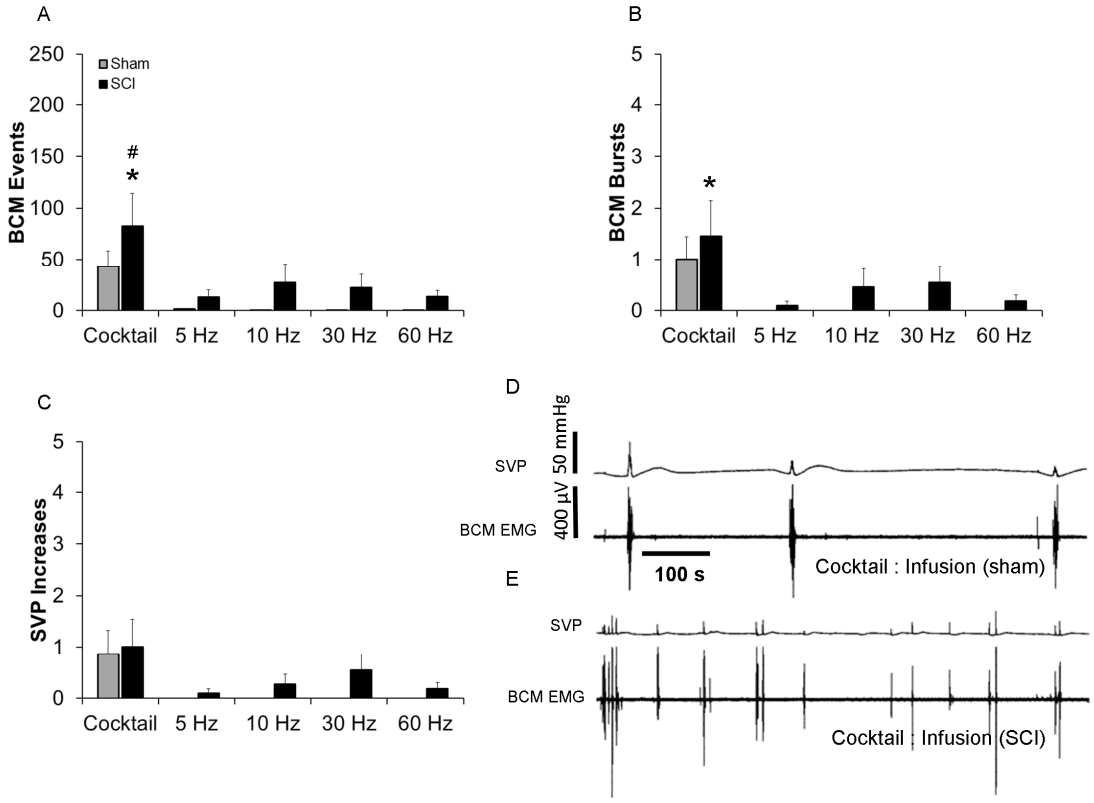

Figure 35 Effects of cocktail infusion on BCM EMG and SVP in sham and SCI male rats

Quantitative analysis of BCM events **(A)**, bursts **(B)** and SVP increases **(C)** following intrathecal infusions of a cocktail of neuropeptides (galanin, deltorphin, GRP and DAMGO) and subsequent DPN stimulations (5 – 60 Hz counterbalanced) and representative BCM EMG and SVP traces in sham (**D**: Cocktail infusion) and SCI (**E**: Cocktail infusion) male rats. * represents significant differences from 5, 10, 30 and 60 Hz DPN stimulations within the same treatment group. # denotes significant differences between treatment groups (sham vs. SCI) within the same DPN stimulation frequency. Each trace shown represents 10 minutes of recordings.

5.5 Discussion

The current study demonstrated that SCI following contusion injury severely disrupted ejaculatory reflexes in male rats. Moreover, the current study sought to determine if intrathecal infusions of four receptor ligands known to be expressed in LSt cells, were able to improve ejaculatory function in SCI males. We had previously shown facilitative effects of these four receptor ligands on ejaculatory reflexes in control animals (Kozyrev et al., 2012; Kozyrev, 2013b, 2013c). Indeed, GRP and DAMGO, but not galanin and deltoprhin had a beneficial impact on ejaculatory function.

Infusions of GRP rescued ejaculatory reflexes in a large subset of male rats with chronic SCI. GRP infusions were most effective in triggering emission and expulsion following drug infusion alone or in combination with subthreshold levels of DPN stimulation (5 – 10 Hz) in both sham and SCI males. Similarly, intrathecal infusions of the MOR agonist DAMGO restored emission in male rats with chronic SCI. Although the effects of DAMGO on expulsion, including the numbers of BCM events and bursts were not statistically significant across the group of SCI males, DAMGO infusions restored expulsion reflexes in a large percentage of SCI males (54 %). Future studies are required to investigate whether lower doses of DAMGO can restore ejaculatory reflexes in response to subthreshold levels of DPN stimulation in male rats with chronic SCI.

In contrast, intrathecal infusions of galanin and deltorphin significantly enhanced ejaculatory reflexes following subthreshold DPN stimulation (5 – 10 Hz) in sham males but had no effect on

emission or expulsion in SCI male rats. One possible explanation for the inefficacy of galanin and deltrophin in the recovery of ejaculatory reflexes in male rats with chronic SCI may be a substantial reduction in delta opioid receptor and galanin receptor expression in the lumbosacral spinal cord as a result of the chronic SCI. Since infusions of GRP and DAMGO, but not galanin or deltorphin were sufficient in restoring ejaculatory reflexes in a subset of SCI males, this suggests that the densities of mu opioid receptors and GRP receptors may remain relatively unchanged in male rats with chronic SCI. Our laboratory has previously observed that galanin immunoreactivity in LSt cells and axons in male rats with chronic SCI was not changed (unpublished observations), but a detailed investigation of neuropeptide or receptor expression has not yet been conducted.

The disruption of descending pathways to the lumbosacral spinal cord following chronic SCI may constitute another reason why DPN stimulation may be ineffective in triggering ejaculation in SCI males (de Groat, 2010). This disruption of descending pathways can result in the denervation of spinal neurons triggering the release of neurotrophic factors which in turn trigger spinal cord plasticity and remodeling, including altered properties of afferent neurons, afferent nerve sprouting and changes in the synaptic connections between these neurons (de Groat, 2010). Indeed, SCI male rats exhibited profoundly impaired DPN-stimulation induced ejaculatory reflexes compared to sham males. The inability to trigger ejaculatory reflexes in response to sensory stimulation in SCI males may be due to a number of factors including neuroplasticity of the sensory pathways to the spinal ejaculation generator as a consequence of the chronic SCI which presumably may alter the sensitivity and/or the efficiency of the DPN to transmit sensory signals to the LSt cells. Electrophysiological studies in cats demonstrated the presence of

alterations in the afferent limb of the micturition reflex pathway as well as remodeling of synaptic connections in the spinal cord associated with the recovery of bladder function after chronic SCI (de Groat et al., 1981; de Groat et al., 1990; Araki and de Groat, 1997; Cheng et al., 1999). Reorganization of the micturition reflex pathway after chronic SCI suggests the possibility of reorganization of the spinal ejaculation generator as well. This reorganization likely involves A δ and C-fibers in the DPN. Both types of fibers have been previously characterized in the DPN (Johnson and Halata, 1991). However, the contribution of each type of fiber to ejaculation is currently unclear. Interestingly, alterations in morphology, neuropeptide expression and function of C-fiber afferents have been observed in the rat after chronic SCI. These changes include increased soma size of bladder afferent neurons in the L6-S1 dorsal root ganglion, (Yoshimura et al., 1998) the precise point of entry into the spinal cord of the DPN, after joining the pudendal nerve (Nunez et al., 1986), as well as increased neuropeptide expression and considerable afferent axonal expansion in the lumbosacral spinal cord (Zvarova et al., 2005; Vizzard, 2006). Based on these observations, it appears that C-fiber bladder afferents contribute to the synaptic remodeling in the lumbosacral spinal cord after chronic SCI. Moreover, the inability to trigger DPN stimulation-induced ejaculatory reflexes in SCI males in the current study suggests that the C-fiber mediated synaptic remodeling in the lumbosacral spinal cord may be the culprit. Future studies will be required to test the hypothesis that selective degeneration of C-fibers in the DPN will restore DPN-stimulation induced ejaculatory reflexes in male rats with chronic SCI.

It is likely that the neuroplasticity resulting after chronic SCI considerably alters the sensory pathways to the spinal ejaculation generator, including the DPN, thereby rendering sensory

stimulation of the DPN an ineffective means of triggering ejaculatory reflexes in male rats with chronic SCI. A future experiment may utilize DPN stimulation to induce activation of the MAP kinase pathway in LSt cells in SCI and sham control animals. A finding that LSt cells in SCI males do not express pERK (Staudt et al., 2010; Kozyrev et al., 2012) or cFos (Truitt et al., 2003) may be indicative of reduced sensory inputs to the LSt cells.

Taken together, the results of the current study demonstrated that infusions of both GRP and DAMGO improved ejaculatory reflexes in a subset of chronic SCI male rats, with greater effects of GRP. However, it should be noted that in the current study, all animals received an acute transection of the mid thoracic spinal cord prior to the intrathecal infusions of the drugs. The rationale for this procedural decision is that ejaculatory reflexes in sham control animals can only be triggered upon removal of the supraspinal influences on the spinal ejaculation generator. Hence, it is currently unknown if GRP or DAMGO treatments will be effective in triggering ejaculatory reflexes in male rats with chronic SCI, but otherwise intact remaining suprasinal connections. Thus, additional preclinical studies will be required before GRP may be considered for its potential therapeutic properties in the treatment of ejaculatory dysfunction in SCI men.

Chapter 6: Systemic infusions of 7-OH-DPAT rescued ejaculatory reflexes in male rats with chronic contusion spinal cord injury

6.1 Introduction

Ejaculation is a complex physiological process, that is composed of two successive phases: emission and expulsion (Marberger, 1974; McKenna, 1999). During the emission phase, seminal fluids are secreted from the accessory sexual organs, including the prostate, seminal vesicles and vas deferens, and moved through the urethra, while the bladder neck and external urethral sphincter shut to deter retrograde ejaculation. During the expulsion phase, the striated perineal muscles, including the bulbocavernosus muscle (BCM), undergo coordinated, rhythmic contractions and in this way vigorously eject seminal contents from the urethral meatus (Bohlen et al., 1980; Gerstenberg et al., 1990; Holmes et al., 1991; Holmes and Sachs, 1991). Ejaculation is radically altered in men with chronic spinal cord injuries (SCI) (Brackett et al., 1998) the majority of whom (85%) are men between the ages of 18-45 (Ohl et al., 1989). Of all SCI men, only 12 – 15 % are able to ejaculate by means of intense vibratory stimulation or electro-ejaculation (Elliott, 2006). Recovery of normal sexual function is the highest priority for paraplegic men, followed by bladder function (Anderson, 2004). However, the neural mechanisms that alter ejaculation following chronic SCI are currently unknown.

Ejaculation is a reflex (Pescatori et al., 1993; McKenna, 1999) controlled by a spinal ejaculation generator, comprised of a set of interneurons located in lamina 10 and the medial aspect of lamina 7 in lumbar segments 3 and 4 (L3-4) in the lumbosacral spinal cord and their projections to spinal autonomic and motor neurons (Truitt and Coolen, 2002). The interneurons, known as lumbar spinothalamic (LSt) cells due to their anatomical positioning in the lumbar spinal cord and thalamic projections (Ju et al., 1987; McKenna, 1997; Coolen et al., 2003; Truitt et al., 2003), are critical for ejaculation. Previous studies from our laboratory have shown that

targeted lesion of these interneurons completely ablates ejaculation while sparing all other components of male sexual behavior in freely-moving male rats during coitus (Truitt and Coolen, 2002; Staudt, 2011) and abolishes ejaculatory reflexes induced by stimulation of sensory inputs from the dorsal penile nerve (DPN) or urethra, or triggered by infusion of the selective dopamine D3 receptor agonist 7-hydroxy-2-(di-N-propylamino) tetralin (7OHDPAT) in anesthetized and spinalized male rats (Staudt, 2011). The mechanisms whereby the spinal ejaculation generator controls ejaculation involve the coordination of sympathetic, parasympathetic, and motor ouflow necessary to trigger emission and expulsion (Carro-Juarez et al., 2003; Coolen et al., 2004; Coolen, 2005; Carro-Juarez and Rodriguez-Manzo, 2007) as well as the integration of the DPN-transmitted sensory signals during mating with this autonomic and motor ouflow to initiate ejaculation (Coolen et al., 2004; Coolen, 2005). Specifically, the LSt cells are postulated to convert sensory signals associated with mating into motor or secretory outputs (Staudt et al.; Truitt and Coolen, 2002; Coolen et al., 2004; Coolen, 2005; Staudt, 2011). LSt cell axons directly project to sympathetic, parasympathetic and motor spinal nuclei in the thoracolumbar and lumbosacral spinal cord implicated in emission and expulsion components of ejaculation (Newton, 1993; Allard et al., 2005; Xu et al., 2006; Sakamoto et al., 2008; Kozyrev et al., 2012; Kozyrev, 2013c, 2013b). LSt cells and axons co-contain the neuropeptides enkephalin, galanin, cholecystokinin and gastrin releasing peptide (GRP) (Ju et al., 1987; Newton, 1993; Nicholas et al., 1999; Coolen et al., 2003; Truitt et al., 2003; Sakamoto et al., 2008; Kozyrev et al., 2012). Release of these neuropeptides is critical for ejaculation, as blockade of each of the neuropeptide receptors in the lumbosacral spinal cord prevents ejaculatory reflexes triggered by DPN stimulation (Kozyrev et al., 2012; Kozyrev, 2013c, 2013b).

Moreover, the neurotransmitter dopamine, has also been shown to play a critical role in ejaculation (Hull et al., 2004; Peeters and Giuliano, 2008). In evidence of this, intracerebroventricular (i.c.v) or systemic infusions of the selective dopamine D3 receptor agonist 7-hydroxy-2-(di-N-propylamino) tetralin (7OHDPAT) dramatically reduces the number of intromissions prior to ejaculation as well as the latency to ejaculation in freely mating male rats (Ahlenius and Larsson, 1995; Ferrari and Giuliani, 1996). In addition, i.c.v administration of 7OHDPAT consistently triggers coordinated, rhythmic contractions of the BCM and concurrent increases in SVP in anesthetized spinal intact male rats (Clement et al., 2007; Kitrey et al., 2007; Clement et al., 2008; Clement et al., 2009a; Staudt, 2011). 7OHDPAT can also trigger ejaculatory reflexes in spinalized anesthetized male rats, demonstrating that it acts on D3 receptors in the lumbosacral spinal cord to mediate ejaculation (Staudt, 2011). Moreover, effects of 7OHDPAT are dependent on intact LSt cells, suggesting that this D3 agonist acts either on LSt cells or on D3 receptor-containing neurons that provide inputs to LSt cells.

Given the facilitatory effects of 7OHDPAT on ejaculatory reflexes, the current study aimed to test whether subcutaneous infusions of the D3 receptor agonist 7OHDPAT can rescue ejaculatory reflexes in anesthetized and spinalized male rats with chronic SCI. It was predicted that subcutaneous infusion of 7OHDPAT will restore ejaculatory reflexes in chronic SCI male rats without added sensory stimulation by acting on D3 receptors in the lumbosacral spinal cord. Additionally, it was predicted that subcutaneous infusions of 7OHDPAT will rescue emission and expulsion reflexes in SCI males following threshold levels of DPN stimulation (30 – 60 Hz). The DPN stimulation model was utilized for these experiments because it is a physiologically relevant model of ejaculation (Clement P, 2011). The DPN stimulation model reliably triggers ejaculatory reflexes in rodents (Pescatori et al., 1993; Staudt et al., 2010; Kozyrev et al., 2012;

Kozyrev, 2013b, 2013c), primates (Herbert, 1973), and men (Wieder et al., 2000) and all experiments are performed on anesthetized male rats.

6.2 Materials and Methods

6.2.1 *Animals*

Adult male Sprague Dawley rats (225-250 grams) were obtained from Charles River (Wilmington, MA, USA) and pair housed in standard housing cages on a reverse 12-hour light/dark cycle with lights off at 9 a.m. Food and water were available at all times. All procedures were approved by the Use and Care of Animals at the University of Michigan and conformed to the guidelines outlined by the National Institutes of Health.

6.2.2 *Spinal Cord Surgeries*

Male rats were divided in a spinal cord injury (SCI) group or sham group. All surgical procedures were performed under ketamine (87 mg/ml) / xylazine (13 mg/ml) anesthesia (I ml/kg i.p. injection), and as described in Chapter 5. The entire back of the males was shaved and cleaned with betadine, iodine and 70 % alcohol solution. Eyes were covered with Lacrilube opthalamic ointment (Allergen, Irevine, CA) to prevent them from drying. All animals received a laminectomy between the 6^{th} and 7^{th} thoracic spinal segments (T6-T7) and SCI male rats received a contusion injury of the spinal cord, performed with the IH-400 impactor (Precision Systems and Instrumentation, LLC, Lexington, KY). The impactor was positioned 1.5 inches above the cord and a force of 200 kdyn was applied to the dura of the exposed spinal cord.

Afterwards, the layers of muscle and skin were sutured separately, with absorbable sutures and wound clips respectively. In order to alleviate pain and prevent bacterial infection animals received subcutaneous injections of the analgesic ketoprofen (5 mg/ml/kg), and the antibiotic Baytril (5 mg/ml/kg), immediately following surgery and for three days thereafter. The sham animals underwent all procedures, including ketoprofen and Baytril treatment, except for the contusion injury. In SCI animals, bladders were expressed twice per day until bladder function returned (typically 7-10 days after injury). Sham animals were handled, but bladders were not expressed.

6.2.3 *Locomotor testing*

Locomotor activity was measured as described in Chapter 5, at 2, 3, and 4 weeks after contusion injury or sham procedures, sham (N= 7) and SCI (N = 13) males were placed in an Open Field Apparatus (Med Associates Inc, Vermont) surrounded by two 16x16 photobeam arays, during the first half of the dark phase. Locomotor activity was measured and expressed ambulatory time in the horizontal plane, and vertical rearing. Both measures of locomotor activity were analyzed using a two-way repeated anova (factors: SCI and time) and Holm-Sidak post-hoc tests.

6.3 Experimental Designs

6.3.1 *General Methods:*

Recording parameters of expulsion and emission: All experiments started four to five weeks following the spinal cord contusion surgeries. Male rats were deeply anesthetized with urethane

(1.5 g/kg, i.p.) and procedures for recordings are identical as we described previously (Staudt et al., 2010; Kozyrev et al., 2012; Kozyrev, 2013b, 2013c). Connective tissues and ligaments enclosing the reproductive organs were carefully removed to expose the muscle of the bulbocavernosus (BCM) and the dorsal penile nerve (DPN). In preparation for electromyographic (EMG) recordings, silver recording electrodes connected to the PowerLab/7SP Data Acquisition System (AD Instruments, Inc., Colorado Springs, CO, USA) were inserted bilaterally into the BCM and a ground electrode was implanted into the muscle of the right thigh. For simultaneous recordings of seminal vesicle (SV) pressure, a marker of the emission component of ejaculation (Clement et al., 2008; Kozyrev et al., 2012; Kozyrev, 2013b, 2013c; Kozyrev, 2013a), a small abdominal incision was performed and the right seminal vesicle was located. SV pressure (SVP) was recorded using a pressure catheter (AD Instruments Inc., model number: SPR-671(1.4 F, Single, Straight, 15 cm, Ny) coupled to a catheter interface cable (AD Instruments Inc., model number: AEC-IOD) and connected to a Bridge AMP (AD Instruments Inc.). The pressure catheter was gently inserted into the lumen of the SV and fixed in place before the start of recordings. The initial electrical stimulation of the DPN comprised square wave pulses of 1 ms duration, 4 V at 30 or 60 Hz for 10 seconds. These established DPN stimulation parameters consistently induce emission and expulsion reflexes in all saline-treated control male rats, reflected in rhythmic increases in SVP and concurrent bursting of the BCM respectively (Staudt et al., 2010; Staudt, 2011; Staudt et al., 2011; Kozyrev et al., 2012; Kozyrev, 2013c, 2013b; Kozyrev, 2013a).

6.3.2 *BCM EMG and SVP Analysis*

For these experiments, SVP and BCM EMG recordings were analyzed for 15 minutes following infusions of saline and 7OHDPAT, and for 90 seconds following each DPN stimulation. Specifically, the numbers of SVP increases and concurrent BCM events and bursts were analyzed following DPN stimulation and subcutaneous infusion of saline and 7OHDPAT. The numbers of BCM events were analyzed using LabChart 7.35 (AD Instruments Inc.) software and all events above baseline activity were incorporated in the analysis (Staudt et al., 2010; Kozyrev et al., 2012). The numbers of BCM bursts and SVP increases were analyzed as previously described (Kozyrev et al., 2012; Kozyrev, 2013b; Kozyrev, 2013a; Kozyrev, 2013c). Specifically, 8 or more successive BCM events above baseline activity were counted as a 'burst' and all simultaneous increases in SVP (greater than baseline) were incorporated in the analysis.

Acute spinal transection: Depending on the experimental design (see below), animals received a complete acute spinal transection before and after 7OHDPAT infusions. A laminectomy was performed at the eighth thoracic segment (T8), caudal to the injury site, and the spinal cord was completely transected.

Drug infusions: 7-Hydroxy-2-(di-n-propylamino) tetralin hydrobromide (7OHDPAT; Sigma Alderich Co. LLC) was dissolved in sterile saline (0.9%) and injected s.c. at dose 1 mg/ml/kg. This dose was previously shown to trigger ejaculatory reflexes in anesthetized, spinalized male rats (Staudt, 2011).

6.3.3 *Experiment 1: 7OHDPAT or saline infusions after acute spinal transection*

This second experiment was a partial repeat of the first experiment, but without the high stimulation frequencies prior to 7OHDPAT infusions. Moreover, each animal received both saline and 7OHDPAT infusions to allow for within animal comparisons. Anesthetized and spinally-transected sham (N = 6) and SCI (N = 6) male rats received DPN stimulation at 30 and 60 Hz in a counterbalanced manner and SVP and BCM EMG were recorded for 90 seconds following each stimulation (trial 1). Next, sterile saline was infused subcutaneously and SVP and BCM EMG were recorded for a 15 minutes. Afterwards, 7OHDPAT was infused subcutaneously and once again SVP and BCM EMG were recorded for 15 minutes following drug infusion. Following drug infusion, the DPN was stimulated at 30 and 60 Hz in a counterbalanced manner and SVP and BCM EMG were recorded for 90 seconds after each stimulation (trial 2; Figure 36A).

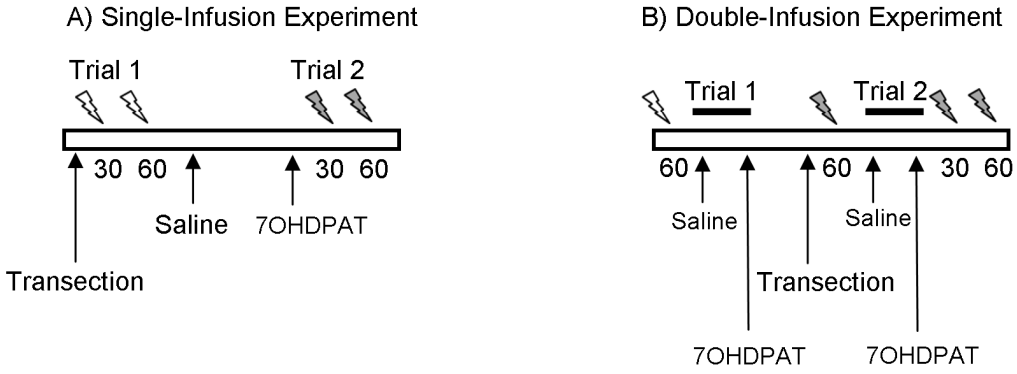

Figure 36 7-OH-DPAT experiments: Timeline of experimental manipulations.

A) In the first experiment, spinalized sham and SCI male rats received 30 and 60 Hz DPN stimulations (indicated by empty bolt arrows) in a counterbalanced manner with five-minute intervals between stimulations and recordings of BCM EMNG and SVP, in Trial 1. Next, animals received saline (arrow) and subsequently 7OHDPAT (arrow), each followed by 15 minutes recording of BCM EMG and SVP, in Trial 2. Next, animals received 30 and 60 Hz DPN stimulations in a counterbalanced order (filled bolt arrows) and BCM EMG and SVP were recorded for 90 seconds following each DPN stimulation, in Trial 3. **B)** In the second experiment, sham and SCI male rats received 60 Hz DPN stimulation (bolt arrow), followed by consecutive saline and 7OHDPAT infusions (arrows: Trial 1) and BCM EMG and SVP were recorded for 15 minutes following each infusion. Next, animals received acute spinal transection and DPN stimulation at 60 Hz (bolt arrow). Subsequently, animals received consecutive infusions of saline and 7OHDPAT (arrows: Trial 2) and BCM EMG and SVP were again recorded for 15 minutes each. Finally, males received DPN stimulation at 30 and 60 Hz

(counterbalanced: Bolt arrows) and BCM EMG and SVP were recorded for 90 seconds following each DPN stimulation.

Statistical analysis: The effects of SCI (Factor: SCI versus sham) on numbers of BCM events and bursts, and SVP increases were compared between saline and 7OHDPAT infusion trials (Factor: drug versus saline) using two-way repeated ANOVA. In separate two way repeated ANOVAs, effects of SCI (factor: SCI versus sham) on responses to each 30 or 60 Hz DPN stimulation were compared for before and after spinal cord transection (factor: spinal transection). Holms Sidak tests were used for all post hoc analyses. 95% confidence level was set for all tests.

6.3.4 *Experiment 2: 7OHDPAT infusions before and after acute spinal transection*

In this experiment, anesthetized and spinally-intact sham (N = 7) and SCI (N = 8) male rats received initial DPN stimulation at 60 Hz to confirm lack of ejaculatory reflexes. Next, all animals received subcutaneous infusion of sterile saline (1ml/kg; 0.9%) and SVP and BCM EMG were recorded for 15 minutes after saline infusion (trial 1: Saline trial). Again, no reflexes are expected under these circumstances. Next, male rats received subcutaneous injections of 7OHDPAT (1mg/ml/kg) and SVP and BCM EMG were recorded for 15 minutes immediately following drug infusion (trial 2: Drug trial). After a rest period of 3 hours, selected to allow the effects of the 7OHDPAT on ejaculatory reflexes to subside, the spinal cord was completely transected at T8. Next, the DPN was stimulated at 60 Hzand SVP and BCM EMG were recorded for 90 seconds following stimulation, in order to verify the completeness of the spinal cord

transection. Next, sterile saline was infused subcutaneously and SVP and BCM EMG were recorded for 15 minutes following saline injection (trial 1: Saline trial). Subsequently, 7OHDPAT was infused subcutaneously and SVP and BCM EMG were recorded for another 15 minutes following drug infusion (trial 2: Drug trial). Finally, the DPN was stimulated at 30 and 60 Hz (in a counterbalanced manner) and SVP and BCM EMG were recorded for 90 seconds after each DPN stimulation with a 5 minute rest period between stimulations (Figure 36B).

Statistical analysis: Numbers of SVP increases, BCM events and bursts were compared within animals between saline or 7OHDPAT treatment, and between SCI and sham groups within saline or 7OHDPAT using a two-way repeated anova (factors: drug and SCI). Data were analyzed separately for before and after spinal transection.

6.4 Results

6.4.1 *Locomotor activity was impaired in SCI male rats*

Locomotor activity was impaired in SCI rats, but not completely absent. Animals displayed comparable levels of ambulatory activity, as there were no significant differences between sham and SCI male rats in time spent in horizontal activity (Figure 37A). However, SCI males had impaired ability to rear and thus time spent on vertical activity (Figure 37B). There were main effects of SCI on vertical activity ($F(1, 59) = 9.361$; $P = 0.007$; Figure 37B). Vertical

activity was significantly reduced in SCI male rats compared to sham male rats three weeks (P = 0.006; Figure 37B) and four weeks (P = 0.005; Figure 37B) following contusion injury, with a trend towards significance 2 weeks following surgery (P = 0.081; 2 tailed test).

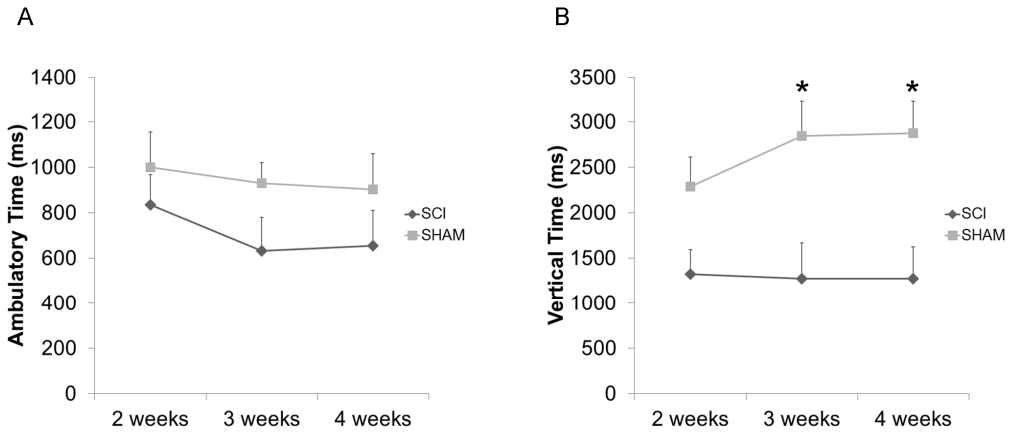

Figure 37 7-OH-DPAT experiments: Locomotor activity in sham and SCI male rats 2, 3, and 4 weeks after surgery.

Locomotor acitivity expressed as the mean time (ms) sham and SCI male rats spent in ambulatory **(A)** and vertical **(B)** locomotion during three 15-minute tests, two, three and four weeks following spinal cord contusion injury (or sham surgery).

6.4.2 *7OHDPAT infusions improved ejaculatory reflexes in chronic SCI male rats after acute spinal transection*

First, DPN stimulation did not trigger ejaculatory reflexes in SCI males, confirming the detrimental effects of SCI we reported previously (Kozyrev, 2013a). The initial 60 Hz DPN stimulation increased numbers of BCM events, bursts, and SVP increases in sham, but not SCI males (Table 2).

Table 2 Initial 60 Hz DPN stimulation facilitates BCM events, bursts and SVP increases in sham but not in SCI male rats.

60 Hz	**SHAM (N=6)**	**SCI (N=6)**
EVENTS	411.5 ± 61.956	27.5 ± 15.718*
BURSTS	5.833 ± 0.801	0.666 ± 0.422*
SVP	2.006 ± 0.571	0.666 ± 0.422*

Data are presented as mean and standard error of the mean for numbers of BCM events, bursts and SVP increases in sham (N=6) and SCI (N=6) male rats. * indicates significant differences between groups (events: $P < 0.001$; Bursts: $P < 0.001$; SVP: $P = 0.005$).

However, 7OHDPAT infusions (Trial 2) triggered both emission and expulsion reflexes in SCI male rats, similar to the effects in sham males (Figure 38). In addition, 7OHDPAT amplified ejaculatory reflexes following 30 and 60 Hz DPN stimulation in SCI males compared

to DPN stimulation without drug infusion delivered following spinal cord transection in trial 1 (Figure 39). Of all sham males tested, 83% responded to 7OHDPAT infusion alone, compared to 100% of SCI males. 'Responders' had at least one SVP increase and accompanying BCM burst following 7OHDPAT treatment. Moreover, 100 % of SCI and sham male rats responded to 30 and 60 HZ DPN stimulation following infusions of 7OHDPAT (Figures 39B, 39D).

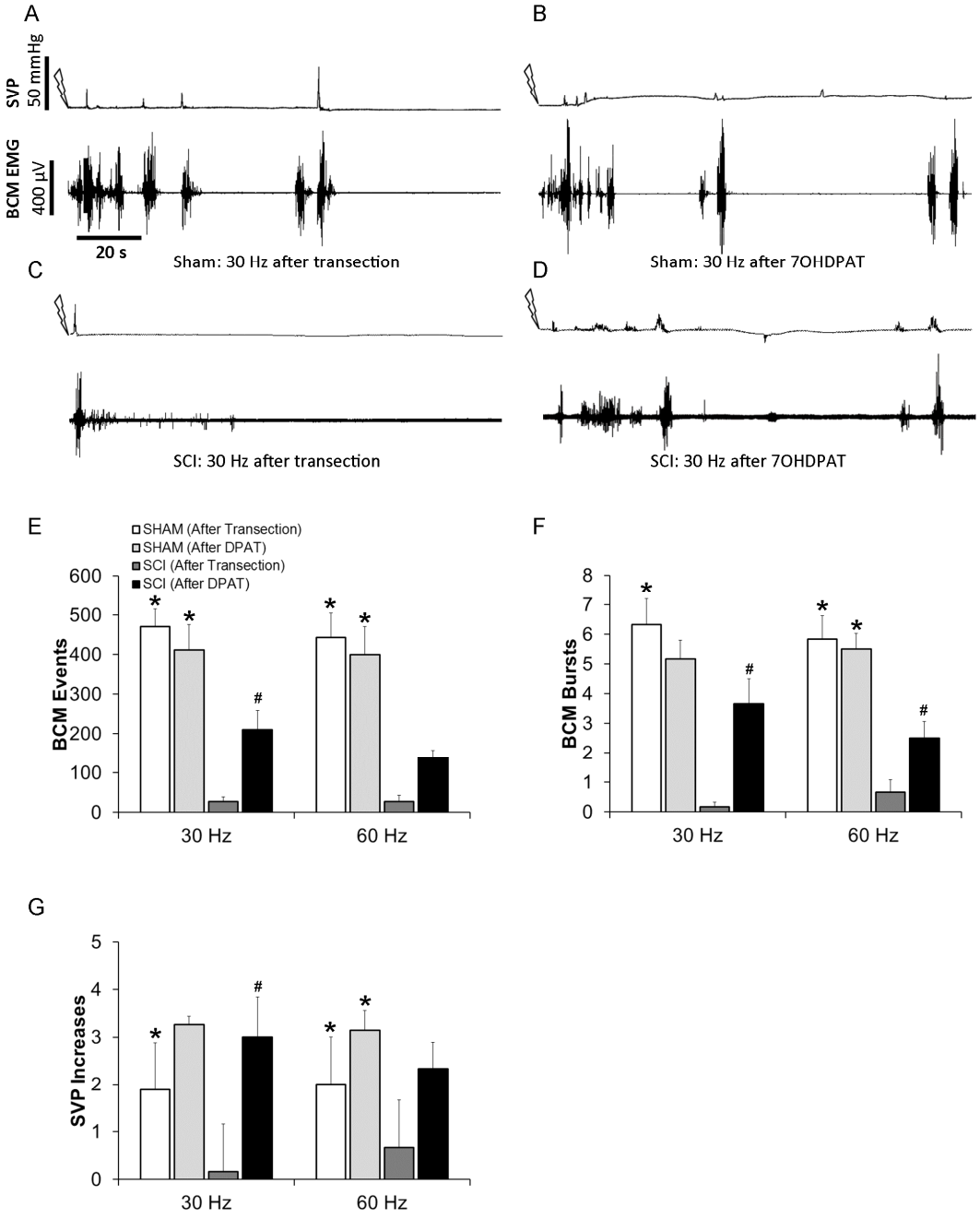
A
SVP
50 mmHg
BCM EMG
400 µV
20 s
Sham: 30 Hz after transection
B
Sham: 30 Hz after 7OHDPAT
C
SCI: 30 Hz after transection
D
SCI: 30 Hz after 7OHDPAT
E
SHAM (After Transection)
SHAM (After DPAT)
SCI (After Transection)
SCI (After DPAT)
BCM Events
30 Hz
60 Hz
F
BCM Bursts
30 Hz
60 Hz
G
SVP Increases
30 Hz
60 Hz

Figure 38 Single-infusion 7-OH-DPAT experiment: Effects of 30 and 60 Hz DPN stimulations on the numbers of BCM events, bursts and SVP increases in sham and SCI male rats.

Representative BCM EMG and SVP traces in response to 30 Hz DPN stimulation after spinal cord transection **(A)** and following 7OHDPAT infusion (**B** – same animal as in A) in a sham male and corresponding traces triggered by 30 Hz DPN stimulation after spinal transection **(C)** and after 7OHDPAT infusion (**D** – same animal as in C) in an SCI male. Quantitative analysis of BCM events **(E)** bursts **(F)** and SVP **(G)** triggered by 30 and 60 Hz DPN stimulations following spinal transection and infusions of 1mg/kg 7OHDPAT. # indicates significant differences from transection (trial 1: control trial) within the same treatment group. * denotes significant differences between treatment groups (sham and SCI) within the same testing trial and stimulation.

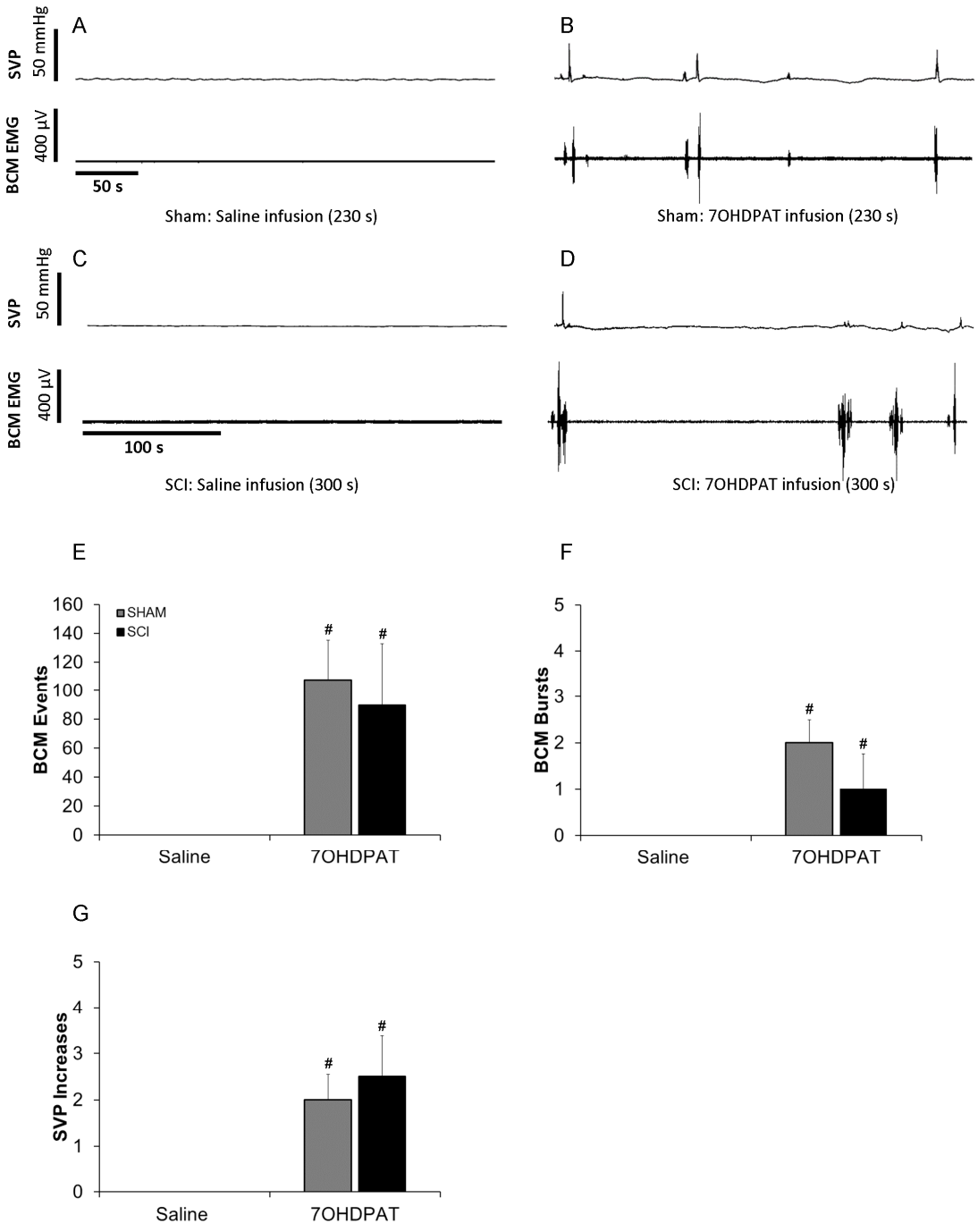
A
B
SVP
50 mmHg
BCM EMG
400 µV
50 s
Sham: Saline infusion (230 s)
Sham: 7OHDPAT infusion (230 s)
C
D
100 s
SCI: Saline infusion (300 s)
SCI: 7OHDPAT infusion (300 s)
E
F
G
SHAM
SCI
BCM Events
BCM Bursts
SVP Increases
Saline
7OHDPAT
#

Figure 39 Single-infusion 7-OH-DPAT experiment: Effects of subcutaneous 7-OH-DPAT infusions on the numbers of BCM events, bursts and SVP increases in sham and SCI male rats

Single-infusion 7OHDPAT experiment: Effects of subcutaneous 7OHDPAT infusion on the numbers of BCM events, bursts and SVP increases in sham and SCI male rats. Representative BCM EMG and SVP traces in response to saline **(A)** and 7OHDPAT **(B** – same animal as in A**)** infusion in a sham male and corresponding saline **(C)** and 7OHDPAT **(D** – same animal as in C**)** infusion traces in a SCI male rat. Quantitative analysis of BCM events **(E)** bursts **(F)** and SVP **(G)** in sham and SCI male rats triggered by infusions of 1mg/kg 7OHDPAT. # indicates significant differences from saline (trial 1: control trial) within the same treatment group.

There were main effects of saline versus 7OHDPAT treatment on the numbers of BCM events and bursts (events: $F(1,23) = 14.851$; $P = 0.003$; bursts: ($F(1,23) = 17.163$; $P = 0.002$;) and numbers of SVP increases ($F(1, 23) = 12.484$; $P = 0.005$), but there were no main effects of SCI on any parameter of ejaculatory behavior. Post hoc analyses revealed that both sham and SCI males had significantly greater numbers of BCM events (sham: $P = 0.014$; SCI: $P = 0.033$) and bursts (sham: $P = 0.013$; SCI: $P = 0.018$) and SVP increases (sham: $P = 0.043$; SCI: $P = 0.023$) following infusions of 7OHDPAT compared to saline infusions; Figure 38). There were no differences between sham and SCI groups in their responses to 7OHDPAT, indicating the D3 agonist fully triggered ejaculatory reflexes in SCI males.

However, only SCI males had significantly greater numbers of SVP increases following 30 Hz DPN stimulation in trial 2 (7OHDPAT infusion; $P = 0.005$; Figure 39G). In addition, there were main effects of trauma on the numbers of SVP increases, BCM events and bursts following 30 Hz (SVP: $F(1,23) = 19.912$; $P = 0.001$; Figure 39G; Events: $F(1,23) = 46.677$; $P < 0.001$; Figure 39E); Bursts: $F(1,23) = 23.100$; $P < 0.001$; Figure 39F) and 60 Hz (SVP: $F(1,23) = 29.091$; $P < 0.001$; Figure 39G; Events: $F(1,23) = 29.748$; $P < 0.001$; Figure 39E; Bursts: $F(1,23) = 47.451$; $P < 0.001$; Figure 39F) DPN stimulation frequencies. Moreover, there were significant interactions between factors of testing trial and trauma on the numbers of SVP increases, BCM events and bursts following 30 Hz DPN stimulations (SVP: $F(1,23) = 7.168$; $P = 0.023$; Events: $F(2,23) = 5.900$; $P = 0.036$; Bursts: $F(2,23) = 12.484$; $P = 0.005$; Figures 39E,

39F, 39G). Post hoc analyses demonstrated that sham male rats had significantly more BCM bursts following 60 Hz DPN stimulation after spinal cord transection in trial 1 compared to infusion of 7OHDPAT in trial 2 (P = 0.031; Figure 39A, 39B, 39F). In contrast, SCI males had significantly greater numbers of SVP increases, BCM events and bursts following 30 Hz DPN stimulation and with a trend for numbers of BCM events following 60 Hz DPN stimulation after 7OHDPAT treatment, in trial 2, than after spinal cord transection, in trial 1 (SVP: 30 Hz: P = 0.005; Events: 30 Hz: P = 0.014; Bursts: 30 Hz: P = 0.004; Events: 60 Hz: P = 0.055; Figures 39C, 39D, 39E, 39F, 39G). Following spinal cord transection in trial 1, sham males had significantly greater numbers of SVP increases, BCM events and bursts following 30 Hz DPN compared to SCI males (SVP: $P < 0.001$; Events: $P < 0.001$; Bursts: $P < 0.001$; Figures 39A, 39C, 39E, 39F, 39G). In addition, sham males had significantly greater numbers of BCM events following 30 Hz DPN stimulation in trial 2, after 7OHDPAT infusions, compared to SCI males (BCM events: P = 0.002; Figure 39B, 39D, 39E). Similarly, sham males had significantly greater numbers of SVP increases, BCM events and bursts following 60 Hz DPN stimulation in both trials 1 and 2 after transection and 7OHDPAT treatment respectively, compared to SCI males (SVP (trial 1): P = 0.001; (trial 2): P = 0.020; Figure 39G; Events (trial 1): $P < 0.001$; (trial 2): P = 0.002; Figure 39E; and Bursts: (trial 1): $P < 0.001$; (trial 2): P = 0.001; Figure 39F).

6.4.3 *7OHDPAT double infusion experiment: Results*

Prior to spinal cord transection in trial 1, subcutaneous injections of 7OHDPAT triggered ejaculatory reflexes in 2/7 sham (29%) and 1/8 SCI (13%) males with intact spinal cords in the absence of DPN stimulation (Figures 40A, 40B, 40C, 40D, 40I, 40J, 40K). Conversely, infusions

of 7OHDPAT after spinal cord transection triggered ejaculatory reflexes in 100% of sham and 88% of SCI male rats (Figures 40G, 40H, 40I, 40J, 40K). There were main effects of infusion on the numbers of BCM events in sham male rats ($F(1, 29) = 5.112$; $P = 0.042$). Post hoc tests demonstrated that infusions of 7OHDPAT (trial 2) in anesthetized, intact sham males significantly increased the numbers of BCM events compared to saline treatment (trial 1: $P = 0.033$; Figures 40A, 40B, 40I). In addition, there was a significant interaction between factors of infusion and trauma on the numbers of SVP increases prior to spinal cord transection ($F(2, 29) = 4.718$; $P = 0.049$). Specifically, sham males with intact spinal cords (prior to transection) demonstrated significantly enhanced numbers of SVP increases after 7OHDPAT infusions in trial 2 compared to saline infusion in trial 1($P = 0.023$; Figures 40A, 40B, 40K). There were also main effects of infusion on the numbers of SVP increases, BCM events and bursts in anesthetized and spinalized sham and SCI male rats (SVP: ($F(1, 29) = 16.136$; $P = 0.001$; Figure 40K); Events: ($F(1, 29) = 14.498$; $P = 0.002$; Figure 40I); Bursts: ($F(1, 29) = 16.136$; $P = 0.001$; Figure 40J). Post hoc tests revealed that spinally-transected sham and SCI males demonstrated significantly greater numbers of SVP increases and BCM bursts following infusions of 7OHDPAT in trial 2 compared to saline infusions in trial 1 (SVP: (sham): $P = 0.042$; (SCI): $P = 0.004$); (Bursts: (sham): $P = 0.042$; (SCI): $P = 0.004$; Figures 40E, 40F, 40G, 40H, 40J, 40K). However, only spinalized SCI males demonstrated significantly greater numbers of BCM events following 7OHDPAT infusions in trial 2 compared to saline injections in trial 1 ($P = 0.004$; Figures 40G, 40H, 40I) while there was a trend towards significance for sham males ($P = 0.071$; Figures 40E, 40F).

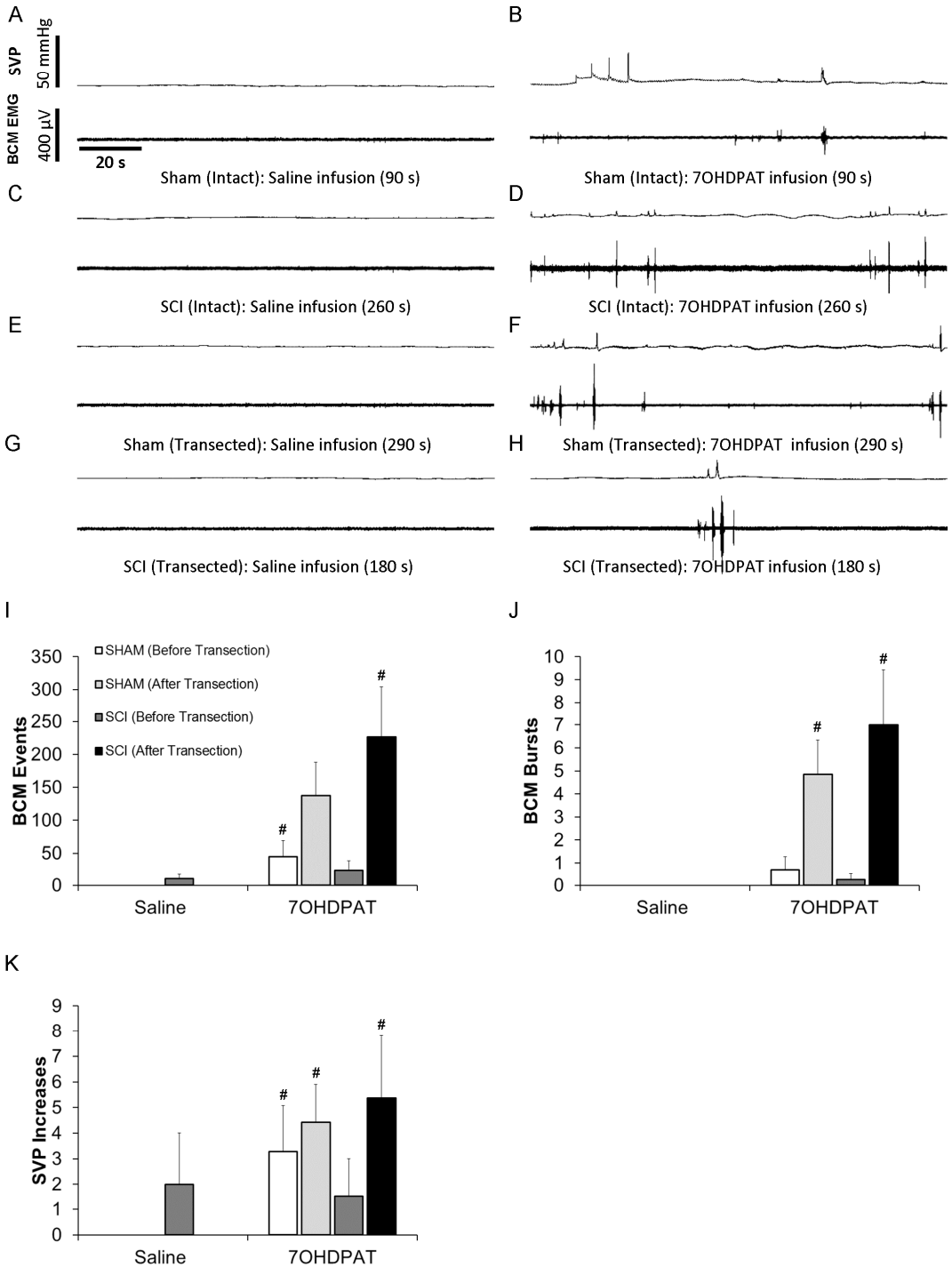
A
SVP
50 mmHg
BCM EMG
400 µV
20 s
Sham (Intact): Saline infusion (90 s)
B
Sham (Intact): 7OHDPAT infusion (90 s)
C
SCI (Intact): Saline infusion (260 s)
D
SCI (Intact): 7OHDPAT infusion (260 s)
E
Sham (Transected): Saline infusion (290 s)
F
Sham (Transected): 7OHDPAT infusion (290 s)
G
SCI (Transected): Saline infusion (180 s)
H
SCI (Transected): 7OHDPAT infusion (180 s)
I
SHAM (Before Transection)
SHAM (After Transection)
SCI (Before Transection)
SCI (After Transection)
BCM Events
Saline
7OHDPAT
J
BCM Bursts
K
SVP Increases

Figure 40 Double-infusion 7-OH-DPAT experiment: Effects of 7-OH-DPAT infusions on the numbers of BCM events, bursts and SVP increases in spinally-intact and transected sham and SCI male rats.

Representative BCM EMG and SVP traces in response to saline **(A)** and 7OHDPAT **(B – same animal as in A)** infusions in an intact sham male and corresponding saline **(C)** and 7OHDPAT **(D – same animal as in C)** infusions in an intact SCI male rat. Representative BCM EMG and SVP traces triggered by saline **(E)** and 7OHDPAT **(F** – same animal as in E**)** infusions in a transected sham and corresponding saline **(G)** and 7OHDPAT (H – same animal as in H) traces in a transected SCI male rat. Quantitative analysis of BCM events **(I)** bursts **(J)** and SVP **(K)** triggered by infusions of 1mg/kg 7OHDPAT in intact and subsequently spinally-transected sham and SCI male rats. # indicates significant differences from saline (trial 1: control trial) within the same treatment group.

6.5 Discussion

These studies demonstrate that systemic administration of the dopamine D3 receptor agonist 7OHDPAT improved ejaculatory reflexes in anesthetized and spinalized SCI male rats. Specifically, 7OHDPAT infusions triggered emission and expulsion reflexes in both sham and SCI male rats in the absence of sensory stimulation. However, an acute spinal transection is required for this effect of the D3 agonist. These results suggest that supraspinal influences inhibit the mechanisms by which D3 receptor agonist 7OHDPAT facilitates ejaculation. In addition, infusions of 7OHDPAT facilitated ejaculatory reflexes in SCI males following threshold (30 – 60 Hz) levels of DPN stimulation. In all experiments, sham males demonstrated considerably

more robust ejaculatory reflexes prior to 7OHDPAT treatment. However, there were no significant differences between sham and SCI males in the numbers of SVP increases, BCM events or bursts triggered by infusions of 7OHDPAT with one exception.

In the single-infusion experiment, SCI males clearly showed severe impairment in ejaculatory reflexes compared to sham males prior to 7-OH-DPAT treatment, as evidenced by the dramatically reduced numbers of SVP increases, BCM events and bursts following DPN stimulation (30 – 60 Hz) in SCI males compared to sham males in trial 1, following spinal cord transection. Undoubtedly, 7-OH-DPAT treatment restored ejaculatory reflexes in SCI males. However, even after 7-OH-DPAT infusions, SCI males still had reduced numbers of SVP increases, BCM events and bursts following DPN stimulation compared to sham males suggesting the presence of an underlying problem with the transmission of sensory information to the spinal ejaculation generator required to activate the LSt cells, and in turn, trigger emission and expulsion. One reason for the altered transmission of sensory information to the lumbosacral spinal cord in SCI males might be the disruption of descending pathways to the lumbosacral spinal cord following chronic SCI (de Groat, 2010). Impaired descending pathways can lead to the denervation of spinal neurons promoting the release of neurotrophic factors leading to synaptic remodeling and plasticity of afferent neurons, marked changes in the properties of these neurons and their connections (de Groat, 2010). Another reason for the impaired DPN-stimulation induced ejaculatory reflexes in SCI males may be the reorganization of sensory pathways, particularly the DPN, resulting in reduced transmission of sensory information from the sexual organs to the dorsal horn of the lumbosacral spinal cord. Indeed, previous studies reported reorganization of sensory fibers of the micturition reflex pathway in cats following chronic SCI (de Groat et al., 1981; de Groat et al., 1990; Araki and de Groat, 1997; Cheng et al.,

1999) indicating that reorganization of afferent fibers may also occur in other reflex pathways in the spinal cord, namely in the spinal ejaculation generator. The DPN is composed of A δ and C-fibers (Johnson and Halata, 1991) but the role of each fiber type in ejaculation is presently unknown. However, previous studies reported changes in soma size, neuropeptide expression and function of C-fiber afferents (Yoshimura et al., 1998) as well as neuroplasticity of sensory fibers in the lumbosacral spinal cord in rats with chronic SCI (Zvarova et al., 2005; Vizzard, 2006). Therefore, C-fiber bladder afferents undergo synaptic remodeling and reorganization following chronic SCI and the inefficacy of DPN stimulation to trigger ejaculatory reflexes in chronic SCI males may be the result of synaptic remodeling of C-fibers in the DPN.

The DPN represents the sensory branch of the pudendal nerve that conveys sensory information associated with copulation to the spinal ejaculation generator and in turn, leads to the activation of LSt cells with ejaculation (Hart, 1968; McKenna and Nadelhaft, 1986; Staudt et al., 2010; Staudt, 2011; Kozyrev et al., 2012). Moreover, stimulation of the DPN produces coordinated and rhythmic contractions of the BCM akin to those observed in male rats during mating (Pescatori et al., 1993) and bilateral lesion of the DPN completely ablates ejaculation in copulating rats (Lodder and Zeilmaker, 1976). Direct stimulation of the DPN results in the neural activation of LSt cells, in anesthetized and spinalized male rats (Staudt et al., 2010; Kozyrev et al., 2012). Specifically, DPN stimulation activates the LSt cells through map kinase signaling (Staudt et al., 2010) and the activation of NMDA receptors (Staudt et al., 2011) and pharmacologically blocking either of these components inhibits ejaculatory reflexes triggered by DPN stimulation in male rats (Staudt et al., 2010, 2011). Anatomically, it is known that fibers of the DPN innervate the dorsal horn and the dorsal central grey (DCG) in lumbosacral spinal cord, in the vicinity of the LSt cells (McKenna and Nadelhaft, 1986; Ueyama et al., 1987; Tang et al.,

1999). However, precisely how sensory information associated with mating or DPN stimulation is transmitted to the LSt cells and more importantly, any direct functional connections between the DPN and LSt cells are presently unknown. Clearly, spinal cord trauma appears to alter the functional properties of the DPN, since DPN stimulation (30 – 180 Hz) alone was ineffective in triggering ejaculatory reflexes in SCI males. However, it is likely that other intraspinal connections, acting through D3 receptors, remain intact after chronic spinal trauma, since systemic infusions of 7-OH-DPAT successfully induced ejaculatory reflexes in 100% of anesthetized and spinally-transected chronic SCI male rats without added sensory stimulation. It is known from previous studies that systemic treatment with 7-OH-DPAT enhances mating by lowering the latency to ejaculation and the number of intromissions required to ejaculate in male rats (Ahlenius and Larsson, 1995). In addition, i.c.v infusions of 7-OH-DPAT have been shown to trigger coordinated BCM bursting (Clement et al., 2007). Furthermore, i.c.v administration of a D2/D3 or a specific D3 antagonist prevented BCM bursting following infusions of 7-OH-DPAT (Clement et al., 2007) indicating that ejaculatory reflexes are triggered by activation of D3 receptors. The medial preoptic area has been shown to play an important role in ejaculation through the activation of D3 receptors since targeted injections of 7-OH-DPAT into this brain region induced ejaculatory reflexes in anesthetized male rats (Kitrey et al., 2007). Finally, previous studies from our laboratory have clearly shown that subcutaneous infusions of 7-OH-DPAT in anesthetized and spinally-transected male rats triggered ejaculatory reflexes (Staudt, 2011). Moreover, LSt lesions blocked ejaculatory reflexes induced by 7-OH-DPAT injections suggesting that D3 receptors are also expressed in the spinal ejaculation generator and that 7-OH-DPAT activates these receptors to induce ejaculatory reflexes (Staudt, 2011). Although the expression of D3 receptors in the spinal ejaculation generator is currently unknown, it has been

suggested that because intact LSt cells are critical for the effects of 7-OH-DPAT on ejaculatory reflexes, D3 receptors may be expressed directly on LSt neurons as well as/or on neurons in the spinal cord that convey sensory signals associated with mating or DPN stimulation to the LSt cells (Staudt, 2011).

Unexpectedly, very few anesthetized but spinally-intact sham and SCI animals responded to systemic infusions of 7OHDPAT with enhanced emission and expulsion reflexes compared to saline infusions (29% of sham and 13% of SCI males). In contrast, 7OHDPAT infusions triggered ejaculatory reflexes in 100% of sham and 88% of SCI males (in the same animals) after complete spinal cord transection. This finding provides further evidence for the presence of D3 receptors in the spinal ejaculation generator since transection of the cord would have limited activation of D3 receptors in supraspinal regions. Although a previous study has demonstrated high densities of D3 receptor binding in the dorsal horn and around the central canal in the lumbar spinal cord (Levant and McCarson, 2001), the expression of D3 receptors in the spinal ejaculation generator is still unclear. Therefore, future studies are required to localize and determine the densities of dopamine D3 receptors in the spinal ejaculation generator of sham and chronic SCI male rats.

In the current study, all SCI males clearly showed evidence of hindpaw paralysis as reflected in the significantly reduced time SCI males engaged in vertical locomotion. In addition, prior to 7OHDPAT treatment, all SCI animals had profoundly diminished BCM events, bursts and SVP increases in response to 30 and 60 Hz DPN stimulations compared to sham males. The current study clearly demonstrated that systemic infusions of 7OHDPAT consistently triggered ejaculatory reflexes as indicated in the coordinated pattern of BCM bursting and concurrent increases in SVP observed in anesthetized and spinalized chronic SCI males. Therefore,

7OHDPAT treatment restored ejaculatory reflexes in SCI males. Indeed, ejaculatory reflexes were significantly enhanced in SCI males following 7OHDPAT infusions compared to saline treatment. However, sham males still showed significantly greater numbers of BCM events, bursts and SVP increases, particularly following 60 Hz DPN stimulation, compared to SCI males suggesting that transmission of sensory information by the DPN is severely altered following chronic SCI and cannot be completely restored by activation of dopamine D3 receptors. Perhaps future anatomical tract tracing studies can reveal the specific changes to the reflex pathways of the spinal ejaculation generator following chronic SCI in male rats.

In conclusion, these data established that systemic administration of the dopamine D3 receptor agonist 7-OH-DPAT restored ejaculatory reflexes in anesthetized and spinalized male rats with chronic SCI. It should be emphasized that 7-OH-DPAT likely triggered ejaculatory reflexes through the activation of D3 receptors in the lumbosacral spinal cord since complete transection of the cord resulted in a robust increase in the percentages of sham and SCI males that responded to 7-OH-DPAT infusions with coordinated, rhythmic bursting of the BCM and concurrent increases in SVP, compared to 7-OH-DPAT treatment prior to spinal transection in the same animals. Furthermore, 7-OH-DPAT infusions facilitated emission and expulsion reflexes in anesthetized and spinalized SCI males. However, even after 7-OH-DPAT treatment, SCI males had significantly reduced ejaculatory reflexes following DPN stimulation compared to sham males indicating that the transmission of sensory information via the DPN is hindered in chronic SCI animals, likely due to the reorganization and plasticity of the DPN following chronic spinal trauma. Finally, the current data suggest that 7OHDPAT may be utilized in the treatment of ejaculatory dysfunction in men with chronic SCI.

Chapter 7: General Discussion

7.1 Summary of Results and Conclusions

Taken together, the findings of these studies determined that activation of GRP, DOR, MOR, galanin, and CCK receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in anesthetized and spinalized male rats. However, the activation of only one of these receptors was not sufficient to trigger ejaculatory reflexes in all male rats in the absence of sensory stimulation suggesting that the co-release of multiple neuropeptides may be required to elicit emission and expulsion reflexes without added sensory stimulation in male rats. Furthermore, these studies demonstrated that activation of MOR and GRP receptors in the lumbosacral spinal cord can rescue ejaculatory reflexes in male rats with chronic contusion SCI. Conversely, the activation of DOR and galanin receptors in the lumbosacral spinal cord failed to restore ejaculatory reflexes in SCI males. These findings suggest that the densities of DOR and galanin, but not MOR and GRP receptors in are severely reduced in the lumbosacral spinal cord following chronic SCI. Lastly, systemic infusions of 7-OH-DPAT significantly improved ejaculatory reflexes in SCI males. However, DPN-induced ejaculatory reflexes remained impaired in SCI males even after treatment with GRP and D3 receptor agonists indicating that the transmission of sensory information via the DPN to the spinal ejaculation generator is severely disrupted following chronic SCI. In conclusion, GRP, MOR, DOR, galanin and CCK receptor antagonists can be used in the treatment of premature ejaculation to delay ejaculation in afflicted individuals. Finally, GRP and the D3 agonist 7-OH-DPAT can be useful in the treatment of ejaculatory dysfunction, specifically to restore anterograde ejaculation, perhaps in conjunction with intense penile vibratory stimulation or electro-ejaculation, in men with chronic SCI.

7.1.1 *Chapter 2: Activation of gastrin-releasing peptide receptors in the lumbosacral spinal is required for ejaculation in male rats*

The main finding of Chapter 2 is that GRP is critically involved in the control of ejaculation in anesthetized and spinalized male rats (Kozyrev et al., 2012). This finding is consistent with a previous report that spinal GRP is involved in the control of male sexual reflexes (Sakamoto et al., 2008). GRP acts on the outputs of LSt cells, specifically on autonomic and motor areas in the lumbosacral spinal cord. Indeed, the expression of GRP was observed in 100% of LSt cells and LSt axons projecting to autonomic nuclei including the IML, CAN and SPN and to motor neurons in the SNB. Furthermore, intrathecal infusions of the GPR receptor antagonist RC-3095 dramatically impaired ejaculatory reflexes induced by DPN stimulation in male rats. This reduction in ejaculatory reflexes was reflected in the significant decrease in numbers of BCM events, bursts and SVP increases following electrical stimulation of the DPN. In contrast, infusions of the GRP receptor agonist GRP $^{20\text{-}29}$ enhanced both emission and expulsion reflexes as evidenced by the amplified numbers of SVP increases and concurrent BCM events and bursts respectively. GRP was particularly enhancing for ejaculation when accompanied by subthreshold levels of DPN stimulation (5 – 10 Hz). These subtle levels of DPN stimulation are normally not sufficient to trigger ejaculatory reflexes in male rats. However, this mild intensity of electrical stimulation was sufficient to trigger emission and expulsion reflexes in conjunction with intrathecal infusion of GRP $^{20\text{-}29}$. This finding indicates the presence of combined effects of endogenous neuropeptide release following sensory DPN stimulation and intrathecal infusions of GRP $^{20\text{-}29}$ that cumulatively trigger activation of GRP receptors in the lumbosacral spinal cord to induce ejaculatory reflexes in male rats. GRP receptor activation is pivotal for DPN stimulation-induced ejaculatory reflexes in anesthetized and spinalized male rats. Indeed, RC-2095 did not

prevent activation of LSt cells induced by stimulation of the DPN. Instead, the GRP receptor antagonist likely blocked GRP receptors in LSt target regions including autonomic and motor areas of the lumbosacral spinal cord. Future studies will be necessary to elucidate the expression and distribution of GRP receptors particularly in autonomic and motor nuclei of the lumbosacral spinal cord that contain axonal projections from the LSt cells.

7.1.2 *Chapter 3: Activation of Mu and Delta Opioid Receptors in the Lumbosacral Spinal Cord is required for Ejaculatory Reflexes in Male Rats*

The primary finding of Chapter 3 is that enkephalin is essential in the control of ejaculatory reflexes in anesthetized and spinalized male rats and acts through the activation of MOR and DOR in LSt cell target areas in the lumbosacral spinal cord. Enkephalin has been previously shown to be expressed by LSt cells and axons (Nicholas et al., 1999). Pharmacological blockade of MOR or DOR in the lumbosacral spinal cord prevented both the emission and expulsion phases of ejaculation triggered by sensory stimulation in male rats. In contrast, the activation of MOR or DOR following infusions of lower doses of the MOR agonist DAMGO and the DOR agonist Deltorphin II significantly enhanced ejaculatory reflexes induced by sub-threshold levels of DPN stimulation (5 – 10 Hz) in male rats. Interestingly, intrathecal infusions of the MOR agonist but not the DOR agonist triggered both emission and expulsion reflexes in a large percentage (75 %) of male rats without added sensory stimulation of the DPN. These findings indicate that activation of MOR but not DOR is sufficient to trigger ejaculatory reflexes in anesthetized and spinalized male rats. However, activation of MOR but not DOR with a higher dose of agonist resulted in an inhibition of ejaculatory reflexes induced by DPN stimulation in

male rats. This finding is consistent with a previous study which found that a high doses of intravenous morphine inhibit rhythmic bursting of the BCM, associated with the expulsion phase of ejaculation in male rats (Carro-Juarez and Rodriguez-Manzo, 2009). Taken together, the current study provides insight into the role of opioid receptors specifically found in the lumbosacral spinal cord, in nuclei comprising the spinal ejaculation generator, in the control of ejaculatory reflexes in male rats.

7.1.3 *Chapter 4: Activation of Galanin and CCK Receptors is Required for Ejaculatory Reflexes in Male Rats*

The main finding of Chapter 4 is that galanin and CCK receptor activation in LSt target regions is required for ejaculatory reflexes in male rats by acting on galanin and CCK receptors in the lumbosacral spinal cord. Moreover, intrathecal infusions of galanin or CCK receptor antagonists galantide and proglumide respectively, abolished DPN stimulation-induced emission and expulsion reflexes in male rats. Conversely, galanin and CCK agonists enhanced ejaculatory reflexes triggered by DPN stimulation. In particular, galanin and CCK agonists were most effective at facilitating ejaculatory reflexes triggered by subthreshold levels of DPN stimulation (5 – 10 Hz) and all male rats demonstrated significantly greater numbers of SVP increases, BCM events and bursts following infusions of either agonist followed by low levels of DPN stimulation. However, infusions of galanin or CCK alone were not sufficient to trigger ejaculatory reflexes in male rats without added sensory stimulation of the DPN. This finding suggests that the release of multiple neuropeptides, including galanin, CCK, enkephalin and GRP, is necessary to trigger ejaculatory reflexes in male rats. However, the release of only one

of these neuropeptides is not sufficient to induce ejaculatory reflexes in all male rats without added sensory stimulation of the DPN. Since infusions of either the galanin antagonist galantide or the CCK antagonist proglumide blocked ejaculatory reflexes but did not prevent the neural activation of LSt cells induced by DPN stimulation in anesthetized and spinalized male rats suggests that galanin and CCK likely act on receptors expressed on LSt target regions, including autonomic preganglionic neurons in the IML, CAN and SPN and on motor neurons in the SNB in the lumbosacral spinal cord to exert their effects on ejaculatory reflexes in male rats. However, the expression of galanin and CCK receptors in the lumbosacral spinal cord remains to be investigated in future experiments. Finally, both galanin and CCK may be used to pharmacologically treat premature ejaculation by increasing the latencies for emission and expulsion during sexual activity in men.

7.1.4 *Chapter 5: Activation of Mu opioid and GRP receptors in the lumbosacral spinal cord can improve ejaculatory reflexes in male rats with chronic contusion SCI*

These studies utilized a model of chronic contusion SCI in male rats. The main finding of these set of studies was that infusion of the GRP receptor agonist GRP $^{20\text{-}29}$ and the MOR agonist DAMGO rescued ejaculatory reflexes in male rats with chronic SCI (4 – 5 weeks). Specifically, intrathecal infusions of GRP $^{20\text{-}29}$ facilitated ejaculatory reflexes in all sham males and 66% of SCI males following subthreshold levels of DPN stimulation (5 – 10 Hz) and triggered emission and expulsion in 62% of sham males compared to 55% of SCI male rats. Similarly, intrathecal administration of DAMGO rescued both emission and expulsion in a large subset of SCI males (54 %). However, intrathecal infusions of DAMGO prevented ejaculatory reflexes triggered by

DPN stimulation in both sham and SCI males, a finding which is consistent with previous reports of inhibitory effects of opioids on ejaculatory reflexes in male rats (Carro-Juarez and Rodriguez-Manzo, 2009). In contrast to GRP and DAMGO, intrathecal infusions of galanin or the DOR agonist deltorphin II did not restore ejaculatory reflexes in SCI male rats. This finding suggests that galanin and DOR receptors may be substantially diminished in the lumbosacral spinal cord following spinal cord trauma in SCI males. In addition, there may be insufficient amounts of neuropeptides released following DPN stimulation in SCI males due to altered transmission of sensory signals to the lumbosacral spinal cord following trauma. Overall, this set of experiments demonstrated that administration of DAMGO and GRP can restore emission and expulsion reflexes in a large subset of male rats with chronic SCI suggesting that the densities of MOR and GRP receptors remain predominantly unchanged following chronic SCI although this hypothesis remains to be investigated in future studies. Finally, GRP can be used to treat ejaculatory dysfunction in men with SCI.

7.1.5 *Chapter 6: Systemic Infusions of 7-OH-DPAT rescued ejaculatory reflexes in male rats with chronic contusion SCI*

The main finding of Chapter 6 was that systemic administration of the dopamine D3 receptor agonist 7-OH-DPAT rescued ejaculatory reflexes in male rats with chronic contusion SCI. Infusion of 7-OH-DPAT induced emission and expulsion reflexes in SCI males without added sensory stimulation of the DPN. Furthermore, 7-OH-DPAT infusions enhanced ejaculatory reflexes triggered by threshold levels of DPN stimulation (30 – 60 Hz) in SCI males. Remarkably, there were no significant differences observed in emission and expulsion reflexes

between sham and SCI males following infusions of 7-OH-DPAT indicating that the distribution and densities of D3 receptors in the lumbosacral spinal cord and ejaculation generator are not affected by chronic contusion SCI in male rats. However, diminished DPN stimulation-induced ejaculatory reflexes persisted in SCI males compared to sham males even after infusion of 7-OH-DPAT suggesting that the transmission of sensory signals following sensory stimulation of the DPN is severely impaired in SCI males. This disruption of sensory transmission to the LSt cells via the DPN may be attributed to several factors including neuroplasticity of the fibers of the DPN resulting in diminished relay of sensory signals from the reproductive organs to the lumbosacral spinal cord. In addition, the denervation of descending pathways to the lumbosacral spinal cord that previously modulated the ejaculatory reflex may have resulted in impaired emission and expulsion reflexes following sensory stimulation. Indeed, previous experiments have found that the destruction of descending pathways innervating the lumbosacral spinal cord leads to the denervation of spinal neurons. The release of neurotrophic factors as a result of the trauma results in neuroplasticity and neurogenesis of afferent neurons, including those of the DPN as well as changes in the connections of these neurons. All of these factors together may contribute to the marked changes in the transmission of sensory information to the ejaculation generator during sexual activity or stimulation of the DPN. Taken together, the results of Chapter 6 demonstrated that systemic infusions of 7-OH-DPAT restored ejaculatory reflexes and enhanced ejaculatory reflexes following threshold levels of DPN stimulation in anesthetized and spinalized SCI male rats by acting on D3 receptors in the lumbosacral spinal cord. In conclusion, 7-OH-DPAT may be incorporated in the treatment of ejaculatory dysfunction in men with chronic SCI.

7.2 Future Directions

The current set of studies have made significant progress in determining the functional role of neuropeptides released from LSt cell axons onto autonomic and motor target areas in the lumbosacral spinal cord in the control of ejaculatory reflexes in male rats. In addition, these experiments have demonstrated that MOR, GRP and the D3 receptor agonist 7-OH-DPAT can restore ejaculation in male rats with chronic SCI. However, many more questions remain regarding the control of ejaculatory reflexes both in intact males and in males after chronic SCI. First, it is imperative to elucidate the expression and densities of GRP, MOR, DOR, galanin, CCK and D3 receptors in the lumbosacral spinal cord, particularly in LSt cell target areas including autonomic preganglionic nuclei (IML, CAN and SPN) and motor neurons of the SNB in order to determine precisely how the neuropeptides released onto LSt target areas exert their effects on ejaculatory reflexes in normal, healthy male rats and in male rats with chronic SCI. We confirmed that lower doses of DAMGO facilitate ejaculatory reflexes following subthreshold levels of DPN stimulation in male rats. Thus, it will be useful to test in future experiments whether intrathecal infusions of lower doses of DAMGO followed by DPN stimulation (5 – 60 Hz) can restore ejaculatory reflexes in male rats with chronic SCI. Finally, it is important to investigate the nature of the altered transmission of sensory inputs to the LSt cells by the DPN following chronic SCI. Specifically, synaptic alterations to the fibers of the DPN need to be examined and tract tracing studies can elucidate changes to the sensory pathway from the DPN to the lumbosacral spinal cord in male rats with chronic spinal injuries.

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Appendix A: Animal protocol approval

UNIVERSITY OF MICHIGAN
UNIVERSITY COMMITTEE ON USE AND CARE OF ANIMALS (UCUCA)

412 VICTOR VAUGHAN SPC 2054
1111 E. CATHERINE ST.
ANN ARBOR, MICHIGAN 48109-2054
[redacted]
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July 1, 2013

Coolen, Joanna
Molec & Integrative Physiology
7732B Med Sci II 5622

Dear Dr. Coolen:

The University of Michigan Committee on Use and Care of Animals (UCUCA) has reviewed the animal use application referenced below. The proposed animal use procedures are in compliance with University guidelines, State and Federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals." This project has been approved.

Committee approval must be obtained prior to changes from what is originally stated in the protocol. An amendment must be approved prior to the implementation of the change. Contact the UCUCA Office for further information.

The United States Department of Agriculture (USDA) and University policy require an annual administrative review of animal use protocols. Your continued animal use approval is contingent upon the completion of this online form. Additionally, your renewal application must be submitted, reviewed and approved in a timely manner prior to the expiration date of the current protocol. You will receive notification prior to the deadlines for both the annual review and protocol expiration.

The University's Animal Welfare Assurance Number on file with the NIH Office of Laboratory Animal Welfare (OLAW) is A3114-01, and most recent date of accreditation by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC, Intl.) is June 12, 2012.

If you receive news media inquiries concerning any aspect of animal use or care in this project, please contact James Erickson, News and Information Services, [redacted] you have security concerns regarding the animals or animal facilities, contact Joseph Piersante, Interim Executive Director of Public Safety and Security, [redacted]

Sincerely,

[redacted]

Dr. Daniel Myers, D.V.M., MPH
Associate Professor and Chairperson,
University Committee on Use and Care of Animals

cc: ORSP

TITLE: NEURAL PATHWAYS MEDIATING SEXUAL REWARD
APPROVAL PERIOD: 03/02/2011-03/02/2014
FUNDING AGENCY: CANADIAN INSTITUTES OF HEALTH RESEARCH
UCUCA APPROVAL NUMBER: 10534-1

Appendix A: Animal Protocol Approval 1

Appendix B: License Agreement

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS
Jul 12, 2013

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Curriculum Vitae

Natalie Kozyrev

Department of Anatomy and Cell Biology
University of Western Ontario
London, Ontario, Canada

Educational Background

PhD **Sept. 2009 – July 2013**
University of Western Ontario
Department of Anatomy and Cell Biology
University of Michigan
Department of Molecular and Integrative Physiology
Supervisor: Dr. Lique M. Coolen

MSc. **Sept. 2007 – August 2009**
Queen's University
Centre for Neuroscience Studies

BSc (Hons.) **Sept. 2003 – June 2007**
University of Manitoba
Department of Psychology

Teaching Experience

Teaching Assistant (September 2009 - April 2009), Western University,
Systemic Human Anatomy, Department of Anatomy and Cell Biology

Scholarships and Awards

(2009 - 2013): Western Graduate Research Scholarship

(2009): International Society for Magnetic Resonance in Medicine (ISMRM) Scientific Meeting & Exhibition Educational Stipend Award.

(2007 – 2009): Queen's Graduate Award

(2008): Queen's Graduate Student Travel Award

(2008): Centre for Neuroscience Graduate Travel Award

(2004 - 2007): Dean's Honor List

(2003): Bergen Family Scholarship

Publications

1. **Kozyrev N,** Lehman MN, Coolen LM (2012). Activation of gastrin-releasing peptide receptors in the lumbosacral spinal cord is required for ejaculation in male rats. J Sex. Med. 9 (5): 1303-1318.

2. **Kozyrev N**, Figley CR, Alexander MS, Richards JS, Bosma RL, Stroman PW (2012). Neural correlates of sexual arousal in the spinal cords of able-bodied men: A Spinal fMRI investigation. J Sex. Marital Ther. 38 (5): 418-435.

In Preparation

1. **Kozyrev N,** Lehman, MN, Coolen LM. Activation of mu and delta opioid receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats.

2. **Kozyrev N,** Lehman, MN, Coolen LM. Activation of galanin and cholecystokin receptors in the lumbosacral spinal cord is required for ejaculatory reflexes in male rats.

3. **Kozyrev N,** Staudt MD, Hryciw T, Brown A, Coolen LM. Systemic infusions of the dopamine D3 receptor agonist 7-OH-DPAT rescued ejaculatory reflexes in male rats with chronic contusion spinal cord injury.

4. **Kozyrev N,** Staudt MD, Hryciw T, Brown A, Coolen LM. Activation of mu opioid receptors and GRP receptors in the lumbosacral spinal cord can improve ejaculatory reflexes in male rats with chronic contusion spinal cord injury.

5. **Kozyrev N**, Figley CR, Alexander MS, Richards JS, Stroman PW. Neural correlates of sexual arousal in the spinal cords of able-bodied and spinal cord-injured women.

Abstracts

1. **Kozyrev N,** Coolen LM (2012). Activation of mu and delta opioid receptors in the lumbar spinal cord is critical for ejaculation. World Meeting on Sexual Medicine, Chicago, IL.

2. **Kozyrev N,** Coolen LM (2011). Gastrin-releasing peptide receptor activation in the spinal cord is required for ejaculation. Society for Neuroscience, Washington, DC.

3. **Kozyrev N,** Coolen LM (2011). Gastrin-releasing peptide in the lumbar spinal cord controls ejaculatory reflexes in male rats. Society for Behavioral Neuroendocrinology, Queretaro, Mexico.

4. **Kozyrev N,** Frohmader KS, Coolen LM (2010). Effects of glutamate in the parvocellular, subparafascicular thalamic nucleus on male rat sexual behavior. Society for Behavioral Neuroendocrinology, Toronto, Canada.

5. **Kozyrev N,** Figley CR, Stroman PW (2009). Heart-rate-based analysis of fMRI data can reveal 'lost' signal intensity changes in the spinal cord associated with distinct phases of the human sexual response. International Society for Magnetic Resonance in Medicine, Honolulu, Hawaii, USA.

6. **Kozyrev N,** Alexander MS, Richards JS, Figley CR, Stroman PW (2008). Mapping a neural model of sexual responses in the human spinal cord. Society for Neuroscience Meeting, Washington, DC, USA.

7. Stroman PW, Figley CR, Ghazni NF, **Kozyrev N** (2008). The enigma of intermediate and ventral spinal cord activity with thermal sensory stimulation: A spinal fMRI investigation. International Society for Magnetic Resonance in Medicine, Toronto, Canada.

8. Ghazni NF, Cahill CM, Pukall CF, **Kozyrev N,** Stroman PW (2008). Altered spinal cord and brainstem activation in response to peripheral sensitization to sensory stimuli: A spinal fMRI study. International Society for Magnetic Resonance in Medicine, Toronto, Canada.

Seminars:

The University of Western Ontario*,* Spinal Cord Control of Sexual Reflexes in Male Rats. Department of Anatomy and Cell Biology Seminar Series (2012).

Queen's University, Neural Correlates of Sexual Responses in the Human Spinal Cord. Centre for Neuroscience Studies, Friday Fights Seminars (2009).

Memberships

Society for Behavioral Neuroendocrinology (2010-2012)
Society for Neuroscience (2008-2012)
Sexual Medicine Society of North America, Inc. (2008-Present)
International Society for Magnetic Resonance in Medicine (2007-2009)

Masters Research Summary

I completed my Master's degree in Neuroscience at Queen's University in Kingston, Ontario in Canada under the supervision of Dr. Patrick W. Stroman. During this time, I conducted studies on men and women to investigate the neural correlates of sexual responses in the intact and injured human spinal cord during sexual arousal and orgasm using functional magnetic

resonance imaging of the spinal cord (spinal fMRI). My thesis work focused on investigating changes in neuronal activity in the human spinal cord in response to sexually salient stimuli. Specifically, I mapped neuronal activity in the spinal cord of men and women in response to viewing erotic films (mental sexual arousal), genital self-stimulation (physical sexual arousal) and the combination of the former and latter component culminating in orgasm.

With recent advancements in fMRI, it is now possible to investigate changes in neuronal activity not only in the human brain but also in the human spinal cord. Now it is possible to visualize and map alterations in neural activity in individuals after injury or trauma to the spinal cord and compare these maps to those obtained from able-bodied individuals. My work in humans represents the very first glimpse into the neural circuitry of sexual arousal and orgasm in the human spinal cord. A manuscript describing the neural correlates of sexual arousal in the spinal cords of able-bodied men was accepted for publication in the Journal of Sex and Marital Therapy (Kozyrev et al., 2011). Additional manuscripts describing the neural correlates of sexual arousal and orgasm in the spinal cords of able-bodied women are in the final stages of preparation.

In August 2009 I went on to pursue a Ph.D in the laboratory of Dr. Lique M. Coolen at the University of Western Ontario, in London, Canada. I was motivated to pursue a Ph.D. because I wanted to continue my work in the spinal cord control of sexual function but in an animal model. I was not satisfied to understand the physiology of sexual function in the spinal cord only at the systems level. My goal was to elucidate the cellular mechanisms of sexual reflexes in the spinal cord.

Ph.D Research Summary

Disorders of male sexual function have a severe negative impact on the quality of life of men of all ages and world-wide. At present, ejaculatory dysfunction represents the biggest challenge, as there are no satisfactory treatments available. Indeed, up to 30% of adult men in North America alone suffer from premature ejaculation. In addition, ejaculatory function is considerably altered following traumatic spinal cord injury and up to 85 % of men are not able to ejaculate after spinal cord trauma. Spinal cord trauma can occur by means of direct damage to the spinal cord, for instance as a result of a motor vehicle accident, sports injury or gunshot wound or by indirect means due to damage of the surrounding blood vessels, tissues and bones. There are more than

12,000 new cases of spinal cord trauma each year in the USA alone and the afflicted individuals are usually young, healthy men between the ages of 15-35. It is well-known that sexual function and ejaculation in particular is disrupted in men after spinal cord injury. Regaining the ability to ejaculate is of the outmost importance to men after spinal cord trauma. However, few studies to date have investigated the spinal cord control of ejaculation and no studies have directly addressed what changes occur in the spinal cord after a chronic spinal cord injury to severely disrupt ejaculation.

The overall goal of my Ph.D. thesis is to investigate the spinal cord control of ejaculation in male rats. The thesis consists of two main parts. In the first, I tested the hypothesis that neuropeptides expressed in the outputs of the spinal cord ejaculation generator are critical for ejaculatory reflexes in a physiologically-relevant model of ejaculation in the absence of supraspinal inputs using anesthetized and spinally transected male rats. Specifically, I tested whether these neuropeptides are required for the emission and expulsion components of ejaculation using specific receptor antagonists. In addition, I tested whether these neuropeptides are sufficient to trigger ejaculatory reflexes in the absence of sensory stimuli using specific receptor agonists. A manuscript describing the work on the role of gastrin-releasing peptide in the lumbosacral spinal cord in the control of ejaculation has recently been published in the Journal of Sexual Medicine (Kozyrev et al., 2012). Additional manuscripts describing the results of these studies are currently in preparation for publication.

The second objective of my thesis is to investigate changes to the spinal cord ejaculation generator following traumatic spinal cord injury. A chronic model of contusion spinal cord injury was utilized for these experiments. Furthermore, I tested whether intrathecal infusions of neuropeptide agonists identified in my first set of studies to facilitate ejaculation, can rescue ejaculatory reflexes in male rats 4-5 weeks following a contusion spinal cord injury.

My studies used an interdisciplinary approach combining techniques in small animal surgery (laminectomy, spinal cord transection, chronic spinal cord contusion injury) neuropharmacology (intrathecal, intracranial and subcutaneous infusions of drugs), electromyography of striated perineal muscle contractions and seminal vesicle pressure recordings, electrical nerve

stimulation, neuroanatomy (immunohistochemistry and microscopy analysis and behavioral neuroscience (recording and analysis of sexual behavior and locomotion).