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External Urethral Sphincter Activity during Micturition in the Adult Female Rat before

and after Spinal Cord Injury

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Abstract of the Dissertation

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The process of micturition is fundamental to life and one of the major ways by which organisms excrete toxic byproducts of metabolism. To function efficiently, micturition requires reciprocal bladder contraction and urethral relaxation that is coordinated by the brainstem. Spinal cord injury (SCI) above the level of the lumbosacral spinal cord results in dysfunction of the lower urinary tract (LUT). One of the most common forms of LUT derangement is detrusor-sphincter dyssynergia (DSD), which is characterized by simultaneous contraction of the bladder and the external urethral sphincter (EUS) muscle due to the impairment of reflex and voluntary sphincter control. SCI patients suffering from this condition exhibit inefficient voiding and urine retention, and are prone to developing bladder and urinary tract infections. Proper bladder maintenance has a negative impact on the quality of life of the individual, thus dysfunction of the LUT following SCI is the subject of intense scientific research. One of the most prevalent animal models for studying micturition function is whole muscle EUS electromyographic (EMG) recordings during continuous flow cystometry (CM) in the adult female rat. The implementation

of this animal model served as the basic experimental setup for the thesis research described in this document.

This dissertation highlights three major research studies. (1) The first study established an *in vivo* model for studying the temporal patterns of EUS activity during micturition in the spinally intact rat using CM and EUS EMG recordings. Once the model was established and could be used to generate reproducible data, the aim of the study shifted to the identification and quantification of major changes in bladder and whole EUS muscle activity following chronic mid-thoracic spinal cord transection in the rat (1 – 8 weeks survival). (2) The second study successfully identified and categorized different EUS motor unit (MU) recruitment patterns during micturition. While this study also focused on quantifying EUS activity in the spinally intact and transected rat, it was done at the level of the single EUS MU in order to enhance our understanding of micturition in the rat before and after SCI. (3) The third and final part of this work concentrated on studying the role of γ-Aminobutyric acid-B (GABA$_b$) receptor activation on micturition. Since baclofen, a GABA$_b$ agonist, has been shown to be effective in ameliorating LUT dysfunction and spasticity after SCI in humans and animals, intrathecal pharmacology was used to examine its effects on EUS activity in the spinally intact and transected rat. Together these three studies were designed to provide a more thorough understanding of micturition and SCI-induced LUT dysfunction in adult rat.

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List of Abbreviations

Abbreviation	Meaning
5-HT	5-Hydroxytryptamine (serotonin)
AHP	Afterhyperpolarization
ATP	Adenosine tri-phosphate
BO	Burst only
BP	Bladder pressure
BS	Bulbospongiosus
CM	Cystometry
CNS	Central nervous system
CPG	Central pattern generator
DC	Direct current
DGC	Dorsal grey commissure
DLAR	Division of laboratory animal resources
DLN	Dorsal lateral nucleus
DMN	Dorsal medial nucleus
DO	Detrusor overactivity
DRG	Dorsal root ganglia/ganglion
DSD	Detrusor sphincter dyssynergia
EAS	External anal sphincter
ECG	Electrocardiography/cardiogram
EMG	Electromyography/myogram
ENG	Electroneurography/neurogram
EPSP	Excitatory post-synaptic potential
EUS	External urethral sphincter
H-reflex	Hoffman reflex
HRP	Horseradish peroxidase
HT	High threshold
IACUC	Institutional animal care and use committee
IC	Ischiocavernosus
IUS	Internal urethral sphincter
L	Lumbar
LUT	Lower urinary tract
LT	Low threshold
LS	Low threshold sustained
MHC	Myosin heavy chain
MHNS	Medium / high threshold not sustained
MHS	Medium / high sustained
MT	Medium threshold
MU	Motor unit
NSAID	Nonsteroidal anti-inflammatory drug
NSCISC	National Spinal Cord Injury Statistics Center
PAG	Periaqueductal gray
PET	Positron emission topography
PIC	Persistent inward current

PMC	Pontine micturition center
PMN	Pudendal motor nerve
S	Sacral
SCI	Spinal cord injury
SD	Standard deviation
SEM	Standard error of the mean
SPN	Sacral parasympathetic nucleus
Type FF	Type fast-fatigable
Type FR	Type fast fatigue-resistant
Type S	Type slow oxidative
VR	Ventral root

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Chapter 1

Introduction

Micturition (more commonly known as urination) is one of the major processes by which organisms expel waste byproducts of biochemical and cellular metabolism from the internal to the external environment. This behavior is quite ubiquitous and has been described in many organisms throughout the animal kingdom including birds, fish, reptiles and mammals. While the primary goal of micturition is more or less universal (i.e. the removal of byproducts of metabolism), other factors may play significant roles. For instance, while elimination of waste in the form of urine is the sole purpose of micturition in humans, gregarious or predatory mammals such as wolves, dogs, cats, and rodents use micturition to mark familiar territory, disseminate sexual pheromones, express dominance, and communicate across distances. Because micturition can serve multiple roles, most vertebrates have evolved the anatomical and physiological capability to both consciously store and periodically evacuate urine. These modes are highly dependent on the development of complex neural circuits that involve all levels of the nervous system to govern anatomical operation. Specifically, the mammalian lower urinary tract (LUT) has two main anatomical components: 1) a reservoir (i.e. the bladder) that functions as a temporary storage unit for urine generated by the kidneys and 2) a conduit or outlet (i.e. the urethra) that allows for flow of urine from the bladder to the outside environment. Therefore, the LUT also has two major physiological functions: The temporary storage and periodic expulsion of urine. Traumatic events such as spinal cord injury (SCI), brain injury, and stroke or

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pathological conditions such as multiple sclerosis can severely disrupt neural control of

micturition and have deleterious effects on the function of the LUT.

The study of micturition: A historical perspective

The early 20th century marked the advent of both the anesthetized and decerebrate cat

preparations that provided invaluable information regarding the neurophysiology and reflex

control of micturition. Probably the most pioneering and celebrated work in the field of

micturition is ascribed to a series of studies in the cat conducted by surgeon and urologist

Fredrick James Fitzmaurice Barrington in the early to mid 20th century (Barrington 1915, 1921,

1925, 1928, 1931, 1941). While autonomic innervation of the cat bladder had already been

comprehensively described anatomically at this time (Langley and Anderson 1895, 1896), reflex

control of micturition was unknown. Thus, of all of Barrington's studies, arguably the most

seminal was that of a 1925 paper entitled "The effect of lesions of the hind- and mid-brain on

micturition in the cat" (Barrington 1925). Two of his earlier publications (Barrington 1915,

1921) indicated that reflex control of micturition in the cat was dependent on both an intact

brainstem and thoracic spinal cord. However, his pivotal 1925 experiments utilized a stereotaxic

instrument (the Horsley-Clarke apparatus) for more precise and less destructive uni- and bilateral

lesions to the dorsolateral pontine tegmentum and surrounding areas of the brainstem in

anesthetized cats. After a thorough physical examination of the lesioned animals and an

interpretation of their observed voiding behavior, he ultimately concluded that the cat brainstem

consisted of two important regions – one responsible for normal evacuation of urine, and a

second essential for the awareness of micturition and the desire to void. The first of these regions

went on to be dubbed the "pontine micturition center" (PMC, also commonly referred to in

literature as Barrington's nucleus) and was later shown to make essential connections with the lumbosacral spinal cord (described in greater detail later). At the time of his 1925 paper, Barrington was accurate in his hypotheses that proper micturition in the cat necessitated the coordination of bladder contraction and urethral relaxation, and that several distinct reflexes mediating the activity of the bladder, internal urethral sphincter (IUS), and external urethral sphincter (EUS) were at play. However, many of his ideas were formulated through observing changes in bladder and urethral pressures during forced fluid flow. By taking advantage of techniques used to record the electrical activity of nerve and muscle, subsequent work made it possible to expand on, refine, and consolidate the reflexes originally posited by Barrington.

Similar to what was demonstrated in the human (Denny-Brown and Roberston 1933a, 1933b, 1935), J.P. Evans confirmed that bladder distention in the anesthetized cat was the main stimulus for afferent activity and depended on the integrity of the pelvic nerve (Evans 1936). Resection of this nerve eliminated bladder stretch-mediated pudendal nerve activation of the EUS. By recording from the pudendal nerve, Evans was also successful in reaffirming one of Barrington's early ideas that the EUS muscle was more important than the bladder neck (i.e. IUS) in maintaining tonic activity of the urethra during bladder filling and for urethral relaxation during micturition (Barrington 1914; Evans 1936). This was corroborated by Garry et al. who, using whole muscle electromyography (EMG), demonstrated that the EUS in the decerebrate cat exhibited low level tonic activity that increased with bladder distention, but eventually disappeared entirely with high intravesical pressures and voiding (Garry et al. 1959). Taken together, these studies shared an underlying theme: distension of the bladder and the activation of sensory afferents triggered a coordinated micturition response in both humans and animals that involved multiple reflexes. However, the exact organization of the reflex pathways (i.e.

whether spinally or centrally mediated) remained uncertain. Perhaps the first compelling

evidence that substantiated Barrington's claim of brainstem-mediated micturition came from the

work of de Groat and Ryall in the late 1960s. During this time, the group was focused on

positively identifying the spinal location of parasympathetic preganglionic neurons innervating

the bladder of the cat (de Groat and Ryall 1968). In one study, they showed that following

bladder distension or pelvic nerve stimulation, sacral parasympathetic reflexes in spinally intact

cats exhibited both long latency extracellular discharges (80 – 120 ms) and intracellularly

recorded excitatory postsynaptic potentials (EPSPs, 65 – 100 ms). However, cats with complete

spinal cord transection exhibited latencies that were far shorter and discharges that were less

robust (de Groat and Ryall 1969). These results suggested the presence of supraspinal

connections to sacral parasympathetic preganglionic neurons. This was firmly corroborated when

Lalley et al. successfully demonstrated that electrical stimulation of the pons in cats resulted in

activation of sacral parasympathetic neurons and contraction of the bladder. Conversely, it was

also possible to record negative field potentials from the rostral pons following stimulation of

pelvic nerve afferents (de Groat 1975; Lalley et al. 1972). It became clear that, at least in the cat,

Barrington's micturition center made connections with the sacral parasympathetic nucleus (SPN)

and was important in normal bladder function.

  About this time, many investigators began utilizing the rat as a model system for studying

the neurophysiological control of micturition. The rat allowed for greater experimental

throughput and had the added advantages of being less costly, less procedurally complex, and

more behaviorally tractable than the cat. Practically speaking, the exact reasons for this decision

could be considered largely academic as use of the rat greatly built upon the wealth of

information already gleaned from the cat. Early on, a number of studies in the rat complemented

the work of Barrington, de Groat, Ryall, and Lalley, among others. For instance, in agreement with the work carried out by Barrington and others in the cat, the PMC of the rat was identified by introducing lesions to the caudal part of the nucleus tegmentalis laterodorsalis (Satoh et al. 1978b) and electrical stimulation of this putative micturition center in the rat evoked excitation in bladder postganglionic neurons and produced bladder contractions (Noto et al. 1989; Kruse et al. 1990). Additionally, using autoradiographic and horseradish peroxidase (HRP) transneuronal tracing techniques, direct anatomical links between the PMC and the sacral spinal cord were established by a number of groups (Hida and Shimizu 1982; Loewy et al. 1979; Satoh et al. 1978a). These data were extremely noteworthy as it became obvious that the same pathways involved in control of micturition were common among multiple species and that investigation could be expanded to include several animal models.

During the late 1970s and throughout most of the 1980s, in addition to CNS regulation of the bladder, other research began to place emphasis on control of the EUS. In the human, EUS motoneurons are located in Onuf's nucleus, named after Bronislaw Onuf-Onufrowicz, the American neurologist who discovered them in 1899 (Onuf 1899). Onuf's nucleus resides bilaterally in lamina IX on the lateral edge of the ventral horn at the S2 – S4 level of the spinal cord and contains motoneurons that, via the pudendal nerve, innervate the perineal muscles: the EUS, the external anal sphincter (EAS) and, in males, the ischiocavernosus (IC) and bulbospongiosus (BS) muscles that are involved in sexual function (de Groat 2006; Fowler et al. 2008; Schroder 1981; Thor and de Groat 2010). In the early to mid 1970s, the close dendritic bundling and general morphology of sacral motoneurons supplying the pudendal nerve in the cat and rat had been described in several communications (Anderson et al. 1976; Dekker et al. 1973; Kerns et al. 1974; Matthews et al. 1971). Using electron microscopy, a more detailed structural

description of Onuf's nucleus in the cat came from Konishi et al. who noted the presence of tightly intertwined transverse and longitudinal (rostrocaudal) dendrites and the segregation of the nucleus into dorsomedial and ventrolateral divisions (Konishi et al. 1978). Later morphological studies in the cat eventually showed that the transverse dendritic projections of these motoneurons in the spinal cord were strikingly similar to those of autonomic preganglionic neurons controlling the bladder (Beattie et al. 1990; Sasaki 1994; Thor et al. 1989). These arrangements were unique in comparison to other spinal motoneurons (i.e. those innervating skeletal muscle), but suggested a close functional relationship between EUS and bladder motoneurons.

Injection of transneuronal dyes and HRP into the perineal muscles of other species led to the discovery of additional homologs of Onuf's nucleus including those in the dog (Kuzuhara 1979; Kuzuhara et al. 1980), the South American monkey (Nakagawa 1980), the Rhesus monkey (Roppolo et al. 1985) and the rat (McKenna and Nadelhaft 1986). Many of these reports were consistent with what was known about Onuf's nucleus in the human; that it 1) was a single dense cell group situated in the sacral ventral horn, 2) consisted of relatively small motoneurons, and 3) exhibited a dual topological organization of neurons (dorsomedial and ventrolateral) that innervated different perineal muscles via the pudendal nerve. However, some important exceptions were noted. For instance, the separation of motoneuron pools into dorsomedial and ventrolateral positions was found only to exist in the cat and monkey. Additionally, a striking difference was discovered in the cellular organization of the rat spinal cord. Injection of HRP into the pudendal motor nerve (PMN) of the adult female rat labeled two separate nuclei, a DLN and dorsal medial nucleus (DMN) that innervated the EUS and EAS muscles, respectively. Also, in contrast to the other species, these nuclei were localized to the lumbar ($L_5 - L_6$) segments of

the spinal cord (McKenna and Nadelhaft 1986). Since IC and BS motoneurons are largely atrophied in the female rat, the main implication of this finding was that each nucleus represented a virtually homogenous population of motoneurons that could be studied independently.

With the spinal and peripheral projections of EUS motoneurons largely understood, studies also confirmed their direct connection with the brainstem, particularly those regions immediately ventral and lateral to the PMC (Ding et al. 1995; Holstege et al. 1986). At this point it was fully realized that, in multiple species, distinct regions of the PMC and associated areas made direct synaptic contacts with both the parasympathetic preganglionics controlling the bladder and EUS motoneurons in Onuf's nucleus. However, in order to achieve a coordinated response during micturition, inhibition of EUS activity during bladder contraction is mandatory. Blok and his colleagues conducted a number of experiments showing that the PMC in the cat made excitatory connections with inhibitory interneurons (i.e. glycinergic and GABAergic) of the dorsal grey commissure (DGC), an area of the lumbosacral spinal cord shown to inhibit EUS motoneurons and produce urethral relaxation (Blok et al. 1997, 1997a, 1998; Sie et al. 2001). Thus, the basic mechanism for the reciprocal relationship between bladder contraction and urethral relaxation became established. Blok and his group were also instrumental in taking advantage of the development of positron emission topography (PET) for studying the activation of different areas of the brain in the human. Using this technique, an increase in blood flow to the dorsomedial pontine tegmentum was noted in human subjects during micturition, the area now considered to be the homolog of the PMC in other animals (Blok et al. 1997b, 1997c). These studies helped further unify the common neural mechanisms and pathways important for micturition in both humans and animals.

Basic anatomy of the lower urinary tract

In mammals, the bladder functions as a compliant storage unit for urine during the time when voiding is not preferred or inappropriate (de Groat 2006; Fowler et al. 2008; Tai et al. 2006). The inner urothelial lining of the bladder needs to maintain a highly impermeable barrier to the constituents of urine (e.g. water, urea, and ions) as a result of the high intravesical pressures associated with long term storage. The luminal urothelium is composed of three principle layers, an apical epithelium, an intermediate layer, and an inner basement membrane. The combination of these layers along with epithelial tight junctions and specialized membrane proteins ensure a remarkably leak-free barrier. Beneath the urothelium exists an interstitial layer of non-neuronal myofibroblasts linked extensively by gap junctions and a deeper layer of smooth muscle cells forming the detrusor. The detrusor smooth muscle receives autonomic innervation and is the machinery responsible for active contraction and relaxation of the bladder during micturition. Interspersed among the urothelium, interstitium, and smooth muscle are efferent and afferent nerves that lie in close apposition to and chemically communicate with each of these layers (Birder et al. 2009; de Groat 2006; Fowler et al. 2008). The ability of the urothelium to respond to local chemicals and physical distension, secrete autocrine/paracrine factors (e.g. adenosine tri-phosphate (ATP), nitrous oxide, and acetylcholine), and elicit electrical signals in afferent nerves support its role as an active sensory organ and not merely a passive barrier to water and solutes.

The distal part of the bladder (i.e. the bladder neck) begins to taper and eventually becomes the urethra, the tract that serves as the conduit for flowing urine. The urothelium described above is also continuous with the lining of the urethra. Parts of the proximal urethra (i.e. near the bladder neck) are also enveloped by thick layers of longitudinal and circular smooth

muscle cells comprising the IUS. The IUS is innervated by the autonomic nervous system and exhibits a basal level of myogenic tone at rest that contributes to the closure of the proximal urethra (Fowler et al. 2008). Additionally, nearly the entire rostrocaudal extent of the urethra is surrounded by somatically innervated striated muscle commonly referred to as the EUS. Contraction and relaxation of the EUS is under both volitional and reflex control, and is extremely important to ensure appropriate storage of urine and efficient micturition (Fowler et al. 2008). Historically, the EUS has been referred to by different names including the striated urethral sphincter, the striated urethralis muscle, and more recently, the urethral rhabdosphincter (Thor and de Groat 2010). To be consistent with recent publications describing the work detailed in this dissertation, this muscle will be referred to exclusively as the EUS.

Afferent and efferent control of the lower urinary tract

The mammalian LUT has two major physiological functions: The temporary storage and periodic evacuation of urine. Both of these phases involve complex interplay between peripheral nerves, the lumbosacral spinal cord, and the brainstem requiring participation of the somatic, autonomic, and central nervous systems (CNS) (de Groat 2006; de Groat and Yoshimura 2006; Fowler et al. 2008; Kuru, 1965; Tai et al. 2006). Three main peripheral nerves cooperate to regulate the LUT: the hypogastric (sympathetic), pelvic (parasympathetic), and pudendal (somatic) nerves. Each of these nerves are mixed and contain both afferent (sensory) and efferent (motor) fibers (Birder et al. 2009; de Groat and Yoshimura 2006, 2009, 2010).

The cell bodies of afferent neurons carrying information from the LUT into the spinal cord are found in the dorsal root ganglia (DRG) of the thoracolumbar (hypogastric) and sacral (pelvic and pudendal) spinal levels. Pelvic and pudendal afferents overlap in the spinal cord and

their dendritic terminals synapse with spinal interneurons in the superficial and lateral dorsal horn, the DGC, and the SPN to participate in local segmental reflexes. Other afferent terminals synapse with spinal projection neurons that ascend to regions of the brainstem such as the periaqueductal gray (PAG) and the PMC that integrate and relay sensory information to higher brain centers (e.g. the medial frontal cortex, insula, and hypothalamus) (de Groat 2006; Fowler et al. 2008; Kruse et al. 1990). The presence of mechanosensitive tension/volume receptors in the bladder wall respond to a variety of sensory stimuli (Iggo 1955; Shea et al. 2000). In humans and other mammals, these receptors consist mainly of two fiber types, Aδ and C-fibers that travel primarily through the pelvic nerve (Birder et al. 2009). Thinly myelinated Aδ fibers transmit sensory information to the CNS in response to mechanical deformation and thus provide information about bladder fullness and are responsible for cortical sensations of urgency. Under normal physiological conditions, unmyelinated "silent" C-fibers do not respond significantly to bladder stretch or contraction, but instead become activated in response to a variety of noxious stimuli such as temperature (hot and cold), high osmolarity, chemical irritation, inflammation, and extremely high intravesical pressures. The vast majority of C-fibers are immunoreactive for neuropeptides and sensitive to capsaicin. Aberrant C-fiber activity has been implicated in a myriad of disorders affecting the LUT including interstitial cystitis, idiopathic overactive bladder, and SCI-induced neurogenic destrusor overactivity (de Groat and Yoshimura 2009, 2010; Fowler et al. 2008). Afferents projecting through the pudendal nerve have also been demonstrated to play important roles in bladder-EUS reflex activation (Shefchyk and Buss 1998). Stimulation of afferents in the proximal urethra (for example through urine leakage) strongly potentiates EUS activation and the maintenance of continence during the guarding reflex. Conversely, as urine flows through the medial and distal urethra during micturition,

activation of pudendal afferents facilitates pelvic nerve-mediated bladder contraction and contributes to voiding efficiency (Peng et al. 2008a, 2008b).

Preganglionic parasympathetic efferent fibers originating from the SPN ($S_2 - S_5$) of the intermediolateral spinal cord travel through the pelvic nerve and synapse with postganglionic parasympathetic neurons within the pelvic ganglion and the wall of the bladder (de Groat and Ryall 1968, 1969; de Groat 1975; de Groat 2006; Fowler 2008). Postganglionic terminals release primarily acetylcholine to activate muscarinic (M_3) receptors, but also non-cholinergic non-adrenergic neurotransmitters that either induce bladder contraction (e.g. ATP via purinergic receptors) or promote urethral relaxation (e.g. nitrous oxide). Preganglionic sympathetic fibers originating in the medial and lateral thoracolumbar spinal cord synapse with postganglionic neurons in the sympathetic chain or peripheral mesenteric ganglia. Postganglionic axons project through the hypogastric and pelvic nerves to innervate the smooth muscle of the bladder and IUS, releasing norepinephrine to activate inhibitory β-adrenergic receptors of the bladder and excitatory α$_1$-adrenergic receptors of the IUS/bladder neck resulting in relaxation and contraction, respectively. In contrast to the autonomic innervations of the bladder and the IUS, the striated muscle of the EUS receives cholinergic innervation from the motor branch of the pudendal nerve whose motoneurons reside in Onuf's nucleus of the sacral spinal cord (Onuf 1899).

Control EUS function: EUS motoneurons

In the human, cat, dog, monkey, hamster, and guinea pig, EUS motoneurons reside in Onuf's nucleus situated in lamina IX of the spinal cord on the lateral edge of the sacral ventral horn. In all examined species, these motoneurons project their axons to the EUS through the

pudendal nerve (Fowler et al. 2008; Kuzuhara 1979; Kuzuhara et al. 1980; Nakagawa 1980; Ropollo 1985; Thor and de Groat 2010; Onuf 1899). However, in the rat, motoneurons innervating the EUS are localized in the $L_5 - L_6$ dorsal lateral nucleus (DLN), the rat homolog of the Onuf cell group (McKenna and Nadelhaft 1986). These nuclei consist of densely packed and relatively uniform small to intermediate sized motoneurons that exhibit tight longitudinal (rostrocaudal) and transverse dendritic bundling. Transverse dendrites project dorsally to the SPN and lateral dorsal horn, dorsomedially to the DGC, and ventrally to the lateral funiculus. This kind of anatomical organization is unusual for spinal motoneurons (i.e. those innervating skeletal muscle), but bears close similarity to parasympathetic preganglionic neurons. It has been suggested that this organization facilitates coordination of bladder and EUS spinal reflexes during micturition (Thor et al. 1989).

Apart from their general histology and anatomy in the spinal cord, EUS motoneurons differ significantly from other spinal motoneurons, particularly those that innervate skeletal muscle (e.g. the pelvic floor and extremities) both synaptically and biophysically (Thor and de Groat 2010). Because the EUS is a perineal muscle and is not attached to bone or tendons, it does not possess proprioceptive receptors such as muscle spindles or Golgi tendon organs. Consequently, EUS motoneurons do not receive monosynaptic type Ia afferent input or exhibit recurrent or disynaptic reciprocal inhibition. EUS motoneurons also differ with respect to their intrinsic electrophysiological properties. Generally, they are smaller and more excitable than other spinal motoneurons, exhibiting more depolarized resting membrane potentials, lower rheobase, higher input resistances, and shorter afterhyperpolarization (AHP) half-decay times (Carp et al. 2010; Collins 2010; Hochman et al. 1991; Sasaki 1991; Yashiro et al. 2010). It is

speculated that these biophysical properties make EUS motoneurons well suited for their role in controlling a muscle required to be tonically active for sustained periods of time.

Neurocontrol of EUS motoneurons is highly complex and likely involves several neurotransmitters and the activity of many receptor subtypes. Pharmacological and immunohistochemical studies have shown that EUS motoneurons within Onuf's nucleus and the DLN of the rat can be influenced by activation of a diverse array of receptors (Conely et al. 2001; Furuta 2009; Mbaki and Ramage 2008; Palea et al. 2004; Pikov and Wrathrall 2002; Ramage 2006; Xu et al. 2007). Ascending and descending communication between the lumbosacral spinal cord and the brainstem, as well as reflex activation of the EUS likely involve the classic fast ligand-gated ion channels (i.e. excitatory glutamatergic and inhibitory glycinergic and GABAergic neurotransmission) (de Groat et al. 1998; Liu et al. 1995). For instance, the amplitude of negative field potentials recorded in the rostral pons of the rat after pelvic nerve stimulation can be markedly attenuated with administration of N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists (Kakizaki et al. 1998). Further, using *in situ* hybridization, the DLN of the rat has been shown to express high levels of NMDA and AMPA receptor mRNA (Pikov and Wrathall 2001) and intravenous administration of NMDA and AMPA antagonists profoundly suppressed EUS EMG activity in these animals (Yoshiyama et al. 1995). In terms of fast acting inhibitory synaptic transmission, glycinergic chloride channels were also demonstrated to be present within Onuf's nucleus in the decerebrate cat. Application of strychnine, a glycine receptor antagonist, replaced the normal inhibition of EUS activity during voiding with tonic EUS activation in a dose-dependent manner (Shefchyk et al. 1998). In the unanaesthetized rat, dietary glycine and intrathecal administration of the GABA reuptake inhibitor tiagabine and the $GABA_a$ receptor

agonist muscimol had inhibitory effects on micturition (Miyazato et al. 2005, 2008a; Pehrson and Andersson 2002). It is obvious from these data that fast ionotropic glutamate, glycine, and GABA mediated neurotransmission play a vital role in normal micturition function. However, there is also a significant neuromodulatory component to EUS motoneuron activity that depends largely on the activation of metabotropic (i.e. G-protein coupled) receptors.

Neuromodulation of motor control has been abundantly researched in rat and cat hindlimb and tail motoneurons. The number of potential neuromodulators is quite extensive, however, the most thoroughly studied are likely the brainstem-derived biogenic monoamines 5-hydroxytryptamine (5-HT, serotonin) and norepinephrine released the Raphe nucleus and locus coeruleus, respectively. These neuromodulators have been shown to increase the overall excitability of tail and hindlimb motoneurons in the rat and cat by altering their intrinsic electrical properties, including resting membrane potential, action potential threshold, rheobase, input resistance, and AHP (Murray et al. 2011; Rank et al. 2011). Additionally, noradrenergic and serotonergic input to spinal motoneurons strongly increases the amplitude of Na^{+1} and Ca^{+2} mediated persistent inward currents (PICs) that are necessary for sustained depolarizations and repetitive self-sustained firing of action potentials (Heckmann et al. 2005, 2008; Li and Bennett 2003; Li et al. 2004a, 2007). Because terminals that release 5-HT and norepinephrine project densely to Onuf's nucleus (Rajaofetra et al. 1992; Thor et al. 1993; Xu et al. 2007) the effects of monoaminergic neuromodulators on the electrical properties of EUS motoneurons and LUT function have been investigated. Recording from neonatal rat EUS motoneurons *in vitro*, Yashiro et al. demonstrated that norepinephrine and phenylephrine, an α_1-adrenergic receptor agonist, promoted EUS motoneuron excitation by directly depolarizing the resting membrane potential, increasing input resistance, and shortening the AHP duration. Further, these effects were blocked

by application of prazosin, an α_1-adrenergic receptor antagonist. While the overall excitability of EUS motoneurons was increased through α_1-adrenergic activation, the group was unable to elicit significant PICs, sustained plateau potentials or repetitive firing (Yashiro et al. 2010). In the adult rat, agonist activation of $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A/B/C}$ receptor subtypes has been shown to enhance EUS activity (Dolber et al. 2007; Cheng and de Groat 2010; Mbaki and Ramage 2008; Ramage 2006), while duloxetine and venlafaxin, both serotonin-norepinephrine reuptake inhibitors (SNRIs) that increase synaptic levels of these neuromodulators, had a similar effect in the cat (Thor and Katofiasc 1995; Katofiasc et al. 2002). Duloxetine in particular has shown clinical efficacy in mitigating the symptoms of stress urinary incontinence in humans (Dmochowski et al. 2003; Zinner 2003). Evidence shows that this is at least partially related to duloxetine's ability to increase EUS muscle tone and EUS motoneuron excitability through activation of α_1-adrenergic and $5\text{-}HT_2$ receptors (Thor 2003, 2004). Taken together, these data highlight the importance of metabotropic neuromodulation in regulating EUS motoneuron activity and LUT function.

Summarizing the process of micturition

In humans and other mammals, micturition is a spinobulbospinal reflex that operates in a switch-like manner producing coordinated bladder contraction and urethral relaxation (de Groat 2006; Fowler et al. 2008; Tai et al. 2006). During the storage phase (i.e. bladder filling), parasympathetic innervation to the bladder is inhibited while sympathetic innervation is active. Combined, these activity patterns result in relaxation of the bladder. Also, during bladder filling, activation of the EUS via the pudendal nerve and IUS via the hypogastric nerve maintain closure of the urethra. The pattern of EUS activation is governed primarily by local circuitry in the

lumbosacral spinal cord to prevent incontinence and is referred to as the guarding reflex (Fowler et al. 2008; Park et al. 1997). After a threshold level of afferent activity is reached, the PMC triggers a coordinated micturition event (Barrington 1925; Fowler et al. 2008; Kruse et al. 1990; Sasaki 2005). During micturition, parasympathetic activity elicits bladder contraction while activity of the IUS (sympathetic) and EUS (somatic) are simultaneously inhibited resulting in urethral relaxation thus permitting the flow of urine. It is important to note that the aforementioned processes may also be elicited through the conscious desire to urinate. Whether micturition is involuntary or voluntary, there is a reversal of the local storage reflexes characterized by simultaneous bladder contraction and urethral relaxation.

Spinal cord injury

SCI is the result of physical trauma to the spinal cord that results in impaired sensory and motor control. Started in 1973, the federally funded National Spinal Cord Injury Statistics Center (NSCISC) boasts the largest repository of SCI data in the world (https://www.nscisc.uab.edu/). According to the NSCISC, as of 2010, the incidence of SCI in the United States is approximately 12,000 new cases each year with a prevalence of 232,000 to 316,000 persons with an average age of 40.7 years. The vast majority (80%) of SCI related incidents befalls men over women and the etiology is almost exclusively traumatic in nature. Motor vehicle accidents account for 42.1% followed by falls (27.9%), violence (15%, primarily gunshot wounds), and recreational sporting activities (8%). SCI is typically classified based on 1) the extent of the injury (i.e. incomplete vs. complete) and 2) the level of the spinal cord affected by the trauma (e.g. cervical, thoracic and lumbosacral). The majority (60%) of SCI incidents are incomplete (i.e. crush injuries) resulting in the partial loss of sensory and motor function below the level of the injury. In addition to the

loss of volitional motor control and sensation, victims will likely experience other complications associated with SCI including, but not limited to, autonomic dysreflexia, spasticity, and impaired sexual, bowel and LUT function.

In humans and animals, complete SCI rostral to the lumbosacral spinal cord disconnects this region from the PMC and higher brain areas. This causes LUT dysfunction characterized first by an acute phase of bladder areflexia due to spinal shock followed by the gradual development of a neurogenic bladder and automatic micturition driven by segmental reflexes (de Groat 1993, 2006; de Groat and Yoshimura 2006; de Groat et al. 1998; Fowler et al. 2008). The reemergence of spinal reflexes and spontaneous bladder contractions occur over the course of several weeks, however, EUS-bladder dysfunction is permanent. While some humans with SCI will experience chronic incontinence (Blaivas 1993), other pathological states frequently appear such as SCI-induced detrusor-sphincter dyssynergia (DSD) and detrusor overactivity (DO). DSD is characterized by dyscoordination between the bladder smooth muscle and the EUS whereby the bladder repeatedly (i.e. DO) contracts against a hyperactive EUS due to disrupted volitional and reflex control (Andersen and Bradley 1976; Tai et al. 2006). These conditions may result in inefficient voiding, urinary retention, persistent and recurrent cystitis, and vesicoureteral reflux with consequent kidney damage in the long term (Castro-Diaz and Taracena Lafuente 2006). Thus, dysfunction of the LUT after SCI is a significant health risk and the associated challenge of bladder maintenance (i.e. urethral cannulation and associated drug or surgical therapies) has a negative impact on the quality of life of the patient. When surveyed, both quadriplegics and paraplegics rated recovering bladder and bowel function as a top priority (Anderson 2004).

To better understand the dysfunction of the LUT after SCI, investigators will often use simultaneous bladder CM and EUS EMG recordings in various animal models (Garry et al.

1959; Mersdorf et al. 1993). CM and EUS EMG recordings from cats (Rampal and Mignard 1975) and rats (Kruse et al. 1993; Leung et al. 2007; Pikov and Wrathall 2001) that have received mid-thoracic SCI show increased tonic EUS activity, inefficient voiding, urine retention, increased bladder capacities, and bladder contractions exhibiting long durations and high intravesical pressures. These observations are qualitatively similar to the pathological state of the human and support the appropriateness of using these models to study DSD and DO (Andersen and Bradley 1976). Even still, while DSD has been characterized in humans, cats, and rats, the underlying mechanisms have not been fully elucidated and thus are the subject of current research. Early on, much of the work investigating the impact of SCI on micturition function was done in the cat; however, in the early 1990s, Kruse et al. introduced a chronic spinal cord transection model in the adult female rat that is now widely used for studying LUT dysfunction following SCI (Kruse et al. 1993). This adult rat SCI model served as the framework for much of the research described in this dissertation and its use will be elaborated on in the following chapter.

Chapter 2

External urethral sphincter and bladder activity during micturition in the intact and spinally transected adult rat

Abstract and overview

The goal of this part of the study was to determine the effect of chronic mid-thoracic spinal cord transection on the time course of EUS and bladder activity associated with micturition events in the rat. Adult female Sprague Dawley rats, either spinally intact or transected ($T_9 - T_{10}$), were anesthetized with urethane and set up for continuous flow urodynamic recording of BP and EUS EMG. Spinal transections were performed under isoflurane anesthesia $1 - 8$ weeks prior to the terminal experiment. Four major differences between intact and transected rats were observed: 1) the frequency of micturition events in intact rats was dependent upon the rate of bladder filling while the bladder contraction and associated EUS activation in transected rats exhibited an intrinsic rhythm that was independent of the rate of bladder filling and post-transection survival time, 2) EUS activation was augmented at the beginning of active bladder contraction in the transected rat, indicating an amplified guarding reflex, 3) phasic EUS activity at the peak of bladder contraction (EUS bursting) in the intact rat was markedly reduced or absent in the transected rat, and 4) the sustained tonic EUS activity following bladder relaxation in the intact rat was absent in the transected rat. These results are discussed in the context of understanding the pathophysiology of SCI-induced DSD.

Background

The main function of the LUT is the coordinated storage and elimination of urine. During the storage phase, the EUS muscle becomes increasingly active and maintains closure of the urethra during bladder filling while the bladder smooth muscle is quiescent. During micturition, this situation is reversed as the EUS muscle is relaxed and the bladder simultaneously contracts (de Groat and Yoshimura 2006; Fowler et al. 2008; Kruse et al. 1990; Kuru 1965). In humans and animals, SCI rostral to the lumbosacral level results in LUT dysfunction characterized first by bladder areflexia followed by the development of a neurogenic bladder driven by segmental reflexes (de Groat et al. 1998; Fowler et al. 2008). The reemergence of spinal reflexes and spontaneous bladder contractions occurs over the course of several weeks (de Groat 1993; de Groat and Yoshimura 2006), however, EUS-bladder dysfunction is permanent. In humans, a pathological condition frequently ensues resulting in SCI-induced DSD. DSD is the result of dyscoordination between the bladder smooth muscle and the EUS whereby the bladder contracts against a hyperactive EUS (Andersen and Bradley 1976; Tai et al. 2006). These conditions may result in urinary retention, cystitis, periodic incontinence, and kidney damage in the long term (Castro-Diaz and Taracena Lafuente 2006).

CM and EMG recordings are routinely used for monitoring bladder and EUS function, both experimentally and clinically. In the spinally intact rat, continuous infusion of saline into the urinary bladder elicits repetitive micturition reflexes and the associated pattern of EUS activity is well documented (Collins and Schuster 2008; Collins et al. 2009; D'Amico et al. 2011; Maggi et al. 1986; Kruse et al. 1993; Mersdorf et al. 1993; Kakizaki et al. 1997; Peng et al. 2008; Streng et al. 2004). During passive filling and active contraction of the bladder, there is an increase in tonic activation of EUS motoneurons (i.e. guarding reflex) leading to an abrupt and

transient period of highly synchronized phasic EUS activity (i.e. EUS bursting) that accompanies and promotes efficient voiding. EUS bursting is then followed by sustained tonic EUS activity persisting well after bladder contraction has ended that can last sometimes for a minute or longer. In contrast, micturition is markedly different in spinalized animals, characterized by increased tonic EUS activity, inefficient voiding, increased bladder capacities, and bladder contractions exhibiting long durations and large bladder pressures (Kruse et al. 1993; Rampal and Mignard 1975).

The purpose of the present study was to quantify the temporal pattern of EUS and bladder activity associated with micturition events in the intact and spinally transected rat. There has been considerable focus on studying the urodynamics of micturition in the adult rat, specifically EUS bursting because of its requirement for efficient voiding (de Groat and Yoshimura 2006; Kruse et al. 1993). For example, several studies have investigated the role of the $5\text{-}HT_{1A}$ receptor subtype in the regulation of EUS bursting (Chang et al. 2007; Cheng and de Groat 2009; Dolber et al. 2007). However, little attention has been placed on quantifying other aspects of EUS activity such as the guarding reflex and sustained tonic EUS activity. In the present study, and in contrast to the intact rat, all transected animals (1 - 8 weeks post-transection) exhibited 1) an intrinsic bladder and EUS rhythm that was largely insensitive to the rate or degree of bladder filling, 2) an enhanced guarding reflex at the beginning of bladder contraction, and 3) inhibition of sustained EUS activity following bladder contraction. Results of this study were reported in abstract form (Collins et al. 2009; Collins and Schuster 2008) and as a publication (D'Amico et al. 2011).

Materials and Methods

Spinal cord transection and animal care

All animals were housed in the Division of Laboratory Animal Resources (DLAR) facility at Stony Brook University where they had free access to food and water in a room with controlled temperature and a 12 hour alternating light-dark cycle. DLAR at Stony Brook University is a fully accredited AAALAC facility and is supervised by trained professional veterinarians and support staff. All experimental procedures were executed in accordance with policies of the Stony Brook University IACUC protocol and the Office of Research Compliance.

Under isoflurane anesthesia (5% induction, 2% maintenance; Webster Veterinary) in 100% oxygen, 27 adult female Sprague Dawley rats (Taconic; 220 - 320 g) underwent complete spinal cord transection after laminectomy at the T_9-T_{10} level. During surgery, animal body temperature was maintained at 37°C using a heating pad. Spinal cord transection was verified by visualizing the ventral and lateral aspects of the vertebral column to ensure complete disconnection. The open cavity separating the two ends was packed with hemostatic Gel foam (Henry Schein) and covered with sterile Parafilm prior to closing. The overlaying paravertebral muscles were sutured and the skin incision closed using wound clips. Immediately after surgery, bladders were manually expressed and animals were given 2 cc 2% dextrose-supplemented Lactated Ringer solution (sc), 0.05 mg/kg of the opioid buprenorphine (im) and 10 mg/kg of the broad spectrum antibiotic enrofloxacin (sc). Postoperatively, rats were housed in shallow cages with high absorbent bedding and had access to food and water *ad libitum.* Animals were treated with enrofloxacin (sc; 5 - 7 days) and buprenorphine (sc; 3 days) and monitored for signs of pain, infection, dehydration and autotomy behavior. Bladders were manually expressed thrice daily

until spontaneous voiding returned (usually 10 - 14 days), after which animals were expressed once daily.

CM and EUS EMG recordings

Both intact (n = 11) and spinally transected rats (1 - 8 week survival; 1 week n = 5, 2 weeks n = 4, 3 weeks n = 4, 4 weeks n = 5, 6 weeks n = 5, 8 weeks n = 4) were anesthetized with urethane (Sigma, 1.2 g/kg; sc). During surgery, animal body temperature was maintained at 37°C using a heating pad and heart rate was monitored via electrocardiogram (ECG). After an incision in the neck, the left or right internal jugular vein was cannulated for fluid administration and a trachea tube inserted to facilitate respiration. After a midline incision in the lower abdomen, the pubic symphysis was removed exposing the underlying EUS muscle. Two fine wire electrodes (A-M Systems; 50 μm insulated stainless steel) were inserted bilaterally into the EUS to measure EMG activity. A flared end tube (PE 90) was inserted into the dome of the bladder and connected in series to a pressure transducer for bladder intravesical pressure (BP) measurement and to a syringe pump for infusion of room temperature 0.9% saline (up to 21 ml/hr).

Refer to Figure 1 for schematics. The EUS EMG signal was amplified through a differential AC preamplifier (A-M Systems) with high and low pass filters (0.1 kHz and 1 kHz). The BP signal was amplified through a PM-1000 transducer amplifier (CWE Inc.) and filtered (DC to 0.5 kHz). Data were sampled at 2 kHz using a DAQ analog to digital converter card (National Instruments M-series), and stored using the IGOR pro/NIDAQmx Tools (WaveMetrics) data acquisition system for later analysis. At the end of the experiment, animals were euthanized by overdose with intravenous administration of urethane.

H-reflex

Hoffman reflex (H-reflex) measurements from the hindlimb were made as described previously (Reese et al. 2006; Yates et al. 2008) and done prior to any pelvic or abdominal manipulation. In intact (n = 4) and transected animals (survival times: 1 week n = 5, 4 weeks n = 5, 6 weeks n = 5, 8 weeks n = 4) two sub-dermal needle electrodes were inserted subcutaneously at the level of the right or left calcaneus for tibial nerve stimulation with the cathode at the proximal position in the heel. Ipsilaterally, one recording electrode was placed in the dorsal interosseus muscle between the fourth and fifth metatarsals and the other in the skin for a reference. A ground was inserted sub-dermally between the stimulating and recording electrodes on the dorsal surface of the foot to minimize stimulus artifact. Suprathreshold stimulation of the tibial nerve produced both short latency M-wave (direct orthodromic) and longer latency H-wave (reflex) responses. In each experiment, the stimulus was set at the minimum intensity needed to achieve maximum H-wave amplitude (typically x1.2 - 1.5 threshold, 30 - 40 V, 0.1 ms duration). H-wave recordings were obtained with stimulation frequencies of 0.2, 0.5, 1, 2, 5 and 10 Hz and averaged (n = 10). The first 3 - 5 responses in each series were discarded to ensure stable amplitude responses during the recording. For each rat, the amplitude of the averaged H-wave response at each stimulation frequency was measured, normalized by dividing by the H-wave amplitude at 0.2 Hz stimulation, and expressed as percent. Thus, H-reflex depression is indicated by normalized H-wave amplitudes less than 100%.

Data analysis

BP and EUS EMG data were analyzed using the computing software IGOR pro. All basic urodynamic measurements in intact and transected rats were made from BP and rectified EUS

EMG records (10 – 15 micturition events) smoothed by software down-sampling to achieve an effective sampling rate of 10 Hz. In addition, for the slope analysis, records were binomially smoothed and normalized using the maximum and minimum values during a series of micturition events. The 15 seconds prior to the peak of BP were averaged (5 – 8 successive micturition events) to capture activity during active bladder contraction. Normalized EUS EMG data were plotted as a function of BP and the first derivative (slope) between 30 – 40% EUS EMG activity was calculated and averaged. In all cases, measurements from successive micturition events were averaged with an "n of 1" reflecting the averaged measurement from one animal. Error bars represent the standard deviation ($\pm$ SD) of the mean and statistical significance was assessed using one-way ANOVA or a two-sample unpaired t-test with the degrees of freedom determined by the number of animals. A $p<0.05$ was considered significant. In the case that significance was met, post hoc tests were carried out to compare between individual groups.

Results

Characteristics of the micturition reflex

The first goal of this part of the study was to quantify the temporal patterns of bladder function in the intact rat and record changes following chronic spinal transection. In intact animals, continuous infusion of saline at a constant rate (5.24 ml/hr) into the bladder produced a sequence of complete voiding contractions at regular intervals (figure 3A). The frequency of bladder contraction increased (decreased cycle period) when the infusion rate was increased from 2.6 to 15.7 ml/hr (figure 2A). In contrast, all transected rats exhibited rhythmic bladder contractions with a frequency that was unresponsive to changes in the rate of bladder filling.

Moreover, there was no significant difference in bladder contraction frequency between animals of different post-transection survival times (figure 2A).

The rate of bladder filling had no significant effect on peak intravesical pressure or bladder contraction duration in the intact rat during voiding contractions (figure 2B and 2C). Bladder contraction duration tended to be longer in transected animals, peaking at the three-week post-transection survival time (figure 2C). Also, bladders were hypertrophied and capable of generating large intravesical pressures (e.g. 2/27 animals achieved pressures above 40 mm Hg; one animal at three weeks reached 50 mm Hg; one animal at two weeks reached 42 mm Hg). Similar to bladder contraction duration, the highest peak pressures were observed in animals three weeks post-transection (figure 2B). As in the intact rat, both bladder contraction duration and peak intravesical pressure were not significantly affected by changes in the rate of bladder filling (figures 2B and 2C).

A continuum of bladder behaviors was observed in the spinally transected rat that was contingent upon the observed voiding efficacy. Starting with an empty bladder and using a low rate of infusion (5.24 ml/hr), in all cases, bladder filling produced rhythmic non-voiding contractions of increasing amplitude eventually leading to small volume voiding contractions. In the majority of animals (n = 13/27), a repetitive state was reached where incomplete voiding occurred at the peak of each contraction (figure 3B). Alternatively, some animals (n = 10/27) would only void at high bladder volumes with large and sustained intravesical pressures. This resulted in a "choked" bladder with slow and intermittent urethral dribbling at high peak and baseline pressures (figure 3C). All rats one week post-transection (n = 5/5) exhibited this behavior. Finally, in a minority of cases (n = 4/27), bladders would produce several rhythmic

non-voiding contractions of increasing amplitude before culminating in a single strong voiding contraction. This pattern would then repeat (figure 3D).

Characteristics of EUS activity during micturition

The effect of chronic spinal cord transection on the time course of EUS activity was examined. Three primary changes in EUS activity were observed in spinally transected rats: 1) EUS activation at the onset of bladder contraction was enhanced, 2) EUS bursting at the peak of bladder contraction was absent or markedly reduced, and 3) sustained tonic EUS activity following bladder relaxation was absent. These changes are described in detail below.

The guarding reflex

Work from this part of the study was focused on quantifying the guarding reflex during the period of active bladder contraction. EUS activity during passive bladder filling during the inter-contraction interval between micturition events was not assessed (see discussion for details). In intact rats, BP and EUS EMG activity increased in parallel at the beginning of each micturition event at the onset of bladder contraction (figure 4A). In contrast, EUS activation was enhanced at the beginning of bladder contraction in all transected rats (example in figure 4B, contrast with 4A). This observation was noticed to be more prominent in animals of longer survival times (4 – 8 weeks, data not shown). As a result, plotting the smoothed and rectified EUS EMG as a function of BP revealed forward (clockwise) hysteresis in transected animals (example in figure 4D).

To analyze the guarding reflex during active bladder contraction, the first derivatives (slope) of EUS activity plotted as a function of BP in intact (n = 9) and transected rats (n = 8)

were measured. Five to 8 successive micturition events from intact and transected animals were aligned at the peak of bladder contraction and then averaged. Data were pooled from transected animals that exhibited a steady state of rhythmic voiding contractions (see figure 3B; 3 weeks n = 2, 4 weeks n = 2, 6 weeks n = 3, 8 weeks n = 1). The activity 15 seconds preceding the peak of BP was specifically chosen in order to capture the period of active bladder contraction (figure 5A). Normalized EUS EMG activity was plotted as a function of BP and the slope between 0.3 – 0.4 (30 – 40%) of EUS EMG activity was averaged (figure 5B). In sharp contrast to intact rats, EUS-BP plots from transected rats were conspicuously non-linear and exhibited saturation (figure 5B). Additionally, the average slope between 0.3 – 0.4 EUS EMG activity was significantly larger in transected rats (figure 5C, intact 0.99 ± 0.12 and transected 3.93 ± 1.69).

To examine the relative timing of the maximum rates of rise, EUS EMG and BP data from 4 – 6 weeks post-transected animals were pooled, normalized and smoothed to achieve values between 0 and 1. The first derivative of this data set was calculated in order to analyze the changes in the slope and measure the maximum rates of rise of EUS EMG and BP activity during the period of active bladder contraction (figure 6). The peak rates of change of EUS EMG and BP activity were measured (figure 6B) and the time offset between these peaks was calculated (figure 6D). The normalized maximum rates of change of BP and EUS EMG activity were the same in both intact and transected rats (figure 6B), however, the relative timing was altered following transection. In intact rats, the maximum rates of change of BP and EUS EMG activity occurred at the same time (time offset = 0.075 ± 0.407s). In contrast, the maximum rate of change of EUS EMG activity in transected rats occurred well before the maximum rate of change of BP (time offset = 3.88 ± 1.31s, figure 6D).

EUS bursting

All intact animals in this part of the study showed normal EUS bursting at the peak of

bladder contraction (example in figure 7A). However, this activity was almost completely

abrogated in transected animals (example in figure 7B). This is consistent with other studies

examining EUS bursting in urethane-anesthetized rats (de Groat and Yoshimura, 2006, Kakizaki,

et al., 1997, Kruse, et al., 1993). In this study, three out of 27 animals with spinal cord

transection exhibited EUS burst-transients at the peak of bladder contraction; specifically, two

out of four animals at three weeks post-transection and one out of five animals at four weeks

post-transection. It should be noted that this bursting was not required for voiding. Quantification

of the burst duration was difficult in transected animals as the phenomenon was not well defined.

Nevertheless, the burst duration was shorter in transected animals. In intact animals, the average

burst duration was 2.45 ± 0.78s, whereas burst-transients in transected animals were $\leq$ 1s.

Because the bursting response was limited in transected rats, the frequency of individual burst

events could not be accurately measured.

Sustained tonic EUS activity

In spinally intact animals, EUS motoneurons manifest sustained tonic EUS activity that

persists well after bladder contraction has ended (figure 4A). This produces counter-clockwise

(reverse) hysteresis when plotting the smoothed and rectified EUS EMG as a function of BP

(figure 4C). In order to quantify the sustained activity, a double exponential function was fit to

the decay in EUS EMG activity (rectified and smoothed) following bladder contraction in both

intact and transected rats (figure 8A and 8B). Generally, both short and long time constants (τ1

and τ2, respectively) could be resolved from EUS EMG records in intact animals with mean

values of $\tau 1 = 1.56 \pm 0.91$s and $\tau 2 = 15.2 \pm 7.12$s (figure 8C). In contrast, EUS motoneurons in transected rats failed to exhibit significant sustained tonic activity (example in figure 8B, see also figure 4B). Under these circumstances only one time constant could be resolved with $\tau 1$ equal to $\tau 2$ ($\tau 1$: 1 week 7.40 ± 1.83s, 2 weeks 6.09 ± 1.24s, 3 weeks 5.44 ± 1.37s, 4 weeks 6.68 ± 1.90s, 6 weeks 5.90 ± 1.69s, 8 weeks 4.81 ± 2.73s; $\tau 2$: 1 week 7.43 ± 1.76s, 2 weeks 5.87 ± 0.34s, 3 weeks 5.56 ± 1.28s, 4 weeks 6.57 ± 2.04s, 6 weeks 5.80 ± 1.61s, 8 weeks 4.79 ± 2.78s). The long time constant is effectively lost, and the short time constant is elevated 2 - 4 fold (figure 8C). A detailed explanation on the significance of both time constants is given in the discussion (see page 37).

H-reflex

The H-reflex is an electrophysiological measure of reflex excitability (Cook 1968; Komiyama et al. 1999; Matthews 1966). In this study, it was used to assess the level of hindlimb hyperreflexia (e.g. spasticity) in the transected rat. Since H-wave measurements were made prior to EUS EMG and CM assessment, it was not possible to correlate different levels of background EUS activity with the degree of frequency-dependent inhibition of the H-wave. Nevertheless, background EUS activity is presumed to be low in intact animals, but variable in transected rats. In the present study, electrical stimulation of the tibial nerve at an intensity sufficient to activate both motoneuron and Ia axons produced a short latency (1.0 - 1.5 ms) direct orthodromic response (M-wave) followed by a longer latency (6.0 - 7.0 ms) reflex response (H-wave) in the ipsilateral dorsal interosseus muscle. In agreement with previous studies (Reese et al. 2006; Thompson et al. 1998; Yates et al. 2008) the H-wave exhibited marked amplitude depression at stimulation frequencies greater than 0.2 Hz. H-wave amplitude depression, computed by

normalizing to the H-wave amplitude at 0.2 Hz, increased as a function of stimulation frequency reaching 95% depression at 10 Hz (highest frequency tested) in intact rats. In contrast, the H-wave amplitude depression was significantly less in transected rats over the same range of stimulation frequencies (55 - 68% depression at 10 Hz). No significant difference was observed between rats of different post-transection survival times (figure 9).

Discussion

Kruse and colleagues first described a spinal cord injury model in the adult female rat showing that mid-thoracic spinal cord transection resulted in inefficient voiding, increased bladder capacity, and a hyperactive EUS (Kruse et al. 1993). The continued refinement and development of new animal models is of paramount importance in understanding the effects of bladder and EUS impairment in SCI-induced LUT dysfunction, particularly DSD. Towards this goal, the objective of this part of the study was to quantify the temporal pattern of bladder and EUS activity associated with micturition events in the intact rat and to characterize and quantify the changes in this activity following chronic spinal cord transection. Four principle observations were made in this part of the study: 1) in intact rats the timing of micturition events was dependent on the rate of bladder filling, while transected rats had bladder and EUS activities that exhibited an intrinsic rhythm independent of bladder filling and post-transection survival time, 2) in intact rats, increases in EUS activity closely paralleled sharp rises in bladder pressure during active bladder contraction. However, EUS activation was augmented at the onset of bladder contraction in transected rats indicative of an enhanced guarding reflex, 3) intact rats exhibited EUS bursting at the peak of bladder contraction associated with voiding, however, this activity

was markedly reduced or absent in transected rats, and 4) the sustained tonic EUS activity

following bladder relaxation in intact rats was absent in transected rats.

Bladder activity

In this part of the study, bladder contraction frequency in intact animals could be

modulated by changing the rate of bladder filling. This affirms that bladder contraction is

dependent on a threshold of pressure driving the level of activity of afferents innervating the

bladder wall. In contrast, continuous infusion of saline into the urinary bladder of transected rats

revealed rhythmic bladder and EUS contractions. The rate of bladder filling only determined

how quickly peak intravesical pressure was reached, but had little or no effect on the intrinsic

bladder contraction rhythm. Thus, the rhythm in transected animals exists independently of

bladder filling, bladder volume, and the type of bladder contraction (voiding vs. non-voiding)

suggesting that the bladder pressure threshold is suppressed following spinal cord transection. In

all transected rats, bladder filling always produced initial low amplitude rhythmic bladder

contractions that eventually gave rise to larger and stronger contractions associated with

incomplete voiding. However, a continuum of bladder behaviors was observed (see results for

details). Fifty percent of the transected animals studied reached a steady state of rhythmic

voiding contractions. However, nearly 40% of the transected animals were unable to achieve

sufficient voiding to prevent over-distention of the bladder (i.e. rising baseline pressure resulting

in bladder "choking" with slow production of saline droplets during voiding). It is interesting to

note that all rats at one week post-transection exhibited this bladder pattern (n = 5/5). This may

be because either: 1) bladders at this early stage are not yet able to generate strong enough

contractions to overcome urethral resistance or 2) the synaptic/neural drive to bladder

parasympathetic preganglionics has not had sufficient time to develop. Nonetheless, it is tempting to hypothesize that bladder choking is due to high urethral resistance resulting in a reduction in voiding efficacy. In support of this, in the minority of cases where bladder filling produced rhythmic contractions of increasing amplitude that culminated in a single strong voiding contraction (figure 2D), the observed voiding tended to be of a greater volume (i.e. continuous streams of saline or rapidly produced saline droplets).

It has been purported that the neurogenic bladder and EUS dyssynergy that arise after SCI are due to a combination of competing bladder-EUS segmental reflexes and the presence of a spinal central pattern generator (CPG) (Tai et al. 2006). In the present study, the insensitivity of transected rats to the rate of bladder filling is suggestive of a functional bladder rhythm CPG that emerges following complete spinal cord transection. The activity of this putative CPG was present as early as one week following transection and did not change significantly with post-transection survival time. An intriguing possibility is that this CPG may be unmasked under additional pathological conditions such as incomplete SCI or peripheral injury to the bladder. Further studies are required to clarify exactly how and where this CPG operates in the spinal cord. A good candidate may be local circuits within the lumbosacral spinal cord where segmental inhibitory and excitatory interneurons receiving afferent input from the bladder wall may modulate sacral parasympathetic preganglionic neurons (Fowler et al. 2008).

The guarding reflex

In the majority of intact rats there is little or no increase in EUS activity during passive bladder filling between micturition events. However, a marked increase in EUS activity is elicited during rapid rises in bladder pressure (e.g. during active bladder contraction and

increases in abdominal pressure). The analysis presented in this part of the study focused on

changes in EUS activity associated with active bladder contraction only. A lack of EUS activity

during passive filling has been noted by other groups (Conte et al. 1991; Streng et al. 2004) and

has been interpreted by some to mean there is little or no guarding reflex in the adult rat

(McMurray et al. 2006). These observations may be due to the fact that only a limited number of

EUS motor units are active during passive filling and their activity may be difficult to detect

using whole muscle EMG recordings. However, on several occasions during this part of the

study, small increases in EUS activity and the recruitment of EUS motor units were noted during

passive bladder filling. Furthermore, in all cases, strong activation of the EUS in the intact rat

always began at the onset of bladder contraction and increased in parallel with bladder pressure.

These observations suggest that the adult rat does indeed exhibit a guarding reflex that

contributes to continence and would likely be important during situations where there is a rapid

increase in abdominal pressure (e.g. coughing and sneezing).

In this part of the study, co-activation of the EUS and bladder in intact rats could be

demonstrated by superimposition of smoothed and normalized EUS EMG and BP records (figure

4A) and the linear relationship when EUS activity was plotted as a function of BP during active

bladder contraction (figure 5B). Additionally, the maximum rates of rise of normalized BP and

EUS EMG activity were similar and occurred at the same time (figure 6A and 6D). However, in

transected rats, the timing of bladder and EUS co-activation was altered. The onset of bladder

contraction produced a strong EUS EMG response with a more rapid time course than the

increase in BP. This was evident in superimposed normalized records of EUS and BP activity

(figure 4B), the relative larger slope of EUS activity (dEUS / dBP) in transected rats (figure 5B

and 5C), and the time offset between the maximum rates of rise of BP and EUS activity (figure

6D). From these data one can conclude that there is an increased gain in the EUS-BP relationship and an enhancement of the guarding reflex during active bladder contraction. It is possible that the more rapid time course of EUS activation seen at the onset of bladder contraction in transected rats reflects a generalized increase in EUS background/baseline activity. However, the level of background activity varied between animals in both intact and transected rats so it is unlikely that it contributed significantly to the differences seen in the time course of activation. Additionally, EUS hyperactivity may reflect a decrease in pre- and post-synaptic inhibition in the spinal cord. This would be consistent with the H-reflex data obtained in the present study where all spinally transected animals exhibited reduced frequency-dependent inhibition of the H-wave. Finally, it is conceivable that the rapid activation of the EUS at the beginning of bladder contraction reflects temporal and spatial alterations in the recruitment pattern of individual EUS motor units. Closer inspection of motor unit organization and timing during micturition in the intact and spinally transected rat would yield new insights into EUS activation. Regardless of the mechanism, it is likely that this enhanced guarding reflex is an important component in LUT dysfunction after SCI in the adult rat and would contribute to urine retention and increased bladder capacity.

EUS bursting

As bladder filling progresses in rats with an intact neuroaxis, a threshold level of bladder afferent activity is reached and carried into the sacral spinal cord via the pelvic nerve. This information is relayed by interneurons to the PMC (also known as Barrington's nucleus) located in the rostral pons and triggers coordinated bladder contraction and EUS relaxation (de Groat and Yoshimura 2006; Kruse et al. 1990; Kuru 1965; Sasaki 2005). In the rat, this is manifested as a

transitory switch from tonic to phasic EUS activity (EUS bursting). In contrast to the characteristic EUS bursting in rats, the EUS relaxes completely during voiding in the spinally intact human (Fowler et al. 2008). However, it is interesting to note that the DSD that develops following SCI (i.e. tonic EUS activation during bladder contraction) is qualitatively similar in unanesthetized humans and anesthetized rats. Evidence is now starting to emerge regarding the regulation of EUS bursting. Chang and colleagues propose that somewhere within the $T_{10} - L_3$ spinal segment is a neural substrate important for mediating EUS bursting and regulating the pelvic-EUS reflex arc in the intact rat (Chang et al. 2007). They find that ablation of this spinal level eliminates phasic EUS activity and perturbs the reflex response following bladder distension. Other studies show that phasic EUS activity similar to what is observed in the intact rat can be evoked in transected animals following administration of the 5-HT_{1A} receptor agonist 8-OH-DPAT, bolstering the argument for the existence of a EUS bursting CPG that is sensitive to exogenous monoamines (Dolber et al. 2007). It is important to note that other work has demonstrated that unanesthetized rats with both incomplete and complete SCI are still capable of producing phasic EUS activity at the peak of bladder contraction (Leung et al. 2007). The authors postulate that anesthesia may interfere with normal EUS bursting. Therefore, the possibility cannot be ruled out that the doses of urethane used in past and present studies was the reason such a small number of animals showed bursting. Nevertheless, taken together, these data suggest that the neural substrate for EUS bursting exists in the spinal cord below the level of injury and remains functional in transected rats. Most likely this EUS bursting CPG is different than the aforementioned bladder rhythm CPG. While it appears that the EUS bursting CPG is inhibited after supraspinal disconnection, the bladder rhythm CPG is unmasked and develops fully after several weeks post-transection.

Sustained tonic EUS activity

Two major time components of the decay in EUS activity following bladder contraction can be resolved in spinally intact rats: $\tau 1$ (short) and $\tau 2$ (long). The short time constant ($\tau 1 \sim$ 1.6s) reflects the decay in EUS activity due to bladder relaxation. However, EUS motoneurons exhibit sustained tonic activity following voiding that persists well after bladder relaxation has ended. This activity is represented by the long time constant ($\tau 2 \sim 15s$) and exists independently of bladder relaxation. Sustained activity in intact rats was evident as reverse hysteresis when EUS activity was plotted as a function of BP (figure 4C) and in the overlay of BP and EUS EMG activity (figure 4A). In transected rats, EUS motoneurons conspicuously failed to exhibit this sustained activity. Instead, the decrease in EUS EMG activity closely paralleled the fall in BP (see figure 4B) and the long time constant could no longer be resolved. Here, the decline in EUS activity is more aptly described by an elevated short time constant.

An attractive candidate mechanism for sustained EUS activity is the presence of persistent inward currents (PICs) in EUS motoneurons. A PIC is depolarizing current thought to be mediated by the combination of low threshold voltage-sensitive sodium and L-type calcium channels. PICs are capable of producing sustained plateau potentials and repetitive firing in motoneurons in both normal and pathophysiological states and are known to be facilitated by descending monoaminergic input (mediated principally by norepinephrine and 5-HT) from the brainstem (Bennett et al. 2001; Bennett et al. 2004; Heckmann et al. 2005, 2008; Li and Bennett 2003; Li et al. 2004). Thus, one can envision that PICs become activated during micturition and produce plateau potentials with sustained firing that persist after bladder contraction has ended. It is possible that spinal cord transection interrupts monoaminergic regulation of EUS motoneurons and results in the abrogation of normal PICs. To test this hypothesis, it would be interesting to

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combine *in vivo* intracellular recordings of EUS motoneurons during micturition and intravenous administration of agents known to inhibit PICs (e.g. L-type calcium channel blockers).

In the present study, there was no evidence for the development of muscle spasticity in the EUS following spinal cord transection. This was surprising since the transected rats exhibited clear signs of hindlimb spasticity (e.g. abrupt hindlimb extension during handling and manual bladder expression) and hyperreflexia (e.g. decreased frequency-dependent H-wave depression). A number of factors may contribute to the development of spasticity including intrinsic motoneuron hyperactivity or a decrease in pre-and post-synaptic inhibition (Elbasiouny et al. 2010; Nielsen, et al., 2007). For example, the development of tail spasticity following spinal cord transection in rats is associated with the intrinsic reemergence of PICs with plateau potentials and sustained firing in tail motoneurons (Bennett et al. 2001; Li et al. 2004). A likely explanation for the lack of EUS spasticity following transection is that EUS motoneurons do not develop constitutively active PICs. Alternatively, the continual level of activity in the EUS may play a role as there have been reports that chronic passive exercise of hindlimbs in spinally transected rats reduces muscle spasticity and hyperreflexia (Reese et al. 2006). A chronically high level of tonic EUS activity in spinalized rats might therefore account for the absence of this behavior. While there is no immediate evidence for the development of classical spasticity in the EUS, it is possible that the decreased inhibition in the spinal cord, as reflected in the H-reflex data, is instead being manifested as the enhanced guarding reflex discussed earlier.

Figures and captions

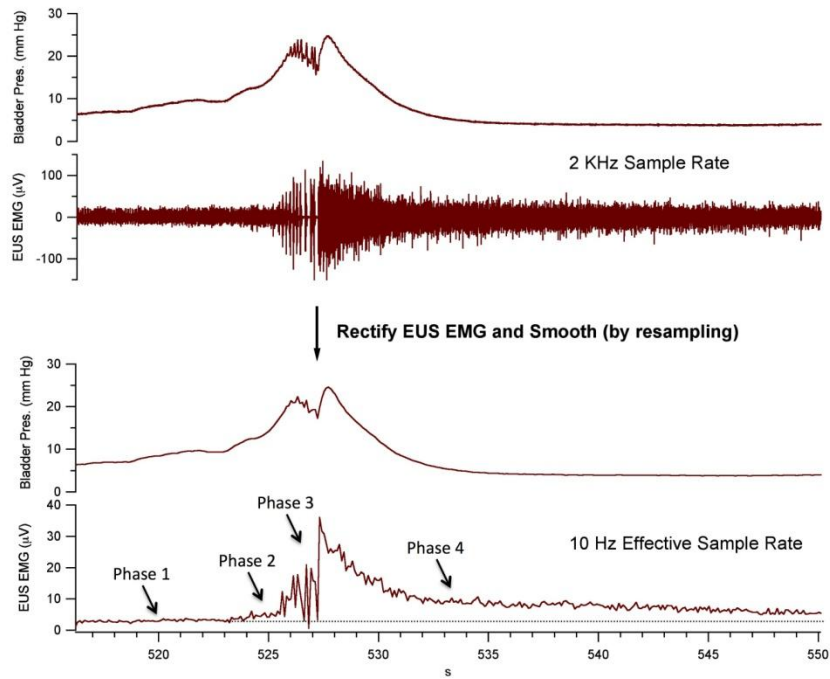**Figure 1.**

    A.
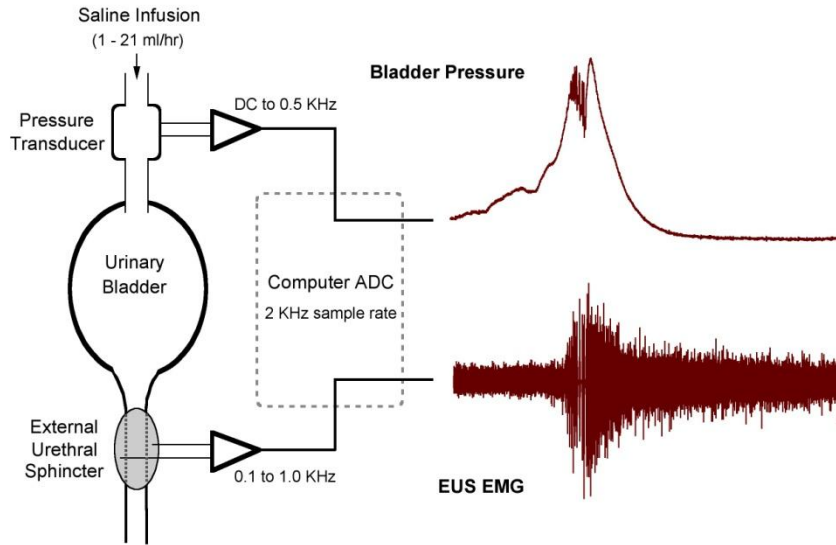
    B.

Figure 1. A. Schematic diagram (left) of flow cystometry set up for simultaneous recording of bladder pressure and EUS EMG (right). Records were acquired from a spinally intact adult female rat. **B.** Example of data smoothing. Raw records were acquired at a sample rate of 2 KHz (top). EUS EMG records were rectified and then both bladder pressure and EUS EMG records were smoothed by software resampling (decimation) to achieve a final effective sample rate of 10 Hz (bottom). Same records as illustrated in **A**.

Figure 2.

A.
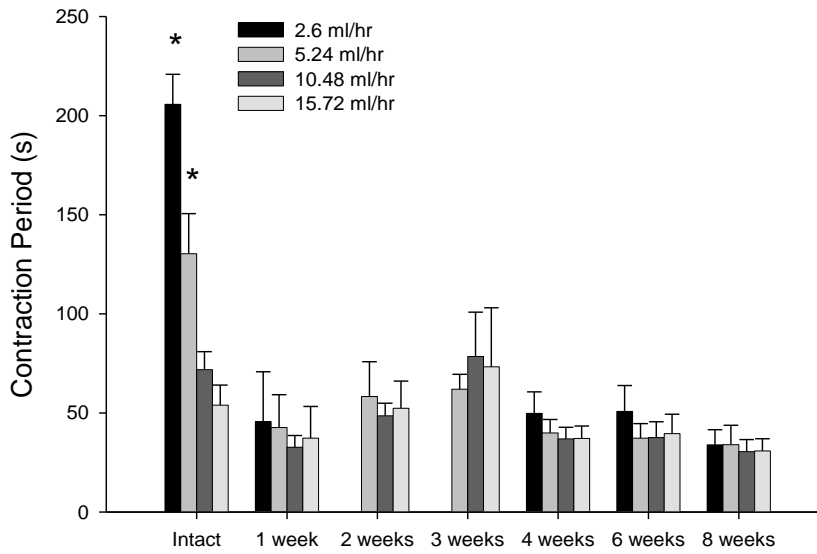
B.
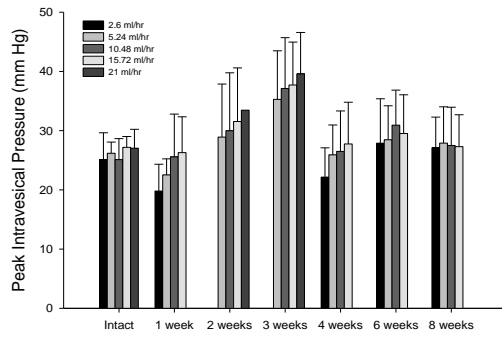
C.

Figure 2. Basic characteristics of bladder activity from intact and transected rats as a function of survival time and the rate of bladder filling. A. In the intact 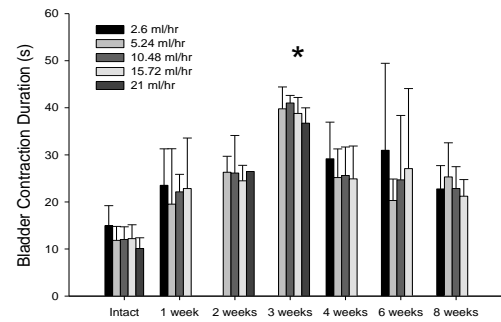rat, the average contraction cycle period (reciprocal of bladder contraction frequency) decreased from 205.7 ± 50s to 48.6 ± 9.7s when the rate of bladder filling was increased from 2.6 to 15.72 ml/hr. In transected rats, bladder contraction frequency from a single animal was fixed and insensitive to the rate of bladder filling. However, some degree of variability was seen from animal to animal with no significant difference as a function of post-transection survival time. Values are shown as mean $\pm$ SD. Single asterisks represent statistical significance of intact animals compared to all transected groups with a $p<0.05$. **B.** Peak intravesical pressure (peak amplitude) measurements from intact and spinally transected rats. The highest pressures were recorded at three weeks post-transection (39.6 ± 7.0 mm Hg as opposed to 27.2 ± 1.8 mm Hg in intact animals). Peak pressure was unaffected by changes in the rate of bladder filling. **C.** Measurement of bladder contraction duration from intact and spinally transected rats. Bladder contraction duration was longer in spinally transected rats compared to intact rats. The duration appeared to increase with time following transection, reaching a maximum at three weeks, however, there was significant variability from animal to animal such that the increased duration was statistically significant only at three weeks post-transection (intact 17.1 ± 4.6s, 3 weeks 42.5 ± 0.8s). Bladder contraction duration was unaffected by changes in the rate of bladder filling. Values are shown as mean $\pm$ SD. Single asterisks represent statistical significance ($p<0.05$) of three week survival group compared with: 1) Intact animals 2) one week survival group and 3) two week survival group. Intact animals (n = 11). Transected animals (1 week n = 5, 2 week n = 4, 3 week n = 4, 4 week n = 5, 6 week n = 5, 8 week n = 4).
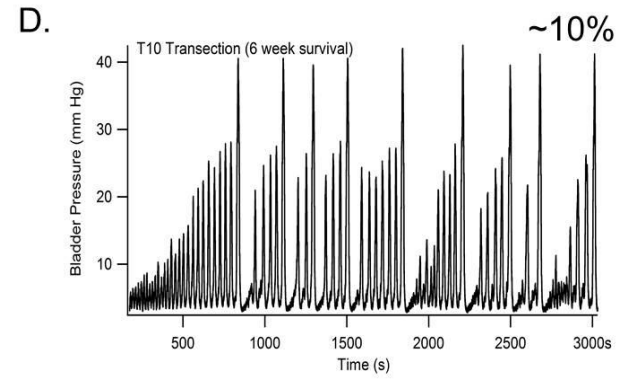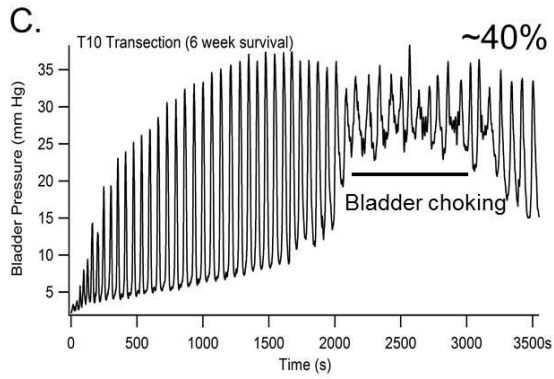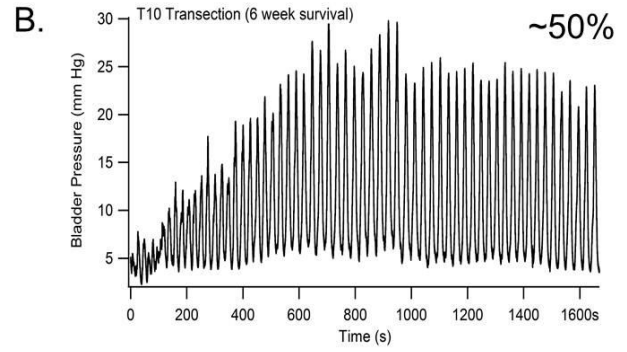
Figure 3.
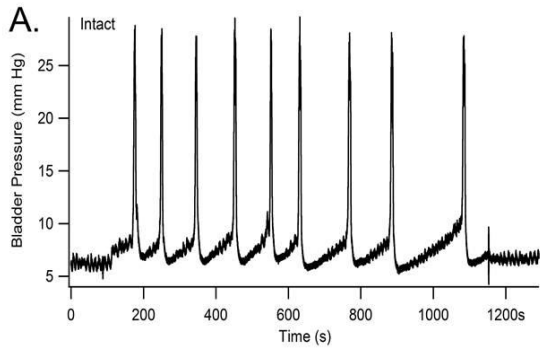
Figure 3. **Bladder pressure records of intact and transected rats during infusion of saline into the bladder (5.24 ml/hr). A**. BP record from an intact rat showing nine typical micturition events of characteristic shape and form. Increases in bladder volume and pressure during infusion led to coordinated bladder contractions with voiding. **B.** A BP record from a rat six weeks post-transection. As bladder filling progressed, small rhythmic non-voiding bladder contractions gradually increased in amplitude eventually producing (starting at ~700s) a small void at the peak of each contraction. **C.** A BP record from a second rat six weeks post-transection. Here, rhythmic bladder contractions increased in amplitude during filling as in **B**, however, this was associated with a slow rise in baseline pressure that led to a "choked" bladder that was over-distended. Under these conditions urethral dribbling was frequently observed (starting at ~1800s). Note the basic intrinsic rhythm persisted. **D.** A BP record from a third rat six weeks post-transection. Here, there was a repeating pattern of micturition events that consisted of a series of small non-voiding bladder contractions of increasing amplitude that ultimately gave way to a strong voiding bladder contraction.

Figure 4.

A. Intact

B. 6 weeks

C. Reverse Hysteresis (Intact)

D. Forward Hysteresis (6 week survival)

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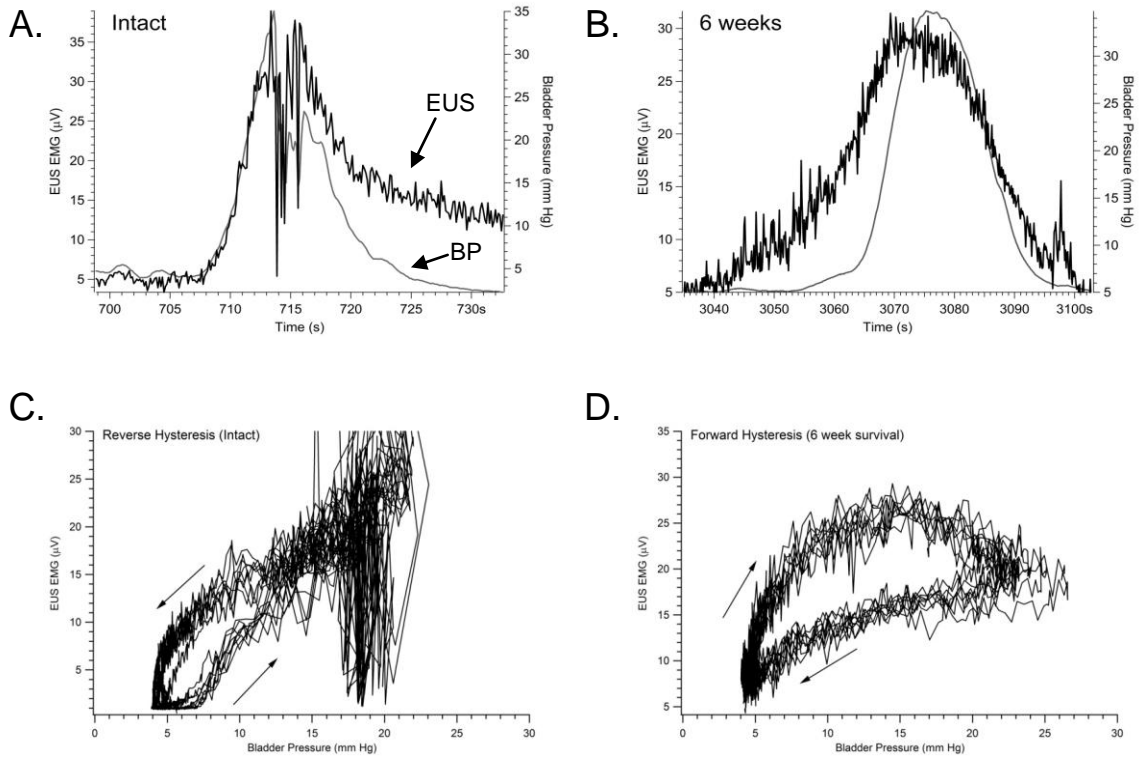
Figure 4. BP and EUS EMG activity in the intact and transected rat. A. Overlay of smoothed and rectified BP and EUS EMG activity during a single micturition event from an intact rat. The increase in EUS EMG activity closely matched the rise in BP at the onset of bladder contraction. However, EUS EMG activity remained elevated after complete relaxation of the bladder. **B.** Overlay of smoothed and rectified BP and EUS EMG activity during a single micturition event from a rat 6 weeks post-transection. In contrast to intact animals, the increase in the EUS EMG activity at the onset of bladder contraction preceded the rise in BP. The decline in EUS EMG activity paralleled the decrease in BP. **C.** Plotting EUS EMG activity as a function of bladder pressure revealed reverse (counter-clockwise) hysteresis in intact rats due to the sustained tonic EUS EMG activity following bladder relaxation **D.** In contrast, the same plot of data from transected animals produced forward (clockwise) hysteresis due to the increased activity at the beginning of bladder contraction and the lack of sustained activity following bladder relaxation. In **C** and **D**, multiple micturition events are represented and arrows indicate direction of hysteresis.

Figure 5.
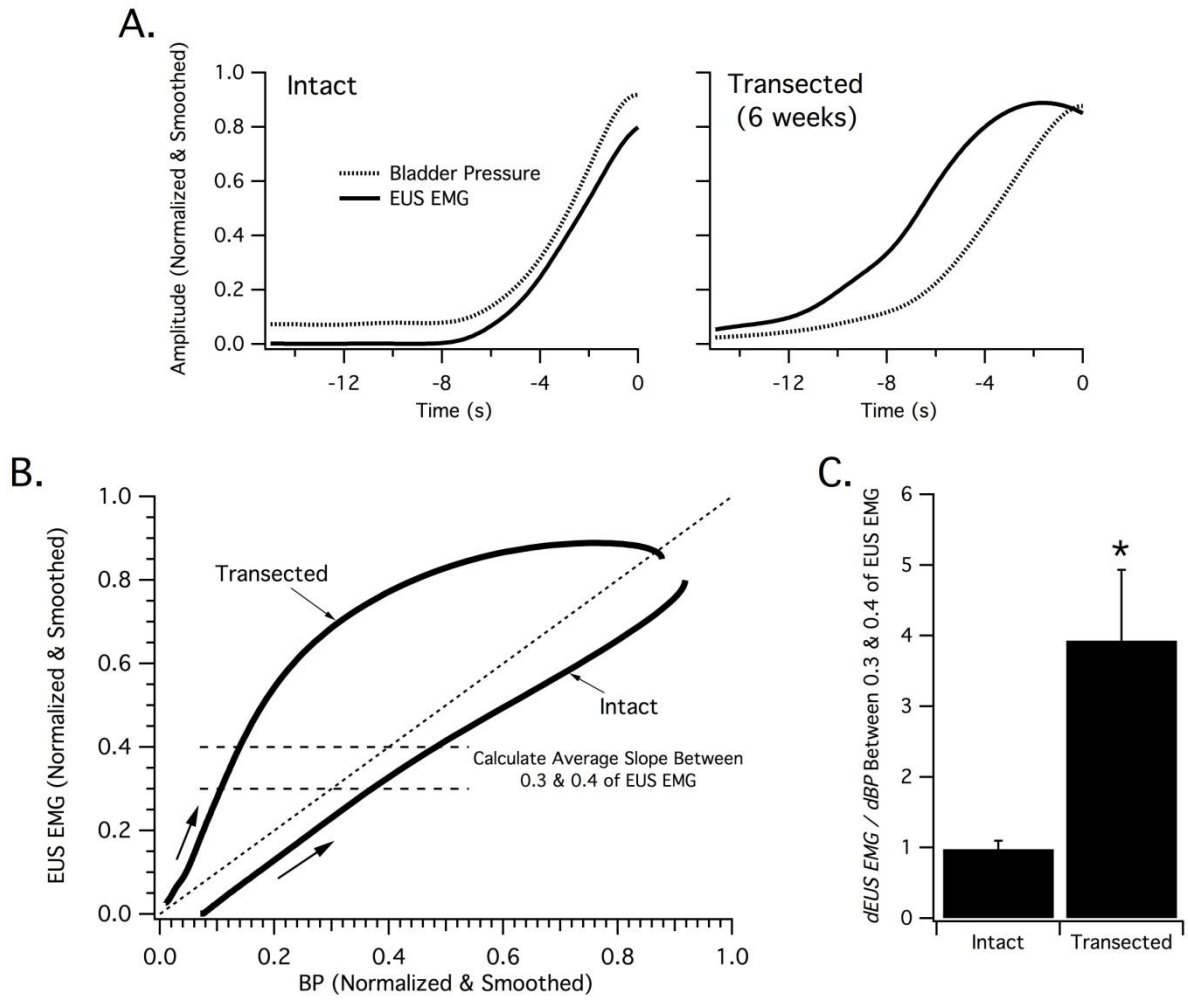
Figure 5. Relationship of BP and EUS activation. BP and rectified EUS EMG records were normalized and smoothed. Fifteen seconds of data prior to the peak of bladder contraction from successive micturition events were aligned at the peak of the BP and averaged in register (t = 0 at BP peak). **A.** In intact rats (left), EUS EMG activity increased in parallel with BP throughout active bladder contraction (average of 5 successive micturition events). In contrast, transected rats (right) exhibited enhanced EUS activation at the beginning of bladder contraction that reached maximum prior to the peak of the BP (average of 8 successive micturition events). **B.** Plotting EUS EMG activity as a function of BP reveals that EUS activity increases linearly with BP throughout most of active bladder contraction in intact rats. In contrast, in transected rats this relationship is nonlinear with enhanced EUS activation at the onset of bladder contraction **C.** The slope of the EUS-BP curve at the onset of active bladder contraction (calculated between 0.3 and 0.4 of maximum EUS activity; dashed lines) is greater in transected rats (3.93 ± 1.69; n = 8) than in intact rats (0.99 ± 0.12; n = 9).

Figure 6.
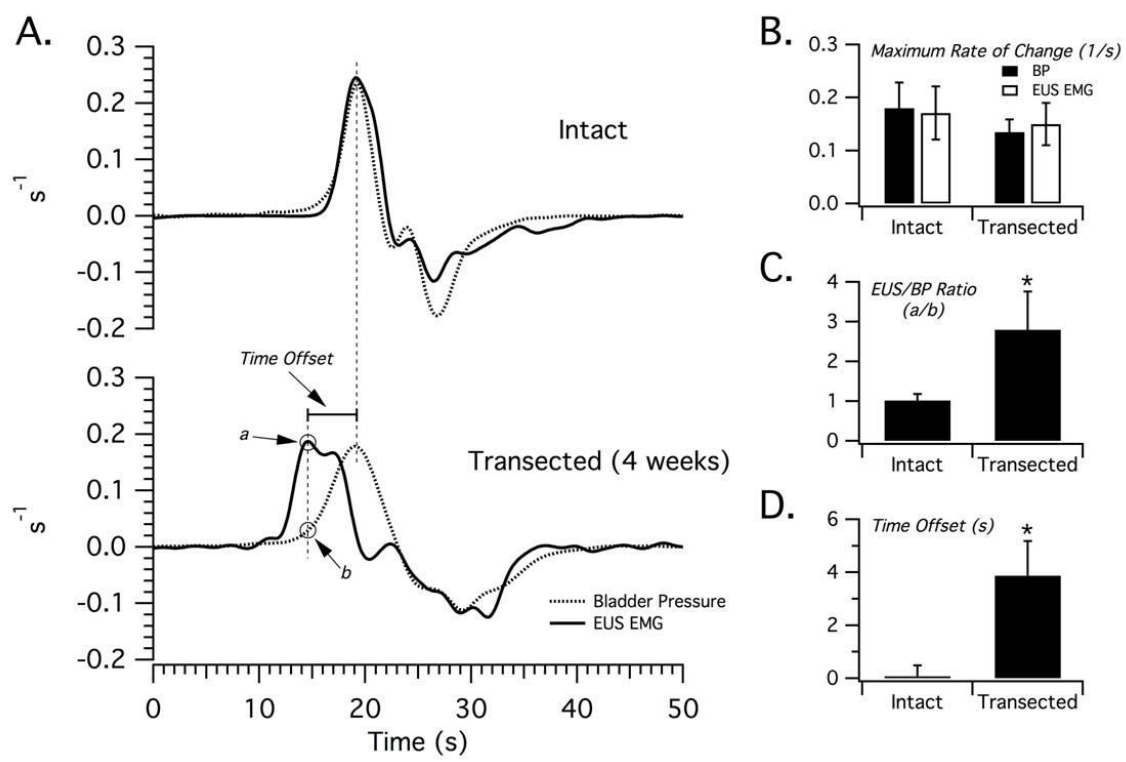
Figure 6. Timing of BP and EUS EMG activity in intact and transected rats. The first derivatives of normalized and smoothed BP and EUS EMG activity in intact and transected rats (4 - 6 weeks). **A.** Examples of first derivative records illustrating how measurements were made. The amplitudes and times of the maximum rates of change were measured (dashed lines). **B.** The maximum rates of change of normalized EUS and BP activity were the same in intact and transected rats **C.** The ratio of the maximum rate of rise of EUS EMG activity to BP activity. **D.** The maximum rates of rise of EUS and BP activity occurred at the same time (time offset = 0.075 ± 0.407s). In transected animals, the maximum rate of rise of EUS EMG activity occurred well before the maximum rate of rise of BP with an average time offset of 3.88 ± 1.31s. Values are shown as mean $\pm$ SD. Single asterisks represent statistical significance of spinally transected animals compared with intact group with a $p<0.05$.

Figure 7.

A.

B.
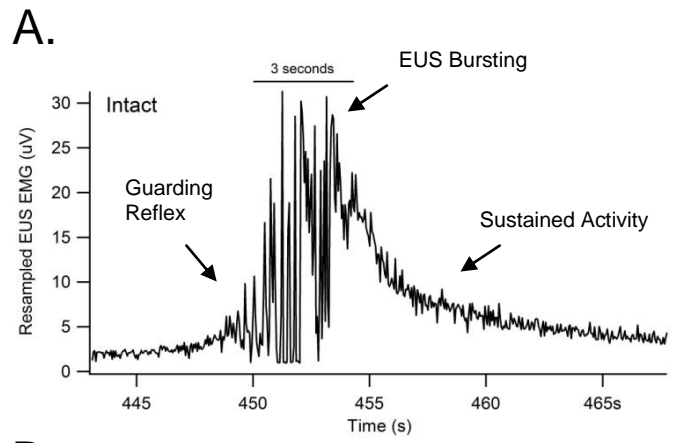
Figure 7. EUS bursting in the spinally intact and transected rat. A. Rectified and smoothed EUS EMG activity of a single voiding contraction that exhibited EUS bursting in an intact rat. Burst duration was approximately 3s **B.** Rectified and smoothed EUS EMG activity of a single voiding contraction with burst transient in a rat three weeks post-transection. Burst duration was approximately 1s.

Figure 8.

A.

B.

C.
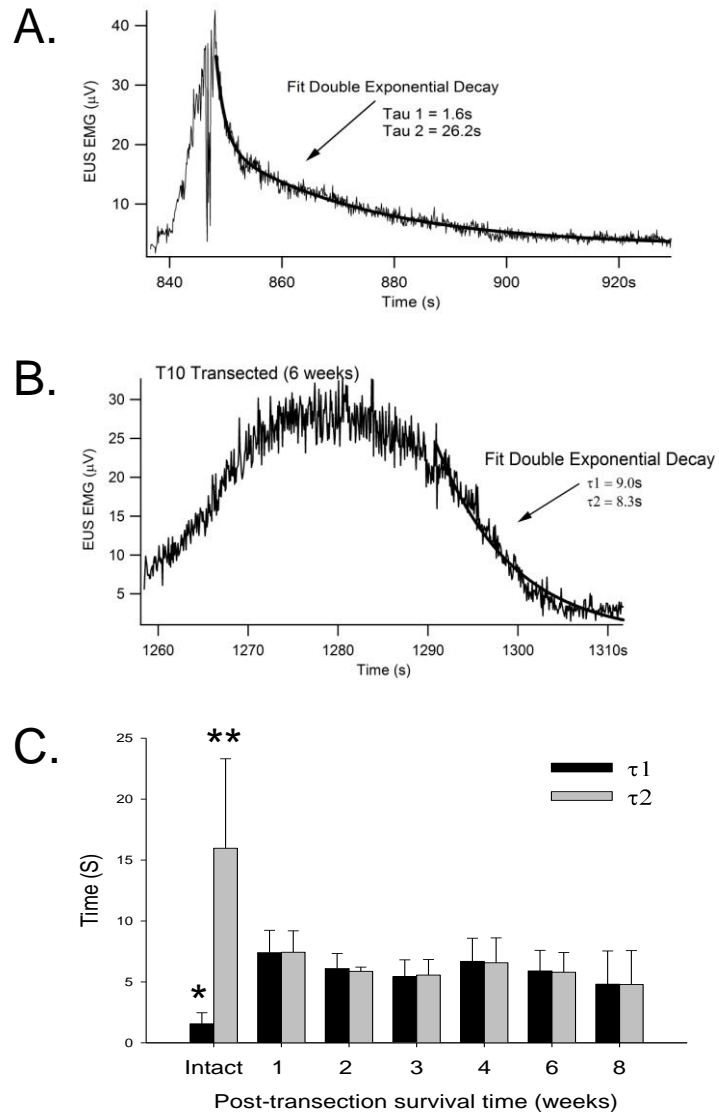
Figure 8. Sustained tonic EUS activity following bladder contraction. A. Example of a smoothed and rectified EUS EMG record from an intact rat during a single micturition event. In t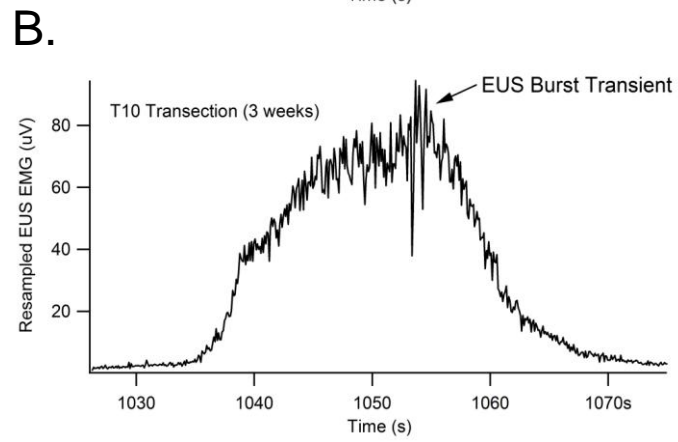his rat, two distinct time constants (short and long) could be resolved with $\tau1 = 1.6$s (short) and $\tau2 = 26.2$s (long). **B**. Example of a smoothed and rectified EUS EMG record from a rat 6 weeks post-transection. EUS EMG activity was fit with a double exponential decay function with $\tau1 = 9.0$s and $\tau2 = 8.3$s. **C**. Quantification of short and long time constants plotted as a function of post-transection survival time. Both time constants could be resolved from EUS EMG records in intact animals with average values of $\tau1 = 1.56 \pm 0.91$s and $\tau2 = 15.2 \pm 7.12$s. In contrast, only a single time constant (i.e. $\tau1=\tau2$) could be resolved in transected rats with a value intermediate between $\tau1$ and $\tau2$ calculated from intact rats. Values are shown as mean $\pm$ SD. Double asterisks represent statistical significance of $\tau2$ values from intact animals compared with all transected groups with a $p<0.05$. Single asterisk represents significance of $\tau1$ values from intact animals compared with all transected groups with a $p<0.05$. Intact animals (n = 6). Transected animals (1 week n = 5, 2 weeks n = 4, 3 weeks n = 4, 4 weeks n = 5, 6 weeks n = 5, 8 weeks n = 4).

Figure 9.
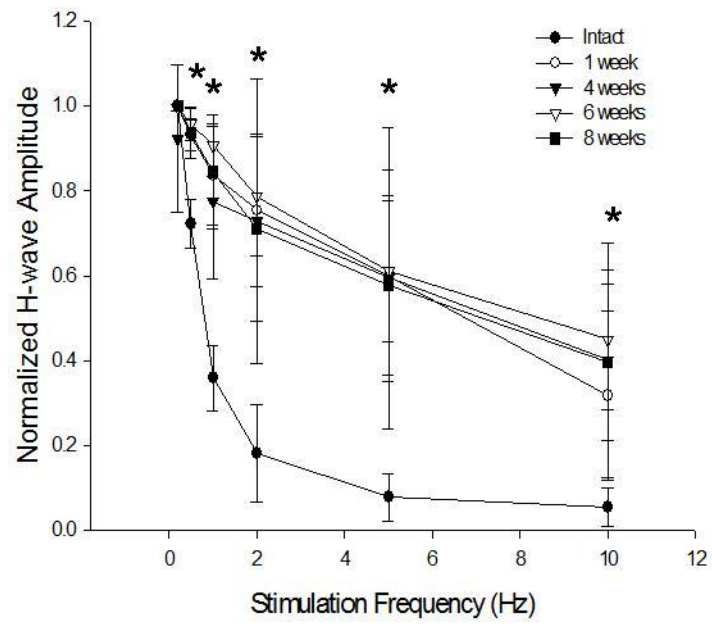
Figure 9. H-reflex from spinally intact and transected rats. Average H-wave amplitudes from intact and transected rats at different stimulation frequencies (0.2, 0.5, 1, 2, 5, and 10 Hz) illustrating frequency dependent H-wave depression. H-wave amplitude depression was significantly greater in intact rats (95% depression at 10 Hz) compared to transected rats (55 - 68% depression at 10 Hz). Data were normalized to the amplitude at 0.2 Hz and shown as mean ± SD. Single asterisks represent statistical significance of spinally transected animals compared with intact group with a $p<0.05$. Intact animals (n = 4). Transected animals (1 week n = 5, 4 weeks n = 5, 6 weeks n = 5, 8 weeks n = 4).

Chapter 3

Classification of external urethral sphincter motor unit recruitment patterns during micturition in the spinally intact and transected adult rat

Abstract and overview

The primary goal of this part of the study was to identify and characterize different EUS MU recruitment patterns in the normal and spinally injured adult rat during repeated micturition events. In the rat, EUS activation during micturition consists of three sequential phases: 1) an increase in tonic EUS activity during passive filling and active contraction of the bladder (guarding reflex), 2) synchronized phasic activity (EUS bursting) associated with voiding, and 3) sustained tonic EUS activity that persists after bladder contraction. These phases are perturbed following spinal cord injury. EUS MU activity was recorded either from the L_5 or L_6 ventral root (intact) or EUS muscle (transected) during continuous flow cystometry in urethane-anesthetized adult female Sprague Dawley rats. In both intact and transected rats, EUS MUs could be classified according to their bladder pressure threshold and timing of activation. Four distinct patterns of EUS MU activity were identified in the intact rat: Low Threshold Sustained, Medium/High Threshold Sustained, Medium/High Threshold Not Sustained and Burst Only. In general, these MUs displayed little frequency modulation during active contraction of the bladder, fired in high frequency phasic bursts consisting of multiple action potentials during EUS bursting, and varied in terms of sustained tonic activity. In contrast, three general patterns of EUS MU activity were identified in the transected rat: Low Threshold, Medium Threshold, and

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High Threshold. These MUs exhibited considerable frequency modulation during active contraction of the bladder, fired at low to moderate instantaneous frequencies, and exhibited no bursting behavior and little to no sustained firing.

Background

The pattern of activation of the external urethral sphincter (EUS) during micturition in the adult rat is well established (de Groat et al. 1998; Kruse et al. 1993; Maggi et al. 1986; Mersdorf et al. 1993; Streng et al. 2004; Van Asselt et al. 1995) and consists of three distinct phases. 1) Prior to voiding, passive filling and active contraction of the bladder result in tonic activation of the EUS muscle (i.e. the guarding reflex) that increases with bladder pressure and functions to maintain continence. 2) At the peak of bladder contraction, the emergence of highly synchronized phasic EUS activity (i.e. EUS bursting) coincides with and promotes efficient voiding. 3) Following EUS bursting, there is a period of extended EUS activity (i.e. sustained tonic EUS activity) that persists past complete relaxation of the bladder (D'Amico et al. 2011).

As a model for studying SCI and its impact on the LUT, many studies have examined changes in bladder and EUS activity following mid-thoracic SCI in the adult rat (Kruse et al. 1993; Leung et al. 2007; Pikov and Wrathall 2001; Pikov and Wrathall 2002; Wrathall and Emch 2006; Yoshiyama et al. 2000). Extensive focus has been given to addressing the deleterious effects of SCI on bladder function, primarily through assessment of relevant cystometric variables (e.g. bladder capacity, voiding volume, residual volume, and voiding efficiency) during micturition. As these variables are highly dependent on the efficacy of EUS bursting, more recent studies have addressed the process of EUS activity using whole EUS muscle EMG in the spinally intact and injured rat. However, other aspects of EUS activity associated with

micturition, such as the guarding reflex and sustained tonic activity, have received much less attention. Previous work from this laboratory quantified EUS activation in the intact rat and demonstrated that chronic spinal cord transection increased the guarding reflex and inhibited sustained tonic EUS activity (D'Amico et al. 2011).

Most of our understanding of EUS activation during micturition in the adult rat is based on whole muscle EMG recordings in combination with continuous flow cystometry. To date, there has been no investigation of EUS activity at the level of the single MU (i.e. a single α-motoneuron and all of the muscle fibers it innervates) during micturition, nor has there been any attempt to characterize MU recruitment patterns in this muscle before or after SCI. Knowledge of EUS MU recruitment in the rat is of paramount importance to fully understanding the role of the EUS in micturition and any changes associated with SCI. While the recruitment order and behavior of individual EUS MUs during micturition in the adult rat has not been investigated, combinations of histochemical and *in vitro* electrophysiological analyses indicate that the rat EUS is composed of different MU types. Immunohistochemical detection of myosin heavy chain (MHC) in the female rat EUS confirmed the presence of both slow and fast muscle fibers, the preponderance of which were of the fast MHC type (Praud et al. 2003). In a more detailed analysis, Buffini and colleagues found the rat EUS to be largely oxidative as a whole and, at the single muscle fiber level, determined it to be a mixed muscle composed of fast, fatigue-resistant (type 2A myosin), fast-fatiguing (type 2B myosin), and slow-oxidative (type 1 myosin) fibers (Buffini et al. 2010). *In vitro* intracellular recordings from EUS motoneurons in the adult rat (Carp et al. 2010) revealed EUS motoneurons with long or short duration afterhyperpolarizations (AHP) indicative of type-S or type-F MUs, respectively (Gardiner 1993; Zengel et al. 1985).

The purpose of the present study was to characterize the recruitment patterns of individual EUS MUs during micturition in the spinally intact and transected adult rat as to better understand the impact of SCI on the process of EUS activation. Consistent with the observation that the EUS is composed of different muscle fiber types, distinct patterns of EUS MU activity were identified in the intact rat based on differences in bladder pressure threshold (i.e. the bladder pressure at which EUS MUs became activated), instantaneous firing frequency, and sustained tonic activity. In contrast, EUS MU activity patterns in the transected rat were less distinct with no EUS bursting and little to no sustained tonic activity. As such, individual EUS MUs were classified almost exclusively on bladder pressure threshold. However, these MUs exhibited greater spike frequency modulation during the guarding reflex. Results of this study have been presented in abstract form (D'Amico and Collins 2010).

Materials and methods

Chronic spinal cord transection and animal care

All terminal experiments and survival surgeries necessitating animal care were done with adherence to the policies detailed by the Stony Brook University IACUC protocol and the Office of Research Compliance. Of the 28 adult female Sprague Dawley rats (Taconic; 200 – 300 g) used in this study, 15 underwent complete spinal cord transection as previously described in Chapter 2 (D'Amico et al. 2011). Briefly, under isoflurane anesthesia (5% induction, 2% maintenance) and using a dorsal approach, the spinal cord was transected at the T_9/T_{10} spinal level. Post-surgical animal care consisted of manual bladder emptying three times a day for 9 – 14 days and postoperative administration of the broad spectrum antibiotic enrofloxacin (10

mg/kg, sc, 5 – 7 days) and either the NSAID Ketorolac (6 mg/kg, sc, 2 days) or the long-lasting

opioid buprenorphine (0.05 mg/kg, sc, 3 days). When animals were capable of spontaneously

voiding on their own (9 – 14 days), bladders were expressed once a day. Animals were inspected

daily for distress, sores, dehydration and hematuria and treated accordingly if discovered.

Single MU recording in the intact rat

Urethane anesthetized (Sigma, 1.2 g/kg, sc) adult female rats with intact spinal cords (n =

13) were set up for continuous flow cystometry with simultaneous physiological recordings of

bladder pressure, EUS muscle EMG, left PMN electroneurography (ENG) and left L_5 or L_6

ventral root (VR) ENG (figure 10). Expiratory tidal $_PCO2$, ECG and core body temperature were

monitored during the course of the experiment, and to help maintain the viability of the animal,

room temperature lactated Ringers solution was continuously infused intravenously at a rate of 1

- 2 ml/hr. Cystometry and continuous infusion of saline into the bladder was done as previously

described in Chapter 2 (D'Amico et al. 2011). To measure whole muscle EMG activity, hooked

fine wire (50 μm insulated stainless steel, A-M Systems) electrodes were inserted

subcutaneously using a 30 gauge needle bilaterally into the EUS muscle under the pubic

symphysis. After closing the incision in the abdomen, the animal was placed in the prone

position in a spinal frame. The combination of an overhead heat lamp and a 37°C water-

circulating heating pad were used to maintain normal core body temperature while the animal

was suspended in the frame. The spinal cord was exposed after laminectomy from vertebrae L_1-

S_1 revealing the full extent of the lumbar roots and dorsal root ganglia. Following the

laminectomy, the left gluteus superficialis muscle in the hip was retracted laterally exposing the

L_6-S_1 trunk and the pudendal motor and sensory nerves above the ischiorectal fossa. The PMN

was dissected free of connective tissue and placed on bipolar platinum electrodes for stimulation and recording. Care was taken to position the electrodes as caudal as possible in the ischiorectal fossa at a site where the PMN only contains axons that innervate the EUS and external anal sphincter (EAS) and distal to the nerves innervating muscles of the pelvic floor (Bremer et al. 2003; McKenna and Nadelhaft 1986). Stimulus intensity was adjusted between $1.5 - 2.0$x threshold for detection of the associated orthodromic EUS EMG response. Under mineral oil, the dura was cut down the midline of the spinal cord and retracted laterally. Using glass nerve hooks, the L_5 or L_6 VR was placed on bipolar platinum electrodes and identified by antidromic stimulation of the ipsilateral PMN. Action potential conduction distance was determined by measuring the length of a suture placed between the PMN and VR electrodes to calculate conduction velocity.

Under mineral oil, the L_5 or L_6 VR was split multiple times in continuity using Dumont forceps (#5, Fine Science Tools) to produce fine root filaments for recording single EUS motor axon activity. Simultaneous recordings of bladder pressure, EUS EMG, left PMN ENG, and left L_5 or L_6 VR filament ENG were digitized and sampled at 20 kHz using a DAQ analog to digital converter card (National Instruments, M-series) during repeated micturition events and stored using the software Igor Pro 6.1 (WaveMetrics). The EUS EMG, PMN ENG and VR filament ENG signals were amplified (EUS EMG gain x1,000, PMN and VR ENG gain x10,000) through an AC differential preamplifier (A-M Systems) with high and low pass filters set to 0.1 kHz and 10 kHz, respectively. The BP signal was amplified through a PM-1000 transducer amplifier (CWE Inc.) and filtered DC to 0.5 kHz.

The identification of EUS MUs in the spinally intact rat

Confidence in the positive identification of EUS MUs in VR filament recordings was dependent on several criteria. 1) Phasic activity in the VR was time-locked to bursting activity in both the PMN and EUS. 2) In 19 of the 64 EUS MUs, it was possible to positively identify through spike-triggered-averaging orthodromic MU action potential propagation through the distal PMN, a site that only contains motor axons that innervate the EUS and EAS in the female rat (Bremer et al. 2003; McKenna and Nadelhaft 1986). Unpublished preliminary data from this laboratory indicate that EAS MUs do not exhibit phasic activity during micturition. 3) Of the 19 EUS MUs resolved in the PMN, all four EUS MU patterns identified in this study were represented. 4) The initial attempt to directly record from the EUS in the intact rat corroborated the existence of the same general EUS MU patterns identified from the VR. Taken together, these factors firmly indicate that most if not all MUs identified from the L5 or L6 VR are EUS specific.

Single MU recording in the spinally transected rat

Rats with chronic spinal cord transection (6 – 10 week survival; n = 15) were anesthetized with urethane and set up for continuous flow cystometry as described above. With the animal supine, the pubic symphysis was removed to expose the full rostrocaudal extent of the EUS. Single EUS MU activity during rhythmic bladder contractions was recorded using a tungsten microelectrode (A-M Systems; 1 MΩ) advanced into the EUS from the ventrolateral surface using a manual micromanipulator (David Kopf Instruments). The BP and EUS EMG signals were amplified as described for the intact rat. However, data were sampled at 40 kHz and the EUS EMG signal was low pass filtered at 20 kHz.

Different recording techniques

As outlined in the previous three sections, two different methods were used in this part of the study to record single EUS MU activity: 1) extracellular recording of L_5 or L_6 VR filaments using bipolar platinum hook electrodes and 2) direct recording from the EUS muscle using a tungsten microelectrode. However, this decision was not made arbitrarily. Initially, attempts were made to record MU activity directly from the EUS muscle to ensure the identity of the MU. However, in spinally intact rats the movement of the muscle during phasic activity associated with voiding had a tendency to dislodge the electrode from the surface of the muscle making it difficult to cleanly resolve MU activity during EUS bursting and to stabilize the amplitude of the signal long enough to accurately measure sustained tonic activity. Consequently, this approach was abandoned in the intact rat in favor of simultaneous extracellular recordings of the L_5 or L_6 VR, PMN, and EUS. This technique proved advantageous as it was possible to use the phasic activity recorded in the VR filament to identify putative EUS MUs. Additionally, in some cases, conduction velocity could be calculated. In transected rats, direct recording from the EUS yielded stable single unit recordings since the absence of EUS bursting mitigated the instability problem. Thus, both of these techniques were attempted in intact and transected rats; however, the recording of single fibers in the VR was more successful in the intact animal, whereas directly recording from the EUS muscle was better suited towards rats with chronic spinal transection.

Data analysis

In spinally intact and transected rats, the timing of action potentials generated by individual EUS MUs was detected using software amplitude/window discrimination (custom

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program in Igor Pro 6.1) and expressed as instantaneous frequency. For intact rats, VR action potential spike times were used to resolve MU action potentials in the ipsilateral PMN and the EUS muscle via spike-triggered-averaging. In addition to verifying propagation through the PMN to the EUS, this allowed calculation of MU latency in the PMN for conduction velocity measurements (n = 19). Conduction latency measurements were not possible in transected rats. Individual MUs in the intact (n = 64) and spinally transected rat (n = 83) were grouped according to their bladder pressure threshold and pattern of activity. Data from EUS MUs of the same group were pooled, averaged and expressed as mean (SD) (standard deviation) (n) in tables 1 and 2. Statistical significance ($p<0.05$) was determined using one-way ANOVA and post-hoc tests in Sigmaplot 11.

Results

EUS MU patterns in the intact rat

The first goal of this study was to determine the organization of EUS MU recruitment in the spinally intact rat during micturition. Motor axon activity of individual EUS MUs (n = 64) was recorded from small L_5 or L_6 VR filaments isolated in continuity. Each MU exhibited phasic activity during voiding that was time-locked to the bursting activity recorded simultaneously in the PMN and EUS. However, there was considerable variability between MUs in the bladder pressure threshold (i.e. the bladder pressure at the onset of MU activity) and the duration of MU activity following complete bladder relaxation (i.e. sustained tonic activity). Based on bladder pressure threshold and duration of sustained activity, EUS MUs in the intact rat could be divided into four distinct groups: 1) Low Threshold Sustained (LS, n = 15/64, 24%), 2) Medium/High

Threshold Sustained (MHS, n = 38/64, 59%), 3) Medium/High Threshold Not Sustained (MHNS, n = 5/64, 8%), and 4) Burst Only (BO, n = 6/64, 9%).

LS MUs were always recruited during passive filling before active bladder contraction and remained active following complete bladder relaxation (i.e. exhibited sustained tonic activity) (figure 11A). MHS MUs were recruited during active bladder contraction prior to or at the onset of EUS bursting and exhibited sustained tonic activity (figure 11B). MHNS MUs were recruited during active bladder contraction prior to or at the onset of EUS bursting and exhibited only brief tonic firing following EUS bursting that was not sustained past complete bladder relaxation (figure 11C). Finally, BO MUs were recruited immediately at the onset of EUS bursting and exhibited no sustained tonic activity following EUS bursting (figure 11D).

Duration of MU activity

In the intact rat, each group of EUS MU exhibited a systematic difference with respect to duration of tonic activity before and after EUS bursting (table 1). LS MUs exhibited the longest total duration of neuronal activity, as well as the longest duration of activity prior to EUS bursting. However, the difference in duration of sustained tonic activity after EUS bursting was not found to be statistically significant between LS and MHS MUs. In contrast, MHNS MUs exhibited brief neuronal activity immediately before and after bursting. By definition, BO MUs exhibited no tonic EUS activity before or after EUS bursting. As such, MHNS and BO MUs exhibited the shortest durations of neuronal activity.

Frequency of MU activity

In the intact rat, EUS MUs exhibited little frequency modulation during the guarding reflex, but reached high instantaneous frequencies during EUS bursting. EUS MU instantaneous frequency was measured: 1) at the time of MU activation (i.e. onset frequency), 2) immediately before, during and immediately after EUS bursting, and 3) at the time of MU inactivation (i.e. off frequency) (see table 1). To get a sense of the contribution that each MU had on the guarding reflex (i.e. during passive bladder filling and active bladder contraction), the instantaneous frequency at activation (onset frequency) was compared with the frequency just before the start of EUS bursting. Also, frequency modulation was calculated by subtracting the onset frequency from the instantaneous frequency before EUS bursting. LS MUs (n = 6/6) were activated during passive bladder filling and showed moderate to large increases in instantaneous frequency with increasing bladder pressure during the guarding reflex. MHS and MHNS MUs were activated during active contraction of the bladder, however, in contrast to LS MUs, only a very small number of MHS MU (n = 4/30) and no MHNS MU (n = 0/5) exhibited significant increases in firing frequency during the period of active bladder contraction (figure 12A and 13A). Since BO MUs exhibited no tonic activity prior to bursting, this measurement was undefined and they were excluded from this analysis.

In whole muscle EMG recordings, EUS bursting in the rat is characterized by phasic bursts of activity (i.e. burst events) alternating with periods of complete silence during which voiding occurs. Since EUS bursting is essential for efficient voiding in the adult rat (Conte et al. 1991; Kruse et al. 1993; Peng et al. 2006, 2008; Yoshiyama et al. 2000), it was imperative to examine MU behavior during this time. In the present study, low threshold EUS MUs (e.g. LS and some MHS MUs) developed a burst-like pattern of activity that gradually became more

synchronized leading to full synchronization of EUS MU activity at the onset of EUS bursting (example in figure 14). Thus, the EUS muscle does not abruptly switch from tonic firing to phasic activity. Rather, the phasic activity builds within the EUS motoneuron pool culminating in complete synchronized burst events and EUS bursting.

During EUS bursting, each MU fired a series of individual burst events. Each burst event was composed of one to five action potentials at two or three dominant frequency ranges (figures 14 and 15). The first action potential invariably had the lowest instantaneous frequency and reflected the frequency of the burst event (6 - 8 Hz). Almost universally, the second action potential occurred at the shortest interval corresponding to the upper range of dominant frequencies (75 – 90 Hz), but with wide variability in terms of individual instantaneous frequencies (as high as 150 Hz). Subsequent action potentials generally occurred at an intermediate frequency comprising the middle dominant frequency range (40 - 55 Hz). LS, MHS and MHNS MUs fired three to five action potentials per burst event (example in figure 16A) and exhibited three dominant frequencies (burst event, middle, upper; figure 15A-C). In contrast, BO MUs fired only one to two action potentials per burst event (example in figure 16B) and consistently exhibited only two dominant frequencies (burst event and middle; figure 15D). The highest maximum instantaneous frequencies were seen in LS MUs (upper dominant frequency range) while BO MUs fired at the lowest maximum frequencies (middle dominant frequency range).

Finally, for LS, MHS, and MHNS MUs, the instantaneous frequency of tonic activity immediately after EUS bursting was compared with the frequency of tonic firing immediately before bursting (table 1). LS MUs fired at similar instantaneous frequencies immediately before and after EUS bursting. In contrast, the instantaneous frequency immediately after EUS bursting

in MHS and MHNS MUs was significantly higher than the instantaneous frequency immediately before bursting. Additionally, the average tonic firing frequency after EUS bursting was similar between LS, MHS and MHNS MUs (35 – 45 Hz). Since, by definition, BO MUs exhibited no tonic activity prior to or immediately after EUS bursting, they were excluded from this analysis.

MU conduction velocity

For 19 EUS MUs in the intact rat it was possible to clearly resolve the MU action potential propagating through both the VR filament and the PMN. In these cases, MU conduction velocity was calculated by measuring the conduction distance and MU action potential latency between the VR and PMN recording electrodes (figure 17A). Unfortunately, it was not possible to calculate a conduction velocity for every MU. This was likely the result of a conduction block distal to the VR recording electrodes that interrupted propagation of the MU action potential to the periphery. While a wide range of conduction velocities was observed among EUS MUs (8 – 31 m/s), there were no statistical differences in the average conduction velocities between LS, MHS, MHNS and BO MUs (table 1). However, the duration of sustained tonic activity appeared to vary inversely with conduction velocity, particularly MHS MUs, such that MUs with slower conduction velocities exhibited longer durations of sustained activity (figure 17B).

EUS MU patterns in the spinally transected rat

The second major goal of this study was to examine individual EUS MU recruitment patterns during micturition in the spinally transected rat and to compare their organization with MUs in the intact rat. Recordings from single EUS MUs (n = 83) were made directly from the ventral surface of the EUS muscle using tungsten microelectrodes. In contrast to the intact rat, no

bursting and little to no sustained tonic activity was observed in these MUs. Consequently,

bladder pressure threshold was the primary criterion used to classify individual EUS MU activity

in the transected rat. Three general patterns of EUS MU activity were identified: 1) Low

Threshold (LT, n = 22/83, 26%), 2) Medium Threshold (MT, n = 47/83, 57%), and 3) High

Threshold (HT, n = 14/83, 17%). LT MUs were always recruited before the beginning of active

bladder contraction (figure 18A) with some (n = 6/22) being tonically active at small bladder

volumes and low bladder pressures. Additionally, a subset of LT MUs (n = 9/22) remained active

past bladder relaxation (i.e. exhibited short duration sustained tonic activity, 1 – 15 s). MT MUs

were recruited during active bladder contraction and HT MUs were recruited near the peak of

bladder contraction (figure 18B and 18C). MT and HT MUs never exhibited sustained activity

and always ceased firing prior to complete bladder relaxation.

Duration of MU activity

In order to compare the timing of activation among different groups of MUs in the

transected rat, the total duration of MU activity was expressed as the percent of the duration of

bladder contraction (i.e. the duration of MU activity equal to the duration of bladder contraction

was considered to be 100%). This normalization was necessary because of the wide variability in

the duration of bladder contractions in different transected rats. An inverse relationship was

noted between bladder pressure threshold and normalized activity duration such that MUs of

higher bladder pressure threshold tended to exhibit shorter durations of activity (see figure 18A-

C and figure 13B). The difference in the average percent activity in terms of MU threshold was

statistically significant (table 2).

Frequency of MU activity

In contrast to the intact rat, EUS MUs in the transected rat exhibited significant frequency modulation during the guarding reflex and no high frequency bursting. The instantaneous frequency of EUS MUs in the transected rat was measured 1) at the time of activation (i.e. onset frequency), 2) at or near the peak of bladder contraction (maximum instantaneous frequency of tonic firing), and 3) at the time of inactivation (i.e. off frequency). To assess the contribution that each EUS MU had on the guarding reflex in the transected rat, the onset frequency was compared with the maximum tonic firing frequency, the latter of which occurred at or just before peak bladder pressure. The increase in frequency from activation to peak bladder contraction was large in LT MUs, moderate in MT MUs and small in HT MUs (figures 12B and 13B). Additionally, an inverse relationship was noted between bladder pressure threshold and maximum instantaneous frequency such that MUs of lower bladder pressure threshold typically reached higher maximum tonic firing frequencies (see figure 18A-C and figure 13B). The average maximum frequency between different EUS MU groups in the transected rat was found to be statistically significant (table 2). It should be noted that the vast majority of MUs (n = 73/83) in the transected rat reached maximum tonic firing before peak bladder pressure (table 2). Among the 10 MUs that did not, nine fired maximally at the peak of bladder pressure and one immediately after.

Discussion

In the present study, individual EUS MUs could be classified based on bladder pressure threshold, firing frequency, and duration of activity following micturition. In the intact rat, four distinct EUS MU patterns of activity were identified: Low Threshold Sustained (LS),

Medium/High Threshold Sustained (MHS), Medium/High Threshold Not Sustained (MHNS), and Burst Only (BO). During EUS bursting, each of these MUs fired multiple action potentials (one to five) within each burst event reaching high instantaneous frequencies. However, they displayed little frequency modulation during active bladder contraction and differed mainly with regard to their bladder pressure threshold and degree of sustained tonic activity. In contrast, three general MU activity patterns could be identified in the transected rat based on bladder pressure threshold: Low Threshold (LT), Medium Threshold (MT), and High Threshold (HT). No bursting behavior and little to no sustained firing were observed in transected rats. During active bladder contraction, EUS MU activity increased irrespective of bladder pressure threshold and reached only low to moderate frequencies.

The guarding reflex

To maintain continence there is an increase in EUS activity during bladder filling. Referred to as the guarding reflex, this response is initiated through activation of afferents in both the pelvic and pudendal nerves and organized segmentally in the lumbosacral spinal cord (Fowler et al. 2008; Thor and de Groat 2010). While the guarding reflex is prominent in humans (Park et al. 1997), its importance in the rat is less appreciated (McMurray et al. 2006). In the rat, the guarding reflex consists of two distinct phases: 1) a small increase in EUS activity as the bladder passively fills and 2) a robust EUS response during active bladder contraction or events associated with rapid rises in bladder pressure (D'Amico et al. 2011). Therefore, the guarding reflex is somewhat limited during passive bladder filling, but quite pronounced when the bladder actively contracts during the initial phase of a micturition event.

In the present study, 16% of EUS MUs in intact rats exhibited significant frequency

modulation as bladder pressure increased. These were predominately the LS MUs that were

recruited well before active bladder contraction. In contrast, MHS and MHNS MUs were

recruited during active bladder contraction, but most did not significantly increase their firing

rate before EUS bursting and voiding. Because MHS and MHNS together constitute the majority

of EUS MUs (~67%), it strongly suggests that the guarding reflex in the intact rat is largely

mediated by the recruitment of *additional* MUs during active bladder contraction as opposed to

increased temporal summation at the level of individual MUs. Nevertheless, one should not

discount the role of frequency modulated LS MUs on the guarding reflex. Because BO MUs

were activated immediately at the onset of EUS bursting, it is unlikely that they contribute in any

way to the guarding reflex.

The classic model for MU recruitment was developed from seminal work on the stretch

reflex in decerebrate cats. It describes an orderly recruitment pattern beginning with MUs of

smaller size and slower conduction velocity followed by larger and faster MUs that generate

stronger force (i.e. the size principle) (Clamann 1993; Henneman 1957; Henneman et al. 1965a,

1965b; Hodson-Tole and Wakeling 2009). Based on the size principle and past studies using

whole muscle EMG recordings of the EUS during micturition in the spinally intact rat, it was

initially anticipated that as bladder pressure increased, additional EUS MUs would become

active and exhibit a smooth temporal increase in instantaneous frequency. Thus, it was surprising

to discover little frequency modulation in the majority of EUS MUs, particularly those recruited

during active contraction of the bladder, a time when stimulation (i.e. the increase in bladder

pressure) was high. However, studies suggest that exceptions to the size principle model do exist.

For instance, motor tasks that require high bouts of muscle activity and the generation of

immediate force (e.g. fast activation of the triceps surae, paw shake in the cat, high-speed cycling in the human, swimming in fish) can result in a preferential recruitment of high threshold MUs (Gollnick et al. 1974; Jayne and Lauder 1994; Nardone et al. 1989; Smith et al. 1980). In the intact rat, a recruitment strategy consisting of the activation of numerous medium/high threshold MUs during active bladder contraction may be more effective at generating rapid increases in EUS muscle tension than a more orderly recruitment accompanied by temporal changes in the rate of MU firing.

In contrast to the intact rat, the majority of EUS MUs in the transected rat exhibited marked increases in spike frequency during active bladder contraction. This was particularly the case for low and medium threshold MUs. Further, nearly all of these MUs reached their maximum tonic firing frequency before peak bladder pressure. A previous study from this laboratory (D'Amico et al. 2011) demonstrated that the gain of the guarding reflex is augmented in rats with chronic spinal cord transection. The present data indicate that this enhanced guarding reflex in the transected rat is largely determined by frequency modulated LT and MT MUs that accelerate and achieve maximal tonic firing during the period of active bladder contraction. This is in contrast to the intact rat where a generalized increase in MU firing frequency during the rise in bladder pressure appears to play less of a role. While HT MUs in the transected rat do share similar characteristics with LT and MT MUs (e.g. frequency modulation), their high bladder pressure threshold, short duration of activity, and low maximum frequency indicate they contribute little to the guarding reflex.

EUS bursting

EUS bursting in the spinally intact adult rat is characterized by highly synchronized whole muscle phasic activity composed of individual burst events that alternate with periods of total quiescence and is essential for efficient voiding (Conte et al. 1991; Kruse et al. 1993; Peng et al. 2006, 2008; Yoshiyama et al. 2000). SCI disrupts this activity resulting in urine retention and increased bladder capacity. Because EUS bursting is essential for efficient voiding in the spinally intact rat, it was of interest to observe single MU behavior during this phase of micturition. In the intact rat, all EUS MUs exhibited synchronized phasic activity during EUS bursting, however, a subset of individual EUS MUs developed burst-like patterns of activity prior to the onset of EUS bursting and voiding. This occurred primarily in LS and MHS MUs and was evident as a progressive transition to phasic activity during the period of active bladder contraction leading up to EUS bursting. The timing of this transition depended on when the individual MU was recruited. For instance, LS MUs became tonically active during passive bladder filling and exhibited a gradual transition to phasic activity. In contrast, MHS and MHNS MUs became active during active bladder contraction prior to EUS bursting, but exhibited a transition that was much more abrupt.

The results of this study indicate the possibility of two separate excitatory synaptic inputs (potentially mediated by two independent interneuron sources) to EUS motoneurons that are simultaneously active and produce different patterns of excitation. The first could be a slow excitatory input that produces smooth depolarization of EUS motoneurons driving them to fire tonically. Superimposed on this slow excitation is a second excitatory input consisting of rapid phasic depolarizations that grow in amplitude and drive the motoneurons to fire in high frequency phasic bursts. This pattern of simultaneous slow and phasic excitation has been

observed in *in vivo* intracellular rat EUS motoneuron recordings during micturition (Collins 2010). It follows that lower threshold EUS MUs would be highly susceptible to both of these excitatory inputs, thus exhibiting tonic firing following by smooth transitions into phasic activity. Conversely, high threshold MUs would be more heavily influenced by phasic excitatory input and consequently exhibit less tonic firing and a more abrupt transition into phasic bursting. In the present study, the development of phasic activity leading up to EUS bursting was apparent in many EUS MUs. However, following EUS bursting, the transition back to tonic activity was not as conspicuous. This was evident not only in single fibers, but in whole muscle EMG recordings where the termination of EUS bursting was much more rapid than its initiation. Some investigators have posited a CPG in the lumbosacral spinal cord that mediates EUS bursting (Chang et al. 2007; Dolber et al. 2007) so it is tempting to speculate that this CPG is responsible for the increasing phasic excitatory drive to the EUS motoneurons. However, it is not clear from the present data what mechanism may be responsible for turning off phasic activity, but one could speculate that it involves the simultaneous removal of tonic inhibition of EUS motoneurons and direct inhibition of the bursting CPG.

During EUS bursting, all MUs contracted simultaneously, firing multiple burst events. However, within a single burst event, the number of MU action potentials varied between one and five and exhibited up to three dominant frequency ranges ($7 - 8$ Hz, $40 - 55$ Hz, and $75 - 90$ Hz) with individual instantaneous frequencies reaching > 150 Hz. Based on previous work that established the EF_{50} (i.e. the frequency at half maximum force generation, 29.1 ± 0.8 Hz) and half-relaxation time (27.5 ± 1.1 ms) of the rat EUS (Buffini et al. 2010), it is evident that the high firing frequencies of individual MUs during EUS bursting are well above those needed for temporal summation of muscle contractions and would most likely produce tetanic contractions

and maximum sphincteric tension. Also, since all individual EUS MUs fired together during EUS bursting, the idea that the whole EUS muscle bursts as a single symmetrical unit during voiding is undoubtedly correct (Thor and de Groat 2010).

The complete inhibition of EUS bursting has been reported in rats with chronic SCI (Kakizaki et al. 1997; Kruse et al. 1993). However, more recent studies document that a spinal mechanism mediating EUS bursting can still become active, albeit less frequently, in the lightly anesthetized or awake rat with incomplete or complete SCI (Cheng and de Groat 2004; Leung et al. 2007). These observations indicate that a EUS bursting mechanism is functional after SCI, yet sensitive to anesthesia. Leung et al. in particular make the distinction between two groups of rats, "tonic" and "phasic", where the former do not exhibit EUS bursting and efficient voiding. This is also consistent with a previous study from this laboratory where very few (~10%) urethane-anesthetized rats with complete spinal cord transection exhibited "burst-transients", described as the brief (~1 s) appearance of phasic activity that did not necessarily coincide with voiding (D'Amico et al. 2011). It is apparent that EUS bursting with efficient voiding after complete SCI is undoubtedly a significant component of the micturition response and the neural substrate mediating this activity is functional in the lumbosacral spinal cord distal to the site of injury. However, it is not ubiquitously expressed under all experimental conditions.

In the present study, no transected animal exhibited EUS bursting. Therefore, analysis and quantification in the transected rat was focused on single EUS MU activity associated with rhythmic bladder contractions. This pattern of bladder-EUS activation has been shown to be present in a significant percentage of rats with SCI and is thought to be an important component of the micturition response (D'Amico et al. 2011; Kruse et al. 1993; Leung et al. 2007). In the transected rat, EUS MUs without bursting activity did not attain comparable maximum

frequencies to those seen during EUS bursting in the spinally intact rat. However, one could hypothesize that, compared to the intact rat, EUS bursting of individual EUS MUs in the transected rat would consist of burst events with a reduced number of MU action potentials and lower maximum instantaneous frequencies. Interestingly, the instantaneous frequency of LS, MHS, and MHNS MUs immediately after phasic activity in the intact rat (35 – 45 Hz) was very close to the highest average tonic firing frequency in the transected rat (LT MUs, ~40 Hz). This suggests that this MU frequency may approximate a maximum limit for tonic firing that can only be overcome by activation of additional excitatory inputs such as a spinal EUS bursting CPG (Chang et al. 2007; Dolber et al. 2007).

Sustained tonic EUS activity

In the intact rat, EUS motoneurons display sustained tonic activity following EUS bursting that can persist for up to a minute or longer. However, this activity is largely inhibited following spinal cord transection (D'Amico et al. 2011). In the present study, MUs from the intact rat varied with respect to derecruitment and sustained tonic activity. Greater than 80% of these MUs (LS and MHS) exhibited a significant level of sustained tonic activity following phasic bursting. Intrinsic PICs are likely candidates for mediating sustained tonic activity in EUS MUs. Ca^{+2} and Na^{+1} PICs are well known to increase the excitability of spinal motoneurons by producing sustained plateau potentials, repetitive self-sustained firing, and bistable behavior in the turtle, cat, and rat (Heckman et al. 2005, 2008; Li et al. 2007; Murray et al. 2011; Rank et al. 2011). Recording intracellularly from rat EUS motoneurons, Carp et al. found that a large population of cells (~40%) exhibited spontaneous tonic firing in the absence of stimulation (Carp et al. 2010). Another study investigating membrane properties of EUS motoneurons in the cat

revealed that these neurons displayed repetitive firing after afferent pudendal nerve stimulation that could be quenched by injection of hyperpolarizing current (Paroschy and Shefchyk 2000). These data support a possible role of PICs in EUS motoneuron activation, however, their existence has not been conclusively demonstrated in adult rat EUS motoneurons. In the present study, it was not possible to identify a specific mechanism for sustained tonic activity; however, it is likely that the putative mechanism is distributed systematically across the EUS motoneuron pool. For instance, sustained tonic activity of EUS MUs was found to vary inversely as a function of conduction velocity such that the longest durations of repetitive firing were generally seen in MUs with the slowest conduction velocities.

In a previous study (D'Amico et al. 2011), sustained tonic EUS activity in the transected rat was rarely seen, ultimately leading to the expectation that little if any sustained firing would be seen at the level of individual EUS MUs. This turned out to be correct. While some LT MUs (n = 9/22) continued to fire after complete bladder relaxation, the duration was short-lived (1 – 15 s). Therefore, EUS stimulation (i.e. bladder stretch and the rise in bladder pressure during active contraction) in the transected rat does not consistently generate prolonged sustained firing in EUS MUs. While in agreement with previous data from this laboratory, it is nonetheless surprising as it stands in stark contrast to work by others who report the presence of PICs, plateau potentials and repetitive firing in briefly stimulated sacrocaudal motoneurons in a chronic SCI rat model of spasticity (Bennett et al. 2001, 2004; Li et al. 2003, 2007; Li and Bennett 2003; Murray et al. 2011; Rank et al. 2011). Taken together, these findings indicate that PICs may be active in EUS motoneurons before, but not after spinal cord transection. Further, the EUS does not exhibit classic signs of spasticity that are regularly seen in the tail and hindlimb muscle after chronic spinal injury.

Comparison between MUs in the intact and transected rat

It is tempting to draw parallels between groups of MUs in the intact and transected rat. While the putative mechanisms that govern the guarding reflex (i.e. MU recruitment), EUS bursting (i.e. phasic CPG or descending brainstem neuromodulators) and sustained tonic EUS activity (i.e. PICs) may be perturbed following spinal cord transection, it is likely that the specific *type* of MU is preserved. Based on bladder pressure threshold, instantaneous frequency, duration of activity, and their similar proportions, one could make the argument that LS, MHS, and BO MUs in the intact rat are correspondingly analogous to LT, MT, and HT MUs in the transected rat (figure 19). In support of this view, LS MUs (24%) in the intact and LT MUs (26%) in the transected rat were both tonically active at low bladder pressures, became activated in advance of active bladder contraction, and exhibited the longest durations of activation and highest maximum instantaneous frequencies among their respective groups. Moreover, LT MUs were the only group in the transected rat to exhibit any level of sustained activity after bladder relaxation while LS MUs in the intact rat always displayed sustained firing. In further support of this view, MHS (59%) in the intact rat and MT MUs (57%) in the transected rat all became activated during active bladder contraction and represented the majority of identified MUs. Finally, BO MUs in the intact and HT MUs in the transected rat shared similar attributes. Both became activated either at or close to the peak in bladder pressure, were active for the shortest periods of time, and exhibited the lowest maximum instantaneous firing frequencies among their respective groups.

However, not all EUS MUs (e.g. MHNS MUs) fit comfortably into distinct niches due to similar behaviors. For instance, similar to BO MUs, several MHNS MUs were of high threshold and became activated at the onset of EUS bursting (i.e. exhibited no tonic firing prior to EUS

bursting). Additionally, the combined percentage of MHNS (8%) and BO MUs (9%) in the intact rat is identical to that of HT MUs in the transected rat (17%), a particularly intriguing observation suggesting that the three groups of MUs are related. On the other hand, MHS and MHNS MUs were tonically active following EUS bursting, whereas BO MUs were not. In this case, one could consider MHS and MHNS MUs as part of a broad continuum of activities. For instance, the variation could be explained through a differential distribution of PIC-mediated sustained tonic activity. Regardless, it is ambiguous whether the small population of MHNS MUs in the intact rat is simply a variation of MHS or BO MUs, and whether they are analogous to MT or HT MUs in the transected rat.

Muscle fiber type

In hindlimb muscle, MUs are typically recruited in an orderly fashion starting with slow oxidative (type S) followed by fast fatigue-resistant (type FR) and, lastly, fast-fatigable (type FF) with each capable of generating increasing force (Clamann 1993; Henneman 1957; Henneman et al. 1965a, 1965b; Hodson-Tole and Wakeling 2009). The EUS in the adult female rat, while highly oxidative and fatigue resistant, is a mixed muscle consisting of both slow (type 1) and fast-twitch (type 2) muscle fibers (Buffini et al. 2010; Praud et al. 2003). Buffini et al. showed that the highest proportion of muscle fibers were of the fast, fatigue resistant type (type 2A, ~37%), followed by fast fatigable (type 2B, ~16%), and slow oxidative (type 1, ~4%). The remaining 30% could not be characterized and remained undefined (Buffini et al. 2010). In addition, EUS motoneurons have been shown to exhibit either short or long duration AHPs (Carp et al. 2010) characteristic of type-F and type-S MUs, respectively (Gardiner 1993; Zengel et al. 1985).

In the present study, EUS MU activity patterns in the intact rat were also found to be heterogeneous with respect to bladder pressure threshold, instantaneous frequency, and sustained tonic activity. LS MUs constituted 24% of those MUs identified in the intact rat. MHS and MHNS MUs together represented the majority of MUs at 67%, and BO MUs made up the remaining 9%. Given the distribution of muscle fiber types in the EUS and the individual activity patterns of MUs in this study, by analogy with hindlimb muscle, one might expect 1) LS MUs to innervate oxidative, slow twitch muscle fibers, 2) MHS MUs to innervate fast, fatigue-resistant muscle fibers, and 3) MHNS and BO MUs to innervate fast-fatigable muscle fibers. If one compares the results of the present study with the work carried out by Buffini et al, it is apparent that the absolute percentages of MU recruitment pattern and muscle fiber type do not match. However, this discrepancy can be reconciled by acknowledging that specific types of MUs differ with respect to the number of muscle fibers innervated. Slow oxidative MUs are generally composed of a small number of individual muscle fibers and, as recruitment progresses, higher threshold MUs with a greater number of muscle fibers are recruited (Clamann 1993; Hodson-Tole and Wakeling 2009).

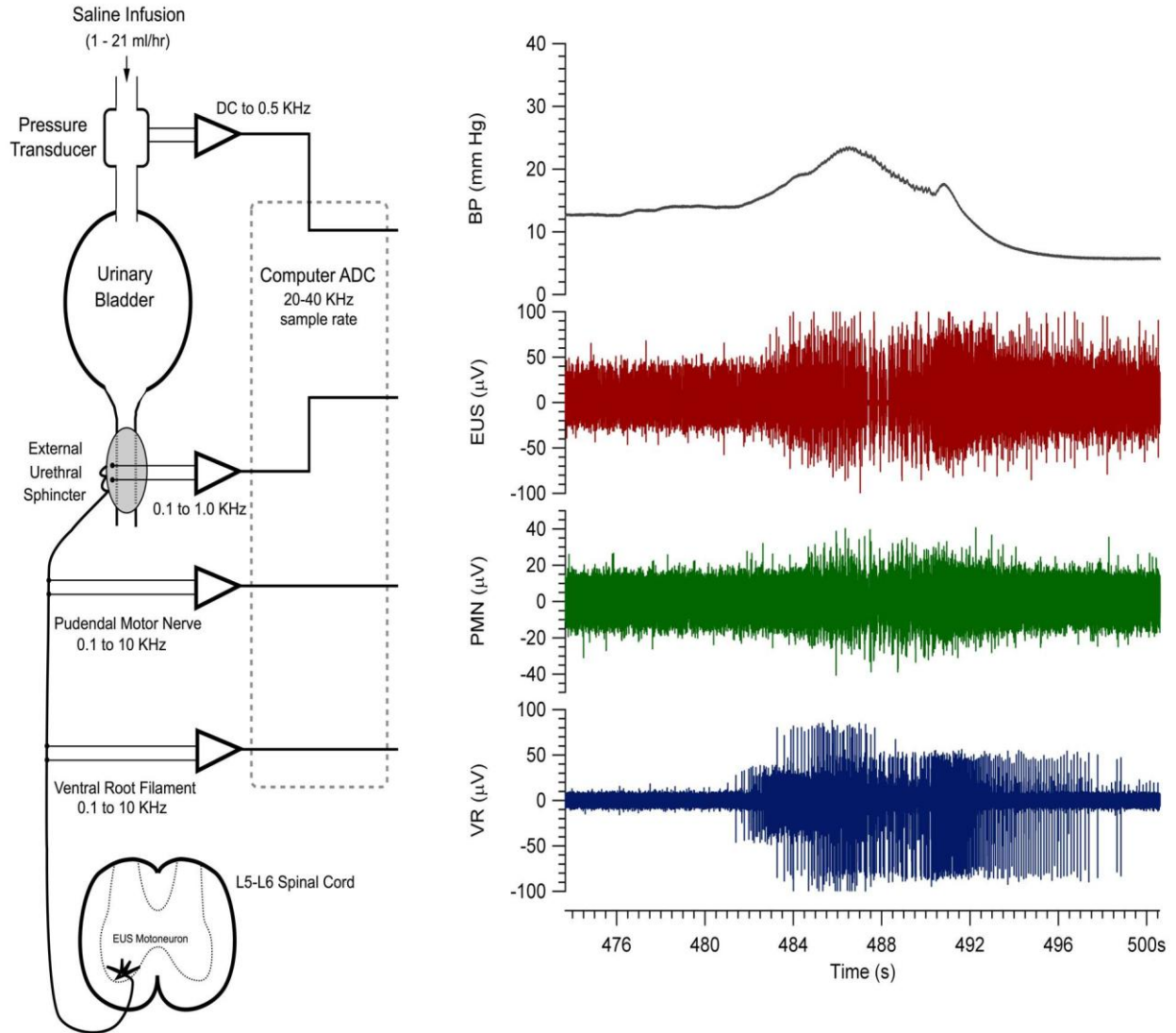# Figures and table captions

Figure 10.

Figure 10. Schematic diagram of *in vivo* extracellular recording setup (left) and data traces (right) illustrating simultaneous recording of bladder pressure (BP) (top trace), external urethral sphincter (EUS) EMG (second trace), pudendal motor nerve (PMN) ENG (third trace) and EUS motor unit activity in an L6 ventral root filament (VR) (bottom trace) in a urethane-anesthetized adult female spinally intact rat during a micturition event evoked by infusion of saline into the bladder.
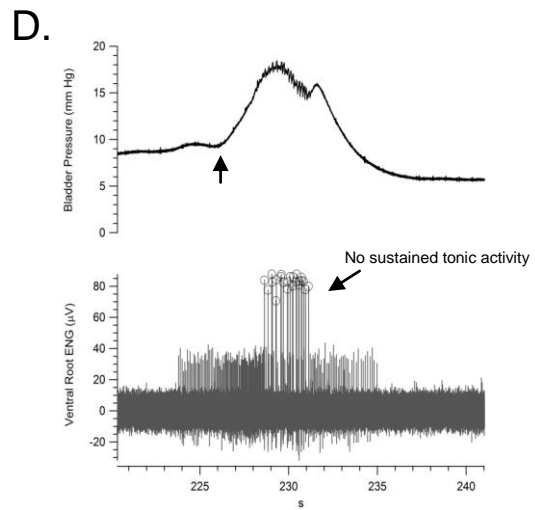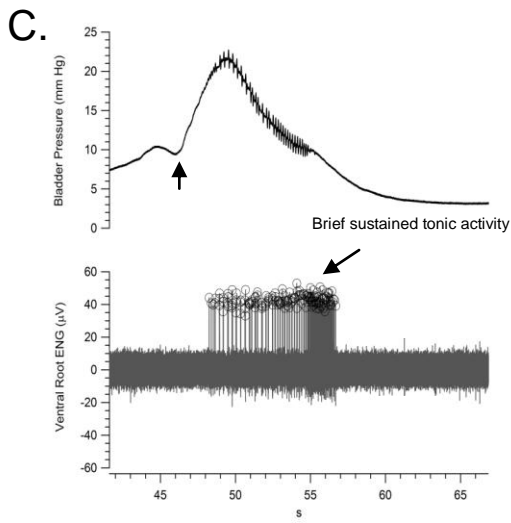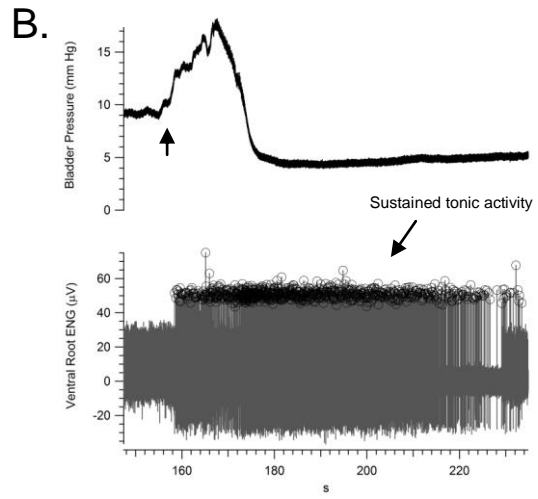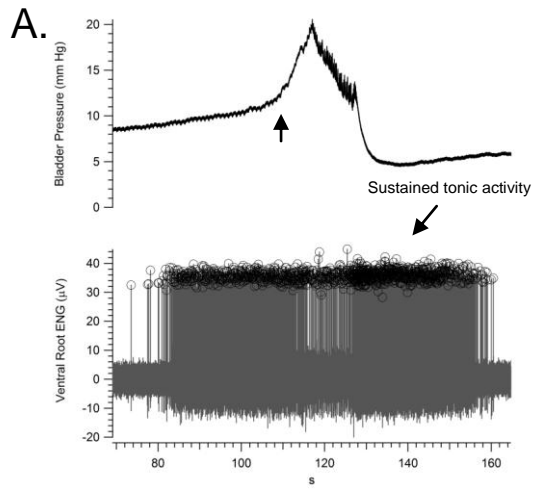
Figure 11.

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Figure 11. Classification of EUS MU activity patterns in the spinally intact rat. Each panel illustrates simultaneous recordings of bladder pressure (top, arrows signify beginning of active bladder contraction) and single EUS MU activity in a L5 or L6 VR filament (bottom) during a micturition event. EUS MUs were detected (black circles) using amplitude/window discrimination. **A.** An example of a Low Threshold Sustained (LS) MU. All LS MUs were recruited before the beginning of active bladder contraction and exhibited sustained tonic activity following complete relaxation of the bladder. **B.** An example of a Medium/High Threshold Sustained (MHS) MU. MHS MUs were recruited during active bladder contraction or just prior to EUS bursting and exhibited sustained tonic activity following complete relaxation of the bladder. **C.** An example of a Medium/High Threshold Not Sustained (MHNS) MU. Similar to MHS MUs, MHNS MUs were recruited during active bladder contraction or just prior to EUS bursting, however, they exhibited tonic activity following EUS bursting that did not persist past complete bladder relaxation (i.e. not sustained). **D.** An example of a Burst Only (BO) MU. BO MUs were recruited immediately at the onset of EUS bursting and, by definition, exhibited no tonic activity after EUS bursting.
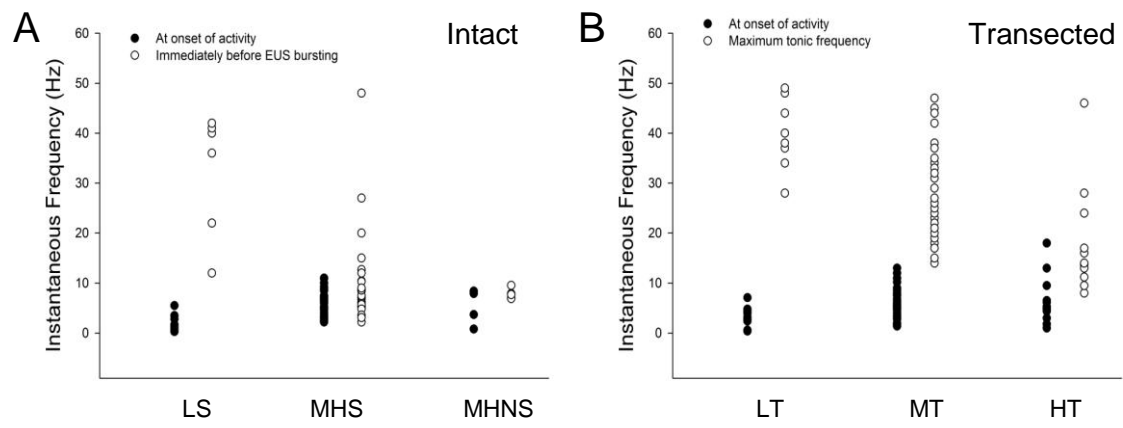
Figure 12.

Figure 12. Increase in instantaneous firing frequency (i.e. frequency modulation) of individual EUS MUs organized by classification in response to increases in bladder pressure during the guarding reflex. A. Instantaneous frequency of individual MUs in intact rats at the time of onset of activity (closed circles) and immediately before EUS bursting (open circles). During the periods of passive bladder filling and active bladder contraction, all LS MUs showed moderate to large increases in instantaneous frequency. In contrast, during the period of active bladder contraction, only a small subset of MHS MUs and no MHNS MU exhibited significant increases in instantaneous firing frequency. **B.** Instantaneous frequency of individual MUs in transected rats at the time of onset of activity (closed circles) and maximum tonic firing (open circles). In contrast to the intact rat, a much greater proportion of EUS MUs significantly increased in instantaneous frequency during passive filling and active contraction of the bladder.
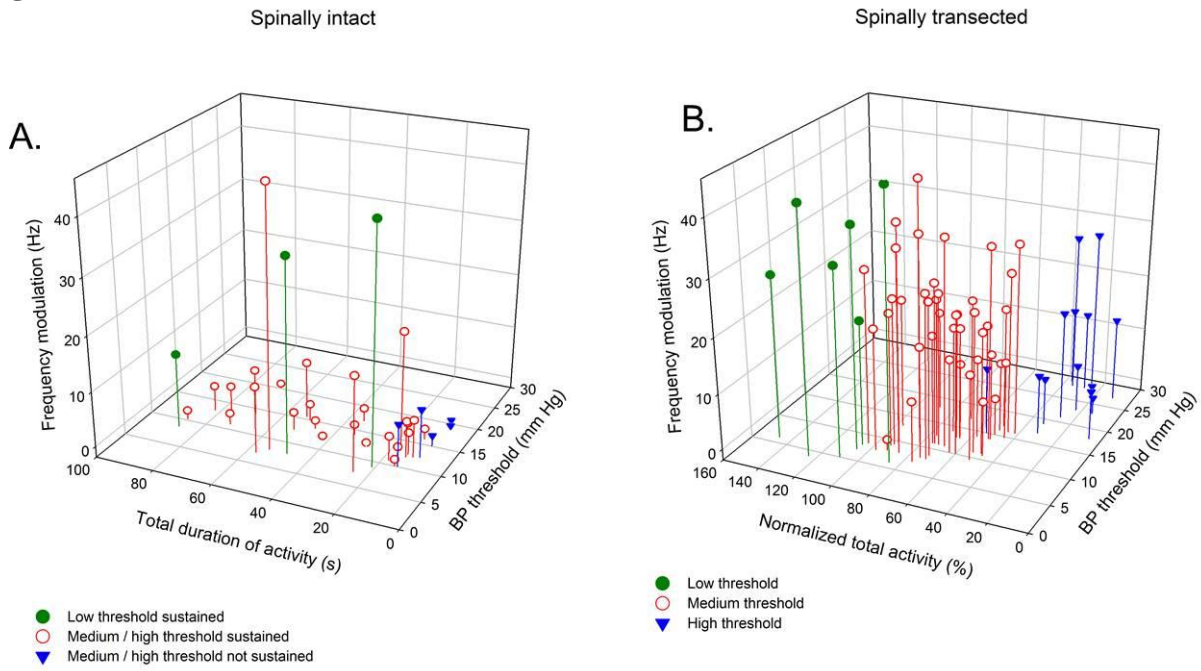
Figure 13.

Figure 13. A. 3D xyz plot of total duration of EUS MU activity (x axis), bladder pressure threshold (y axis) and frequency modulation (z axis) in the spinally intact rat. All LS MUs (green circles) and very few MHS MUs (open red circles) exhibited significant frequency modulation during active bladder contraction. No MHNS MUs (blue triangles) exhibited frequency modulation. Additionally, no relationship was found between the degree of frequency modulation, total duration of EUS MU activity, and bladder pressure threshold in the spinally intact rat. **B.** 3D xyz plot of normalized total activity (x axis, duration of EUS MU activity was normalized to bladder contraction duration), bladder pressure threshold (y axis), and frequency modulation (z axis) in the spinally transected rat. All EUS MUs groups (LT green circles, MT open red circles, and HT blue triangles) exhibited frequency modulation. An inverse relationship was observed between bladder pressure threshold and both the degree of frequency modulation and normalized total EUS MU activity.

Figure 14.
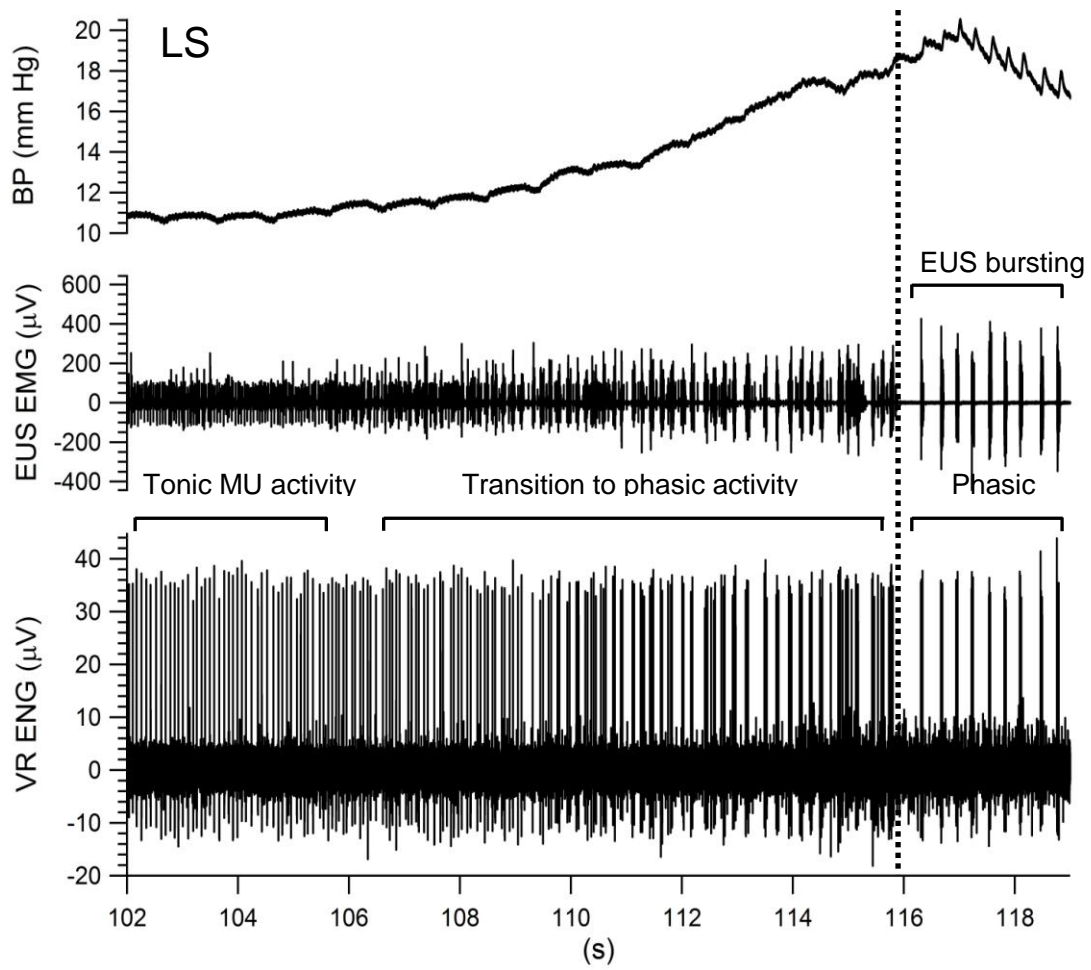
Figure 14. EUS MUs in the spinally intact rat exhibit a transition from tonic to phasic activity during the time leading up to EUS bursting. In an individual LS MU, simultaneous recordings of bladder pressure (top panel), whole muscle EUS EMG (middle panel) and single MU activity (bottom panel) are shown during the initial phase of a micturition event. During the time leading up to EUS bursting and voiding (vertical dashed line) there is a gradual transition from tonic to phasic (i.e. burst-like) EUS muscle and MU activity. Complete phasic activity of MU action potentials was achieved at the onset of EUS bursting. Similar patterns of activity were seen in MHS MUs (not shown) that became activated during active bladder contraction.

Figure 15.

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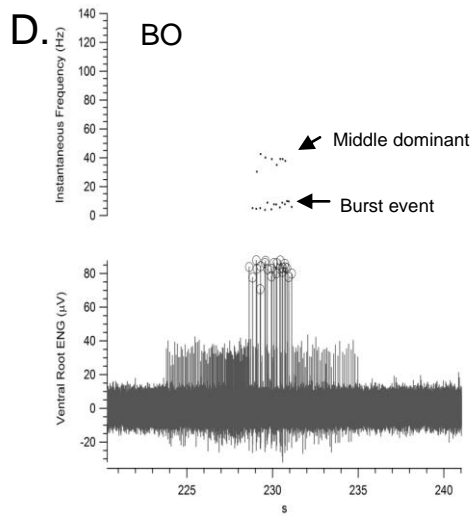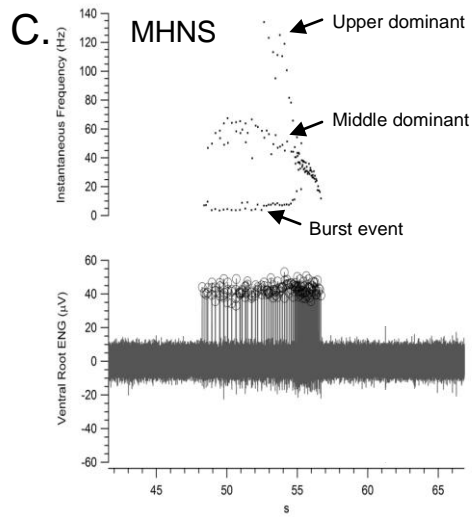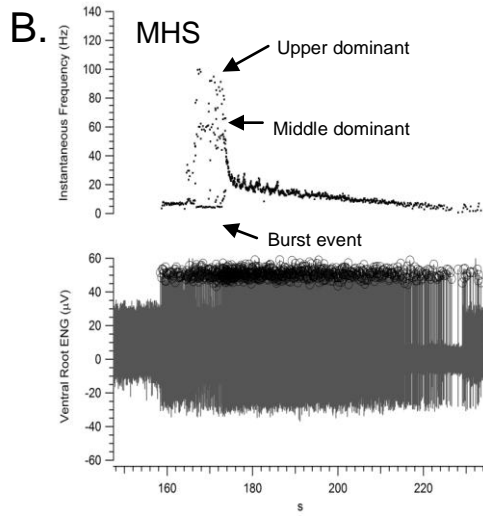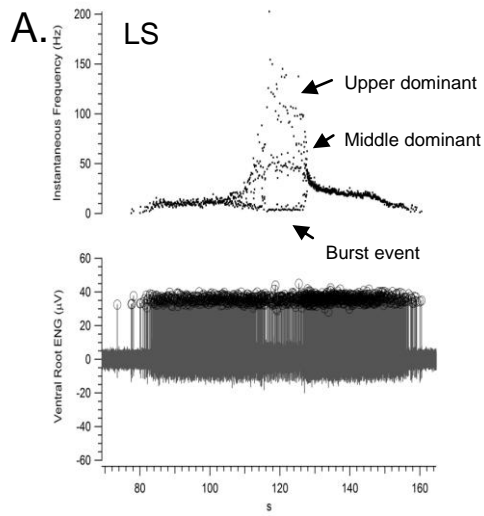
Figure 15. EUS MUs in the spinally intact rat exhibit two to three dominant firing frequencies (burst event, middle, and upper) during EUS bursting. Each panel illustrates single EUS MU activity in a L5 or L6 VR filament (bottom) expressed as instantaneous frequency (top) during a micturition event. **A-C.** Examples of LS MU, MHS, and MHNS MUs. Each of these MU groups exhibited three dominant frequencies during EUS bursting. **D.** An example of a BO MU. BO MUs consistently exhibited only two dominant frequencies during EUS bursting (burst event and middle).

Figure 16.
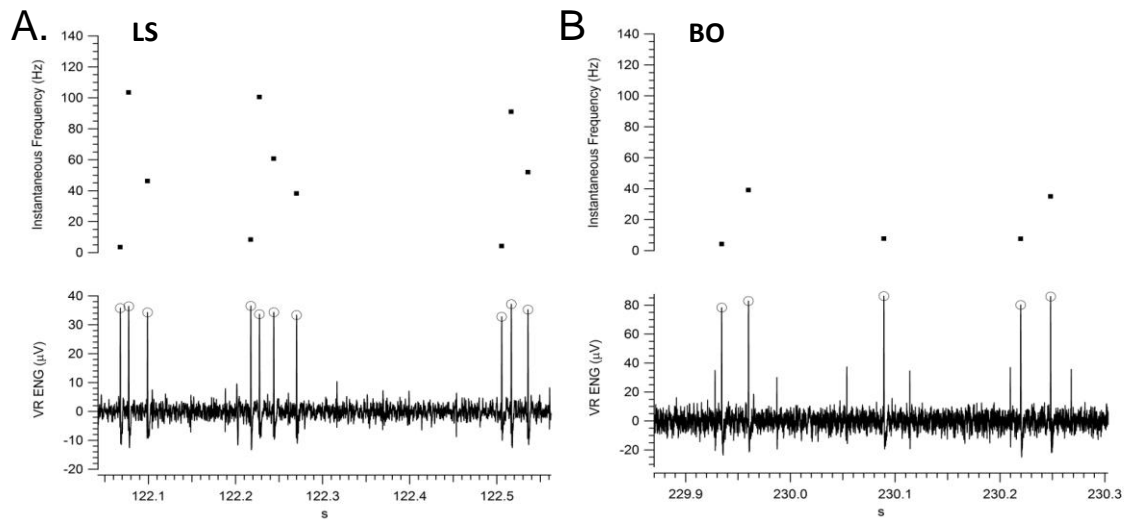
Figure 16. EUS MU activity during EUS bursting in the spinally intact rat. Both panels illustrate single EUS MU activity during three individual burst events (bottom) expressed as instantaneous frequency (top). **A.** An example of individual burst events from a LS MU that generated three or four individual action potentials per burst event. The highest instantaneous frequency was generated by the initial doublet (i.e. the second action potential) within a burst event with consecutive action potentials generating lower instantaneous frequencies. **B.** An example of individual burst events from a BO MU that generated one or two individual action potentials per burst event. In contrast to the other three groups, the instantaneous frequency of the second action potential was significantly lower in BO MUs.

Figure 17.
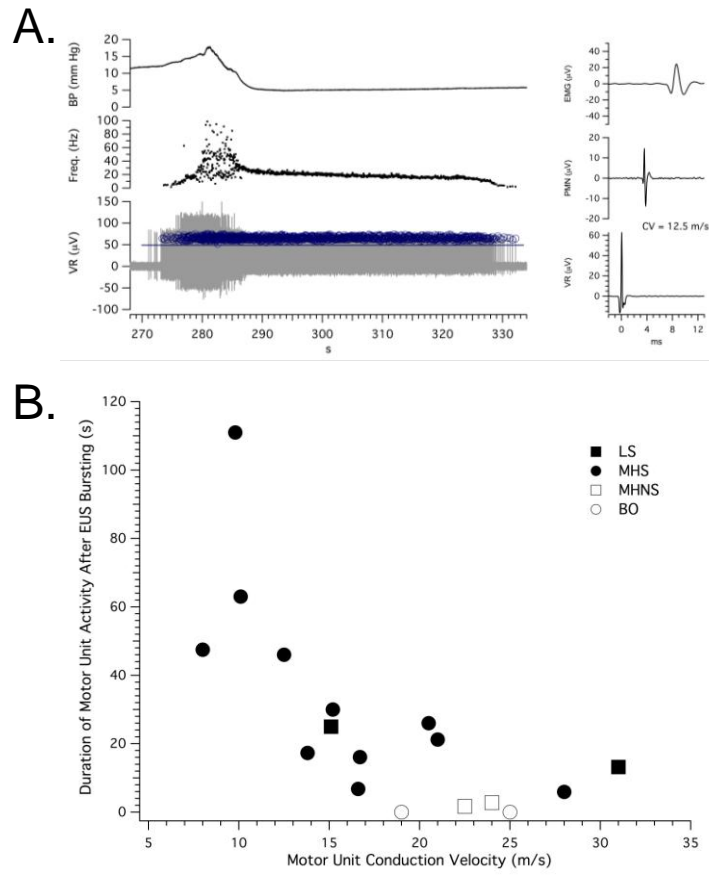
Figure 17. Calculation of EUS MU conduction velocity in the spinally intact rat. A. Simultaneous recording of bladder pressure (left top) and EUS MU activity in an L6 VR filament (extracellular, left bottom) during micturition in a spinally intact rat. A single MHS EUS MU was identified (black circles) with amplitude/window discrimination and the timing information used to calculate instantaneous frequency (left middle) and to resolve the single unit action potentials in the VR filament, pudendal motor nerve (PMN) and EUS muscle via spike-triggered-averaging (right). Conduction time between the VR and PMN recording sites was used to calculate MU conduction velocity. **B.** Plotting the duration of sustained activity following EUS bursting as a function of conduction velocity revealed an inverse relationship, particularly within MHS MUs.

Figure 18.
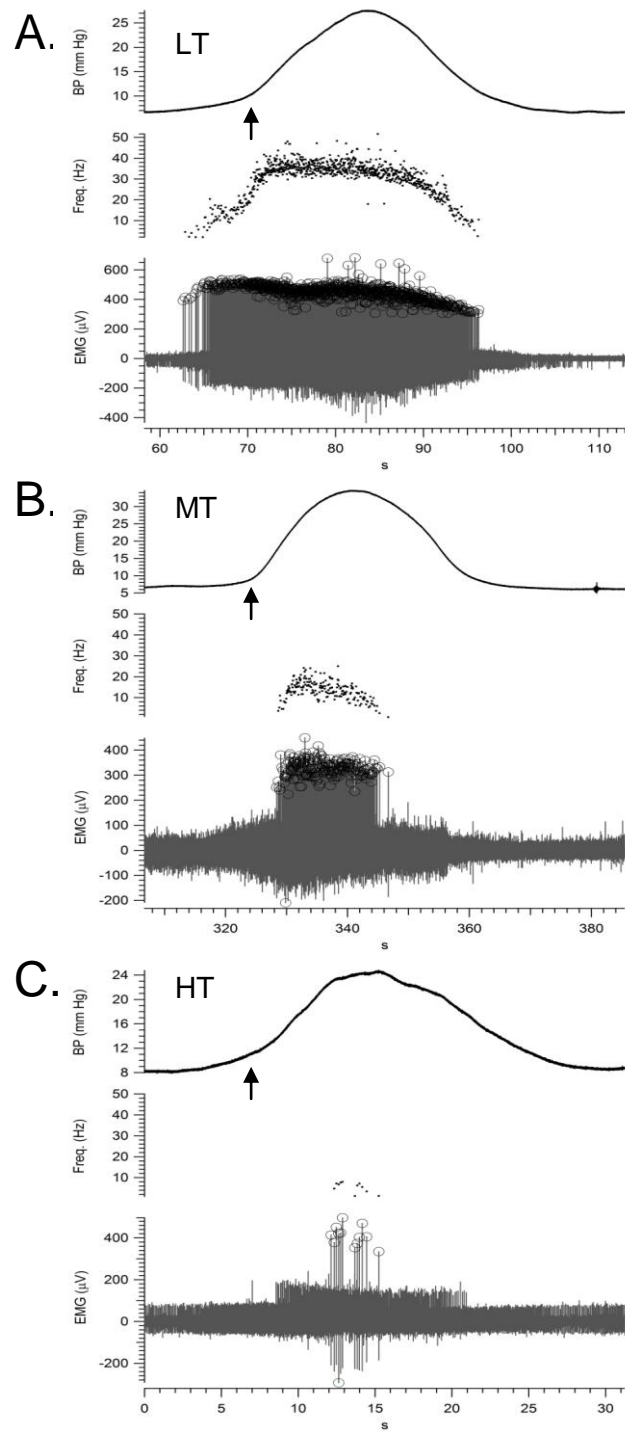
Figure 18. Classification of EUS MU activity patterns in the spinally transected rat. Each panel illustrates simultaneous recordings of bladder pressure (top, arrows signify beginning of active bladder contraction) and single MU activity recorded directly from the EUS muscle (bottom) during a micturition event. EUS MUs were detected (black circles) using amplitude/window discrimination and expressed as instantaneous frequency (middle). **A.** An example of a Low Threshold (LT) MU. LT MUs were recruited before the beginning of active bladder contraction, fired at moderate maximum instantaneous frequencies, and exhibited little to no sustained tonic activity following complete relaxation of the bladder. **B.** An example of a Medium Threshold (MT) MU. MT MUs were recruited during active bladder contraction, fired at low to moderate maximum instantaneous frequencies, and exhibited no sustained activity. **C.** An example of a High Threshold (HT) MU. HT MUs were recruited near or at the peak of bladder contraction, fired at low maximum instantaneous frequencies, and exhibited very short durations of activity.

Figure 19.

Figure 19. EUS MU classification in the spinally intact and transected rat. Based on their similar proportions (%), bladder pressure thresholds, and patterns of activation, EUS MUs in intact and transected rats are likely analogous. Given this criteria, solid black arrows denote a likely association whereas dotted arrows indicate a potential association.

Table 1.

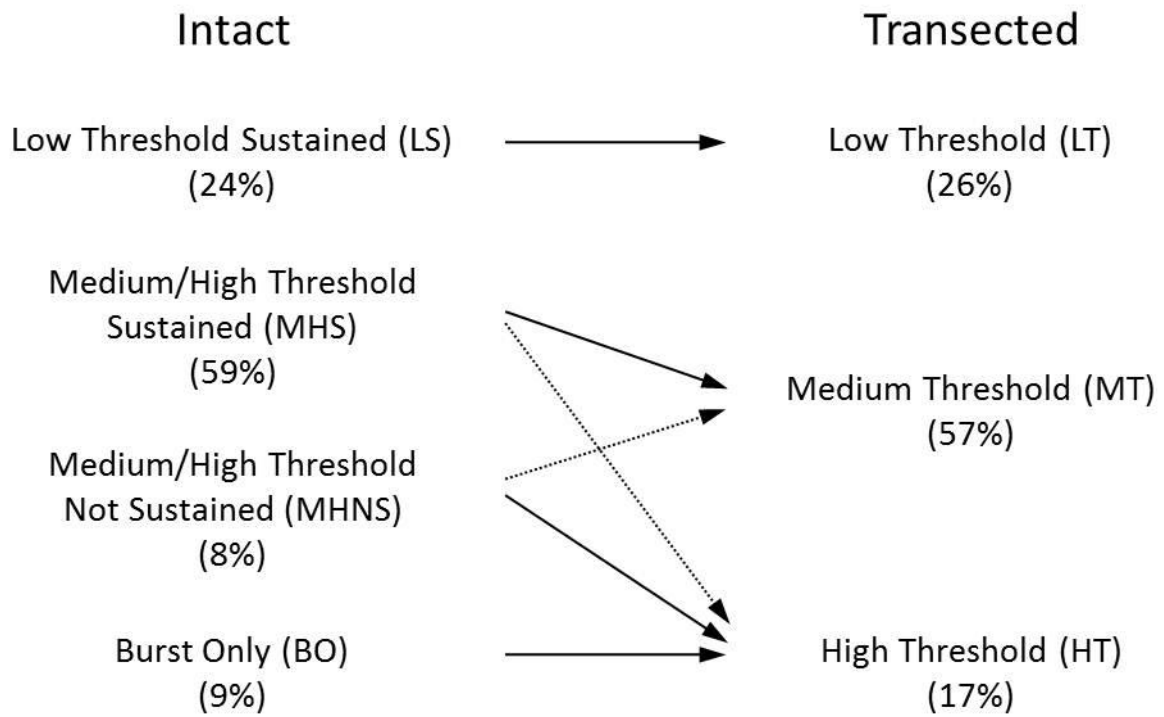|	Low Threshold Sustained	Medium / High Threshold Sustained	Medium / High Threshold Not Sustained	Burst Only
Bladder pressure threshold (mm Hg)	**7.0** (SD 1.2) (14)† ‡	**12.0** (SD 3.1) (34)†	**15.3** (SD 4.2) (5)‡	**17.3** (SD 2.3 (6)†
Total duration of motor unit activity (s)	**106.0** (SD 88.9) (11)†	**40.9** (SD 24.6) (37)†	**10.0** (SD 2.3) (5)†	**5.9** (SD 2.4) (6)†
Conduction Velocity (m/s)	**22.5** (SD 9.2) (4)	**16.7** (SD 6.0) (11)	**22.5**; **24.0** (2)	**19.0**; **25.0** (2)

Before EUS Bursting

Duration of motor unit activity (s)	**47.1** (SD 55.5) (11)*	**2.5** (SD 2.5) (37)	**1.5** (SD 1.4) (5)	N/A
Onset frequency (Hz)	**1.7** (SD 1.6) (11)*	**6.0** (SD 2.4) (37)	**5.8** (SD 3.4) (5)	N/A
Frequency immediately before burst (Hz)	**32.3** (SD 12.5) (6)*	**9.8** (SD 8.8) (29)	**7.8** (SD 1.1) (5)	N/A

During EUS Bursting

# of burst events	**30.4** (SD 25.6) (5)	**34.3** (SD 20.8) (27)	**36** (SD 4.6) (5)	**25.8** (SD 10.4) (6)
# of action potentials per burst event	**3.4** (SD 0.9) (3)	**3.0** (SD 0.6) (24)	**2.6** (SD 0.5) (5)	**2.2** (SD 0.3) (6)*
Total # of action potentials	**107.0** (SD 74) (3)	**102.9** (SD 75.8) (24)	**95** (SD 24.8) (5)	**58.1** (SD 30.7) (6)
Burst event frequency (Hz)	**7.7** (SD 1.7) (4)	**8.5** (SD 1.4) (27)	**7.9** (SD 1.0) (5)	**6.9** (SD 0.9) (6)
Middle dominant frequency (Hz)	**44.6** (SD 5.1) (4)	**47.5** (SD 6.6) (23)	**53.6** (SD 3.4) (5)	**43.3** (SD 4.9) (6)
Upper dominant frequency (Hz)	**91.3** (SD 20.4) (3)	**81.5** (SD 13.5) (17)	**88.1** (SD 9.1) (3)	**67** (1)
Maximum instantaneous frequency (Hz)	**169.0** (SD 51.3) (3)	**126.5** (SD 48.4) (20)	**148.5** (SD 31.8) (5)	**63.5** (SD 13.3) (6)*

After EUS Bursting

Duration of motor unit activity (s)	**42.0** (SD 37.8) (11)	**33.5** (SD 23.3) (38)	**2.4** (SD 1.1) (5)*	N/A
Frequency immediately after burst (Hz)	**37.4** (SD 2.9) (5)	**42.3** (SD 9.0) (26)	**34.6** (SD 12.3) (5)	N/A
Off frequency (Hz)	**3.9** (SD 2.6) (11)	**3.4** (SD 1.7) (37)	**3.7** (SD 1.2) (5)	N/A

Table 1.Quantification of EUS MUs in the spinally intact rat. Values in the table are given as mean (SD) (n) where "n" is the number of MUs. For a given parameter, mean values marked with * are statistically different from the other group means with a p<0.05. For a given parameter, mean values marked with † or ‡ are statistically significant from one another with a p<0.05.

Table 2.

	Low Threshold	Medium Threshold	High Threshold
Bladder pressure threshold (mm Hg)	**5.8** (SD 1.7) (16)†	**12.1** (SD 3.3) (47)†	**23.1** (SD 4.5) (14)†
Onset frequency (Hz)	**3.4** (SD 2.0) (10)	**6.1** (SD 3.3) (43)	**6.2** (SD 4.80 (13)
Bladder pressure off (mm Hg)	**8.0** (SD 3.8) (15)†	**17.0** (SD 5.2) (47)†	**25.2** (SD 5.5) (14)†
Off frequency (Hz)	**5.3** (SD 3.5) (10)	**5.0** (SD 3.4) (43)	**6.1** (SD 4.8) (13)
Maximum instantaneous frequency (Hz)	**40.2** (SD 6.8) (12)†	**27.8** (SD 8.5) (42)†	**17.8** (SD 10.1) (13)†
Peak bladder pressure (mm Hg)	**21** (SD 8.0) (11)	**25.3** (SD 5.6) (47)	**28.6** (SD 5.0) (14)
Bladder pressure at max. frequency (mm Hg)	**17.8** (SD 7.6) (9)	**22.4** (SD 5.6) (42)	**26.5** (SD 5.6) (13)
Duration of motor unit activity (s)	**39.0** (SD 25.0) (14)	**19.8** (SD 8.3) (47)	**5.7** (SD 2.9) (14)
Duration of bladder contraction (s)	**30.0** (SD 14.0) (16)	**29.8** (SD 13.6) (47)	**22.2** (SD 4.0) (14)
Normalized duration of activity (%)	**126.0** (SD 28) (11)†	**68.3** (SD 17.5) (47)†	**25.5** (SD 12.7) (14)†
Sustained activity (s)	**3.6** (SD 4.6) (9)	**N/A**	**N/A**

Table 2. Quantification of EUS MUs in the spinally transected rat. Values in the table are given as mean (SD) (n) where "n" is the number of MUs. For a given parameter, mean values marked with † are statistically different from one another with a p<0.05. Normalized duration of activity was calculated by dividing the duration of MU activity by the duration of bladder contraction and multiplying by 100.

Chapter 4

The effect of GABA$_b$ receptor activation on bladder and external urethral sphincter activity in the spinally intact and transected rat

Abstract and overview

The purpose of this study was to determine the effect of GABA$_b$ receptor activity on bladder and EUS activation in the spinally intact and transected rat. Micturition in the adult female rat is composed of three distinct phases of bladder-EUS activity: 1) At the onset of bladder contraction, the increase in bladder pressure results in tonic activation of the external urethral sphincter (EUS) muscle (i.e. the guarding reflex) that functions to maintain continence, 2) at the peak of bladder contraction, EUS activity rapidly switches to highly synchronized bursts (termed EUS bursting) that coincide with and facilitate voiding, and 3) a period of sustained tonic EUS activity that persists well past complete bladder relaxation. In contrast, rats with chronic spinal cord transection exhibit an intrinsic bladder-EUS rhythm, an increased guarding reflex, reduced EUS bursting, and little or no sustained tonic EUS activity. These changes are speculated to contribute, at least in part, to the dysfunction of the LUT in rats with SCI. In the weeks following SCI in humans and animals, motoneuron systems below the level of injury may develop spasticity, a motor disorder characterized by exaggerated muscle spasms. Baclofen, a GABA$_b$ receptor agonist, is often prescribed as an antispasmodic agent for patients that experience these debilitating symptoms, but its effects on micturition are still being investigated. To study the effect of GABA$_b$ receptor activation on micturition, baclofen (0.01 - 1 ug) and the

GABA$_b$ receptor antagonist CGP 54626 hydrochloride (50 – 150 ug) were administered intrathecally to the ventrolateral $L_6 - S_1$ spinal cord in urethane-anesthetized adult female Sprague Dawley rats with or without spinal cord transection during continuous infusion of saline into the bladder. In both intact and transected rats, baclofen elevated the threshold bladder pressure for active bladder contraction, but had little to no effect on peak intravesical pressure and bladder contraction duration. Additionally, micturition frequency could be modulated in both intact and transected rats. The overall EUS response was attenuated in the majority of animals with spinally intact rats exhibiting a reduction in the gain of the guarding reflex, EUS burst duration, burst event number, and sustained tonic EUS activity. Transected rats also exhibited a reduction in the guarding reflex, but showed additional signs of improved micturition function. In spinally intact rats, the dose-dependent effects of baclofen on EUS activity could be blocked with intrathecal CGP.

Background

Over a period of several weeks following SCI, motoneuron systems below the level of injury undergo neuroplasticity. As a consequence, patients will go on to develop spasticity, a motor disorder described as a "velocity-dependent increase in the stretch reflex" that is characterized by hyperreflexia, clonus, exaggerated tendon jerks, and intense involuntary muscle spasms that hamper rehabilitation and are often severely debilitating (Adams and Hicks 2005; Elbasiouny et al. 2009; Lance 1980; Nielson et al. 2007). Using various animal models, a number of different pathophysiological mechanisms for spasticity have been proposed including decreased descending neuromodulatory inhibition, reduced presynaptic inhibition, and increased excitability of interneurons and α-motoneurons (Elbasiouny et al. 2009; Nielson et al. 2007).

Baclofen, a GABA$_b$ receptor agonist, is often used as an antispasmodic agent in patients that experience the muscle spasticity and hypertonia (increased muscle tone) that arise after SCI (Rekand 2010; Mullarkey 2009). Its effectiveness is largely due to GABA's principle inhibitory action in the spinal cord (Curtis and Lacey 1998; Macdonald and Barker 1979; Macdonald and Young 1981) that may work on several of the aforementioned mechanisms. Specifically, baclofen exerts its actions primarily on the presynaptic terminals of sensory afferents. Binding of baclofen to the metabotropic G-protein coupled GABA$_b$ receptor results in the inhibition of calcium-mediated neurotransmitter release (e.g. glutamate) and activation of K^{+1} channels leading to hyperpolarization of the neuron. Additionally, baclofen has also been shown to have postsynaptic inhibitory effects (Li et al. 2004b).

In addition to its potent muscle relaxant and antispasmodic properties, clinical reports of human subjects with SCI suggest baclofen also improves micturition function (Hachen and Krucker 1977; Steers et al. 1992). Thus, the effects of baclofen on micturition in animal models with and without LUT dysfunction have been investigated. For instance, in the spinally intact unanesthetized rat, intrathecal and intravaneous administration of baclofen and tiagabine, a GABA reuptake inhibitor, were shown to decrease micturition volume, micturition frequency, and micturition pressure (Pehrson and Andersson 2002; Pehrson et al. 2002). Further, the effects of baclofen could be blocked or reversed with CGP62359, a selective GABA$_b$ receptor antagonist (Pehrson et al. 2002). These results suggest that GABA has an overall inhibitory effect on micturition in the normal rat and is endogenously active in the spinal cord. Similar results with baclofen were also seen in rat models of DO induced by cerebral infarction or intravesical administration of oxyhemoglobin (Kanie et al. 2000; Pehrson et al. 2002).

As described in Chapters 2 and 3, following SCI, the LUT becomes functionally compromised often leading to DSD and DO (Castro-Diaz and Taracena Lafuente 2006). DSD and DO result from the disruption of descending communication between supraspinal sites and the lumbosacral spinal cord and are characterized by repeated uncoordinated contractions of the bladder and EUS. Like spasticity, this is a significant health risk in patients afflicted with SCI and thus is the subject of research for the development of new and effective therapies. Recent studies from Miyazato and colleagues suggest that both $GABA_a$ and $GABA_b$ receptor activation are capable of depressing both DSD and DO in the unanaesthetized female rat and that these effects can be specifically blocked with the appropriate antagonist (Miyazato et al. 2008a, 2008b; Miyazato et al. 2009). Given the effects of baclofen on micturition in humans and animals, elucidating the role of GABA in regulation of the LUT is an important research endeavor. Many studies have focused on the measurement of cystometric variables that assess bladder function; however, other aspects of micturition such as the guarding reflex and sustained tonic EUS activity have yet to be investigated thoroughly.

Materials and methods

Spinal cord transection and animal care

Adult female Sprague Dawley rats (Taconic, 200 – 300 g) received complete mid-thoracic spinal cord transection under isoflurane anesthesia as previously described in Chapters 2 and 3. However, in order to help minimize trauma of spinal innervations of the upper urinary tract, the transection level was changed from T_{10} to T_8. Additionally, immediately following surgery, animals were housed together in pairs as to facilitate recovery through social enrichment. During the first week of recovery, animals were kept in shallow cages with special

bedding, but eventually transferred to normal cages and housed similar to uninjured animals. All rats receiving spinal cord transection were checked daily for signs of distress and urinary tract infection.

Drugs

The selective GABA$_b$ receptor agonist baclofen (Sigma) was dissolved in 0.1 N NaOH (stock solution, 10 mg/ml) and stored for no longer than two weeks at 4°C as per the manufacturer's recommendation. The day of the experiment, baclofen was serially diluted to the appropriate concentrations (0.01 – 1 ug/ul) in 37°C 0.9% saline and kept at room temperature until needed. The highly selective GABA$_b$ receptor antagonist CGP 54626 hydrochloride (Tocris Bioscience) was dissolved in 100% DMSO (stock solution, 20 ug/ul) and kept frozen at -20°C in 25 ul aliquots. A single aliquot of CGP was thawed the day of the experiment immediately before use.

Experimental set up

Spinally intact (n = 6) and transected rats (n = 11, 6 – 8 weeks survival) were anesthetized with urethane (1.2 g/kg, sc) and set up for continuous flow CM with simultaneous EUS EMG recordings as previously described in Chapters 2 and 3. Briefly, an abdominal incision was made and a PE 90 catheter inserted into the dome of the bladder for intravesical measurements and continuous infusion of physiological saline. The entire rostrocaudal extent of the pubic symphysis was cleared of muscle and, using a 27G 1/2 gauge syringe needle, two bilateral holes were created at the rostral end of the bone. Two Teflon coated seven-stranded stainless steel wire electrodes (A-M systems) were exposed 1 – 2 mm at the end, bent at a 90 –

180 degree angle, and advanced through the two holes to make contact with the proximal EUS. After checking for EUS EMG signal strength and integrity by gently pressing on the bladder, the wires were firmly secured using crazy glue followed by a silicon-based sealant Kwik-Cast (World Precision Instruments). The abdomen was closed by suturing the peritoneum and stapling the skin using wound clips. The animal was then placed in the prone position and elevated slightly using nose and tail clamps to reduce pressure on the abdomen. A dorsal laminectomy was performed removing vertebrae $L_3 - L_1$ exposing the $L_6 - S_1$ spinal segments. Through a small hole in the dura, a beveled PE-10 cannula connected to a 25 ul glass Hamilton syringe was inserted intrathecally and directed to the ventrolateral $L_6 - S_1$ spinal cord for administration of drug. After catheter implantation, the entire cavity was sealed with 2.5% agar.

Intrathecal application of baclofen and CGP

In spinally intact and transected rats, bladder pressure and EUS EMG data were recorded simultaneously for a period of $30 - 40$ minutes during continuous infusion of saline into the bladder ($5.2 - 10.5$ ml/hr) to establish a steady baseline. To control for any volume-induced or vehicle specific effects, $5 - 15$ ul of vehicle (either 0.9% saline or 100% DMSO) was injected into the spinal cord prior to the application of drug. Following the baseline recording, increasing doses of the $GABA_b$ receptor agonist baclofen (0.01 - 1 ug dissolved in 0.9% saline) were administered intrathecally in a volume of $1 - 10$ ul followed by a $10 - 15$ ul saline flush. After each dose, data were collected for $30 - 40$ minutes. To determine endogenous $GABA_b$ receptor activity, increasing doses of CGP ($2.5 - 5$ ul, $50 - 150$ ug in 100% DMSO) were administered followed by a $15 - 20$ ul saline flush. After each dose, data were collected for $30 - 60$ minutes. To confirm baclofen selectivity, 50 ug CGP and 0.25 ug baclofen were given simultaneously

during the final recording of data collection. At the end of the experiment, animals were euthanized by overdose with intravenous administration of urethane.

Data collection and analysis

Data were collected as detailed in Chapter 2. The EUS EMG signal was amplified (gain 1000x, 0.1 kHz high pass filter, 1 kHz low pass filter) using a differential AC preamplifier and the BP signal amplified through a PM-1000 transducer amplifier and filtered DC to 0.5 kHz. Data were sampled at 2 kHz using a DAQ analog to digital converter card and stored using the acquisition software Igor Pro version 6.1. Raw EUS EMG and BP data were rectified and smoothed by software down-sampling to achieve an effective sampling rate of 10 Hz. To quantify the gain of the guarding reflex, EUS bursting, and sustained tonic EUS activity within a single animal, data from $5-10$ micturition events were pooled and averaged (n = 1). Data were analyzed using the same software tools described in the methods and materials section of Chapter 2, but with some modifications and additions. Sustained tonic EUS activity was quantified differently by computing the integrated area of the decay in rectified and smoothed EUS EMG activity following EUS bursting. Since the area under the curve tended to generate more consistent measurements, this method was used as an alternative to fitting a double exponential decay function to the decrease in EUS activity. Similarly, the total EUS activity during a micturition event was also quantified by taking the integrated area of the entire rectified and smoothed EUS EMG response. There was also a slight modification in how the gain of the guarding reflex was calculated. In Chapter 2, the slope of the BP-EUS input-output relationship was measured between $30-40\%$ of normalized and smoothed EUS EMG activity. However, since BP is the independent variable, the slope was measured between ~$20-65\%$ of normalized

and smoothed BP in this part of the study. Rectified and smoothed data from each animal were averaged and normalized by dividing by the pre-drug baseline (i.e. the baseline was assigned a value of one or 100%). Normalized values from multiple animals were then averaged for each dose. Error bars represent ±SEM and statistical significance was assessed using one-way ANOVA and post hoc tests in Sigmaplot 11.

Results

Spinally intact rats

In the spinally intact rat (n = 6), baclofen (0 – 0.1 ug) was administered intrathecally to the ventrolateral $L_6 - S_1$ spinal cord to quantify dose-dependent effects on cystometric parameters and characteristics of EUS EMG activity during repeated micturtion events. Analysis of cystometric parameters included: bladder contraction duration, bladder contraction period (i.e. micturition frequency), peak intravesical pressure (i.e. the bladder pressure at the peak of contraction), and bladder pressure threshold (i.e. the bladder pressure at which active bladder contraction was triggered). In terms of EUS EMG activity, analysis was focused on the gain of the guarding reflex, EUS bursting, and sustained tonic EUS activity.

Baclofen had little to no significant dose-dependent effect on either peak intravesical pressure or bladder contraction duration (figure 20B and C). However, in a linear fashion, baclofen elevated the threshold for active bladder contraction up to ~200% at a dose of 0.1 ug (figure 20A). With respect to bladder contraction period, baclofen had a biphasic effect. For instance, at low doses of baclofen (0.01 – 0.025 ug), the frequency of micturition was reduced (increased contraction period) by ~30% at 0.025 ug. However, as the dose of baclofen was

subsequently increased (0.05 – 0.1 ug), the frequency of bladder contractions accelerated with the bladder contraction period falling by ~60% at 0.1ug (figure 20D).

The guarding reflex in the adult rat, as defined in this dissertation, is an increase in tonic EUS activity during active bladder contraction. Quantification of the gain of this response (i.e. EUS EMG plotted as a function of BP) is dependent on a number of criteria such as EUS EMG electrode placement, a high EUS EMG signal to noise ratio, and a low level of background EUS EMG activity during the inter-contraction interval between micturition events. Because of these variables, a reliable quantification of the guarding reflex was done on three of the six spinally intact rats (n = 3/6) in this part of the study. Intrathecal baclofen decreased the gain of the guarding reflex in a dose-dependent manner up to 65 – 70% at 0.25 ug (figure 21C). Similar dose-dependent reductions after baclofen application were seen in 1) the total EUS response, 2) sustained tonic EUS activity, 3) the number of EUS burst events, and 4) the total duration of EUS bursting. While EUS burst number and duration decreased in parallel by a maximum of ~40 – 50% at a dose of 0.1 ug, baclofen had little to no effect on the frequency of burst events (figure 21B). The total EUS response and EUS sustained tonic activity also decreased in parallel over the same dose range, but faster and to a greater extent than EUS burst duration and event number (maximum of ~90% reduction at 0.25 ug baclofen) (figure 21A).

In the spinally intact rat, intrathecal administration of the $GABA_b$ receptor antagonist CGP antagonized the effects of baclofen. Although a systematic dose-response of CGP was not evaluated, single doses in the range of 50 – 150 ug CGP increased the average burst duration and burst event number by ~1.5 and 1.8 fold, respectively, but had little or no effect on the total EUS response, sustained tonic EUS activity, and EUS burst event frequency. Additional cumulative doses of CGP (100 – 200 ug total) further increased EUS burst duration and burst event number

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to 2.5 and 3.1 fold, respectively, even in the presence of 0.25 ug baclofen. Again, under these conditions, the total EUS response, sustained tonic EUS activity, and EUS burst event frequency exhibited no significant change (figure 22).

Spinally transected rats

In the spinally transected rat (n = 11), baclofen (0 – 1 ug) was administered intrathecally to the ventrolateral $L_6 – S_1$ spinal cord to assess dose-dependent effects on the same cystometric parameters analyzed in the spinally intact rat (e.g. bladder contraction duration, bladder contraction period, peak intravesical pressure, and bladder pressure threshold). Baclofen had little to no effect on peak intravesical pressure and bladder contraction duration (figures 23B, 23C, 24B, 24C). However, the threshold for active bladder contraction increased in a dose-dependent manner by a maximum of ~40% at 1 ug (figures 23A and 24A). Additionally, baclofen decreased the frequency of micturition (i.e. increased bladder contraction period) four-fold at 0.1 ug (figures 23D and 24D). Some of these results are in contrast to what was seen in the spinally intact rat. For instance, over the same dose range in the intact rat, the bladder pressure threshold was far more sensitive to baclofen while micturition frequency exhibited a biphasic response.

In the spinally transected rat, close attention was given to the effect of intrathecal baclofen on the guarding reflex. In a dose-dependent manner, the gain of the guarding reflex could be suppressed by ~90% with intrathecal baclofen (0 – 1 ug) (figure 25). These results are similar to those seen in the spinally intact rat (compare figures 25 and 21C). In addition to the guarding reflex, interesting, albeit less quantifiable, observations were made regarding the effect of baclofen on bladder-EUS function in the transected rat. As described in Chapter 2, a

significant proportion of spinally transected rats developed a "choked" bladder ", a state with sustained high bladder pressure accompanied by intermittent urethral dribbling and continuous activation of the EUS. In this part of the study, a small subset of rats (n = 3/11) exhibited bladder choking. In each of these animals, intrathecal baclofen reduced the frequency of bladder contractions, decreased the inter-contraction baseline pressure (i.e. bladder choking), and decreased EUS activity (example in figure 26).

In Chapter 2, irrespective of bladder choking, EUS bursting was almost universally absent. Only a very small percentage of spinalized rats (n = 3/27) showed transient signs of bursting. However, other investigators have reported that both unanaesthetized and lightly anaesthetized rats with SCI can exhibit robust EUS bursting and efficient voiding (Cheng and de Groat 2004; Leung et al. 2007). Interestingly, during the latter part of this project, the incidence of urethane-anesthetized spinally transected rats that exhibited EUS bursting and efficient voiding was elevated compared to what was reported in Chapter 2 (n = 6/11 as opposed to 3/27). Further, this EUS bursting was more pronounced and better defined than what was initially observed (figure 27A and B). Nonetheless, the average burst duration and number of burst events in the spinally transected rat were significantly shorter and lower, respectively than in the spinally intact rat (figure 27C and D). Three spinally transected rats exhibited EUS bursting before application of intrathecal baclofen (example in figure 28A and B) and three exhibited EUS bursting after baclofen. In four of these six transected rats, high doses of baclofen qualitatively appeared to either promote or enhance EUS bursting (example in figure 28C and D).

Discussion

There has been much work dedicated to elucidating the role of GABA in regulating

micturition. However, much of this research has concentrated on the effects of GABA$_{a/b}$ receptor

agonists and antagonists on bladder function through the assessment of cystometric variables.

Baclofen, a clinically effective antispasmodic and muscle relaxant, has been used for this

purpose in both human clinical trials and animal models. However, in both spinally injured and

uninjured animals, far less attention has been placed on the effects of baclofen on EUS activity

during micturition. In this part of the study, baclofen-mediated activation of GABA$_b$ receptors in

the lumbosacral spinal cord of the spinally intact and transected adult female rat had obvious

inhibitory effects on bladder and EUS function.

In the spinally intact rat, baclofen had no significant effect on either the duration of

bladder contraction or peak intravesical pressure. However, baclofen clearly increased bladder

contraction threshold and had a biphasic effect on micturition frequency. In terms of EUS

activity, baclofen suppressed, in a dose-dependent manner, the gain of the guarding reflex, EUS

bursting, and sustained tonic EUS activity. Baclofen's primary mechanism of action is to inhibit

calcium-mediated neurotransmitter release from afferent terminals (i.e. presynaptic inhibition),

but it has been shown to directly inhibit motoneurons as well (Curtis and Lacey 1998; Li et al.

2004b; Macdonald and Barker 1979; Macdonald and Young 1981; Mullarkey 2009; Rekand

2010). As others have speculated (Kanie et al. 2000; Pehrson et al. 2002), presynaptic inhibition

of Aδ afferent terminals is likely responsible for elevating the bladder pressure threshold

necessary to elicit an active bladder contraction in the spinally intact rat. This is probably

accomplished by indirectly inhibiting the activity of projection interneurons that ascend to the

PAG and PMC. Additionally, an increase in the threshold for active bladder contraction more

than likely expands the inter-contraction interval between bladder contractions, delaying micturition. This would account for the initial decrease in micturition frequency at low doses of intrathecal baclofen (0 – 0.025 ug) seen in this study. However, since baclofen also works by hyperpolarizing neurons in the spinal cord, a direct inhibitory effect on the parasympathetic preganglionics innervating the bladder should not be discounted. As the dose of baclofen was further increased above 0.025 ug, the frequency of micturition accelerated. This may seem paradoxical at first given baclofen's proposed mechanisms of action. However, this effect was probably the result of either indirect or direct inhibition of the spinal EUS bursting CPG. In the spinally intact rat, EUS bursting is absolutely essential for efficient voiding, both physiologically and under experimental conditions (Conte et al. 1991; Kruse et al. 1993; Peng et al. 2006, 2008; Yoshiyama et al. 2000). Inhibition of EUS bursting with baclofen would result in the retention of fluid, and the increased bladder volume would lead to more frequent micturition events, thus explaining the acceleration in micturition frequency. According to the data obtained in this study, baclofen suppressed the EUS burst duration and the number of burst events per micturition event in the spinally intact rat starting at 0.05 ug. This is the same dose of baclofen where a decrease in micturition frequency was first observed.

In addition to EUS bursting, baclofen also attenuated the gain of the guarding reflex, the total EUS response, and sustained tonic EUS activity in a dose-dependent manner. Baclofen suppressed the guarding reflex and sustained tonic EUS activity to the same extent (by approximately 65 – 75%) over the same dose range (0 – 0.1 ug). However, the average duration of EUS bursting, number of EUS burst events and EUS burst event frequency were far less sensitive to the effects of baclofen and did not decrease to the same extent. This suggests that $GABA_b$ receptor activation is important for both EUS bursting and sustained tonic activity, but

that the localization of GABA action in the spinal cord or the underlying mechanisms may be different. In order to determine the specificity of baclofen and confirm involvement of $GABA_b$ receptor activation on EUS activity, the potent and selective $GABA_b$ receptor antagonist CGP 54626 hydrochloride was administered intrathecally either alone or in combination with baclofen during continuous flow cystometry. In this part of the study, baclofen-mediated inhibition of EUS activity could be blocked with CGP confirming the specificity of baclofen. Additionally, CGP alone or in the presence of baclofen significantly increased both EUS burst duration and the number of EUS burst events, but had little effect on the EUS burst event frequency and no effect on sustained tonic EUS activity. This suggests that endogenous tonic $GABA_b$ receptor activity is important for EUS bursting, but not sustained tonic activity. Additionally, this supports the possibility that the reduced sensitivity of EUS bursting to baclofen may be the result of competition with tonic levels of endogenous GABA at the $GABA_b$ receptor.

Baclofen is a proven clinically effective antispasmodic for the management of spasticity in patients with SCI. Some patients and studies have reported that baclofen also improves micturition function. It is therefore imperative to determine the effects of baclofen on bladder and EUS activity in the chronic spinally injured rat. In contrast to the spinally intact rat, urethane-anesthetized rats with chronic spinal cord transection exhibit four main changes: 1) an intrinsic bladder-EUS rhythm that is largely insensitive to the rate of bladder filling (i.e. neurogenic DO), 2) an increased gain in the guarding reflex, 3) reduced EUS bursting, and 4) little or no sustained tonic EUS activity. These changes are speculated to contribute, at least in part, to the dysfunction of the LUT in rats with SCI. In this part of the study, intrathecal baclofen had no significant effect on peak intravesical pressure or bladder contraction duration in the spinally transected rat. This was similar to what was seen in the spinally intact rat. However,

some major differences were observed. While the threshold for active bladder contraction increased in a dose-dependent manner with intrathecal baclofen in the transected rat, the magnitude of this increase was far less than what was observed in the intact rat, even after administration of a ten-fold higher dose of baclofen. This suggests that, in the spinally transected rat, spinal $GABA_b$ receptor activation on presynaptic Aδ afferent terminals is not playing a prominent role in reflex-mediated bladder-EUS contraction because either 1) Aδ afferent activation is inhibited or 2) $GABA_b$ receptors are less sensitive to baclofen/fewer in number. Since the levels of GABA and glutamic acid decarboxylase, the enzyme that synthesizes GABA from glutamate, are reduced in the spinal cord following spinal injury (Miyazato et al. 2009), it is possible that $GABA_b$ receptors are also down-regulated.

Other investigators have reported that intrathecal administration of baclofen reduces the frequency of non-voiding contractions in the awake spinally injured rat (Miyazato et al. 2008a, 2008b; Miyazato et al. 2009) and in animal models that mimic DO caused by cerebral injury or cystitis (Kanie et al. 2000; Pehrson et al. 2002). In agreement with these studies, the present data showed that intrathecal baclofen decreased the frequency of both voiding and non-voiding bladder contractions in a dose-dependent manner. Following SCI in the rat and cat, C-fiber afferent activity that is normally repressed under physiological conditions is believed to contribute significantly to reflex bladder-EUS contractions (de Groat and Yoshimura 2010). Since $GABA_b$ receptors are present in greater number on C-fiber than Aδ terminals in the spinal cord and Aδ afferent activation plays less of a role in micturition following SCI, it may explain why the biphasic response to baclofen seen in the spinally intact rat was largely absent in rats with spinal transection. However, it should be mentioned that high doses of baclofen in a few animals did produce overflow incontinence with frequent bladder contractions. These animals

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probably accounted for the wide variability in bladder contraction period at the higher end of the dose-response curve. Nevertheless, these results further highlight the beneficial effects of baclofen on reducing DO in spinal cord injured animals.

Both urethane-anesthetized and conscious rats with chronic spinal cord transection have been shown to exhibit several different EUS and bladder activity patterns (Cheng and de Groat 204; D'Amico et al. 2011; Leung et al. 2007). For instance, the majority of rats with complete spinal cord transection exhibit an intrinsic EUS-bladder rhythm without EUS bursting. However, a significant proportion of these animals exhibit "bladder choking." This phenomenon is characterized by the repeated inability of the bladder to generate sufficient intravesical pressures to overcome urethral resistance and produce even small voids (D'Amico et al. 2011). As a consequence, the bladder continues to fill and exhibits high sustained intravesical pressures. Experimentally, this could be a reflection of severe DSD. The situation can be exacerbated further since rats with chronic spinal cord transection have an enhanced guarding reflex that likely contributes to urine retention. Data from this part of the study suggests that intrathecal baclofen improves micturition function through its effect on EUS activity. For instance, three animals (n = 3/11) in this part of the study exhibited bladder choking with high background EUS activity. Administration of baclofen eliminated the bladder choking and reduced the inter-contraction bladder pressure. This was likely due to a combination of decreasing the frequency of micturition (as discussed earlier) and inhibiting EUS motoneuron activity in the lumbar spinal cord. Decreasing the frequency of micturition would allow the bladder sufficient time to relax in between contractions, while inhibition of EUS motoneuron activity would reduce urethral resistance, permitting more effective voids. Another putative mechanism by which baclofen may improve micturition function in the spinally injured rat is through a reduction in the gain of the

guarding reflex. The guarding reflex during active contraction of the bladder is vitally important for maintaining continence and preventing urine leakage in both humans and animals. Therefore, any method by which it is reduced following SCI could potentially be beneficial. Presumably, lowering EUS activity and urethral resistance during active bladder contraction would reduce the need for dangerously high intravesical pressures.

Rats with chronic SCI have been also shown to exhibit several different patterns of EUS activity. For instance, the complete inhibition of EUS bursting had long been reported in rats with chronic SCI (Kakizaki et al. 1997; Kruse et al. 1993). However, more recent studies document that a spinal mechanism mediating EUS bursting can still become active, albeit less frequently (40 – 50% of spinalized rats), in the lightly anesthetized or awake rat with incomplete or complete SCI (Cheng and de Groat 2004; Leung et al. 2007). These results indicate that anesthesia likely interferes with EUS bursting. Consistent with these results, in Chapter 2, it was reported that in the initial part of this project a very small percentage of urethane-anesthetized spinal cord transected rats showed signs of EUS bursting (n = 3/27). However, in the latter part of this study, a significant proportion of spinally transected rats exhibited pronounced EUS bursting and efficient voiding (n = 6/11). This was initially confounding, however, several things must be considered that may explain this discrepancy. For instance, in this part of the study: 1) the transection level was made more rostrally at spinal T_8 instead of T_{10}, 2) less surgical manipulation was done in the abdominal and pelvic regions during experimental preparation (e.g. the pubic symphysis was not removed), and 3) bladder pressure and EUS EMG recordings were done with the animal in the prone vs. the supine position. It is difficult to determine what the exact cause for the discrepancy was, but it likely involves a combination of these factors. In three of the six rats (n = 3/6) with spinal cord transection in this part of the study, EUS bursting did not

appear until after administration of relatively high doses of intrathecal baclofen (0.1 – 0.5 ug). This is interesting considering that these doses in the spinally intact rat had a profound inhibitory effect on EUS bursting and micturition. However, it is still not possible from these data to unequivocally determine if baclofen provoked this response. Regardless, the observation that a high percentage of urethane-anesthetized rats with complete SCI exhibited EUS bursting, even in the presence of intrathecal baclofen, is extremely interesting and warrants further investigation.
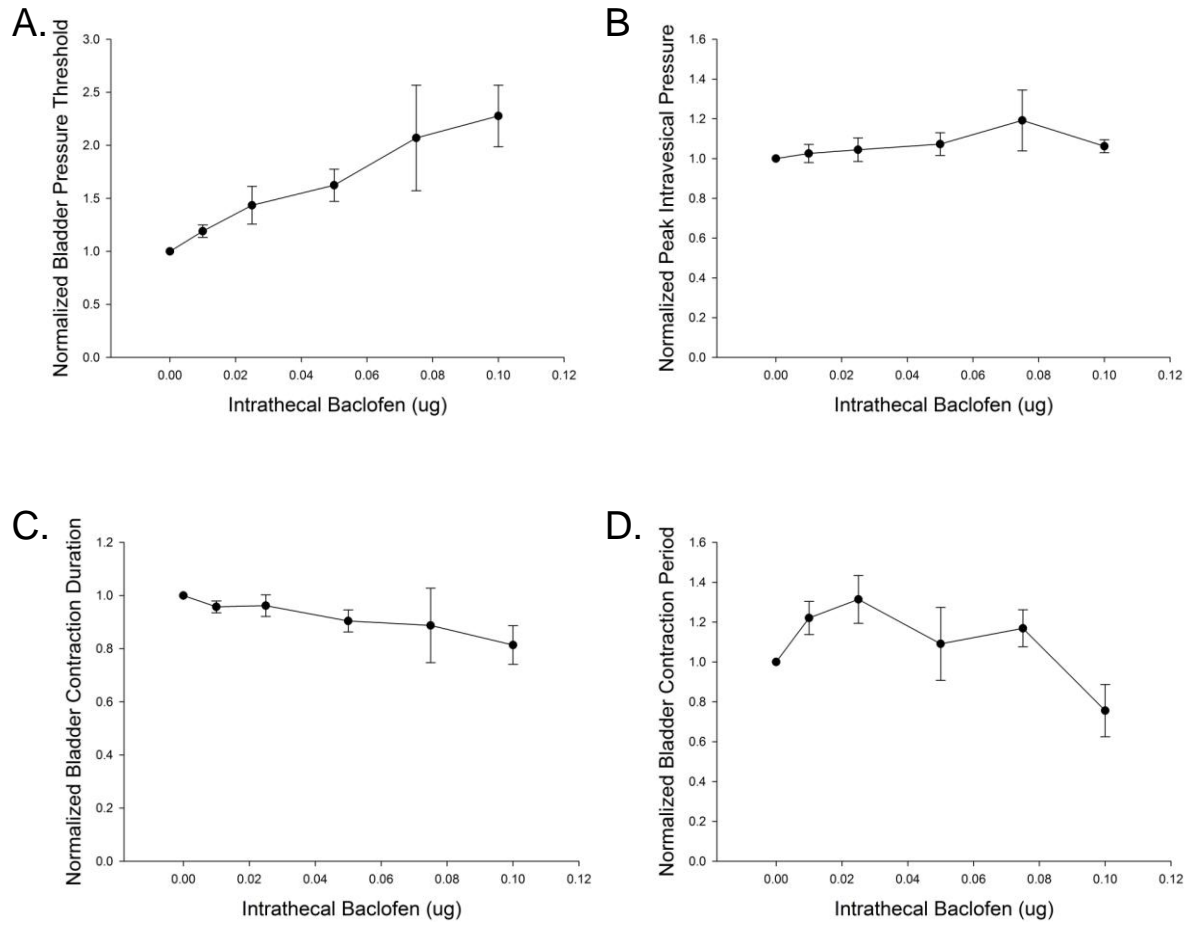
Figures and captions

Figure 20.

Figure 20. Dose-dependent effects of intrathecal baclofen on bladder function in the spinally intact rat. A. The threshold for initiating active bladder contraction was elevated linearly as the dose of baclofen was increased from 0 – 0.1 ug. **B.** However, increasing doses of intrathecal baclofen over the same dose range had no obvious effect on peak intravesical pressure (peak bladder pressure). **C.** Increasing doses of intrathecal baclofen mildly decreased the duration of bladder contraction by approximately 20%. **D.** The frequency of micturition could be decreased (increased bladder contraction period) by approximately 30% at low doses of intrathecal baclofen (0 – 0.025 ug). However, bladder contraction period began to decrease at higher doses (>0.025 ug) falling to approximately 70% of control.
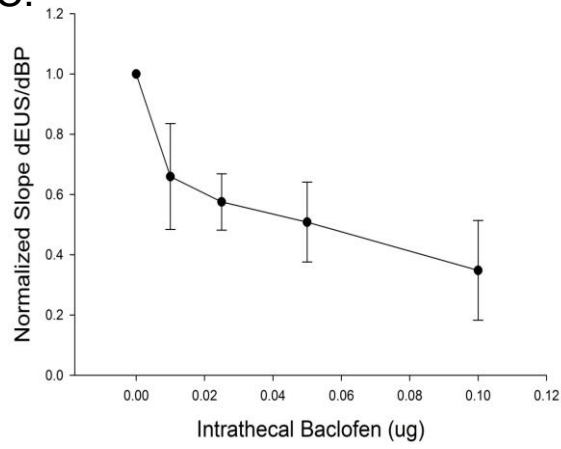
Figure 21.
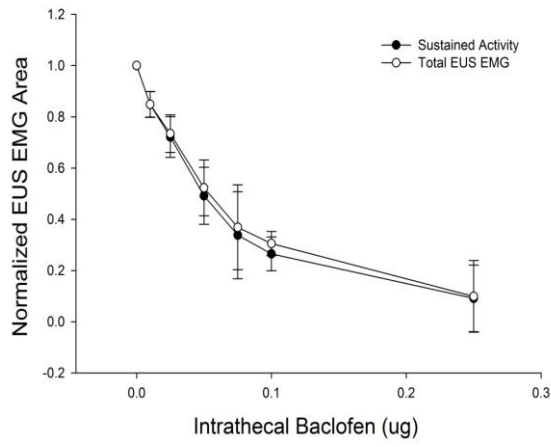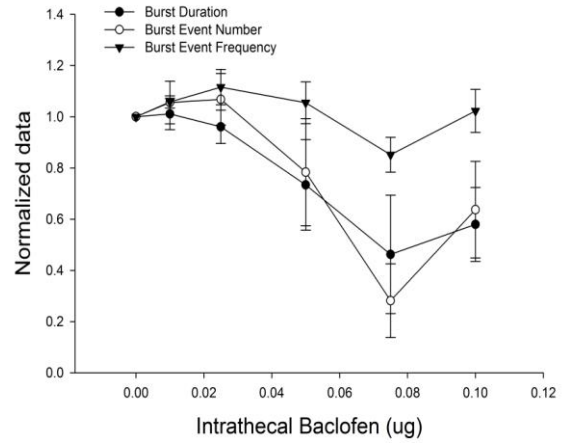
Figure 21. Dose-dependent effects of intrathecal baclofen on EUS EMG activity in the spinally intact rat. A. Increasing doses of intrathecal baclofen (0 – 0.25 ug) decreased, in parallel, total EUS EMG activity (open circles) and sustained tonic EUS activity (black circles) by ~90% . **B.** Similarly, intrathecal baclofen decreased burst duration (black circles) and the number of burst events (open circles) in parallel by 45 – 50%. However, the burst event frequency was not significantly affected by intrathecal baclofen. **C.** Increasing doses of intrathecal baclofen (0 – 0.25 ug) decreased the gain of the guarding reflex by ~70%.
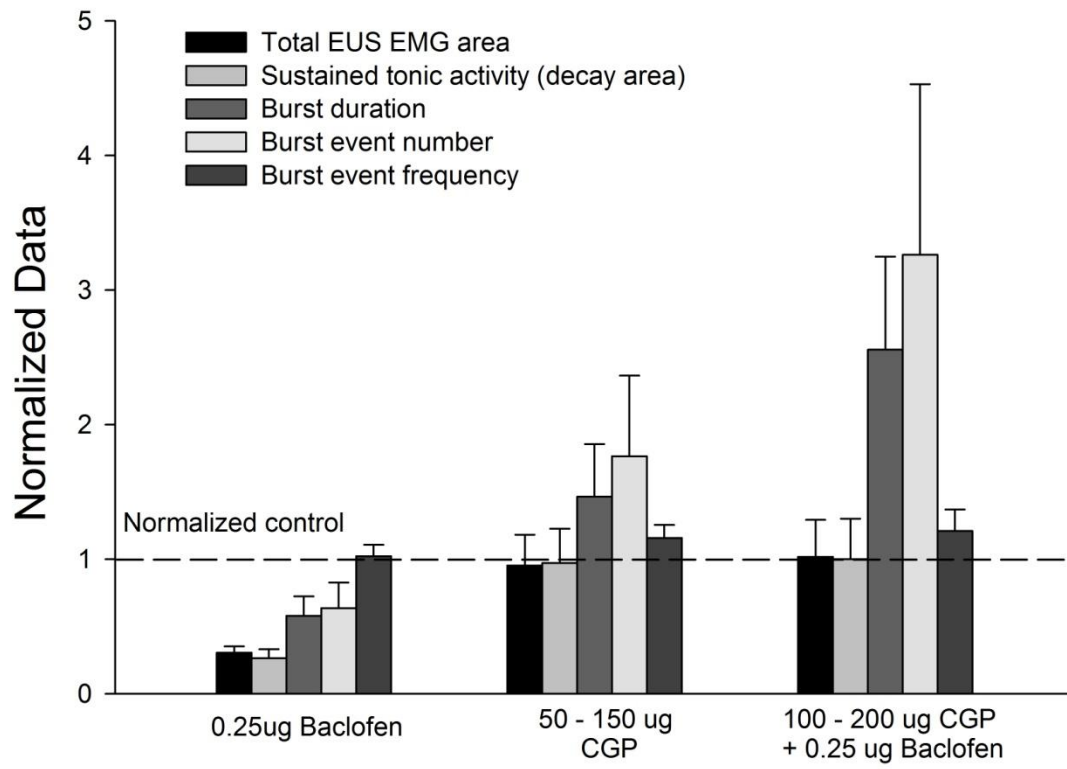
Figure 22.

Figure 22. Effect of intrathecal CGP on EUS EMG activity in the spinally intact rat.
Intrathecal administration of the $GABA_b$ receptor antagonist CGP (50 – 150 ug) modestly increased the total average burst duration and number of burst events above baseline. However, sustained tonic EUS activity (decay area) and EUS burst event frequency exhibited no change. Burst duration and burst event number could be further elevated with the addition of 50 ug CGP in the presence of 0.25 ug baclofen. Sustained tonic EUS activity and EUS burst event frequency remained unaffected.

Figure 23.
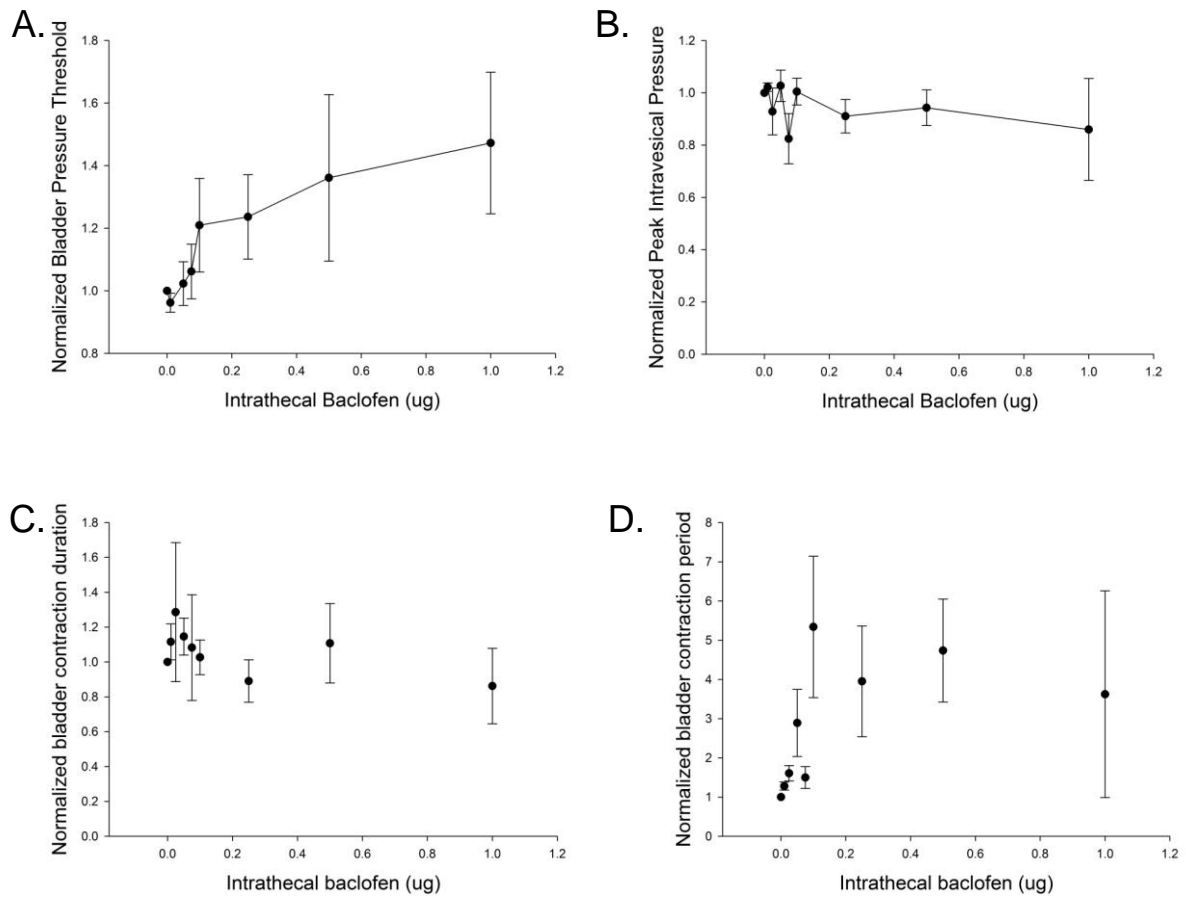
Figure 23. Dose-dependent effects of intrathecal baclofen on bladder function in the spinally transected rat. A. Increasing the dose of intrathecal baclofen elevated bladder contraction threshold by a total of ~40% at 1 ug. **B.** However, baclofen had no significant effect on peak intravesical pressure. **C.** Intrathecal baclofen had little to no effect on the duration of bladder contraction. **D.** Intrathecal baclofen decreased the frequency of micturition (i.e. increased bladder contraction period) by ~4-fold at a dose of 0.1 ug.

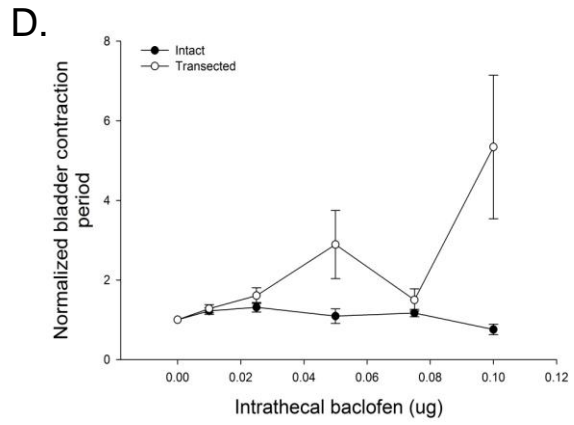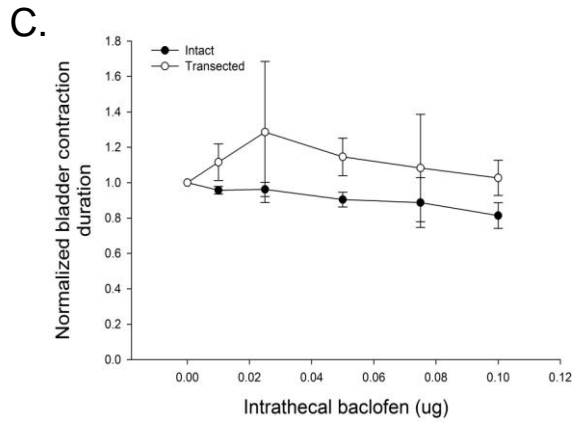**Figure 24.**
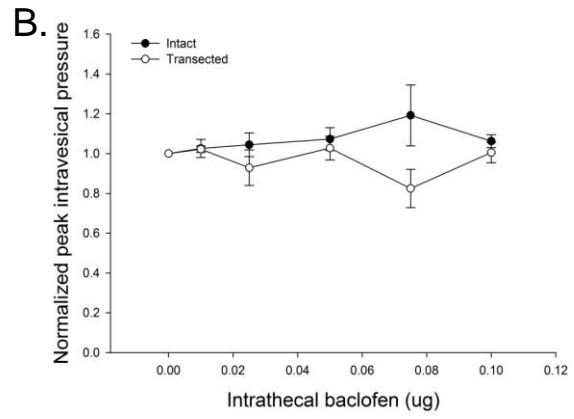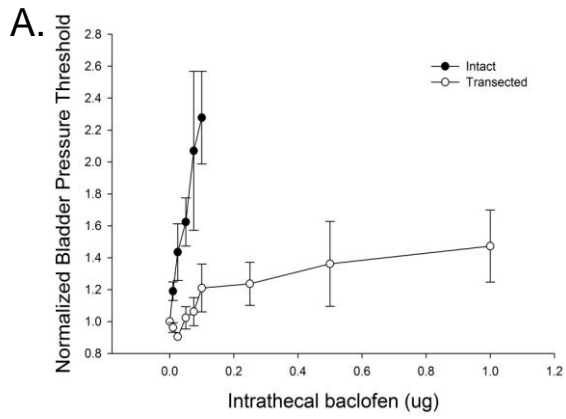
Figure 24. Comparison of the effects of intrathecal baclofen on the spinally intact and transected rat. A. Intrathecal baclofen increased the threshold for active bladder contraction in both spinally intact and transected rats. However, in the intact rat, this increase was far greater and occurred over a smaller dose range. **B.** Baclofen had little to no effect on peak bladder pressure in both the intact and transected rat over the same dose range. **C.** Overall, baclofen mildly decreased the duration of bladder contraction in the intact rat by ~20%, but had little to no effect on bladder contraction duration in the transected rat. **D.** Baclofen had a much more profound effect on bladder contraction period in the spinally transected rat. In contrast to the intact rat, the initial increase in bladder contraction period at low doses of baclofen was more pronounced. Additionally, the bladder contraction period continued to rise as the dose of baclofen was increased and did not exhibit a biphasic response.

Figure 25.
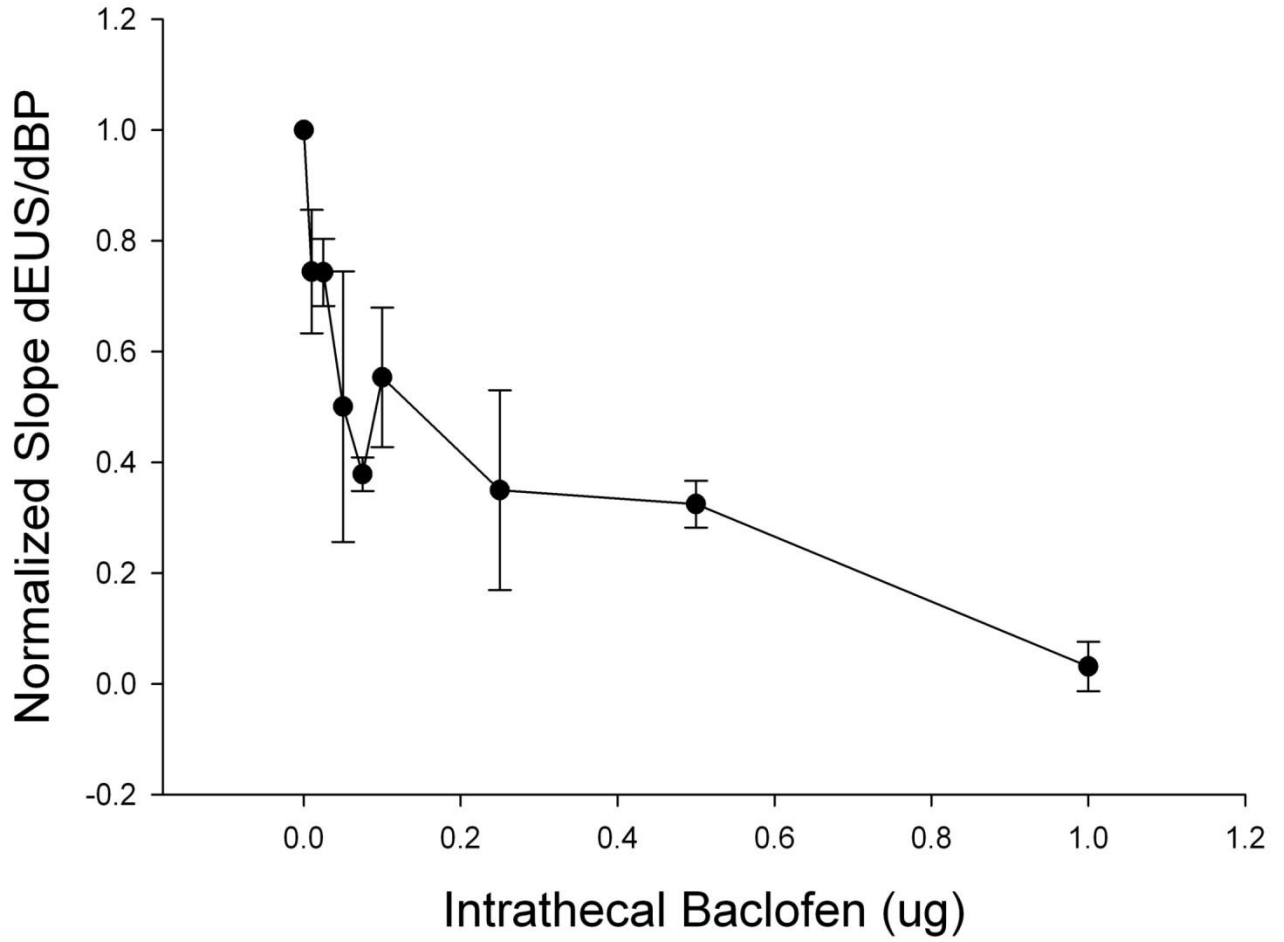
Figure 25. Effect of intrathecal baclofen on the guarding reflex in the spinally transected rat. Intrathecal administration of baclofen to the ventrolateral $L_6 - S_1$ spinal cord of the spinally transected rat depressed the gain of the guarding reflex (dEUS/dBP slope measured between ~20 – 60% BP) during active bladder contraction in a dose-dependent manner by ~90%.

Figure 26.
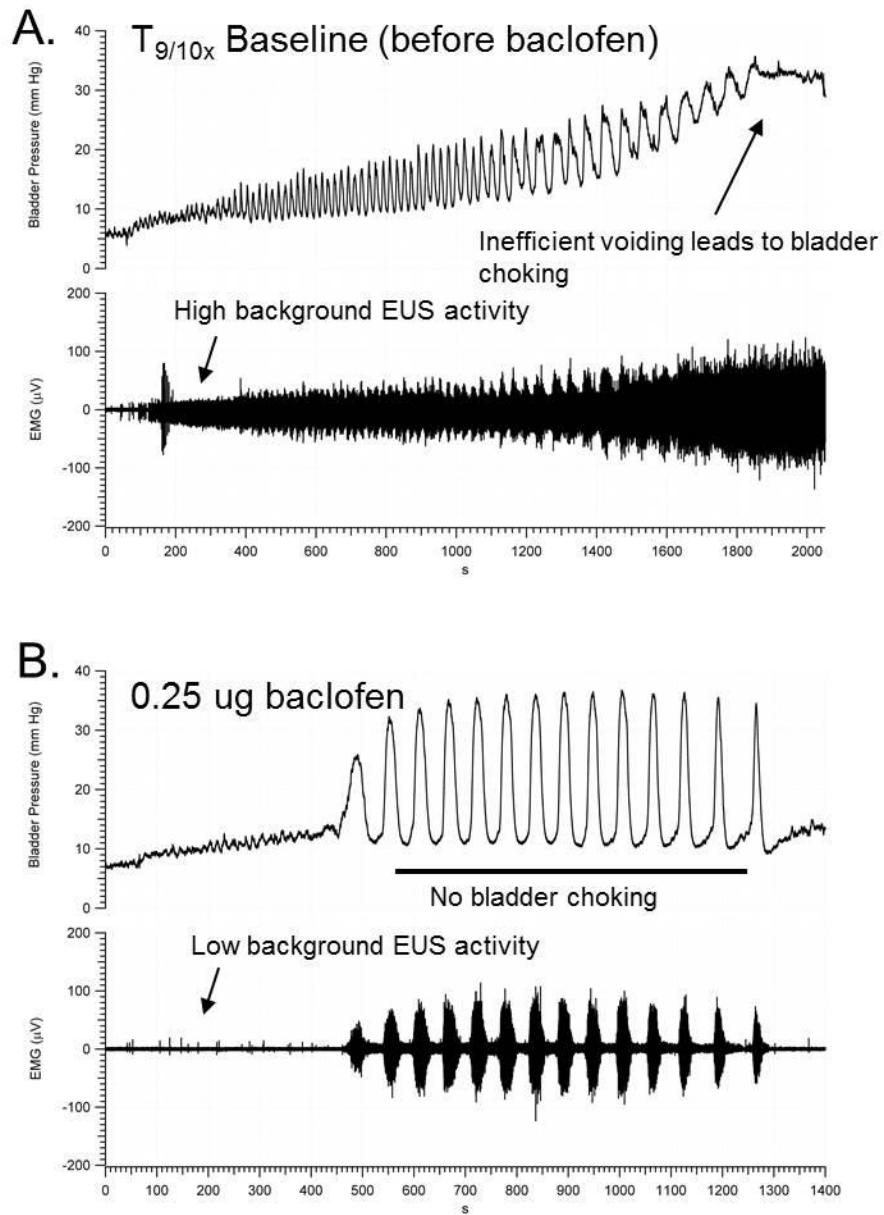
Figure 26. Intrathecal baclofen improved micturition function in the spinally transected rat. A. Example of a spinally transected rat (6 weeks post-transection) during continuous infusion of saline into the bladder with simultaneous recordings of bladder pressure (top panel) and EUS EMG activity (bottom panel). In this animal, the EUS is hyperactive even at low bladder pressures. As the bladder continues to fill, the inter-contraction bladder pressure (i.e. baseline pressure between contractions) rises until the bladder no longer produces well defined bladder contractions (i.e. bladder choking). **B.** Same animal in **A** following administration of 0.25 ug intrathecal baclofen. Peak and pre-contraction baseline bladder pressures were unaffected. However, EUS EMG activity and the inter-contraction bladder pressure were reduced and bladder choking abolished.

Figure 27.
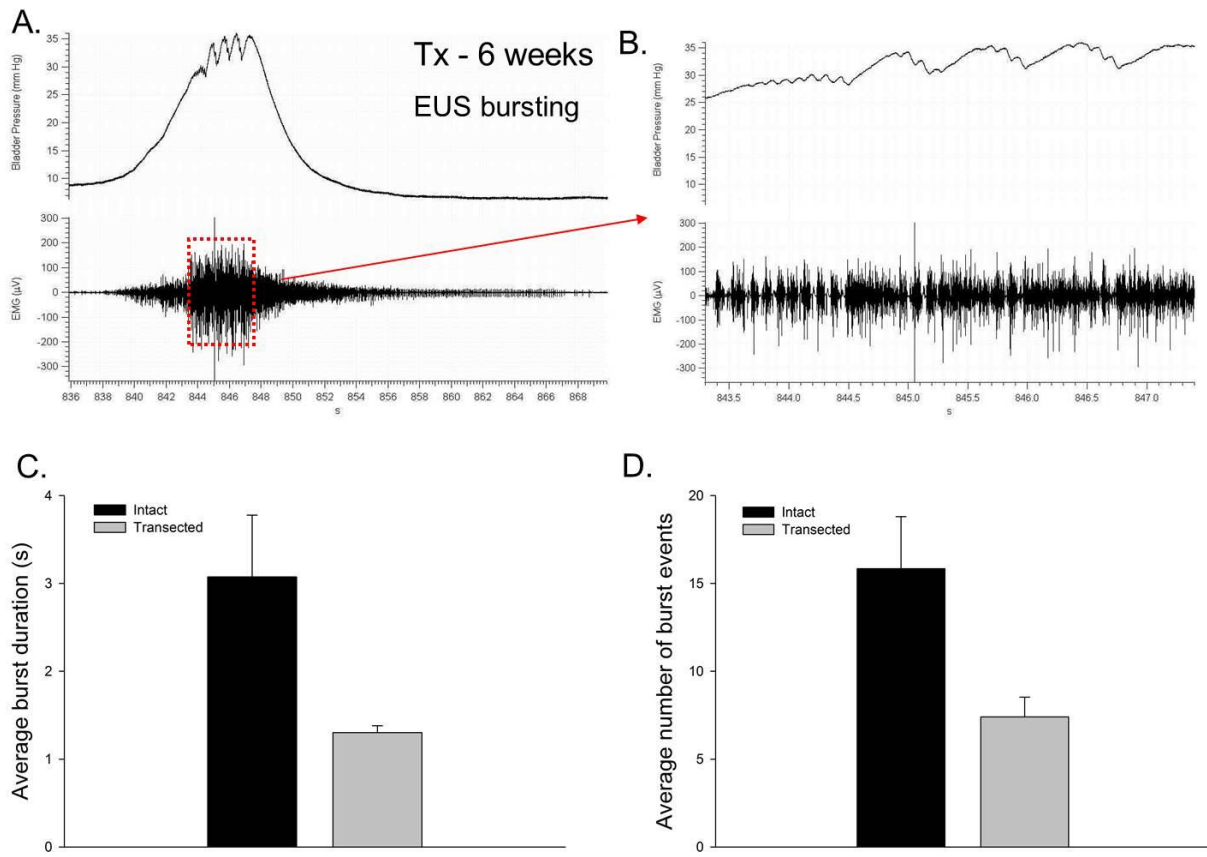
Figure 27. EUS bursting in spinally transected rats. A. A single micturition event in a spinally transected rat (6 weeks post-transection) that consistently exhibited EUS bursting during infusion of saline into the bladder. **B.** An expanded view (red box) of EUS EMG activity and bladder pressure at the peak of contraction. Multiple EUS EMG burst events with corresponding bladder pressure waves are clearly visible. **C.** The average duration of EUS bursting in intact rats (n = 6) was ~3-fold longer than in transected rats (n = 3). **D.** The average number of burst events per micturition event was ~3-fold larger in spinally intact rats (n = 6) than in transected rats (n= 3).

Figure 28.
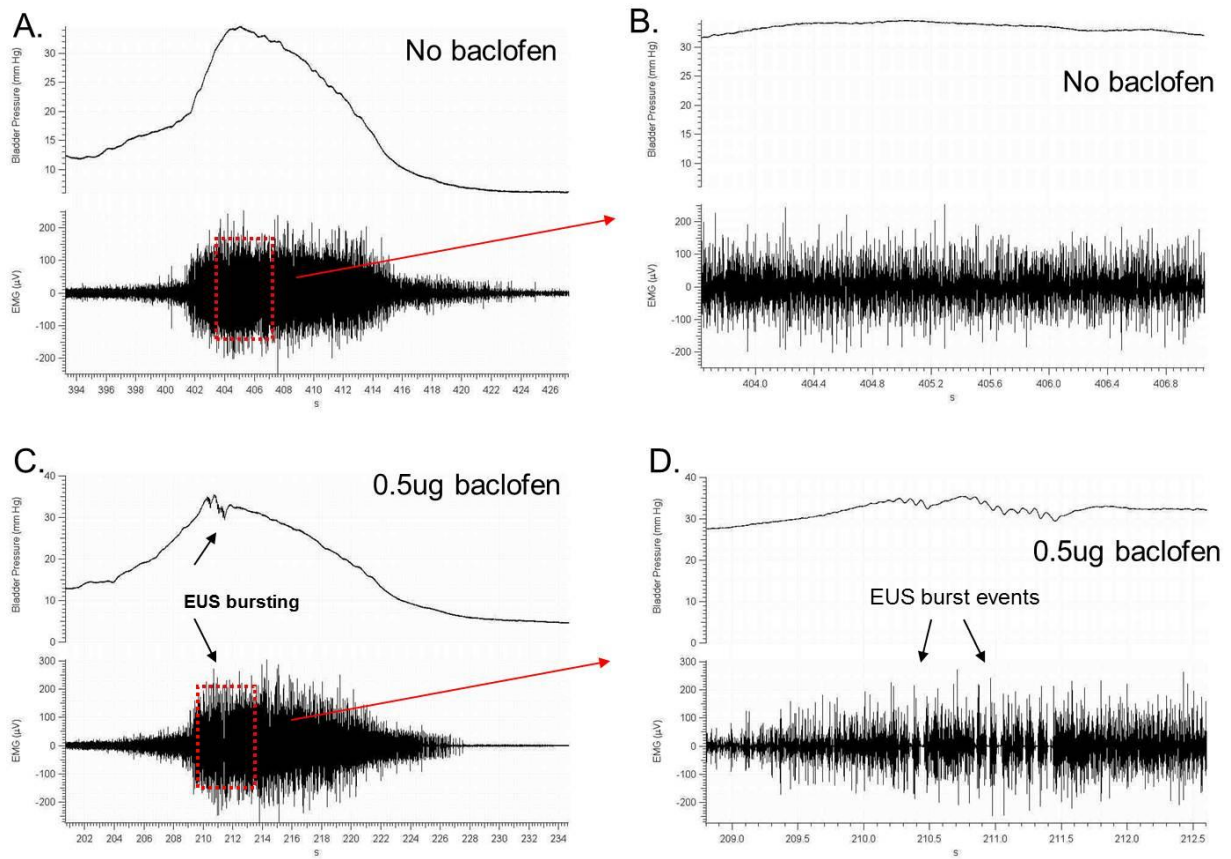
Figure 28. Baclofen may promote EUS bursting in spinally transected rats. A. A single micturition event from a spinally transected rat (6 weeks post-transection) during continuous flow cystometry with simultaneous recordings of bladder pressure (top panel) and EUS EMG (bottom panel). The recording shows no EUS bursting prior to intrathecal administration of baclofen. **B.** Expansion (red box) of the same record shown in A. **C.** An example of a single micturition event from the same transected rat in A and B after administration of 0.5 ug intrathecal baclofen. EUS bursting could be detected in both the bladder pressure and EUS EMG records. **D.** Expansion (red box) of the same record shown in C. Individual EUS burst events and bladder pressure waves are clearly visible.

Final thoughts and conclusions

The overarching goal of the research presented in this dissertation was to examine activation of the EUS in the adult female rat as to better understand its role in micturition and to better ascertain the impact of SCI on LUT function. The first part of the study aimed to implement an *in vivo* rat model for quantifying the temporal pattern of EUS and bladder activity during micturition events. Chronic spinal transection resulted in a variety of bladder-EUS activity patterns that revolved around an intrinsic bladder rhythm (i.e. bladder-EUS rhythm CPG) that was largely insensitive to the rate of bladder filling. With respect to EUS activation, three primary changes were noted and quantified in transected rats: 1) the gain of guarding reflex was enhanced, 2) EUS bursting was reduced, and 3) EUS motoneurons no longer exhibited significant sustained tonic activity following bladder relaxation. While more research is needed to uncover the physiological mechanisms underlying these changes, these data and their quantification represent a good foundation for future studies using the adult rat model in an effort to better understand SCI-induced LUT dysfunction.

The second part of this study also aimed to quantify EUS activity during micturition before and after SCI, but at the level of the single MU. This was the first comprehensive study of single EUS MU activity during micturition in the spinally intact adult female rat and the first to reveal the heterogeneous nature of EUS MU recruitment consistent with the presence of different muscle fiber types. Further, this was the first documentation of single EUS MU activity in the adult rat following SCI. While EUS MUs exhibited a broad continuum of bladder pressure

thresholds, once activated, MUs in intact and transected rats exhibited very different recruitment patterns. All EUS MUs in the intact rat displayed marked high frequency phasic activity during EUS bursting with most exhibiting sustained tonic activity following bladder relaxation, but little frequency modulation during active contraction of the bladder. These activity patterns facilitated the categorization of EUS MUs into distinct groups. In contrast, EUS MUs in the transected rat were less distinctive with no EUS bursting and little to no sustained tonic activity. However, these MUs exhibited a greater acceleration in tonic firing during active contraction of the bladder. The increased frequency modulation of MUs in the transected rat is hypothesized to account for the enhanced guarding reflex seen at the level of the whole muscle. These results will undoubtedly help crystallize our understanding of how the EUS muscle functions during micturition and provide clues as to how SCI may alter its normal operation.

The purpose of the third and final part of the project was to determine the role of $GABA_b$ receptor activity on bladder and EUS activation in the spinally intact and transected rat. Baclofen was chosen as the primary agent due to its high selectivity at the $GABA_b$ receptor and because it is widely used clinically to treat patients with SCI-induced spasticity. While the effects of baclofen on bladder and micturition function have been thoroughly researched in humans and animals, less is known about its impact on distinct EUS activity patterns before and after SCI. Intrathecal baclofen had an overall inhibitory effect on bladder and EUS activity in spinally intact and transected rats. Also, micturition in the latter appeared to qualitatively improve micturition. The results in this part of the study further highlight the therapeutic potential of baclofen in mitigating bladder and EUS dysfunction following SCI, in addition to implicating GABA as an important neurotransmitter/neuromodulator in regulating different aspects of the micturition reflex.

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