NOVEL METHOD TO STUDY AUTONOMIC NERVOUS SYSTEM FUNCTION AND EFFECTS OF TRANSPLANTATION OF PRECURSOR CELLS ON RECOVERY FOLLOWING SPINAL CORD CONTUSION INJURY

DISSERTATION
Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University
By

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2006

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ABSTRACT

Disruption of bladder and sexual function are major complications following spinal cord injury (SCI). To investigate these behaviors in a rat model of SCI, we developed a method to monitor micturition and erectile events by telemetry. Pressure monitoring has been described for recording penile erections in awake rats and involves placement of a catheter into the corpus cavernosum of the penis. We developed a variation on this technique involving pressure monitoring within the bulb of the corpus spongiosum penis (CSP). Using this technique we can record both erectile and micturition events. This technique was validated in 10 male rats and we demonstrated that telemetric recording of CSP pressure provides a quantitative and qualitative assessment of both penile erections and micturitions. Subsequently we monitored CSP pressures in 7 male rats subjected to SCI. We demonstrated that monitoring of CSP pressure in conscious rats is a valuable and reliable method for assessing recovery of autonomic function. Although recovery of micturition occurs in rats following incomplete SCI, recovery is limited and voiding remains inefficient. Moreover, changes consistent with spasticity and/or hyperreflexia were determined through waveform analysis.
To repair the injured spinal cord cell transplant strategies are being developed since they appear to reduce secondary damage and promote regeneration. We examined the effect of transplanting glial restricted precursor (GRP) cells and elevating cyclic adenosine monophosphate (cAMP) concentrations on recovery of autonomic functions following SCI in 53 rats. When compared to an operated control group no beneficial effects of this cell transplantation technique were found with regards to behavioral outcomes. Histopathological analysis showed survival, migration, and differentiation of GRP cells within the spinal cord. Transplanted GRP cells were well integrated in the spared host tissue and animals that had received GRP cells had more tissue throughout the lesion region and at the lesion center. Although elevation of cAMP appeared to reduce the area of spinal cord occupied by graft tissue, cAMP favored differentiation of GRP cells into oligodendrocytes. The transplantation technique used here did not affect serotonergic input to the neurons in the lumbosacral spinal cord that regulate micturition and sexual behavior.
This thesis is dedicated to my parents

Robert and Frances Nout

who taught me the value of education and perseverance.

“Carpe Diem”
ACKNOWLEDGMENTS

None other than Stephen Reed can top the list of people I would like to take the opportunity here to thank. Thank you for teaching me the details of equine neurology and for encouraging me to work with Drs. Beattie and Bresnahan. Thank you for your continuous support and advice throughout the past 6 years. I have enjoyed working with you tremendously and I hope we will continue to do so for years to come. I would like to thank Michael Beattie and Jacqueline Bresnahan, my advisors, for taking a chance on a veterinarian and allowing me to be part of their laboratory. It has been an extremely educational and rewarding experience in an area I consider to be very meaningful to veterinary and human medical progress. Thank you for this inspiring opportunity. I would like to thank Markus Schmidt for teaching me so much about autonomic behaviors and methods of examining these. I have enjoyed the enthusiasm and energy that you have brought towards all aspects of these experiments. Thank you for image preparation, your expertise, and support.

The work presented in this thesis could not have been completed without the input of Amy Tovar, Rochelle Deibert, Crystal Forrider, Esther Culp, John Komon, and the rats. Amy, I want to thank you very much for the many most
excellent ideas you have put forward towards conducting these experiments and for teaching me, an equine internist, the techniques of rat microscopic surgery. I also want to thank you for your support inside the lab and at J.R. Miggs. I would like to thank Rochelle and Crystal for their help with behavioral testing, animal care, and tissue processing. Ladies, your work has been truly fabulous! Esther, I would like to thank you for all the hours of data analysis you have performed for these experiments. I have appreciated your hard work and I would like to thank you for always being available for questions and updates. I would like to thank John Komon for help with histological analyses and excellent image preparation. I would like to thank Margot Mayer-Proschel, Christoph Proschel, and Mark Noble from University of Rochester Medical Center for providing me with the GRP cells. I thank the rats for their participation in these experiments; rats are indeed very friendly creatures and play such an important role in advancement of medical research. They deserve appropriate protection through institutional animal care and use regulations such as present at The Ohio State University.

I would like to thank Lyn Jakeman for her enthusiasm, interest, and input in my work. You have been there through first year NGSP presentations, my candidacy examination, and finally this thesis. I would like to thank Richard Burry and William Muir for their insights in aspects of histology and cardiovascular regulation, respectively. I would like to thank both of you for your support during my candidacy examination.

I would also like to thank Dana McTigue and Ping Wei for their help with immunohistochemical staining techniques and Brian Kemmenoe and Kathy
Wolken for their expertise and help while teaching me to use the confocal microscope in the Campus Microscopy and Imaging Facility.

Now I would like to thank John Gensel, Brandon Miller, Fang Sun, Adam Ferguson, and Randolph Christensen for their help and support in the lab during the past years. John, Brandon, and Adam, I have always enjoyed your company and all our conversations inside and outside the lab. Guys, keep up the good work and Adam, rock on! I would also like to thank Georgeta Mihai, Megan Detloff, and Richa Tripathi for their support and friendship during the past years. It has been great meeting all of you and communicating and working with you.

I would like to acknowledge advice, support, and friendship from Kenneth Hinchcliff, Ramiro Toribio, Colin Schwarzwald, and Catherine Kohn. Ken, thank you for introducing me to basic research, for teaching me to write, and for continued much appreciated advice! Ramiro and Colin, thank you for all your encouragement and friendship! Of course I have loved our visits to Rose and Thistle and Brazenhead and I hope we will continue to frequent these places. I would also like to thank my friends Cheryl Sofaly, Christopher O'Sullivan, Sue Hunter, Laura Werner, Wyatt Winchell, Jamie Murphy, Cheney Meadows, Phillip Lerche, Andrew Phipps, Christopher Premanandan, Wendy Fife, Mark Zuck, Allison Stewart, Monica Figueiredo, Emma Rowe, Kevin Corley, Kristy Amer, and members from the Columbus Running Club. I appreciate all the support and I look forward to meeting up with all of you who have left me behind in Columbus! I would also like to take this chance to thank my friends in The Netherlands, Eva Wolking, Stephen Postma, Angela and Arjen Ruiter, Inge Luning, Saxa van
Eijnsbergen, Annet van Uchelen, Corine Visser, Edwin Enzerink, and my Dutch friends abroad, Nienke Zwank-Wijnia, Martine de Wit, and Ingrid Segboer. Jullie hebben allemaal bijgehouden waarmee ik me heb bezig gehouden de afgelopen paar jaar, en ik wil jullie bedanken voor jullie steun en alle gezellige reunies die we hebben kunnen hebben tijdens mijn bliksem bezoeken aan Wageningen.

Finally I want to thank my family in The Netherlands and Canada, my brother Remi Nout, Maartje Schaap, and my parents for their unfailing love and support. Remi en Maartje, bedankt voor alle gezelligheid in Amsterdam en Remi, wees niet bang, jij blijft natuurlijk de enige echte Nout – dokter. Lieve ouders, vanaf het moment dat ik dierenarts wilde worden hebben jullie mij niet alleen altijd volledig gesteund, maar hebben jullie je ook actief ingezet en mij veel mogelijkheden geboden zodat ik nu mijn dromen heb kunnen realiseren. Wie had ooit gedacht dat ik mijn vaders voorbeeld zou volgen in het onderzoek?? Jullie hebben me zien vertrekken naar de UK, Duitsland, Tanzania, Virginia en nu Ohio en gelukkig zijn jullie me vaak in de VS komen opzoeken. Dat er nog veel vakanties zullen volgen! Ik wil jullie bij deze ontzettend bedanken voor jullie liefde, steun, vakantie lol, en voor alles waar ik nu niet eens aan denk.

Thank you!

Dank jullie wel!
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CHAPTER 1

INTRODUCTION

Acute traumatic damage to the spinal cord occurs in most species, but is most commonly seen in humans, dogs, and horses. In dogs the most common causes of spinal cord injury (SCI) are intervertebral disk extrusion and traumatic penetrating or non-penetrating injuries (LeCouteur and Grandy, 2000). Explosive protrusion of an intervertebral disk can cause both concussion and compression of the spinal cord. Disk extrusion, a condition that occurs in many breeds of dogs, may occur at any site within the vertebral column but is most commonly seen in the thoracolumbar and cervical area of the vertebral column. It has been reported that 1 – 2% of all dogs admitted to veterinary hospitals have injuries to the spinal cord resulting from disc disease (Hoerlein 1953; Priester 1976, Webb et al., 2004) but total incidence of SCI in hospitalized dogs has not been reported. In horses, trauma to the central nervous system is the most common cause of neurologic disease, accounting for 22% of neurologic disorders in one study (Feige et al., 2000). In this same study, 50% of horses with traumatic central nervous system disease were diagnosed with lesions in the cervical spinal cord. Spinal cord injury occurs predominantly in young horses and is most
commonly the result of falls (Matthews and Nout, 2004; Reed, 1994). In horses the spinal cord is well protected in the thoracic and lumbar vertebral column, leaving the cervical and sacral vertebral column the most susceptible to trauma. There are no reports on incidence of SCI in horses; however, since in general SCI is the result of high velocity / high impact forces, prognosis for life is guarded. In humans, it is estimated that the annual incidence of SCI, not including those who die at the scene of the accident, is approximately 40 cases per million population in the U.S., or approximately 11,000 new cases each year. The number of SCI patients in the U.S. who were alive in July 2005 was estimated to be approximately 250,000. Between 2000 and July 2005 vehicular crashes accounted for the majority (47.5%) of reported SCI cases, followed by falls (22.9%) and violence (13.8%) (The National SCI Statistical Center, 2005).

Spinal cord injury results not only in paraplegia or tetraplegia, but also leads to systemic and metabolic alterations secondary to disruption of autonomic nervous system homeostasis. Currently, in a time of increasing emotional value of many of our pets, treatment and care of SCI and other recumbent patients has become an important aspect of veterinary medicine (Nout and Reed, 2005). Although recovery from incomplete SCI in small animals is good, with the majority of dogs regaining motor function, animals with more severe injuries have a much poorer prognosis (Kinzel et al., 2005; LeCouteur and Grandy, 2000; Olby, 1999; Rossmeisl et al., 2005). Furthermore, mobility of paraplegic dogs is improved through development of pet wheelchairs and dog carts; however, it is dysfunction of eliminative behaviors, or incontinence, which poses more serious
problems in the care of these animals. Despite advances in the development of assistive devices these additional consequences of SCI pose threats that ultimately may lead to the decision that favors humane euthanasia. In one study examining long-term (> 6 months) functional outcome after severe SCI in 87 dogs, the prognosis for paraplegic dogs without deep pain perception because of trauma was guarded, while dogs with disk herniation had a better chance of recovering motor function (Olby et al., 2003). A third of the dogs that recovered motor function had intermittent incontinence. Persistent loss of deep pain perception did not preclude recovery of motor function, but such dogs remained incontinent.

In humans, since 2000, the average age at injury was 38 years and 79.6% of spinal cord injuries occurred in males. Thus, primarily young male adults are affected. Since 2000, the most frequent neurologic category at the time of discharge from hospital was incomplete tetraplegia (34.5%), followed by complete paraplegia (23.1%), complete tetraplegia (18.4%), and incomplete paraplegia (17.5%) (The National SCI Statistical Center, 2005). This indicates that 76 – 93.5% of SCI patients are left with incomplete function below the waist which includes inability to walk, loss of bladder and defecatory control, and altered sexual function. In fact, regaining bladder/bowel function was the first or second highest priority for 39.7% of quadriplegics and 38% of paraplegic. Similarly, regaining sexual function was the first or second highest priority to 28.3% of quadriplegics and 45.5% of paraplegics (Anderson, 2004).
These changes profoundly reduce the quality of life for injured individuals and negatively impact society by reducing productivity in injured individuals. The average yearly health care, living expenses, and the estimated lifetime costs that are directly attributable to SCI vary greatly according to severity of injury but exceed $7 billion dollars per year in the U.S. The additional indirect costs including losses in wages, fringe benefits, and productivity average $56,964 per person per year (Center, 2005; DeVivo, 1997).

Damage inflicted to the spinal cord occurs in 2 distinct phases. So-called “primary injury” refers specifically to the mechanical disruption of axons as a result of compression, penetration, laceration, shear, and/or distraction (Tator and Fehlings, 1991). Primary damage results in cell membrane disruption and rapid cell death which is followed by a second period of progression of tissue damage and cell death that can last several hours to days after initial insult (Allen, 1911, 1914; Beattie et al., 2000a; Beattie et al., 2000b; Tator and Fehlings, 1991). This is referred to as “secondary injury”. Many well-characterized mechanisms of cell damage have been shown to play a role during this period of secondary damage, including 1) vascular compromise leading to reduced blood flow, loss of autoregulation, loss of microcirculation, vasospasm, thrombosis, and hemorrhage; 2) electrolyte shifts, permeability changes, loss of cellular membrane integrity, edema, and loss of energy metabolism; 3) biochemical changes including neurotransmitter accumulation, arachidonic acid release, free radical and prostaglandin production, and lipid peroxidation; and 4) apoptosis (Amar and Levy, 1999; Beattie et al., 2000a; Hall and Wolf, 1986;
Tator, 1996, 1998; Tator and Fehlings, 1991). Secondary expansion of the lesion cavity that occurs through necrotic and apoptotic pathways is associated with the invasion of immune cells and activation of the resident microglial population. The period of ongoing secondary injury is considered to be the phase in which therapeutic measurements could be taken to halt the process of ongoing cell loss.

Endogenous repair has been shown to take place following a contusion lesion. Proliferation of ependymal cells that lie around the central canal has been shown to occur in fetal mammals, but also in adult rats (Beattie, et al., 1997; Mothe and Tator, 2005; Namiki and Tator, 1999; Wallace et al., 1987; Vaquero et al., 1981). These cells appear to add tissue bridges referred to as trabeculae that begin to fill the lesion cavity (Bresnahan, 1978; Bresnahan et al., 1987; Bresnahan et al., 1976; Noble and Wrathall, 1989). Glial cells and invading sensory axons can follow these cellular tracks. Over time, these trabeculae can support growth of axons from within the central nervous system, such as the corticospinal tract and fibers from the brainstem reticular formation (Beattie, et al., 1997; Hill et al., 2001). In distinct contrast to the limited distribution of neuronal stem cells, glial precursors are widely distributed in the developing and mature mammalian central nervous system. Furthermore glial progenitor cells that can give rise to both astrocytes and oligodendrocytes exist predominantly in the outer rim of the spinal cord (Horner, et al., 2000). Proliferation of endogenous glia and/or glial precursor cells is thought to play an important role in restoring chronic function to the injured cord (Beattie et al., 1997; Beattie et al., 2002;
McTigue et al., 2001; Zai and Wrathall, 2005). It is likely that oligodendrocyte precursor cells rather than surviving mature oligodendrocytes contribute to the intrinsic remyelination after demyelinating insult (Cao et al., 2002).

Oligodendrocytes, the cells that wrap axons in myelin and allow them to function, undergo apoptosis during the delayed phase of death after SCI and since each oligodendrocyte myelinates 10 – 20 different axons, the resulting loss of myelination and conductance through that segment has a large effect on the loss of function (McDonald et al., 2004). Although remyelination occurs, this intrinsic repair is insufficient to overcome the insult. However, perhaps by increasing the number of these cells in the vicinity of the lesion, functionally significant repair could be obtained. Currently the use of various cell transplant procedures is being widely investigated in the field of SCI research. Rationales for using transplantation strategies are 1) the functional reconstruction of neuronal circuits; 2) the production of neurochemically active substances; and 3) remyelination of axons (Bjorklund and Lindvall, 2000; Okano, 2002).

Although it is currently possible to make axons sprout from surviving but damaged neurons, it is not yet possible to make long axons grow and form appropriate reconnections, however, this ultimate goal may be achievable through ongoing studies and investigations into the potential of stem cell transplantation in neurologic diseases. At this time, it appears that optimizing the function of neuronal circuits that survive SCI is probably the most achievable strategy (McDonald et al., 2004). Many current investigations attempt to do this through enhancement of glial cell function. Immature astrocytes promote axonal
growth and perhaps promote survival after injury (Silver, 1993), whereas immature oligodendrocytes provide remyelination after injury (Kohama et al., 2001; McTigue et al., 2001).

It has now become possible to isolate pluripotent cells from embryos and using treatments with growth factors, push these cells into lineage-specific states that produce either neuronal or glial-specific progenitor cells (Mayer-Proschel et al., 1997; Wu et al., 2002). For cell replacement in neurological conditions, experimental data to date suggests that lineage-committed immature neuronal or glial progenitor cells, rather than multipotential neural stem cells or mature neurons and glial cells, are most likely to result in predictable phenotypic differentiation and potential functional outcome. Key in these treatment strategies is the ability of cells to integrate into the neural circuitry in a functional manner and consequently contribute to the correction of functional deficits.

To improve integration and function of transplanted cells a wide variety of bio-molecular therapies are being investigated. For example neurotrophic factors, such as neurotrophins, have been found to promote a variety of neural responses such as survival and outgrowth of nerves and spinal cord nerve regeneration (Schmidt, C. E. and Leach, 2003). Inducing up-regulation of neurotrophins, regeneration-associated genes, and antiapoptotic factors and / or blocking inhibitory biomolecules in the nervous system through use of genetically modified cells are methods used to complement cell transplantation strategies. Elevation of cyclic adenosine monophosphate (cAMP) concentrations has been shown to play an important role in regeneration of the adult central nervous
system (Spencer and Filbin, 2004) and in overcoming inhibitory aspects of the injured spinal cord milieu (Gao et al., 2004). Recently, elevation of cAMP through administration of the phosphodiesterase IV inhibitor rolipram in conjunction with transplantation of embryonic spinal tissue or Schwann cells resulted in improved functional outcome and promoted axonal regeneration after SCI (Nikulina et al., 2004; Pearse et al., 2004).

Our studies were undertaken to contribute to improving quality of life for all involved in SCI. The main focus of our research was dysfunction of eliminative and sexual function and effects of a cutting-edge transplantation strategy on these body systems. In the following investigations we demonstrate the value of a novel method to assess autonomic behavior in freely moving rats and use that model to investigate the use of a cell transplantation technique in experimental SCI. Since the spinal cord injured population considers eliminative and sexual function to be very important in quality of life and these are important functional outcomes in veterinary spinal cord injured patients, we chose bladder and sexual functions to be part of the behavioral outcomes in our rat model of SCI. Using a telemetric technique to measure corpus spongiosum penis pressure we demonstrate the ability to assess details of micturition and erectile function in conscious male rats. We then use this technique to assess recovery of these functions after an intervention that includes a combination of transplantation of glial restricted precursor cells and elevation of cAMP concentrations.
CHAPTER 2

ALTERATIONS IN ELIMINATIVE AND SEXUAL REFLEXES AFTER SPINAL CORD INJURY

2.1 General characteristics

Both eliminative and sexual functions are compromised following spinal cord injury (SCI) (Comarr, 1970; Frenckner, 1975; Pedersen, 1983). Clinical representations with regards to bladder and bowel function are detrusor hyperreflexia, detrusor sphincter dyssynergia, detrusor areflexia, gastric dilatation, ileus, fecal incontinence and constipation. Inability to achieve psychogenic and reflexogenic erections and ejaculatory dysfunction in men and failure of vaginal lubrication in women are aspects that have a significant impact on sexual life after SCI (Karlsson, 2005). Disruption of supraspinal input to the neuronal circuits that control these functions is the main cause of the autonomic sequelae of SCI.

The eliminative and sexual function systems have both autonomic and somatic components, and are organized by sympathetic neuronal pools in the caudal thoracic/upper lumbar cord, parasympathetic neuronal pools in the sacral cord and somatic motor pools in the caudal lumbar/upper sacral cord. The
anatomical organization of these systems is co-extensive, their functions overlap, and response to injury is similar. Although the basic afferent and efferent limbs of the reflex components are segmentally organized, the loss of brainstem and forebrain descending input results in loss of voluntary control and in abnormal functioning of these systems including the development of dyssynergias and spasticity (Nout, Y. S. et al., 2005a). Before reviewing aspects specific to the different eliminative and sexual functions, in the following paragraphs the characteristics that are similar for these functions are discussed. The most important components of the innervation of rectum, lower urinary tract, and genital tract are through the pelvic, hypogastric, and pudendal nerves.

**Neuroanatomy**

The pelvic plexus is a crossroad of autonomic nerves and ganglia supplying the rectum, the lower urinary tract, and the genital tract (Paxinos, 1995). In the male rat, the main components of this plexus are the right and left pelvic ganglia, also referred to as the hypogastric ganglia, or the major pelvic ganglia. These ganglia are positioned on either side of the prostate, ventral to the rectum, and caudal to the ureter and vas deferens, and are unusual among autonomic ganglia, since they contain both parasympathetic and sympathetic postganglionic neurons. The main afferents to the pelvic ganglia are the hypogastric nerve and the pelvic nerve (Paxinos, 1995).

The majority of fibers comprising the hypogastric nerve originate in the intermediolateral cell column of the thoracolumbar cord, (thoracic (T)11 – L2
(Baron and Janig, 1991). These preganglionic sympathetic neurons send their axons to sympathetic ganglion cells of the bladder in the sympathetic chain (T12 – L6), pelvic ganglia, and to the inferior mesenteric ganglia (Vera and Nadelhaft, 1992). The caudal continuation of the inferior mesenteric ganglion forms the hypogastric nerve, which carries the majority of the sympathetic input to the pelvic plexus. A small number of hypogastric fibers have their cells of origin located in either the sympathetic chain or the inferior mesenteric ganglion.

The pelvic nerve carries the parasympathetic input and originates from the last lumbar (L6) and first sacral (S1) spinal nerves in the rat (Purinton et al., 1973). The pelvic nerve also carries a small portion of sympathetic fibers to the pelvic viscera. Numerous small efferents arise from the pelvic ganglion and supply the rectum, the ureter, the vas deferens, the seminal vesicle, the prostate, the bladder, and the urethra. In the female rat the pelvic ganglion, which is also referred to as the paracervical or Frankenhauser ganglion, is smaller than that in males and lies against the lateral wall of the uterine cervix. Afferent neurons with fibers in the pelvic nerve are located in the L6-S1 dorsal root ganglia in the rat and carry sensory information from the descending colon, bladder, urethra and sex organs (Nadelhaft and Booth, 1984). These fibers enter the tract of Lissauer, encircle the dorsal horn with a medial component terminating in the dorsal commissural gray and the lateral component terminating in the sacral parasympathetic nucleus.

The pudendal nerve arises from L5-L6 in the rat and carries efferent fibers to the coccygeus, internal obturator, ventral and dorsal bulbospongiosus (also
termed bulbocavernosus), ischiocavernosus, external urethral and anal sphincter muscles, and afferent fibers from the penis, prepuce, scrotum, and ventral-proximal tail (McKenna and Nadelhaft, 1986). The sacral plexus is the complex formed by the bridge-like structure connecting the pudendal nerve with the lumbosacral trunk, and two nerve branches emerging from it, one innervating the proximal half of the scrotal skin, and the other, known as the motor branch, innervating the muscles at the base of the penis (Pacheco et al., 1997). These branches are only considered as a part of the sacral plexus because they integrate axons from both the lumbosacral trunk and pudendal nerve.

In rats, retrograde tracer studies of the pudendal nerve have demonstrated the presence of two motor nuclei in the ventral horn gray matter of the L5-L6 spinal cord segments, the dorsomedial and dorsolateral cell columns (Figure 2.1; Breedlove and Arnold, 1980; McKenna and Nadelhaft, 1986; Vera and Nadelhaft, 1992). These nuclei are the homologues to the nucleus of Onuf, which is found in other species, and contains motor neurons innervating the anal and urethral sphincters, bulbospongiosus and ischiocavernosus muscles, all located in one cell group. McKenna and Nadelhaft (McKenna and Nadelhaft, 1986) also demonstrated that in the female rat, urethral sphincter motor neurons accounted for almost all the dorsolateral nucleus motor neurons, and anal sphincter motor neurons accounted for almost all the dorsomedial motor neurons; the ischiocavernosus and bulbospongiosus muscles are vestigial in the female rat. In the male, neurons innervating the external anal sphincter and bulbospongiosus muscles are found intermingled in the dorsomedial nucleus. In
contrast, in the dorsolateral nucleus, the urethral sphincter neurons are located in the lateral portion of the nucleus and the ischiocavernosus neurons are located in the medial portion (Collins et al., 1992; Hermann et al., 1998; McKenna and Nadelhaft, 1986). There are significantly more neurons in both nuclei in the male than in the female and the neurons are larger in the male. However, perineal muscle tracer injections have established that there is no difference between males and females in the number of motor neurons innervating the external anal or urethral sphincters (McKenna and Nadelhaft, 1986).

Figure 2.1 A-F: The caudal lumbar and rostral sacral spinal cord contains the neuronal circuitry that organizes the parasympathetic components of bladder, bowel and sexual function. The following abbreviations are used in this figure:

A. The cord at this level contains the SPN and the associated somatic motor columns innervating the pelvic floor musculature, the DM, and DL nuclei.
B,C. The L6 segment contains the DM and DL nuclei that are characterized by a lack of myelinated fibers; this is most easily seen in the DL. The boxed area in B is shown at higher magnification in C. In this particular section, bulbospongiosus motor neuron cell bodies are retrogradely labeled with horseradish peroxidase (HRP) and can be easily identified in the higher power image shown in C. This is a plastic embedded section containing the labeled bulbospongiosus motor neurons cell bodies; diaminobenzidine was used to visualize the horseradish peroxidase in the neurons. After processing the tissue for HRP, it was stained en bloc with osmium and uranyl acetate and embedded in plastic for subsequent sectioning for electron microscopy. Myelinated fibers appears black (note the darker staining of the ventral white commissure just above the DM nuclei and in the white matter of the dorsal lateral and ventral funiculi). The retrogradely labeled neurons also appear dark in this section (arrow in C).
D. This micrograph shows a 1.0 μm thick, toluidine blue stained section of the
Figure 2.1 continued

DM area enclosed by the circle in C. Two motor neuron cell bodies are shown in cross section; the cell on the left has a clear nucleus, clumps of rough endoplasmic reticulum in the cytoplasm and a large primary dendrite extending dorsally (toward the letter D). The other cell body has clumps of rough endoplasmic reticulum and short primary dendrites that extend mostly out of the plane of section. There is a high density of cross-cut dendrites which appear as small white circles; these are rostrocaudally oriented bundles of dendrites. Black myelinated fibers can be seen at the top of the micrograph and a couple fibers can be seen to traverse the nucleus between the two cell bodies. The magnification is too low to identify the HRP stained elements in the cytoplasm. E,F. These low power dark field micrographs of adjacent sections show the extensive dendritic arborizations of bulbospongiosus motor neurons retrogradely labeled with horseradish peroxidase from muscle injections (Tetramethyl benzidine processing). The bundles extend into the DCG region that receives pudendal and pelvic nerve afferent input, into the DL nucleus that contains motor neurons of the ischiocavernosus and external urethral sphincter muscles, and over to the contralateral DM nucleus. (From: Nout, Y. S. et al., 2005a).
These nuclei have a number of special anatomic features (Figure 2.1) similar to the phrenic nucleus, a motor nucleus in the cervical spinal cord that innervates the diaphragm. These nuclei lack myelinated fibers making them appear translucent, similar to the substantia gelatinosa. The motor neurons exhibit extensive dendritic bundling (McKenna and Nadelhaft, 1986; Roney \textit{et al.}, 1979; Sasaki, 1994; Schroder, 1980); the rostrocaudally oriented dendritic bundles can be seen in Figure 1D. Dense bundles of dendrites from bulbospongiousus motor neurons can be seen in Figure 1E and F (arrows), interconnecting the dorsomedial nuclei on the two sides as well as the dorsomedial and dorsolateral nuclei on the same side. A dorsally extending group projects into the dorsal commissural gray (Figure 1E&F). Peshori \textit{et al.} (1995) demonstrated that motor neurons in the contralateral dorsomedial nucleus could be transneuronally labeled with wheatgerm-agglutinin horseradish peroxidase after a unilateral muscle injection and dye-coupling has been demonstrated between bulbocavernosus motor neurons by Matsumoto et al. (Matsumoto \textit{et al.}, 1988). At the ultrastructural level, the dendrites are connected with tight junctions or puncta adherentia (Nout, Y. S. \textit{et al.}, 2005a; Ramirez-Leon and Ulfhake, 1993) as well as through presynaptic dendrites (Nout, Y. S. \textit{et al.}, 2005a). The synaptic arrangements in this region are also somewhat unique in that axon terminals contacting more than one dendrite is common ((Nout, Y. S. \textit{et al.}, 2005a; Ramirez-Leon and Ulfhake, 1993). The dense innervation of this region by γ-amino butyric acid (GABA)-containing elements is also of note (Li \textit{et al.}, 1995; Ramirez-Leon and Ulfhake, 1993). It is likely that these anatomical arrangements
can provide for a more synchronized activation of motor neurons controlling pelvic floor musculature (e.g. the sphincters and erectile musculature).

The pudendal motor neuron dendrites occupy most of the ventral horn and reach dorsally beyond the central canal (Hermann et al., 1998; Peshori et al., 1995; Sasaki, 1994). In the cat, the sphincter motor neuron dendritic arbors have been shown to extend into the regions where functionally associated preganglionic neurons in the sacral parasympathetic nucleus are located (Beattie et al., 1990). For example, the external anal sphincter motor neurons project dendrites into the dorsal band of the sacral parasympathetic nucleus where the colonic preganglionics are located and the external urethral sphincter motor neurons into its lateral band where the bladder preganglionics reside. Similarly, the bulbospongiosus motor neurons in the rat extend profuse dendrites into the dorsal commissural region (Figure 1E) where interneurons important in sexual reflex function are located (Nadelhaft and Booth, 1984; Peshori et al., 1995; Nadelhaft and Vera, 2001).

Pudendal nerve afferent neurons are located in the L6 and S1 dorsal root ganglia (Nadelhaft and Booth, 1984). In both sexes, pudendal afferent fibers in the spinal cord are located in the dorsal columns, the medial half of Lissauer's tract, the extreme medial edge of the dorsal horn, both ipsilaterally and contralaterally, and in a large terminal field in the dorsal gray commissure. No afferents have been detected in the intermediate or ventral gray (McKenna and Nadelhaft, 1986).
**Supraspinal control mechanisms**

Although in the rat eliminative and sexual reflex patterns are mainly organized at the spinal level, there is evidence for supraspinal centers that may modify the functional response. These supraspinal inputs strongly appear to influence the magnitude and coordination of these reflexes, and generally this descending supraspinal input is inhibitory to the lumbosacral reflexes.

Retrograde tracer injections into the ventromedial gray of the lumbar spinal cord labeled supraspinal input primarily from the vestibular nuclei, the gigantocellular reticular nuclei, the medullary raphe nuclei, and the hypothalamic paraventricular nuclei (Marson *et al.*, 1992; Marson and McKenna, 1990; Monaghan and Breedlove, 1991; Shen *et al.*, 1990). The ventrolateral gray of the lumbar spinal cord has supraspinal input from the dorsolateral pontine tegmental region, also referred to as Barrington’s nucleus or the pontine micturition center (Ding *et al.*, 1995). Furthermore, transneuronal tracing studies using pseudorabies virus injections directly into the muscles involved in pelvic floor reflexes have consistently confirmed these same regions to be the source of supraspinal input (Marson and McKenna, 1996; Tang *et al.*, 1999; Vizzard *et al.*, 2000). Some studies have focused on supraspinal input originating from the nucleus raphe obscurus in the brainstem and the region in the ventrolateral medulla referred to as the gigantocellular – lateral paragigantocellular complex. Projections from this latter area are widespread and include fibers in close apposition to external anal sphincter and bulbospongiosus motor neurons, and although targets from the nucleus raphe obscurus are much more restricted, they also include contacts
between nucleus raphe obscurus terminals and bulbospongiosus and external anal sphincter motor neurons (Hermann et al., 1998; Hermann et al., 2003). More specifically, fibers from the nucleus raphe obscurus ramify in close apposition to both bulbospongiosus and external anal sphincter motor neurons and terminals of nucleus raphe obscurus projections in the lumbosacral spinal cord have been shown to co-localize with serotonin, thyrotropin releasing hormone, and substance P (Ramirez-Leon et al., 1994).

In fact, the nucleus raphe obscurus is thought to be an overall regulator of autonomic functions controlled by both cranial and spinal autonomic efferents. Rostral portions of the nucleus raphe obscurus project to the dorsal vagal complex, which controls proximal digestive functions (McCann et al., 1989), whereas caudal portions of the nucleus raphe obscurus project to the caudal intermediolateral cell column, sacral parasympathetic nucleus, and related somatic motor neurons, which control distal digestive and other pelvic autonomic functions. Also, the nucleus raphe obscurus maintains direct projections to both autonomic and somatic regions and to regions of the intermediate gray, which contain putative interneurons that contribute to the organization of pelvic floor reflexes. The nucleus raphe obscurus is thus in a position to modulate autonomic preganglionic and functionally related skeletal motor neuron activity (Hermann et al., 1998).

Electrical stimulation of the nucleus raphe obscurus results in reduction of spontaneous anorectal activity, providing evidence for a direct brainstem inhibitory circuit (Holmes et al., 1997a). Also, nucleus raphe obscurus lesions
elicit transient increases in anorectal reflex activity (Beattie et al., 1996; Holmes et al., 2002) and disrupt male copulatory behavior in rats (Yamanouchi and Kakeyama, 1992). However, nucleus raphe obscurus lesions do not have an effect on measures of ex copulatory penile reflexes (Holmes et al., 2002). In contrast, lesions of the gigantocellular – lateral paragigantocellular complex did not affect measures of external anal sphincter activity but altered penile reflexes significantly (Holmes et al., 2002). It has been shown that the bulbospongiosus muscle displays electromyographic activity during the passage of urine (Sachs and Leipheimer, 1988; Schmidt, M. H. et al., 1995), and it is suggested that the nucleus raphe obscurus projections may be specific to pudendal eliminative reflexes, while other descending brainstem projections are specific to sexual reflexes (Holmes et al., 2002).

Projections from the gigantocellular – lateral paragigantocellular complex are diffuse, descending to all levels of the spinal cord affecting sensory, motor, and autonomic control circuits beyond those for pudendal reflexes. The gigantocellular – lateral paragigantocellular complex may globally modulate multiple behaviors with somatic and autonomic components such as reproductive reflexes, acoustic startle, and nociception as proposed by (Hermann et al., 2003)

Neurotransmitters implicated in the descending supraspinal control of pelvic and pudendal motor neurons include serotonin, thyrotropin-releasing hormone, and GABA. Medullary thyrotropin-releasing hormone neurons diffusely project to the ventral horn of the spinal cord and sympathetic neurons within the intermediolateral cell column (Appel et al., 1987; Hirsch and Helke, 1988). When
applied intrathecally at the level of the L5-L6 spinal cord, thyrotropin-releasing hormone resulted in contraction of the internal anal sphincter, predominantly through activation of the pelvic nerve. Although sectioning of the hypogastric nerve had no effect on thyrotropin-releasing hormone-induced internal anal sphincter activity, a role for thyrotropin-releasing hormone activation of sympathetic preganglionic neurons should not be excluded since the pelvic nerve has been shown to contain both sympathetic and parasympathetic fibers (Hulsebosch and Coggeshall, 1982). It is suggested that the relative roles of the pelvic and hypogastric nerves in internal anal sphincter contractility differ with changes in activity of rectal afferents (Holmes et al., 1995). Also, pudendal motor neurons are under spinal descending inhibitory control. Similar to the effect thyrotropin-releasing hormone has on the internal anal sphincter, high doses of thyrotropin-releasing hormone applied to the lumbosacral spinal cord resulted in an increase in firing rate of the external anal sphincter (Holmes et al., 1997b). Thus, thyrotropin-releasing hormone appears to be involved in maintaining fecal continence. Other studies have shown that thyrotropin-releasing hormone plays a critical role in modulation of gastrointestinal function (McCann et al., 1989). Thyrotropin-releasing hormone has been shown to increase gastric secretion and motility, intestinal transport, and is thought to play a role in stress-induced increases in defecation (Miyata et al., 1992). Although thyrotropin-releasing hormone appears to have an excitatory effect on external anal sphincter motor neurons, intrathecally applied thyrotropin-releasing hormone has an inhibitory effect on penile erections, suggesting that although external anal sphincter and
bulbospongiosus motor neurons are co-mingled within the same spinal nucleus, they are discretely and differentially regulated by separate neural circuits (Holmes et al., 1997b).

**Eliminative functions following spinal cord injury**

Following acute injury to the spinal cord an initial period of “spinal shock” occurs, that is characterized by areflexia and generally lasts for approximately 24 hours in humans (Ditunno et al., 2004). Disruption of spinal cord tracts proximal to the lumbosacral cord, leads to loss of supraspinal control over normal eliminative and reproductive behaviors. These functions, which reflect coordinated activity of both somatic and autonomic components of the nervous system, are important therapeutic targets as they represent critical problems for the spinal cord injured population, and have been less well studied than locomotion in models of SCI (Anderson, 2004). After the initial post injury period of spinal shock the basic spinal reflexes control these functions independent from supraspinal input. During the recovery phase from spinal shock (1 day – 12 months) a gradual return of reflexes may take place as well as the development of hyperreflexia and spasticity (Ditunno et al., 2004). Interestingly in the rat, the initial loss and subsequent recovery of functions that have autonomic components show a similar pattern of recovery as locomotor function (Holmes et al., 2005) suggesting commonality of underlying recovery mechanisms.

In rat models, in addition to loss of control of eliminative and sexual function, the lumbosacral reflexes develop hyper-reactivity, including increased
external anal sphincter contractions after distention and hyper-reflexia of erections in response to slight tactile stimuli (Holmes et al., 1998; Holmes et al., 2005). This is consistent with the finding in humans, in which removal of supraspinal control leads to spasticity and dyssynergia of urethral and anal sphincters. In addition to hyperreflexia of the external anal sphincter, it is well recognized that hyperreflexia of the detrusor occurs after SCI, as well as detrusor-sphincter-dyssynergia.

We suggest that in addition to these muscles, other pelvic floor muscles, and specifically the bulbospongiousus musculature develops hyperreflexia and spasticity following SCI. This change in reflex activity is reminiscent of hyperreflexia observed in other segmentally mediated reflexes (Bose et al., 2002). Interestingly, McKenna et al. (McKenna et al., 1991), observed simultaneous contraction of all pelvic floor musculature (bulbospongiosus, ischiocavernosus, external anal sphincter and external urethral sphincter) during the urethrogenital reflex after spinal cord transection. This reflex is induced by stimulation of the urethra and these authors suggest that the motor program is controlled by a spinal pattern generator in the absence of descending input and that the spinal circuitry is responsible for the rhythmic, coordinated bursting activity of all these muscles. Certainly, the extensive co-mingling of the dendrites of these motor neurons in the spinal cord (as well as their electrotonic and synaptic coupling) could be an anatomical substrate for this and other coordinated activities (Coolen et al., 2004; Giuliano and Rampin, 2004).

A closer examination of the bulbospongiousus muscle activity after
contusion injury of the spinal cord (Nout et al., 2005) in awake behaving rats, shows that the loss of descending input produces an exaggerated contraction pattern in this muscle similar to that seen in the external anal sphincter. This activity was observed during erectile events as well as during micturition. Using pressure recordings from the corpus spongiosum penis it is possible to reliably assess both micturition and erectile events (Nout, Y.S. et al., 2004; Schmidt, M.H. et al., 2004). Following spinal cord damage, increased corpus spongiosum penis mean pressures and increased duration of micturition as well as of erectile events occur. Increased corpus spongiosum penis pressures indicate bulbospongiosus muscle spasticity, since pressure within the bulb of the corpus spongiosum penis is directly dependent on the activity in the surrounding bulbospongiosus musculature (Schmidt, M. H. et al., 1995).

In addition to loss of descending inhibitory control of spinal reflex pathways, plasticity within the spinal cord is thought to contribute to recovery but also to development of exaggerated spinal reflex responses. For example, Weaver and her colleagues have shown that sprouting of dorsal root afferents containing calcitonin gene-related peptide may contribute to the development of autonomic dysreflexia after severe SCI (Weaver et al., 2001). Similarly, calcitonin gene-related peptide fibers contributing to the parasympathetic components of bladder, bowel, and sexual reflex function have also been demonstrated to sprout after SCI in cats (Beattie et al., 2000b). Spinal cord injury, in this species, produces a chronic (measured at 6 weeks) denervation of identified parasympathetic preganglionic neurons (Beattie et al., 1993); the proportion of
the somatic and proximal dendritic membrane of these efferent neurons
contacted by synaptic terminals is significantly reduced, the size of the terminals
is smaller, and glial coverage is reciprocally increased. In additional studies, an
increase in the proportion of the membrane area contacted by calcitonin gene-
related peptide immunoreactive terminals appears to increase (Beattie et al.,
2000) suggesting that the effect of such input might be even more functionally
significant after SCI. Interestingly, the synaptic inputs to Onuf’s nucleus in the
same studies, showed similar synaptic rearrangements, i.e. reduced size of
terminals apposed to Onuf’s motor neurons after SCI (Beattie et al., 1993;
Behrmann et al., 1993), and an increase in their terminal coverage by GABA
immunoreactive terminals (Beattie et al., 2000b). The plasticity that is seen in this
area of the cord forms an important target for potential therapeutic interference.
For example, it has been shown that interference in the development of the
primary afferent plasticity affected the development of autonomic dysreflexia
(Weaver et al., 2001) and detrusor-sphincter-dyssynergia (Seki et al., 2004).
Recently, Cameron et al. (Cameron et al., 2004) presented a strategy for
modulation of post-traumatic spinal plasticity in both sacral afferents and
propriospinal projection neurons via targeted gene therapy in a model of
autonomic dysreflexia.

2.2 Micturition

When compared to defecatory and sexual function, urinary function has
been studied most extensively in animal models of SCI. Upper urinary tract
function relates to the production of urine in the kidneys and continuous supply of
urine into the bladder through two ureters. Lower urinary tract function involves
storage and complete controlled emptying of urine from the bladder. The lower
urinary tract is comprised of the bladder, internal and external sphincters, and
urethra. The smooth muscle in the bladder wall is termed the detrusor muscle.
Detrusor muscle contraction is mediated primarily by parasympathetic
stimulation. Parasympathetic control of the detrusor muscle originates from the
preganglionic axons in the pelvic nerve. The hypogastric nerve supplies the
sympathetic input to the detrusor muscle. Sensory information from the bladder
wall travels within the pelvic nerve to the spinal cord. The internal sphincter is
located at the junction of the bladder and urethra and is, similar to the bladder
wall, also comprised of smooth muscle. Similar to the detrusor muscle, the
internal sphincter receives sympathetic and parasympathetic innervation;
however, in the internal sphincter sympathetic stimulation causes contraction.
The external sphincter is comprised of striated muscle that surrounds the urethra
and is innervated through the pudendal nerve.

The storage function of the bladder is mediated predominantly through two
components of the sympathetic innervation. The first is closure of the bladder
outlet through contraction of the bladder neck and internal sphincter which is
mediated through α-adrenergic receptors. The second is relaxation of the bladder
wall which is mediated through β-adrenergic receptors. Under normal
circumstances filling of the bladder occurs with little increase in bladder pressure.
As bladder filling increases, sensory input from the bladder wall to the sacral
spinal neurons increases until the threshold for the micturition reflex is reached. For voiding the bladder, a coordinated process is required that involves detrusor muscle contraction in combination with relaxation of the internal and external sphincters, urethra, and pelvic floor musculature. Under normal circumstances this process is under voluntary control and occurs through well-regulated and integrated autonomic and somatic reflexes, but after SCI changes occur at the level of the bladder and spinal cord that result in malfunction of the lower urinary tract.

The complete loss of descending input initially causes an absence of bladder emptying which is gradually replaced by spinal reflex circuit activity that is initiated by a different population of afferents (e.g. c-fiber afferents) than in the normal condition (de Groat et al., 1998; de Groat et al., 1990; de Groat and Yoshimura, 2005). In most cases, reflex detrusor activity reappears 2 – 12 weeks after the spinal shock phase (Chancellor and Blaivas, 1996; Fam and Yalla, 1988). The effect of incomplete SCI on lower urinary tract function depends on the severity of the primary injury (Wrathall and Emch, 2005). While some recovery of this function occurs, residual deficits remain in the coordination of the autonomic efferents to the detrusor muscle and the somatic efferents to the external urethral sphincter muscle (so-called detrusor-sphincter-dyssynergia) producing inadequate bladder emptying. When the supraspinal, coordinated control of these systems is lost, the resulting bladder dysfunction is termed “neurogenic bladder impairment” or a “neurogenic bladder” (Potter, 2005).

Detrusor-sphincter-dyssynergia is thought to be the main reason for the
observed increased voiding pressures and decreased voiding efficiency, and has been documented in rats (Cheng and de Groat, 2004; Kruse et al., 1993; Pikov et al., 1998; Yoshiyama et al., 2000). Although external urethral sphincter bursting activity can be mediated by spinal reflex mechanisms, the bursting activity that is seen after SCI is abnormal, leading to shorter urethral opening times and this presumably contributes to the inefficient voiding and increased voiding pressures (Cheng and de Groat, 2004). Also, the amplitude from external urethral sphincter electromyographic recordings has been shown to increase following SCI (Pikov and Wrathall, 2001). Suppression of external urethral sphincter activity results in improvement of all voiding parameters (Kruse et al., 1993; Yoshiyama et al., 2000). In addition to dyssynergia, detrusor hyperreflexia has been well characterized in rats following SCI (Mitsui et al., 2003) and is thought to be due to a lack of supraspinal inhibition with or without an increase of afferent signaling. Moreover, enlargement of the bladder may result in plasticity of afferents, further contributing to this hyperreflexia (de Groat et al., 1998). The significance of this syndrome in the recovery of micturition, however, is unknown. Recovery of micturition function has been observed to occur through amelioration of detrusor-sphincter-dyssynergia without noticeable change of detrusor hyperreflexia (Mitsui et al., 2003). The authors of this report suggest that the improved external urethral sphincter function and lack of concomitant improved detrusor muscle function, reflects comparatively easier recovery of somatic nerves vs. autonomic nerves (Mitsui et al., 2003).

Studies in animals indicate that recovery of bladder function after SCI is
dependent on reorganization of reflex pathways in both the peripheral and central nervous systems. The ultimate goal of research is restoration of normal function, however providing better management of the neurogenic bladder is an important objective.

2.3 Defecatory function

Defecatory function after SCI has been least studied compared to micturition and sexual function, however, exhibits similar responses to the loss of descending control causing inadequate voiding, fecal impaction and chronic constipation in man (Cosman et al., 1991; Frenckner, 1975) due, perhaps, to dyssynergia and external anal sphincter spasticity (Holmes, 2005; Holmes et al., 1998).

The most distal portion of the gastrointestinal tract is formed by the rectum and anus. Innervation of the rectum is similar to that of the colon, but innervation of the anus is more complex (for a comprehensive review, see Janig and McLachlan, 1987). The anus controls passage of fecal matter by the degree of constriction of the internal and external anal sphincters (Gonella et al., 1987; Schuster, 1968). The internal anal sphincter is formed by thickening of circular smooth muscle that lies immediately inside the anus. Similar to the rest of the gastrointestinal smooth muscle, the internal anal sphincter contains a myenteric plexus within the tunica muscularis. Furthermore, the internal sphincter muscle receives autonomic innervation via the pelvic plexus. The external anal sphincter is composed of striated muscle that surrounds the internal anal sphincter and
extends distal to it. The external sphincter is controlled by nerve fibers in the pudendal nerve, which in the rat has motor, sensory and autonomic components (Holstege and Tan, 1987; Katagiri et al., 1986; Paxinos, 1995; Rexed, 1954). While the internal anal sphincter is the main mechanism for continence, accounting for the majority of the resting pressure, subconsciously the external sphincter is usually kept constricted unless conscious signals inhibit constriction (Gonella et al., 1987). Motor units of the external anal sphincter have been shown to be tonically active (Gonella et al., 1987; Krier, 1985), whereas the other pudendal motor units are effectively quiescent (Holmes et al., 1994).

The rectum and anal canal are supported by the pelvic diaphragmatic musculature, which participates in the functions of fecal continence and defecation. Paired rectococcygeal muscles originate on each side of the rectum and attach dorsally to the base of the tail. These smooth muscles are innervated by autonomic fibers from the pelvic plexus and shorten the rectum during defecation to assist evacuation of feces. Laterally the illeocaudalis and pubocaudalis and the coccygeus muscles surround the rectum. These striated muscles are innervated by the L6 to S1 nerves in the rat (Bremer et al., 2003) and help to compress the rectum during defecation.

In normal animals and humans, defecation is initiated by defecation reflexes (Gonella et al., 1987; Guyton and Hall, 2000). One of these reflexes is an intrinsic reflex mediated by the local enteric nervous system in the rectal wall. Distention of the rectal wall initiates afferent signals that spread through the myenteric plexus to initiate peristaltic waves in the descending colon, sigmoid,
and rectum, forcing feces toward the anus. As the peristaltic wave approaches the anus, the internal anal sphincter is relaxed by inhibitory signals from the myenteric plexus. A rectoanal inhibition reflex occurs in which as the rectum fills and the internal anal sphincter relaxes, the external anal sphincter contracts involuntarily and defecation only occurs following further rectal distension inducing concomitant external anal sphincter relaxation (Gonella et al., 1987).

This local intrinsic defecation reflex, however, is by itself relatively weak and usually must be fortified by another type of defecation reflex, a parasympathetic defecation reflex that involves the sacral segments of the spinal cord. When nerve endings in the rectum are stimulated by distension, signals are transmitted first into the spinal cord and then reflexively back to the descending colon, rectum, and anus by way of parasympathetic nerve fibers in the pelvic nerves. These parasympathetic signals greatly intensify the peristaltic waves and relax the internal anal sphincter and thus convert the intrinsic myenteric defecation reflex from a weak effort into a powerful process of defecation.

The afferent defecation signals entering the spinal cord initiate other effects such as closure of the glottis and contracture of the abdominal wall muscles to force fecal contents of the colon downward and at the same time cause the pelvic floor to relax downward and pull outward on the anal ring to evaginate feces. In the rat reflex defecation is mainly organized at the spinal level but there is evidence for supraspinal centers that may modify the functional response (Maggi et al., 1988). These are described in more detail in the general section of this chapter.
In human patients with SCI, loss of input from proximal to the lesion to the bowel results in reduced transit through the bowel. Additionally, coordination of autonomic and somatic defecation reflexes is lost secondary to disruption of supraspinal control pathways. Compressive destruction of the conus medullaris of the spinal cord can destroy the distal segments of the cord where, in humans, the cord defecation reflex is integrated, and this almost always paralyzes defecation. More frequently the spinal cord is injured more proximal, between the conus medullaris and the brain, in which case the voluntary portion of the defecation act is blocked while the basic spinal cord reflex for defecation is still intact. As described above, there is evidence for development of increased anal sphincter resting pressure or hyperreflexia after SCI (Holmes et al., 1998; Holmes et al., 2005; Sun et al., 1995). A greater understanding of the role of pelvic reflexes in controlling evacuation and continence is required for development of neuromodulation strategies to improve quality of life after SCI (Chung and Emmanuel, 2005; Lynch and Frizelle, 2005).

2.4 Sexual function

Spinal cord injury has an enormous impact upon sexual function of both men and women. Male fertility and sexual function are greatly impaired by SCI with less than 5% of men able to procreate without medical intervention (Talbot, 1955; Thomas, 1983). In women laboratory studies have shown that 44% of women with SCI were able to achieve orgasms compared to 100% of able-bodied controls (Sipski et al., 2001). Regaining sexual function is the highest
priority for paraplegics as demonstrated in a recent survey (Anderson, 2004).

Our understanding of the complex neural pathways and mechanisms underlying sexual function and behavior is limited, but sexual function requires participation of autonomic and somatic nerves and integration with two interconnected portions of the spinal cord, which in turn make connections with the brainstem and cerebral cortex (Giuliano and Rampin, 2000, 2004; Hubscher, 2005; McKenna, 2000; Steers et al., 1988). Similarly as described for micturition and defecation function, the main peripheral nerves involved in sexual function are the pelvic, hypogastric, and pudendal nerves.

Penile erection is a vascular event associated with tumescence of the cavernous bodies and is only a single component of the complex sexual behavior. For a detailed review see (Giuliano and Rampin, 2000, 2004). In humans, erection occurs in response to tactile, visual, and imaginative stimuli. In animals olfactory and auditory cues are also important stimuli. Parasympathetic innervation, through the pelvic nerve, provides the major excitatory input, responsible for vasodilation of the penile vasculature and erection. Sacral preganglionic fibers travel to the pelvic plexus in the pelvic nerve. The cavernous nerve exits the pelvic plexus and innervates different penile structures. Since the pelvic plexus receives sympathetic input as well, the cavernous nerve contains parasympathetic and sympathetic fibers, providing the vasodilator and vasoconstrictor input to penile smooth muscle. Although the sympathetic nervous system appears to play a role in detumescence of the penis, this pathway may facilitate erections after injury to parasympathetic pathways. Sympathetic
preganglionic nerve fibers to the penis originate from neurons in the intermediolateral cell column and intercalated nucleus of the T9-L2 spinal cord segments. These preganglionic neurons receive input from supraspinal sites and pelvic viscera. Sympathetic axons travel to the penis through the pelvic, cavernous and pudendal nerves.

The external genitalia and perineum are innervated by somatic myelinated and unmyelinated afferent fibers. These fibers convey information to the lower lumbar and upper sacral segments of the spinal cord via the pudendal nerve and via branches of the pelvic, genitofemoral, ilioinguinal, and anococcygeal nerves (Hubscher, 2005). These autonomic afferents travel in the dorsal nerve of the penis, which is one of the branches of the pudendal nerve. Afferent input from the penile skin, prepuce, and glans conveyed by the dorsal nerve of the penis initiates and maintains reflexogenic erections.

The reproductive structures innervated by the hypogastric nerve are involved in contraction and movement of substances. The hypogastric nerve is believed to play a role in sperm motility and ejaculation. Sectioning of this nerve does not have an effect on mating behavior (Hubscher, 2005). For a detailed review on regulation of ejaculation see (Coolen et al., 2004).

The sacral spinal cord is essential for penile erection. Destruction of the sacral spinal cord or its outflow, including the conus medullaris, abolishes erections. Reflexive erections are mediated by a sacral spinal reflex with a sensory input through the dorsal nerve of the penis, and motor pathways
consisting of sacral parasympathetic nerves conveyed by the pelvic and eventually cavernous nerves. Supraspinal excitatory and inhibitory mechanisms within the brain and spinal cord coordinate and integrate these neural inputs. In the cat and rat, somatic afferents from the pudendal and cavernous nerves terminate in the medial spinal cord (laminae III and IV) and dorsal commissure (lamina X) of the sacral spinal cord. Afferents in the cavernous nerve terminate in Lissauer’s tract (lamina I), the lateral and medial collateral pathways and just dorsal to the parasympathetic preganglionic nucleus.

After SCI, sexual reflexes are disrupted similar to micturition and defecatory reflexes. Two distinct control mechanisms induce penile erections in humans: reflexogenic and psychogenic. Spinal cord injury above the sacral level will not only preserve this reflex but can even enhance it, especially if the lesion is complete, such as seen in priapism (Elliott, 2005). The ability to have reflex erections is lost if the sacral spinal cord is injured or if the pudendal and / or pelvic nerves are damaged (Elliott, 2005). Psychogenic erections occur in men with an intact nervous system in response to various stimuli and men with injuries to their sacral spinal cord are dependent on intact psychogenic pathways for erectile activity. Reflexogenic and psychogenic mechanisms probably act synergistically to determine erectile response. Although 95% of men with complete spinal cord injury and upper motor neuron lesions experience penile erections, the erection is often of poor quality or poorly sustained (Brown et al., 2005). Clinical literature has indicated that thoracolumbar contusion produces transient priapism following tactile stimulation, suggestive of the presence of
descending inhibitory pathways that normally suppress this segmental excitatory reflex. In humans, loss of descending input results in facilitated spinally mediated reflex erectile activity but disruption of ejaculatory function and seminal fluid composition (Basu et al., 2004; Higgins, 1979). Although currently an erection can be provided through safe and effective erection enhancement methods, neither ejaculatory dysfunction nor penile sensation and orgasm can be reversed (Elliott, 2005).

2.5 References


Li Q, Beattie MS, Bresnahan JC: Onuf's nucleus (ON) motoneurons (MNs) have more GABA-ergic synapses than other somatic MNs: a quantitative immunocytochemical study in the cat. Presented at Society for Neuroscience, 1995.


3.1 Introduction

In animal models of spinal cord injury (SCI) emphasis in general lies on examination of locomotor function. Outcome measurements examining various aspects of locomotor function exist in large numbers. However, in humans, impairment of lower urinary tract function following SCI and its subsequent complications are highly prevalent and clinically very important (Anderson, 2004; Noreau et al., 2000; Schmidt and Schmidt 2004). The state of urologic function is critical and continued investigation of pathophysiology and treatments to improve recovery of this aspect of autonomic function are, therefore, essential.

Existing methods to examine micturition function in rats include cystometric and electromyographic (EMG) techniques (Conte et al., 1988; Kruse et al., 1993; Pikov et al., 1998; Pikov and Wrathall, 2001) and monitoring animals in metabolic cages (Haas et al., 1997; Sakamoto et al., 2000; Schmidt, F. et al., 2001). Limitations of these techniques are use of anesthesia or heavy sedation and placement of catheters in bladder or urethra, both of which are known to
alter the micturition reflex (Cannon and Damaser, 2001; Conte et al., 1988; Yoshiyama et al., 1999). Furthermore, animal well-being may be in jeopardy when animals are placed on a wire mesh surface in metabolic cages for extended periods of time, particularly when animals are paralyzed. Existing methods to study sexual function in rats include animal observation in either copulatory (Hart, 1968) or non-copulatory (Sachs et al., 1994) settings and ex copulatory reflex erection tests (Holmes et al., 1988; Sachs, 1985). Limitations of these tests include difficulty in accurately recognizing various aspects of rat sexual behavior and non-physiologic testing situations.

Investigators of penile erection physiology have designed a technique to monitor erectile events in awake rats by continuously recording pressures within the corpus cavernosum of the penis (CCP) (Giuliano et al., 1994) or the corpus spongiosum of the penis (CSP) (Schmidt, M. H. et al., 1994). During erections blood enters the cavernous sinuses of the CCP and CSP causing expansion of the spongy erectile tissue and the penis to become engorged and erect. The exact roles that the ischiocavernosus (IC) and bulbospongiosus (BS) muscles, two muscles of the pelvic floor that lie over the CCP and CSP respectively, play in this process is not fully elucidated; however both of these muscles have been shown to display electromyographic (EMG) activity during visually confirmed erections (Beckett et al., 1973; Beckett et al., 1975; Holmes et al., 1991; Holmes and Sachs, 1992; Schmidt, M. H. et al., 1995) as shown in Figure 3.1.
Figure 3.1: Electromyographic activity of the BS and IC muscles during erectile events. Examples of erectile events involving several glans erections and flips of the penile body are shown. Note that the entire erectile event is associated with an increase in baseline CSP pressure. Glans erections (E1, E2, E3) were directly associated with BS muscle bursts and flips of the penile body (arrowheads) were associated with IC muscle bursts. CSP = corpus spongiosum penis; BS = bulbospongiosus muscle; IC = ischiocavernosus muscle; EMG = electromyogram (From: Schmidt et al., 1995)

Contraction of the IC muscles is considered critical for the development of penile rigidity and contraction of the BS muscle is considered essential for ejaculation. To what extent the BS muscles are involved in penile rigidity, and what the role of BS muscle contraction is during non-ejaculatory erections is not clear (Schmidt, M. H. and Schmidt, 1993).

When CCP and CSP pressures are evaluated during erections, one can distinguish a vascular-subsystolic tumescence phase that is followed by
development of suprasystolic pressure peaks. The subsystolic tumescence phase is considered a purely vascular event characterized by passive filling of the tissues with blood. In CSP pressure recordings this phase has been shown to have the same pulsatile frequency as electrocardiogram recordings and is thus a direct reflection of arterial blood flow (Schmidt, M. H. et al., 1995). The suprasystolic pressure peaks, however, are considered to be a result of muscular contractions that consequently augment penile rigidity. Pressure peaks that occur in the CCP are considered secondary to IC muscle contractions (Beckett et al., 1973; Beckett et al., 1974; Purohit and Beckett, 1976) and pressure peaks in the CSP have been shown to occur secondary to BS muscle contractions (Beckett et al., 1975; Schmidt, M. H. et al., 1995; Schmidt, M. H. et al., 1994). Muscular pressure peaks generated in the CCP reach pressures of 714 mmHg in rats (Bernabe et al., 1999), 6530 mmHg in stallions (Beckett et al., 1973), 7434 mmHg in dogs (Purohit and Beckett, 1976), and 1100 mmHg in humans (Meehan and Goldstein, 1983). Muscular pressures generated in the CSP remain far below those of the CCP (approximate maximum of 600 – 700 mmHg), supporting the concept that during erections, pressures in the CCP are generated in a closed cavernous system, whereas the cavernous system of the CSP remains an open vascular system (Schmidt, M. H. and Schmidt, 1993). Since the pressures that are generated within these cavernous spaces are so large, it is essential that nonsaturating pressure transducer systems are used (Schmidt, M. H. and Schmidt, 1993; Schmidt, M. H. et al., 1995).
Traditionally, monitoring of CCP pressure is considered the standard method for evaluating erectile function since BS muscle activity and thus CSP pressure is primarily associated with ejaculatory function. However, erections appear to be consistently associated with pressure changes in the CSP (Schmidt, M. H. et al., 1995; Schmidt, M. H. et al., 1994) suggesting that monitoring of CSP pressure is also a valid method of assessing erectile events. Moreover, monitoring CSP instead of CCP pressure carries two distinct advantages. First, the bulb of the CSP is more vascular and contains less dense fibrous tissue than the CCP allowing easier implantation and longer recording times and second, preliminary data suggests that CSP recording allows for simultaneous recording of erectile (Schmidt, M. H. et al., 1995) and micturition events (Schmidt, M.H. et al., 2004).

Monitoring CCP and CSP pressure is currently performed using telemetry. The technique of telemetry is based on transmission of AM radio waves that are recorded, stored, and processed by software. More specifically, for recording penile erections using the technique described by Giuliano et al. (1994) and Schmidt et al. (1995) a pressure transducer is implanted in the CCP or CSP, respectively and pressure changes are detected by the transducer and transmitted to a receiver unit through AM radio waves. The receiver unit collects these signals and transmits them to a computer system with software that processes and stores the collected data (Figure 3.2).
The goal of this study was to verify that pressure waveforms obtained from the CSP by telemetry were consistently associated with either micturitions or penile erections. Furthermore we hypothesized that a positive relationship exists between the duration of the micturition pressure waveform and volume of urine expelled. Here we demonstrate the use of CSP pressure monitoring in the assessment of micturition and sexual function in 10 normal, freely-moving male rats.
3.2 Materials and Methods

Subjects

Ten adult, male Long-Evans hooded rats (Simonsen Laboratories, Gilroy, CA, USA) age 70 ± 3 days (AVG ± SE), were used in this study. Rats were housed individually in plastic cages, maintained on a 12 hour light/dark cycle, and had free access to food and water. All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with NIH guidelines and recommendations.

Surgical Procedures and Post-Operative Care

Transducer implantation was carried out aseptically under deep anesthesia induced by intraperitoneal administration of xylazine (TranquillVed™, Vedco Inc., St. Joseph, MO, USA; 10 mg/kg) and ketamine (ketamine HCl, Abbott Laboratories, N.Chicago, IL, USA; 80 mg/kg). Anesthetic plane was determined by withdrawal to foot pinch. A pre-operative dose of cefazolin (Ancef, Abbott Laboratories, N.Chicago, IL, USA; 50 mg/kg) was administered subcutaneously. Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA, USA) was applied to the eyes prior to surgery and body temperature was maintained at 37.5 ± 0.5°C using a rectal thermal probe and heating pad.

The rat was placed in dorsal recumbency and surgical sites were shaved and cleaned with betadine. A 3 cm skin incision was made on midline on the raphe scroti and a 3 – 4 cm skin incision was made parallel to midline in the right
inguinal area. A telemetric pressure transducer catheter (TA11PA-C40, Data Sciences International, St. Paul, MN, USA) as shown in Figure 3.3 was implanted in the bulb of the CSP as described previously (Schmidt, M. H. et al., 1995).

![Figure 3.3: TA11PA-C40 pressure transducer used in these studies.](image)

Briefly, the battery of the transducer was placed subcutaneously in the lower right abdomen and secured to the external oblique abdominal muscle with 2 sutures. The catheter was tunneled subcutaneously to the perineal area. Testes were retracted and BS muscle and CSP were exposed (Figure 3.4).
With minimal retraction of the BS muscles, a guide hole was made into the distal bulb of the CSP with a 21 gauge needle. The open tip of the catheter was placed into the bulb of the CSP and secured with biological glue and 1 suture (Figure 3.4). The scrotal incision was then closed in 1 layer and the inguinal incision was closed in 2 layers. The rat was allowed to recover. Figure 3.5 demonstrates some aspects of this surgical procedure. Telemetric studies were commenced 4 – 5 days post operatively.
Figure 3.5 A-C: Transducer implantation procedure. In **A** the scrubbed surgical area is shown. **B** shows the transducer battery in place and the catheter being held with forceps. In **C** the surgical area is shown approximately 14 days post operative.

**CSP Pressure Recording**

Physiological telemetry data were recorded on a PC computer using a Spike2 data acquisition program (version 3.1, Cambridge Electronic Design Limited, Science Park, Milton Road, Cambridge CB40FE, U.K.) (Figure 3.6 A). Level of saturation of our system is 400 – 500 mmHg. The 10 animals underwent 2 x 24 hour CSP pressure recording while housed in a plastic cage with a wire mesh floor. Colored paper was mounted on a scale underneath the mesh floor of the cage and this was recorded on video. This made it possible to see when a micturition event occurred and the volume was based on change in scale readout (1ml = 1mg). The receiver unit of the telemetry system was placed on top of the cage. This experimental condition is shown in Figure 3.6 B. To avoid time discrepancies, the camera time was recorded in a simultaneously running event channel of the acquisition program and used as the start time. In this way the
time and approximate volume of each micturition could be determined and correlated to the pressure wave characteristics. During the dark hours a red light was placed over the colored paper to facilitate detection of micturition events.

Four rats were recorded for a 24 hour period weekly for 2 months in order to determine quality of chronic recordings. These recordings were performed while animals were housed in their regular cages as shown in Figure 3.6 A.

![Figure 3.6 A,B: Experimental condition for telemetry recording. In A the condition for recording CSP pressure in freely moving rats is shown. A cage is placed on top of a receiver and is connected to a computer system that collects and displays pressure waves. In B the condition is shown that was used to verify simultaneous occurrence of a micturition event on video and computer readout. As shown the receiver unit is placed on top of the cage allowing urine to fall on the scale.](image_url)

CSP pressure recording during behavior tests was performed to verify simultaneous occurrence of observed erectile events and wave forms characteristic of erections on the computer readout. This was done by placing the
receiver unit underneath either the restraining cylinder during ex copulatory erection tests or underneath the cage in which mating tests were performed.

For all rats CSP pressure data were analyzed for the quantity, quality, and time of occurrence of micturitions during the 24 hour period. During erection tests observed erectile events were recorded on the event channel during simultaneous data acquisition.

For 7 animals the duration of each micturition event was obtained from CSP wave forms for 1 (2 rats) or 2 (5 rats) 24 hour time periods and was directly compared to the volume of that micturition obtained from the scale readout.

**Behavioral Tests**

*Ex copulatory reflex erection tests:*

Ex copulatory reflex erection tests were performed twice in 10 awake animals as previously described (Hart, 1968; Holmes *et al.*, 1988; Schmidt, M. H. *et al.*, 1995). Briefly, rats were placed on a board in dorsal recumbency with the head and anterior torso in a loose-fitting restraining cylinder. The abdomen was secured to the board with masking tape. This is shown in Figure 3.7 A. The head and anterior torso could move freely in the cylinder. The telemetric receiver unit was placed directly under the board. The preputial sheath was retracted, which is the stimulus to elicit reflex erections, and maintained in retracted position by placing the glans penis through a hole in a small piece of masking tape fastened to the abdomen. Once the sheath was retracted, the reflex test lasted for 20 minutes. Rats were habituated to this procedure prior to surgery (2x10min and
2x20min) and the animals quickly adapt to the testing situation. Events were visually scored according to previously described criteria (Schmidt, M. H. et al., 1995): E1, weak glans engorgement; E2, moderate glans engorgement involving some dilation of the distal glans; E3, intense flaring or cup of the distal glans (Figure 3.7 B); F1, dorsiflexion of the penile body; F2, dorsiflexion or flip of the penile body greater than 90º with respect to the body of the rat. These reflexes were scored with a numerical keypad as events 1 – 5, respectively, and were recorded on the event channel during simultaneous data acquisition.

Figure 3.7 A,B: Ex copulatory reflex erection test. In A a rat is shown in the restraining cylinder. In B an erectile event (E3) is shown.

*Non-contact erection tests:*

Non-contact erection tests were performed twice in a glass aquarium (51 x 30 x 29), which was divided in half by a sheet of wire mesh. The male rat was
allowed 5 minutes to adjust to the new environment prior to the start of the test. An estrous female rat was placed in one compartment and the male in the other. The female rat had been administered estradiol cypionate (200μg = 0.1 ml SQ) 24 hours prior to the test and progesterone (500μg = 0.1 ml SQ) 6 hours before the test to ensure receptive behavior. Before the test, female receptivity was verified with non-experimental male rats. The rats were observed for 30 minutes. Events (1: visible erections, 2: grooming of body parts, 3: grooming of genital area without visible erections) were recorded in the event channel as described above. This test was performed with the telemetric receiver unit placed directly under the aquarium.

Mating tests:

Mating tests were performed twice in a glass aquarium (51 x 30 x 29). The male rat was allowed a 5 minute adjustment period prior to introduction of a female rat in estrous. After the male and female rats were placed together they were observed for 30 minutes. The female rat had received the same hormonal treatment as described for the non contact erection test. Events (1: mounts, 2: intromissions, 3: ejaculations, 4: grooming of body parts, 5: grooming of genital area without visible erections) were recorded in the event channel as described above.

This test was performed with the telemetric receiver unit placed directly under the aquarium.
Statistics:

Data are presented as means ± standard error of the mean (SE). To compare the number of micturitions detected by telemetry with those detected by video and to determine the relationship between duration and volume of micturition, regression analysis was performed. Agreement between the two methods was assessed by calculating bias (mean difference between the two methods) and limits of agreement (2 x standard deviation = 2SD) (Bland, J.M. and Altman, 1986). The Bland and Altman statistical method is the most appropriate tool to compare agreement between repeated measurements by each of 2 methods on the same subjects. Also, Bland and Altman plots are useful to reveal a relationship between the differences and the averages, to look for systematic bias, and to identify possible outliers (Bland, J.M. and Altman, 1986; Bland, J. M. and Altman, 1999). Analysis of variance (ANOVA) was used to identify significant differences in CSP waveform characteristics of erectile events recorded during the 3 different behavioral contexts. The statistical computations were performed with software packages (Sigmastat 3.0, SPSS, Chicago, IL and SPSS 12.0, SPSS, Chicago, IL).

3.3 Results

Rats were 70 ± 3 days and weighed 300 ± 10 g for transducer implantation. Complications that occurred around the transducer implantation site were minor and included mild swelling, loss of hair, and scabbing, all of which healed with time.
For all 10 rats micturition and erectile events were easily identified from computer readouts and the characteristics of 3 types of events are described below. Quality of chronic recordings remained stable in 3 of the 4 rats we followed weekly for an 8 week period. Explanting of the transducer of the 4th rat revealed that the catheter tip was no longer in the CSP.

**Micturition**

Telemetrically obtained CSP pressure waveforms during a micturition event are characterized by a smooth, flaccid base line pressure on top of which a pulsatile pattern occurs with a highly conserved frequency of 9.1 ± 0.3 Hz and mean maximum pressure of 93.8 ± 8.9 mmHg. After-peaks (3.2 ± 0.3) are typically seen occurring every 3 – 5 seconds (Figure 3.8 A,B). In Figure 3.8B the phase during which urine is expelled and the afterpeaks are illustrated. During micturition events we do not recognize a vascular component in the CSP pressure waveforms; however, we recognize the muscular component of BS muscle contractions in the form of CSP pressure peaks. This is further demonstrated in Figure 3.9 which shows BS EMG recordings obtained during micturition events. Detailed characteristics of CSP pressures during micturition events are summarized in Table 3.1.

The number of micturitions seen on video and recorded by telemetry is shown for all rats for 2 x 24 hour periods (Figure 3.10). In Figure 3.11 the total number of micturitions (A) and the average number of micturitions per 24 hours (B) that were seen on video and recorded by telemetry are shown.
2 weeks post implantation

Figure 3.8 A,B: CSP pressure waveform obtained by telemetry during micturition. In B the phase of expulsion of urine and the afterpeaks are indicated.
Figure 3.9 A-C: Electromyographic activity in the BS muscle during a micturition event. This figure shows BS muscle firing during the phase of urine expulsion. A – C are same tracings shown at different scales to clarify that BS muscle bursts occur at the same time as CSP pressure peaks. Data obtained by Dr. Markus Schmidt.

<table>
<thead>
<tr>
<th>Event analysis</th>
<th>Afterpeak analysis</th>
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<tr>
<td>Duration</td>
<td>AUC</td>
</tr>
<tr>
<td>3.6 ± 0.2</td>
<td>119 ± 11.5</td>
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Table 3.1: Micturition waveform characteristics in the adult Long-Evans rat. Analysis of 24 hour micturition events. Micturition waveform characteristics include duration (seconds), area under the curve (AUC)(mmHgxseconds), mean and maximum pressures (P)(mmHg), frequency (Hz) of the expulsion phase and number, duration (seconds), AUC (mmHgxseconds), and mean P of the afterpeaks.
Figure 3.10: Number of micturitions. Total number of micturitions per 24 hours shown for each rat detected by telemetry and by video observation.

Figure 3.11 A,B: Total number of micturitions seen on video and detected by telemetry. In A the total number is shown and in B the mean + SE of micturitions per rat per 24 hours is given.
Linear regression analysis between telemetry and video observed number of micturitions resulted in $R = 0.977 \ (p<0.001; \ F=364)$. The bias was -1.9 and the upper and lower limits of agreement were 7.5 and -11.3, respectively (Figure 3.12). One rat (1264) had 1 measurement outside the limits of agreement (-15).

![Bland-Altman: Micturitions/24h](chart.png)

**Figure 3.12:** Bland and Altman plot of the number of micturitions per 24 hours detected by video or telemetry. The average number of micturitions per 24 hours per rat (for 9 rats 2 x 24 h and for 1 rat 1 x 24 h) was used for this analysis.

The volume per micturition was $0.95 \pm 0.12 \text{ ml/urination}$. For 7 rats we determined the relationship between micturition pressure wave form duration and volume of urine expelled. This is shown in Figure 3.13A. Linear regression analysis revealed $R = 0.823 \ (p<0.001; \ F=545)$. The formula for the positive relationship between the two parameters is shown in Figure 3.13B after combination of all data.
Figure 3.13 A,B: Relationship between duration and volume of micturition. The volume and duration of micturitions is shown for individual animals (A) and for the combined data (B).
Sexual Function

Telemetrically obtained CSP pressure waveforms during full erectile events are characterized by development of a tumescence pressure of at least 30 mmHg above baseline on top of which 1 or many suprasystolic pressure peaks occur. At least 1 peak must reach a pressure of 100 mmHg above the tumescence pressure for the event to be classified as a full erectile event (Figure 3.14). The limit of 100 mmHg over baseline pressure is based on being 1.5x standard deviation below the average maximum pressures during E1 events (200 mmHg) (Dr. Markus Schmidt, personal communication). The limit of 100 mmHg over baseline pressure is at 1.5x standard deviation below 200 mmHg. Events that reach pressures of 130 – 140 mmHg are thus considered partial erectile events. Partial erectile events are similar to full erectile events with the exception that peaks never reach 100 mmHg over the tumescence pressure (Figure 3.15). These events clearly demonstrate the vascular subsystolic phase of erections due to cardiogenic vasodilatory mechanisms. In contrast, the full erectile events have characteristics of both the vascular subsystolic component and the muscular suprasystolic component.

During ex copulatory reflex erection tests 100% of observed erectile events corresponded with CSP waveform patterns characteristic of erectile events. Partial erectile events occurred infrequently and were associated with partial filling of the base but not with erectile events.
Figure 3.14: CSP pressure waveform obtained by telemetry during a full erectile event.

Figure 3.15: CSP pressure waveform obtained by telemetry during a partial erectile event.
The characteristic muscular generated suprasystolic pressure peaks seen in the CSP waveforms occurred during full erectile events with glans engorgement (E1 – E3). Figure 3.16 and Table 3.2 illustrate characteristics of CSP waveforms recorded during the different contexts studied.

Figure 3.16: CSP pressure waveforms recorded during 3 behavioral contexts.

During non contact mating tests CSP pressure waveforms characteristic of full erectile events were observed during visible erectile events associated with grooming of the genital area. Furthermore, identically looking CSP pressure
waveforms were seen while the male rat was engaged in grooming or exploratory behavior, in spite of no visually confirmed observation of erections. During mating tests suprasystolic peaks were seen associated with various components of mating such as mounting, copulation, and intromission. However these erectile events were of significantly shorter duration (p<0.05) and therefore did not have the characteristic appearance of erectile events seen in other contexts such as during grooming or ex copulatory reflex erection tests. Furthermore, during noncontact and mating tests, more events were identified by telemetry than by observation alone.

<table>
<thead>
<tr>
<th>Context</th>
<th>Duration AUC</th>
<th>Mean P</th>
<th>Max P</th>
<th>Number Peaks AUC</th>
<th>Duration</th>
<th>Mean P</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hour</td>
<td>25 ± 2.5 1401 ± 154</td>
<td>54 ± 1.1 320 ± 14</td>
<td>4.2 ± 0.2 0.73 ± 0.03</td>
<td>76 ± 3.6 108 ± 3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reflex</td>
<td>54 ± 1.3 2855 ± 584</td>
<td>55 ± 3.1 333 ± 26</td>
<td>6.2 ± 1.2 0.82 ± 0.05</td>
<td>86 ± 10 106 ± 7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-contact</td>
<td>13 ± 2.3 622 ± 165</td>
<td>45 ± 5.1 235 ± 41</td>
<td>2.8 ± 0.9 0.50 ± 0.09</td>
<td>49 ± 8.1 94 ± 7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mating</td>
<td>4.2 ± 1.1 227 ± 54</td>
<td>56 ± 3.4 274 ± 22</td>
<td>1.7 ± 0.5 0.54 ± 0.05</td>
<td>55 ± 5.7 106 ± 5.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2: Erectile event waveform characteristics in the adult Long-Evans rat. Analysis of CSP pressure waveforms during erectile events. Waveform characteristics analyzed include duration (seconds), area under the curve (AUC)(mmHgxseconds), mean and maximum pressures (P)(mmHg), number of peaks of the event, and duration (seconds), AUC (mmHgxseconds), and mean P of the individual peaks. Significance levels: * p<0.05; ** p<0.01.
In this study we demonstrate the accuracy of CSP pressure recording as a modality for measuring frequency and quality of micturition and erectile events in awake freely moving male rats. Furthermore duration of micturition appeared predictive of micturition volume.

Transducer implantation is a minimally invasive surgery with no major complications encountered during our study period of 2 months. Analysis of pressure wave-forms that were recorded by telemetry is straightforward and the 3 types of events, micturition, full erectile and partial erectile events, are easily distinguished and recorded. When pressures are analyzed and compared to other studies and/or compared between different behavioral contexts, it is important to determine the pressure at which the recording system saturates. We do not believe saturation effects AUC since the period at which pressures could be higher than the saturation pressure would be very small.

Based on BS EMG and CSP pressure recordings it appears that pressure changes in the CSP during micturition events are due to muscle contractions of the BS muscle. This represents a new finding, since traditionally the BS muscle is only associated with ejaculation and its role in micturition has not been investigated. Indeed, coordinated activation of the external urethral sphincter (EUS) and contraction of the detrusor muscles are considered the main processes involved in micturition (Pikov and Wrathall, 2001). We suggest that in addition to the role the BS muscle plays in expelling seminal fluids, it likely also plays a role in expelling urine. We propose that the BS muscle and the EUS
contract simultaneously. Thus, EUS muscle contractions may prevent backflow of urine into the bladder while simultaneous BS muscle contractions assist in expelling urine. Interestingly, the fact that BS muscle activity is important during both micturition and erectile events is also demonstrated by the fact that, after spinal cord injury in rats, it is not uncommon to see micturition events directly followed by erectile events (Nout and Schmidt, unpublished observation). Reflex patterns in the pelvic musculature are altered following spinal cord injury (Nout, Y. S. et al., 2005a) and a consequence of this may be that BS muscle firing during micturition enables the triggering of a subsequent reflex erectile event.

BS muscle contractions during micturition occur in a rapid pulsatile manner and result in pressure peaks within the CSP characterized by a similar pulsatile pattern. In contrast, the CSP pressure increase seen during partial erectile events is consistent with a vascular pattern; the low amplitude pulsatile pattern has a frequency that is directly associated with that of an electrocardiogram (Schmidt, M. H. et al., 1995). The technique of CSP pressure determination directly measures this vascular component and forms an indirect measurement of the muscular component of the CSP pressure. CSP pressure changes during full erectile events reflect a combination of vascular subsystolic and muscular suprasystolic components. The vascular phase is characterized by development of a tumescence pressure that is increased from the baseline pressure. Suprasystolic pressure peaks occur on top of this tumescence pressure and are associated with BS muscle contractions. The fact that during full erectile events much higher pressures occur within the CSP than during
micturition is likely due to blood engorgement of the cavernous space during erection whereas there is no filling of the CSP during micturition.

Simultaneous recording of CSP pressures and video-taping of animals demonstrated an excellent agreement between the occurrences of micturition events at the same time as the occurrence of a CSP pressure waveforms characteristic for micturition. Slightly more micturitions were detected by telemetry versus video observation resulting in an R of 0.98 instead of 1. We speculate this is due to the fact that some micturition events were missed on video either due to changing of the video tape while recording or due to poor lighting during dark resulting in inability to see urine fall on the colored paper.

While animals were housed on wire mesh we found an average frequency of 30 ± 4 micturition events per 24 hours. One other study that used metabolic cages to determine frequency of micturition in male Sprague-Dawley rats found they urinated approximately 21 times per 24 hours (Schmidt, F. et al., 2001). However, while the rats in our study had not been accustomed to the study condition, the rats in Schmidt’s study had been acclimatized to the metabolic cages during a 7 day period prior to testing. When we examined micturition characteristics of male rats housed in their regular cages we found a micturition frequency of 26 ± 2 per 24 hours (Nout, Y. S. et al., 2005b), which is closer to what Schmidt et al (2001) reported. In this study we found a mean volume per micturition of 0.95 ± 0.12 ml. This is slightly less than what Schmidt et al (2001) reported. They found an average micturition volume of approximately 1.3 ml. However, when combining these results, the total volume of urine expelled per
24 hours would be similar between the two studies. The rats in our study apparently urinated smaller volumes more frequently, perhaps related to the different housing environment or strain differences. Another study reported a smaller volume (0.6 ml) per micturition in male Wistar rats examined in metabolic cages (Haas et al., 1997). These rats has a smaller body weight (mean: 250 g) compared to the rats we used (mean: 300 g) and the rats used by Schmidt et al (2001) (mean: 399 g). It is likely that smaller rats produce a smaller volume per micturition. Since the number of micturition per 24 hours and the volume of micturitions in this study compared well to what has been found in rats that were observed in metabolic cages, we believe transducer implantation does not affect the micturition reflex.

Telemetric monitoring of erectile events appears a consistent and accurate method for detecting these events in conscious single housed rats and in awake rats during behavioral tests. From this study it was evident that erectile events are difficult to detect by eye in rats during non contact and particularly during mating tests. Typically in behavioral testing conditions, we infer intromission from a specific behavioral pattern although we observe neither intromission nor erection. We suggest that the use of CSP pressure analysis is more inclusive and less likely to miss important data. Critics may suggest we are dealing with false positive erectile events; however, there was a 1 to 1 relationship between visually confirmed erections during ex copulatory reflex erection tests and characteristic CSP pressure changes. Moreover, we never observed erectile events without CSP pressure changes.
Significant differences were present in CSP pressure waveforms during erectile events recorded in different behavioral contexts. This highlights the importance of context-specificity of erectile events and suggests interpretation of the various erectile events by telemetry should occur within the context of specific experimental conditions. The differences in CSP pressures may be due to differences in erectile physiology between the 3 behavioral contexts studied here. Perhaps this reflects the fact that reflex erections are likely to be more spinally mediated and erections that occur during intromission likely have more supraspinal input. Further study of the characteristics of CSP waveforms during erectile events in non contact and/or mating tests and their relationship with observed behavior may improve our understanding of context specificity of erectile events. Future research involving pressure monitoring in both CSP and CCP is required to further elucidate the roles of both cavernous systems and of the muscles controlling them during erectile events.

Telemetric monitoring of CSP pressure for assessment of micturition and erectile events has clear benefits over traditional methods of examining these functions in freely moving animals. CSP pressure monitoring allows simultaneous assessment of micturition and erectile function, which is important for scientists interested in more than 1 autonomic functional outcome. This method allows examination of animals housed in a natural habitat and determination of events that are in general very hard to observe by eye or video recordings. Furthermore, recordings obtained by telemetry not only provide us with quantifiable data such as number of events per 24 hours, but also allows us to determine other
parameters of importance in assessment of these events, such as duration and pressures that occur during these events. Since the bulb of the CSP is more vascular than the CCP, implantation of transducers is easier and failure rate is less. Furthermore, ability to perform long-term measurements is an advantage of CSP monitoring versus CCP monitoring.

In conclusion, changes of CSP pressure wave-form characteristics, detectable by telemetry, are a valuable tool for detecting micturition and erectile events in conscious freely moving rats. Micturition and erectile function are predominantly regulated by the autonomic nervous system and damage to these components following spinal cord injury results in alterations of these important physiologic functions. We anticipate the use of telemetric recording of CSP pressure will aid in future studies examining changes in autonomic outcome following SCI.

3.5 References


CHAPTER 4

TELEMETRIC MONITORING OF CORPUS SPONGIOSUM PENIS PRESSURE IN CONSCIOUS RATS FOR ASSESSMENT OF MICTURITION AND SEXUAL FUNCTION FOLLOWING SPINAL CORD CONTUSION INJURY

(Published: J Neurotrauma 22 (4) 429 – 441)

4.1 Introduction

Spinal cord injury (SCI) results in disruption of motor, sensory, and autonomic function. In research models, recovery following SCI is generally assessed by examination of motor function, leaving autonomic function largely ignored. However, in humans, impairment of urogenital tract function following SCI and its subsequent complications are highly prevalent and clinically very important (Anderson, 2004; Noreau et al., 2000). The state of bladder function and sexual reflexes is critical and continued investigation of pathophysiology and treatments to improve recovery of these autonomic functions are, therefore, essential.

Most studies that have examined micturition function following SCI in rat models have been conducted using cystometric and electromyographic (EMG)
techniques in anesthetized animals or post-mortem examination of bladder characteristics (Kruse et al., 1993; Pikov et al., 1998; Pikov and Wrathall, 2001). However, both anesthesia and placement of catheters in bladder or urethra are known to alter the micturition reflex (Cheng et al., 1995; Yoshiyama et al., 1999). Investigators of penile erection physiology have developed a technique in which pressures within the corpus cavernosum or corpus spongiosum of the penis can be monitored continuously in conscious animals (Giuliano et al., 1994; Schmidt, M. H. et al., 1995; Schmidt, M. H. et al., 1994). Unlike in recordings from the corpus cavernosum, continuous monitoring of pressure in the bulb of the corpus spongiosum of the penis (CSP) in awake, freely moving rats allows for simultaneous recording of erectile (Schmidt, M. H. et al., 1995) and micturition events (Schmidt, M.H. et al., 2004). Monitoring of CSP pressure can be performed over extended periods of time (8 – 12 weeks) in conscious animals, and the procedures have recently been validated in normal rats (Schmidt, M.H. et al., 2004).

In the normal rat, micturition is regulated by spinal and supraspinal neural pathways and is mediated through contraction of the bladder detrusor muscle accompanied by coordinated activation of the external urethral sphincter (EUS) (Kakizaki et al., 1997; Yoshiyama et al., 1999). Unlike in humans, micturition in normal rats is characterized by a pulsatile flow pattern that occurs through rapid, brief contractions and relaxations of the EUS, in addition to the simultaneous burst firing activity apparent in the bulbospongiosus (BS) muscles (Schmidt et al., 2004). Following incomplete SCI, recovery of micturition occurs in rats and is
sufficiently effective that manual bladder expression is no longer required after a variable time following SCI. However, this recovery is limited and voiding remains inefficient with long-term changes occurring in muscle EMG patterns, micturition frequency, volume, and bladder weight (Chancellor et al., 1994; Cheng et al., 1999; Kruse et al., 1993; Yoshiyama et al., 2000; Yoshiyama et al., 1999).

Initially a period of spinal shock occurs during which the bladder is areflexive and overflow incontinence may be present. This is followed by the development of involuntary reflex micturition involving detrusor and EUS muscle activity, which are mediated by spinal reflex pathways (Cheng and de Groat, 2004; Kruse et al., 1993). The returning detrusor muscle contractions may be hyperreflexic (Osborn et al., 1990) and inefficient voiding occurs if the EUS contracts simultaneously with the detrusor muscle, a condition referred to as detrusor-sphincter-dyssynergia (DSD). Although bursting activity of the EUS can be mediated by spinal mechanisms, it is less robust in rats with SCI than in rats with an intact neuraxis (Cheng and de Groat, 2004). Interestingly, the initial loss and subsequent recovery of reflex functions, which are autonominally mediated, show a similar pattern of recovery as locomotor function (Holmes et al., 2005) suggesting commonality of underlying recovery mechanisms.

The present study evaluates the use of CSP pressure monitoring as an assessment tool for measuring recovery of autonomic nervous system function after SCI. More specifically, we investigated the use of chronic telemetric monitoring of CSP pressure in awake, freely moving rats to determine recovery
of both micturition and erectile events following a moderate spinal cord contusion injury.

4.2 Materials and Methods

Subjects

Seven adult, male Long-Evans hooded rats (Simonsen Laboratories, Gilroy, CA, USA) age 71 ± 2 days (AVG ± SE), weight 301 ± 7 g, were used in this study. Rats were housed individually in plastic cages, maintained on a 12-h light/dark cycle, and had free access to food and water. All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with NIH guidelines and recommendations.

Surgical Procedures and Post-Operative Care

Surgical procedures were carried out aseptically under deep anesthesia induced by intraperitoneal administration of xylazine (Tranquilized™, Vedco Inc., St. Joseph, MO, USA; 10 mg/kg) and ketamine (ketamine HCl, Abbott Laboratories, N.Chicago, IL, USA; 80 mg/kg) for transducer implantation, and pentobarbital (Abbott Laboratories, Chicago, IL, USA; 50 mg/kg) for SCI. Anesthetic plane was determined by withdrawal to foot pinch. A pre-operative dose of cefazolin (Ancef, Abbott Laboratories, N.Chicago, IL, USA; 50 mg/kg) was administered subcutaneously. Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA, USA) was applied to the eyes prior to surgery and
body temperature was maintained at 37.5 ± 0.5°C using a rectal thermal probe and heating pad.

For transducer implantation the rat was placed in dorsal recumbency and surgical sites were shaved and cleaned with betadine. A 3 cm skin incision was made on midline on the raphe scroti and a 3 – 4 cm skin incision was made parallel to midline in the right inguinal area. A telemetric pressure transducer catheter (TA11PA-C40, Data Sciences International, St. Paul, MN, USA) was implanted in the bulb of the CSP as described previously (Schmidt, M. H. et al., 1995). Briefly, the battery of the transducer was placed subcutaneously in the lower right abdomen and secured to the external oblique abdominal muscle with 2 sutures. The catheter was tunneled subcutaneously to the perineal area. Testes were retracted and BS muscle and CSP were exposed. A guide hole was made into the CSP with a 21 gauge needle and the open tip of the catheter was placed into the bulb of the CSP and secured with biological glue and 1 suture. The scrotal incision was then closed in 1 layer and the inguinal incision was closed in 2 layers. The rat was allowed to recover. Telemetric studies were commenced 4 – 5 days post-operatively.

A 12.5 g-cm spinal cord contusion injury (SCI) was delivered with a MASCIS/NYU device as previously described (Gruner, 1992). Briefly, a dorsal laminectomy at T9 – T10 was performed and the spinal cord, with an intact dura mater, was impacted with a 10 g rod from a height of 12.5 mm. Post-operative care included administration of cefazolin subcutaneously for 7 days, twice daily bladder expression for 7 days and then once daily for 3 – 4 days, access to 1.2%
liquid vitamin C (Vedco, Inc., St. Joseph, MO, USA) prepared in sugar water, and fluid administration if animals had evidence of hematuria.

**CSP Pressure Recording**

Physiological telemetry data were recorded on a PC computer using a Spike2 data acquisition program (version 3.1, Cambridge Electronic Design Limited, Science Park, Milton Road, Cambridge CB40FE, U.K.). Animals underwent 2 x 24h CSP pressure baseline (BL) recording while housed in their cages prior to SCI. Following SCI, CSP pressures were recorded (24 h per day) on days 1-7, 10, 14, 17, 21, and 24. In addition to this, micturition volume was determined for a period 2 x 24 h prior to SCI and for 5 consecutive hours on days 3, 4, 5, 7, 10, 14 and 21 following SCI.

To determine micturition volume, rats were placed in a plastic cage with a mesh floor. Colored paper was mounted on a scale underneath the mesh floor of the cage and this was recorded on video. This made it possible to see when a micturition event occurred and the volume was based on change in scale readout (1ml = 1mg). The receiver unit of the telemetry system was placed on top of the cage. To avoid time discrepancies, the camera time was recorded in a simultaneously running event channel of the acquisition program and used as the start time. In this way the time and approximate volume of each micturition could be determined and correlated to the pressure wave characteristics. Data were collected for 6 rats.
For 7 animals CSP pressure data were analyzed for the total number of micturitions, full erectile events (tumescence pressure of at least 30 mmHg above baseline on top of which at least 1 suprasystolic pressure peak of 100 mmHg above the tumescence pressure occurs), and partial erectile events (similar to full erectile events with the exception that peaks never reach 100 mmHg over the tumescence pressure) in a 24-hour period. For 3 animals a detailed waveform analysis was performed for 24 hour periods at BL and days 7 and 21 following SCI. Data analyzed were duration of the events, area under the curve (AUC), total number of pressure peaks, peak frequency and mean and maximum pressures. Furthermore, a peak analysis was performed. For micturition events this included the number of afterpeaks, and afterpeak duration, AUC, and mean and maximum pressures. For erectile events this included the duration, AUC, and mean and maximum pressures of the peaks that occurred during those events.

Behavioral Tests

Ex copulatory reflex erection tests were performed in 6 awake animals as previously described (Hart, 1968; Schmidt, M. H. et al., 1995). Briefly, rats were placed on a board in dorsal recumbency with the head and anterior torso in a loose-fitting restraining cylinder. The abdomen was secured to the board with masking tape. The head and anterior torso could move freely in the cylinder. The telemetric receiver unit was placed directly under the board. The preputial sheath was retracted, which is the stimulus to elicit reflex erections, and maintained in
retracted position by placing the glans penis through a hole in a small piece of
masking tape fastened to the abdomen. Once the sheath was retracted, the
reflex test lasted for 20 min. Rats were habituated to this procedure prior to
surgery and the animals quickly adapt to the testing situation. Events were
visually scored according to previously described criteria (Schmidt, M. H. et al.,
1995): E1, weak glans engorgement; E2, moderate glans engorgement involving
some dilation of the distal glans; E3, intense flaring or cup of the distal glans; F1,
dorsiflexion of the penile body; F2, dorsiflexion or flip of the penile body greater
than 90° with respect to the body of the rat. These reflexes were scored with a
numerical keypad as events 1 – 5, respectively, and were recorded on the event
channel during simultaneous data acquisition. Reflex testing was performed
twice prior to SCI and on days 1, 3, 7, 14, and 21 following SCI.

Open-field walking was evaluated before and after transducer
implantation, and at 48 hours, 5, 8, 16, and 22 days following SCI using the 21-
point BBB Locomotor Rating Scale (Basso et al., 1995).

**Histology**

On day 25 after SCI, rats were deeply anesthetized with ketamine/xylazine
followed by transcardiac perfusion with 0.9% NaCl and 4% formalin. The lesion
center of the spinal cord was isolated and kept in formalin (4%) for 24 hours and
then in sucrose (30%) for 24 – 48 hours. The tissue was frozen at –80°C until
further analysis. Lesion centers were sectioned transversely on a cryostat and
stained with luxol fast blue for myelin and cresyl violet to reveal Nissle substance.
Sparing of the white matter in the lesion center was determined with computer software (MCID™, Imaging Research Inc., St. Catherines, Ontario, Canada).

Statistics

Data are presented as means ± standard error of the mean (SE). A one-way repeated measures analysis of variance (ANOVA) was used to analyze the CSP pressure data. The null hypothesis was rejected at \( \alpha = 0.05 \). Significant differences identified by the ANOVA were isolated using the Student-Newman-Keuls method for pairwise multiple comparison post-hoc test. To better characterize the waveform changes over time following SCI, we performed trend analyses on the detailed waveform data. Locomotor recovery data were analyzed with the Friedman repeated measures ANOVA on Ranks followed by Dunn’s method post-hoc test. The statistical computations were performed with software packages (Sigmastat 3.0, SPSS, Chicago, IL and SPSS 12.0, SPSS, Chicago, IL).

4.3 Results

Rats were 71 ± 2 days and weighed 301 ± 7 g for transducer implantation. Rats were 82 ± 2 days and weighed 333 ± 5 g at the time of SCI. Complications that occurred around the transducer implantation site were minor and included occasional mild swelling, loss of hair, and scabbing. One animal was sacrificed on day 5 after SCI due to bladder rupture secondary to manual bladder expression.
Micturition

Baseline data resulted in an average of 26 ± 2 micturition events per 24 hours per rat. The average volume of urine expelled per micturition was 1.0 ± 0.2 ml. CSP pressure waveforms during a micturition event were characterized by a smooth baseline pressure on top of which a pulsatile pattern occurred with a frequency of 9.0 ± 0.2 Hz. This waveform was generally followed by 3 – 4 afterpeaks (Figure 4.1). Following SCI bladders were manually expressed twice daily for 7 days and then once daily for 3 – 4 days. First spontaneous micturition events following SCI were seen by telemetry on days 2 (Rats 4 and 5), 3 (Rats 1 and 3), 4 (Rat 6), and 5 (Rat 2, 7). First micturition events detected following SCI were recognized by the characteristic pulsatile pattern, however, when compared to BL, the amplitude was low and afterpeaks were absent (Figure 4.2). A gradual return of the micturition motor pattern was seen, and 24 – 48 hours after onset of micturition events, afterpeaks were present again (Figure 4.2). Recovery of the total number of micturition events is shown in Figure 4.3. Total number of micturition events on day 24 following SCI was 19 ± 3.
Figure 4.1: Representative waveforms of erectile and micturition events. Waveforms characteristic of full erectile and micturition events at baseline and on day 7 and day 21 following SCI. CSP pressure in mmHg (y-Axis) over a 25 second interval (x-Axis). (Bars = 2.9s for erections and 3.8s for micturitions)
Figure 4.2: Representative micturition waveforms following SCI. Waveforms characteristic of return of micturition over the first 24 – 48 hours after the onset of the first micturition following SCI. Initially (acute recovery) a low amplitude pulsatile pattern is seen, after which (subacute recovery) an increased amplitude of the pulsatile pattern occurs. Finally, afterpeaks return following the pulsatile waveform, to complete the recovery of the micturition motor pattern. CSP pressure in mmHg (y-Axis) given for a 24 second interval (x-Axis).
Video recording of rats on days 3, 4 and 5 following SCI revealed overflow incontinence present in 5 of 6 rats on day 3 and in 2 rats on day 4. Overflow incontinence, characterized by continuous dribbling of urine in combination with a (partial) full bladder, was not detectable by telemetry. All micturition events that were seen on video corresponded with the CSP pressure wave characteristic of micturition, detectable by telemetry. Urine volumes (ml) following SCI were smaller than before SCI on days 3 (0.0 ± 0.0), 4 (0.5 ± 0.3), and 5 (0.8 ± 0.3) before they gradually increased to reach an average volume of 2.1 ± 0.1 on day 21, which was significantly larger than before SCI (Figure 4.4).
Figure 4.4: Urine volume per micturition. Urine volume per micturition (ml) at baseline (BL) and up to 21 days following SCI (n = 6 until day 6, then n = 5). *: significantly different from BL; ▲: significantly different from day 3; ♦: significantly different from day 4; ■: significantly different from day 5.

Although the micturition CSP pressure waveforms following SCI remained similar to those seen before SCI, some differences were noted on days 7 and 21 (Figure 4.1). Table 4.1 shows detailed waveform characteristics for BL and days 7 and 21 following SCI. Significant linear trends were demonstrated towards increased micturition duration, AUC, mean pressure, number of peaks, and peak frequency over time (Fs > 18.5, p < 0.04). Although there were no significant changes in afterpeak analysis on days 7 and 21, there was a transient absence of afterpeaks during recovery.
Table 4.1: Micturition waveform characteristics. Micturition waveform characteristics (means ± SE) at baseline (BL) and on days 7 and 21 following SCI (n = 3). * : significant linear trends.

<table>
<thead>
<tr>
<th>Day</th>
<th>Duration (s) *</th>
<th>AUC (mmHg x s) *</th>
<th>Mean Pressure (mmHg) *</th>
<th>Number of Peaks *</th>
<th>Peak Frequency (Hz) *</th>
<th>Number of Afterpeaks</th>
<th>Afterpeak Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>3.6 ± 0.3</td>
<td>127 ± 12.5</td>
<td>35 ± 1.9</td>
<td>32 ± 2.0</td>
<td>9.0 ± 0.2</td>
<td>3.4 ± 0.3</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>7</td>
<td>6.1 ± 0.3</td>
<td>263 ± 25.5</td>
<td>43 ± 2.0</td>
<td>57 ± 1.6</td>
<td>9.9 ± 0.3</td>
<td>3.7 ± 1.0</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>21</td>
<td>6.5 ± 0.7</td>
<td>302 ± 37.1</td>
<td>46 ± 1.6</td>
<td>66 ± 6.4</td>
<td>10.2 ± 0.1</td>
<td>3.9 ± 0.4</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

Erectile Events

The number of total erectile events with the proportion of full and partial erectile events is shown in Figure 4.5. During full erectile events a tumescence pressure develops of at least 30 mmHg above baseline on top of which 1 or many suprasystolic pressure peaks occur. At least 1 peak must reach a pressure of 100 mmHg above the tumescence pressure for the event to be classified as a full erectile event (Figure 4.1). Before SCI the number of full erectile events was 53 ± 4 per 24 hours. Following SCI the number of full erectile events was significantly less than BL for all time points, except for on day 2 (Figure 4.5). Although the CSP pressure waveforms characteristic for full erectile events seen following SCI remained similar to those observed before SCI, some changes were seen on days 7 and 21 (Figure 4.1).
Figure 4.5: Total number of erectile events following SCI. Total number of erectile events per 24-hour period given with proportions of full and partial erectile events at baseline (BL) and up to 24 days following SCI (n = 7 until day 6, then n = 6). *: significantly different from BL and day 2. ▲: significantly different from days 1–24.

Table 4.2 shows detailed waveform characteristics for BL and days 7 and 21 following SCI. Significant linear trends were demonstrated towards increased AUC (F = 4655, p < 0.00) and maximum pressure (F = 30.3; p < 0.03) of the full erectile events over time, and towards increased duration (Fs = 42.92, p < 0.03), AUC (Fs = 53.56, p < 0.02), and average maximum pressure (Fs = 36.95, p < 0.03) of the individual suprasystolic pressure peaks over time. Furthermore, a marginally significant linear trend was seen towards an increase in duration of full erectile events (Fs = 17.76, p = 0.052).
Table 4.2: Characteristics of full erectile event waveforms. Full erectile event waveform characteristics (means ± SE) at baseline (BL) and on days 7 and 21 following SCI (n = 3). *: significant linear trends.

Partial erectile events are similar to full erectile events with the exception that peaks never reach 100 mmHg over the tumescence pressure. The number of partial erectile events prior to SCI was 8 ± 2 per 24 hours. Following SCI this decreased and the total number of partial erectile events remained significantly less than at BL throughout the study period (Figure 4.5).

**Reflex Erection Tests**

Following SCI, latency (time of first erectile event following sheath retraction) significantly decreased at all time points (Figure 4.6 A). The number of erectile events that occurred during the testing period increased significantly on days 7 and 21 following SCI. Moreover, the number of suprasystolic pressure
peaks that occurred within such events, increased significantly from BL on days 1, 3, 7, and 21 following SCI (Figure 4.6 B,C).

**Figure 4.6 A-C:** Reflex erection test results. Reflex erection test results at baseline (BL) and up to day 21 following SCI (n = 6 until day 6, then n = 5). Latency, or time to first erectile event (A). Total number of pressure peaks and total number of erectile events (B). Number of pressure peaks per erectile events (C). *: significantly different from BL.
**Locomotor Function**

The 12.5 g-cm SCI resulted in an initial profound paraplegia in all 7 rats characterized by a mean BBB locomotor score of 2.6 ± 0.7 at 48 hours after injury. Gradually, rats showed recovery of locomotor function with frequent to consistent weight-supported stepping and no forelimb-hindlimb coordination by 22 days following SCI. A mean BBB score of 10.7 ± 0.3 was reached at that time (Figure 4.7).

![Locomotor recovery](image)

**Figure 4.7:** Recovery of locomotion function. Recovery of locomotor function according to the BBB Locomotor Rating Scale (n = 7 until day 6, then n = 6). *: significantly different from BL; ▲: significantly different from 48 hours.

**Histopathology**

No residual gray matter was observed at the lesion center, but a thin rim of white matter remained. White matter sparing was determined in transverse sections of the spinal cord at the level of the lesion center, and occupied 19.4 ± 2.5% of the cross sectional area (Figure 4.8).
Figure 4.8: Spared tissue at lesion epicenter. Spared tissue at lesion epicenter shown for 6 animals, assessed by examination of transverse sections stained with luxol fast blue and cresyl violet.

4.4 Discussion

In this study we demonstrate that CSP pressure recording is a valuable method for assessment of autonomic nervous system function following SCI. Moreover, this technique allows determination of many parameters of micturition and erectile events in conscious animals and enables us to distinguish subtle differences within these events. Transducer implantation is a minimally invasive surgery with no major complications encountered during our study period of 24 days following SCI. Analysis of pressure waveforms is straightforward and the 3 types of events (micturition, full erectile and partial erectile events) are easily distinguished, since they occur on top of a baseline pressure that barely
fluctuates and generally lies between 0 – 10mmHg. The detailed waveform analysis is more complex; however, further development of the analysis software may facilitate this in the future. This technique has been previously validated for use in detection of erectile events (Schmidt, M. H. et al., 1995) and micturitions (Schmidt, M.H. et al., 2004), and the present data further validate the use of this technique in rats following SCI through simultaneous recording of CSP pressures and both video recording of micturition events and performance of reflex erection tests ((Nout et al., 2004).

The moderate SCI that rats were subjected to in this study resulted in locomotor and histopathologic changes consistent with what has been shown previously for this level of injury (Basso et al., 1995, 1996). Continuous dribbling of urine was seen on video recordings within the first 3 – 4 days following SCI, which is similar to what has been shown in previous studies (Chancellor et al., 1994). As expected, overflow incontinence did not result in changes of CSP pressures and was thus not detectable by telemetry. However, a return of the autonomic/somatic motor pattern characteristic of the pre-lesion pulsatile flow of urine was easily identifiable using the telemetric device by monitoring pressures within the bulb of the CSP. Although first micturition waveforms were seen on days 2 – 5, normalization of the micturition frequency and return of the afterpeaks, only occurred around days 5 – 6, suggesting that twice daily post-operative manual bladder expression for a period of at least 6 days is necessary for this severity level of SCI. The total number of micturitions over a 24-hour period remained lower than at BL; however, this was not statistically significant.
The most significant findings related to micturition in the present experiment were a 2-fold increase in volume per micturition at 10 days following SCI and a 2-fold increased duration of micturition. Furthermore, we found significantly increased mean pressures during micturition events at 7 and 21 days following SCI. These results are similar to what has been shown by other investigators. Yoshiyama et al. (1999) demonstrated a strong correlation between duration of bladder wall contraction and voided volume, both of which were significantly increased following SCI. Furthermore, they showed a 4-fold increase in volume threshold to induce micturition, determined by cystometrograms, at 14 – 21 days following spinal cord transection. Chancellor et al. (1994) showed an almost 2-fold increase in urine volume per micturition at 1 month following a 50 g-cm SCI and found an increase in total voided volume per 24 hours without significant change in water intake. In addition, Mitsui et al. (2003) demonstrated an increase in volume per micturition at 14 days post injury, however this difference did not become significant until 28 days post injury. In contrast, a study that utilized cystometrograms for assessment of urodynamics demonstrated a decrease in voided volume at 12 – 14 days following a 25 g-cm SCI, although by that time the total bladder volume based on post-mortem analyses was increased 8-fold (Pikov et al., 1998).

A significant decrease in voiding efficiency resulting in increased residual urine and increased bladder capacity is commonly seen in rats following SCI. Multiple investigators have shown increased bladder weights, bladder wall muscle hypertrophy, and increased voiding pressures associated with this
decrease in voiding efficiency (Pikov et al., 1998; Pikov and Wrathall, 2001; Yoshiyama et al., 1999). Bladder weights may increase approximately 4-fold by day 12 – 14 following a mild SCI (Pikov et al., 1998).

Detrusor-sphincter-dyssynergia (DSD) is thought to be the main reason for the observed increased voiding pressures and decreased voiding efficiency, and has been documented in rats (Cheng and de Groat, 2004; Kruse et al., 1993; Pikov et al., 1998; Yoshiyama et al., 2000). The degree of chronic impairment of detrusor-EUS coordination is positively correlated with the severity of the SCI (Pikov and Wrathall, 2001). Tonic EUS activity, reflecting closure of the urethral outlet could be considered equivalent to DSD in rats, whereas EUS bursting activity, reflecting opening and closure of the urethral outlet reflects normal coordinated detrusor-sphincter function. Although EUS bursting activity can be mediated by spinal reflex mechanisms, the bursting activity that is seen after SCI is abnormal, consisting of shorter urethral opening times and this presumably contributes to the inefficient voiding and increased voiding pressures (Cheng and de Groat, 2004). Also, the amplitude from EUS EMG recordings has been shown to increase following SCI (Pikov and Wrathall, 2001). Suppression of EUS activity results in improvement of all voiding parameters (Kruse et al., 1993; Yoshiyama et al., 2000).

In addition to DSD, detrusor hyperreflexia has been well characterized in rats following SCI (Mitsui et al., 2003) and is thought to be due to a lack of supraspinal inhibition with or without an increase of afferent signaling. Moreover, enlargement of the bladder may result in plasticity of afferents, further
contributing to this hyperreflexia. The significance of this syndrome in the recovery of micturition, however, is unknown. Recovery of micturition function has been observed to occur through amelioration of DSD without noticeable change of detrusor hyperreflexia (Mitsui et al., 2003). Also, it has been shown that the intercontraction interval of the detrusor muscle may be reduced following SCI (Pikov et al., 1998). When using cystometrograms, reduction of the pressure threshold for micturition (Yoshiyama et al., 1999) and subsequently a quicker onset of micturition after filling of the bladder (Mitsui et al., 2003) is seen, potentially as a result of detrusor hyperreflexia. However, volume threshold was significantly increased and intravesicular pressures during voiding were 85% higher in rats following SCI which, accompanied by reduced urine flow rates, resulted in a significant decrease of voiding efficiency (Yoshiyama et al., 1999).

Hyperreflexia of the external anal sphincter (EAS) reflex has been shown to occur following SCI (Holmes et al., 1998), in addition to DSD and detrusor hyperreflexia, and is thought to be the main contributor to the defecatory dysfunction that is seen following SCI. We suggest that in addition to these muscles, other pelvic floor muscles, and specifically the BS musculature may develop hyperreflexia or spasticity following SCI. This change in reflex activity is reminiscent of hyperreflexia observed in other segmentally mediated reflexes (Bose et al., 2002). In the current study we found trends towards increased mean pressures, increased duration, increased AUC, and increased peak frequencies of micturition events following SCI. These trends of increased CSP pressures may serve as objective indicators of BS muscle spasticity, since pressure within
the bulb of the CSP is directly dependent on the surrounding BS musculature. The telemetric technique employed in the present study allows long-term follow up of these parameters in conscious rats.

The most significant changes related to erectile events observed in this study were a decreased number of both full and partial erectile events per 24-hour period following SCI, and a shortened latency to induction of erectile events during reflex erection tests. Furthermore we found significant trends towards increased duration, AUC, and maximum pressures of the events as well as trends towards increased peak duration, peak AUC, and peak maximum pressures, which may result from BS muscle spasticity or hyperreflexia and may prove a useful outcome measurement in future studies in addition to waveform changes of micturition events. Normal, uninjured Long-Evans rats typically display a long erection latency, as seen in our study (Holmes et al., 2001). The significant reduction of erection latency that we observed following SCI is a result of decreased supraspinal inhibitory input and is consistent with other reports (Hart, 1968; Holmes et al., 2001; Hubscher and Johnson, 2000). Interestingly, the total number of full and partial erections per 24-hour period detected by telemetry decreased following SCI. We hypothesize that the number of partial erectile events decreased due to disinhibition of supraspinal input allowing almost all initiated erectile events to develop into full erections. We had expected to find a large variation in the total number of erections following SCI secondary to disinhibition of supraspinal input combined with dragging of the abdomen and genital area following SCI. It has been shown that movement following SCI
affects the number of erectile events recorded in rats. Rats that move more and consequently drag their abdomen more, have more erectile events than rats that move little (Schmidt, M. H. et al., 1999). Little stimulation of the genital area is necessary to elicit erectile responses following SCI (Hart, 1968), as also demonstrated by the decreased latency in reflex erection tests. We suspect that the decreased number of erectile events on day 1 following SCI was due to generalized post-operative immobility.

Penile erection is only a single component of the complex male sexual behavior and, in animals, can occur in response to tactile, visual, olfactory, and auditory stimuli. In rats, erectile events have been demonstrated to occur during copulatory tests (Hart, 1968), exposure to an estrous female (Sachs et al., 1994), ex copulatory reflex tests (Hart, 1968; Sachs, 1985), and paradoxical sleep (Schmidt, M. H. et al., 1999; Schmidt, M. H. et al., 1994). Although grooming is commonly seen associated with erectile activity, grooming tends to follow the onset of erections, and has not been shown to elicit erectile events in freely moving rats (Sachs et al., 1988). However, one of the possible explanations of our finding of reduced erectile events following SCI is the assumption that erections occur secondary to genital grooming, which reduces markedly following SCI. This could then result in a reduction of erectile events following SCI. Also, ascending sensory pathways from the male genitalia, which are bilaterally located within the dorsal quadrant at the midthoracic level of the spinal cord, are damaged in this model of SCI (Hubscher and Johnson, 1999). The other explanation for a reduction of erectile events following SCI would be through
disruption of descending excitatory mechanisms in addition to disruption of inhibitory tracts. Although, most studies have concluded the descending tracts that modulate sexual reflexes to be inhibitory in nature, recent studies have demonstrated the significance of descending excitatory tracts particularly from the paraventricular nucleus of the hypothalamus (Giuliano and Rampin, 2000).

In conclusion, the current study shows the utility of chronic CSP pressure monitoring for evaluation of recording of both micturition and erectile function after SCI. The measures described reflect coordinated functioning of both somatic and autonomic components of the nervous system, and have been little studied in models of SCI. These functions are important therapeutic targets as they represent critical problems for the spinal cord injured population (Anderson, 2004). This technique may provide valuable objective outcome measurement data in future studies.

4.5 References


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CHAPTER 5

EFFECTS OF TRANSPLANTING GLIAL RESTRICTED PRECURSOR CELLS AND ELEVATING cAMP CONCENTRATIONS ON AUTONOMICALLY REGULATED BEHAVIOR AND HISTOPATHOLOGICAL OUTCOMES IN MALE RATS FOLLOWING SPINAL CORD CONTUSSION INJURY

5.1 Introduction

Impairment of autonomically mediated functions, such as urogenital tract function, following spinal cord injury (SCI) and its subsequent complications are highly prevalent and clinically very important (Hicken et al., 2001; Noreau et al., 2000). Detrusor-sphincter dyssynergia (DSD), cystitis, vesicoureteral reflux, pyelonephritis, renal stone formation, bladder cancer, and impotency are some of the disorders that arise due to lack of normal micturition and sexual function. Although improvement in bladder care of spinal cord injured people over the years has led to a reduction of bladder disorders and death due to renal disease (Ku et al., 2005), urologic care continues to be a challenge. In a recent survey amongst paraplegic and quadriplegic people, improving bladder and bowel
function and regaining sexual function were considered most important for quality of life (Anderson, 2004).

Innervation of the eliminative and sexual organ systems consists of both autonomic and somatic components. In most species, motor neurons that innervate the anal and urethral sphincters, bulbospongiosus and ischiocavernosus muscles, are all located in one cell group referred to as Onuf’s nucleus. In rats, homologues of this nucleus are the dorsomedial (DM) and dorsolateral (DL) nuclei located within the L5-L6 spinal cord (Breedlove and Arnold, 1980; McKenna and Nadelhaft, 1986; Vera and Nadelhaft, 1992). Loss of autonomic regulation of eliminative and sexual functions following SCI results from disruption of ascending afferent pathways in the spinal cord and disruption of descending input to the lumbosacral and sacral spinal cord. Recently, we developed a method to assess recovery of micturition and erectile function in conscious freely moving rats by monitoring corpus spongiosum pressure (CSP) by telemetry (Nout et al., 2005). Using this technique, we demonstrated that following incomplete SCI, recovery of micturition occurs in rats; however, this recovery is limited and voiding remains inefficient. Moreover, changes consistent with the development of spasticity and/or hyperreflexia were determined through waveform analysis. This is consistent with the long-term changes occurring in muscle EMG patterns, micturition frequency, volume, and bladder weight others have shown (Chancellor et al., 1994; Cheng et al., 1999; Kruse et al., 1993; Yoshiyama et al., 2000; Yoshiyama et al., 1999).
In order to repair the injured spinal cord, research is focused on reducing secondary degeneration and promoting regeneration. The adult rat spinal cord has an endogenous progenitor cell population that, according to some studies, is mainly located in the ependymal region around the central canal (Beattie et al., 1997; Mothe and Tator, 2005, Namiki and Tator, 1999), and according to others is present throughout the spinal cord (Horner et al., 2000). The response of these cells after injury, however, is insufficient and does not lead to adequate recovery following SCI (Beattie et al., 1997; Horner et al., 2000; Mothe and Tator, 2005; Namiki and Tator, 1999). Explanations that have been presented for this insufficient endogenous response include insufficient numbers of these cells, the hostile milieu of the lesion center, lack of neurotrophic stimulation and absence of permissive substrates, and presence of inhibitory factors; these have been reviewed by others (Cao et al., 2002b; Horner and Gage, 2000; Popovich et al., 1999; Schmidt, C. E. and Leach, 2003). Cell transplant strategies have the potential of reducing secondary damage and promoting regeneration through many mechanisms such as replacement of lost cells and production of permissive substrates for regeneration (Cao et al., 2002a; McDonald et al., 2004; Okano, 2002; Zhang, 2003). At this time, it appears that optimizing the function of neuronal circuits that survive SCI is the most achievable strategy (McDonald et al., 2004), for example through enhancement of glial cell function. Oligodendrocytes and astrocytes are the major supportive cells within the central nervous system and since they are responsible for myelination of axons and production of trophic factors in addition to providing a surface for axonal growth,
it is reasonable to hypothesize that replacement of this cell population will positively affect regenerative processes.

Immature glial cells have been shown to reduce the inhibitory character of the lesion environment and promote axonal regeneration (Hill et al., 2004; Houle and Reier, 1988; Reier and Houle, 1988; Smith and Silver, 1988). Immature oligodendrocytes provide remyelination after injury (Kohama et al., 2001; McTigue et al., 2001), whereas immature astrocytes promote axonal growth and perhaps promote survival after injury (Silver, 1993). A recent study supports the idea of ensuring both of these cell types, oligodendrocytes and astrocytes, are replaced, since oligodendrocyte precursors failed to remyelinate the spinal cord in the absence of astrocytes (Talbott et al., 2005). After transplanting pluripotent stem cells into normal or lesioned adult rat spinal cord most of these cells had differentiated into astrocytes and no neurons or oligodendrocytes were seen (Cao et al., 2001). This suggests that it may be necessary to induce progenitor cells to differentiate into a certain lineage prior to transplantation. Studies have shown beneficial effects of transplanting glial progenitor cells into the injured spinal cord both in terms of behavior and histopathology (Han et al., 2004; Keirstead et al., 2005; Lee et al., 2005). Our laboratory has previously shown that glial restricted precursor (GRP) cells derived from transgenic rats harboring the heat-stable human placental alkaline phosphatase gene (Kisseberth et al., 1999; Rao et al., 1998) survive and differentiate when transplanted into the spinal cord immediately following injury. Also, under those conditions, GRP cells altered the
lesion environment through reducing astrocytic scarring and through reduced expression of inhibitory proteoglycans (Hill et al., 2004).

Since other studies have shown that the optimal time window for transplanting cells into the lesion site is around day 9 after SCI (Ogawa, Y. et al., 2002b; Okano, 2002), for the current study we use a delayed transplant strategy. Additionally, evaluation of most transplant studies indicates that for optimal results it seems necessary to add substances that promote repair or reduce inhibitors to these cell transplantation techniques. In the current study we elevate cyclic adenosine monophosphate (cAMP) concentrations since this has recently been shown to have beneficial effects on axonal regeneration following SCI (Gao et al., 2004; Nikulina et al., 2004; Pearse et al., 2004; Spencer and Filbin, 2004).

The present study evaluates the effects of GRP cell transplantation and elevation of cAMP concentrations on autonomic outcome measurements and histopathological features. More specifically, we used chronic telemetric monitoring of CSP pressure in awake, freely moving rats to determine recovery of both micturition and erectile events following different interventions including delayed transplantation of GRP cells and elevation of cAMP concentrations through systemic phosphodiesterase IV (PDE-IV) inhibitor and local dibutyryl cAMP (db-cAMP) administration after a moderate spinal cord contusion injury.
5.2 Materials and Methods

Study Design

Fifty-two adult, male Long-Evans hooded rats (Simonsen Laboratories, Gilroy, CA, USA) were used in this study; forty-six rats were age 71 ± 2 days (mean ± SE) and weighed 293 ± 1 g and 6 rats were approximately age 155 days and weighed 469 ± 14 g. This last group served as an end-point age-matched uninjured control (AM control) group for collection of telemetric and histopathological data (Table 5.1). One rat (age 71 days) was used to verify one of the immunohistochemical staining protocols.

Forty-five rats were divided in 4 groups (Table 5.1) in order to investigate the effects of GRP cell transplantation and elevation of cAMP concentrations following SCI. The operated control (OP control) group consisted of 11 rats; these rats were subjected to SCI, received 0.45% NaCl in dimethyl sulfoxide (DMSO) subcutaneously (SQ) by osmotic pump for 14 days, and received 3x3.3μl phosphate buffered saline (PBS, pH=7.4) injected into 3 sites of the lesion region and 2x0.25μl 0.7% NaCl at 0.5cm rostral and 0.5cm caudal to the lesion center. The GRP control group consisted of 11 rats that received GRP cells only; these rats were subjected to SCI, received 0.45% NaCl in DMSO SQ by osmotic pump for 14 days, and received 2-3x10^6 GRP cells in 10μl PBS divided into 3 sites in the lesion region and 2x0.25μl 0.7% NaCl at 0.5cm rostral and 0.5cm caudal to the lesion center. The cAMP control group consisted of 12 rats that received cAMP only; these rats were subjected to SCI, received the PDE-IV inhibitor rolipram (0.5mg/kg/day) in DMSO SQ by osmotic pump for 14
days, and received 3x3.3μl PBS into 3 sites of the lesion region and 2x0.25μl 50mM db-cAMP at 0.5cm rostral and 0.5cm caudal to the lesion center. Eleven rats were administered GRP cells and cAMP (GRP cAMP); these rats were subjected to SCI, received rolipram (0.5mg/kg/day) in DMSO SQ by osmotic pump for 14 days, and received 2-3x10⁶ GRP’s in 10μl PBS divided into 3 sites in the lesion region and 2x0.25μl 50mM db-cAMP at 0.5cm rostral and 0.5cm caudal to the lesion center.

Table 5.1: Outline of experimental groups. For each group age, total number (n), and interventions are shown. Furthermore, the number of animals with transducers and the number of animals that were available for histopathology are shown. Also, the number of animals from which the lesion center was sectioned transversally versus longitudinally is given. AM control = age-matched control, OP control = operated control.
Rats were housed individually in plastic cages, maintained on a 12-h light/dark cycle, and had free access to food and water. All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with NIH guidelines and recommendations.

**Figure 5.1:** Time line for surgical manipulations and administration of treatments. SQ = subcutaneous, IP = intraperitoneal.

**Surgical Procedures and Post-Operative Care**

Surgical procedures (Figure 5.1) were carried out aseptically under deep anesthesia. Anesthetic plane was determined by withdrawal to foot pinch.

Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA, USA) was
applied to the eyes prior to surgery and body temperature was maintained at 37.5 ± 0.5°C using a rectal thermal probe and heating pad. For all surgical procedures, a pre-operative dose of cefazolin (Ancef, Abbott Laboratories, North Chicago, IL, USA; 50 mg/kg SQ) was administered.

**Transducer implantation:** Transducers were implanted in 7 out of 11 OP control rats, 7 out of 11 GRP control rats, 7 out of 12 cAMP control rats, 9 out of 11 GRP cAMP rats, and 5 out of 6 AM control rats (Table 5.1). Anesthesia was induced by inhaled isoflurane (IsoFlow, Abbott Laboratories, North Chicago, IL, USA; 2-3%). For transducer implantation the rat was placed in dorsal recumbency and surgical sites were shaved and cleaned with betadine. A 3 cm skin incision was made on midline on the raphe scroti and a 3 – 4 cm skin incision was made parallel to midline in the right inguinal area. A telemetric pressure transducer catheter (TA11PA-C40, Data Sciences International, St. Paul, MN, USA) was implanted in the bulb of the CSP as described previously (Nout et al., 2005; Schmidt, M. H. et al., 1995). Briefly, the battery of the transducer was placed subcutaneously in the lower right abdomen and secured to the external oblique abdominal muscle with 2 sutures. The catheter was tunneled subcutaneously to the perineal area. Testes were retracted and bulbospongiosus muscle and corpus spongiosum penis (CSP) was exposed. A guide hole was made into the CSP with a 21 gauge needle and the open tip of the catheter was placed into the bulb of the CSP and secured with biological glue and 1 suture. The scrotal incision was then closed in 1 layer and the inguinal incision was closed in 2
layers. The rat was allowed to recover. Telemetric studies commenced 4 – 5 days post operatively. Rats had access to 20ml/day of water containing maple syrup (60ml/L).

Spinal cord injury and osmotic pump placement: Anesthesia was induced by intraperitoneal (IP) administration of pentobarbital (Abbott Laboratories, Chicago, IL, USA; 50 mg/kg). On “day 0”, 6 days following transducer implantation, a 25 g-cm spinal cord contusion injury (SCI) was delivered with a MASCIS/NYU device as previously described (Gruner, 1992). Briefly, a dorsal midline incision was made and a dorsal laminectomy at thoracic vertebra (T)9 –T10 was performed. The spinal cord, with an intact dura mater, was impacted with a 10 g rod from a height of 25 mm. After closing the muscle layers, an Alzet® osmotic pump (model 2ml2; Durect Corporation, Cupertino, CA, USA) was placed subcutaneously to deliver either rolipram (Sigma, St. Louis, MO, USA; 0.5mg/kg/day) in DMSO (Sigma, St. Louis, MO, USA; cAMP control and GRP cAMP groups) or 0.45% NaCl in DMSO (OP control and GRP control groups) for 14 days. These pumps were primed overnight and release a set volume of 5µl/hour. The skin was apposed with skin staples and the animal was allowed to recover.

Post-operative care included continued administration of cefazolin SQ for 12 days, and once or twice daily animal check and bladder expression as needed throughout the remainder of the study. Furthermore, animals were administered lactated Ringer’s solution SQ (Abbott Laboratories, North Chicago, IL, USA) if there was evidence of dehydration or hematuria, and nutritional supplementation
in the form of Nutri-Cal (Evsco Pharmaceuticals, Division of Vétoquinol USA Inc., Buena, NJ, USA) if animals lost more than 10% of bodyweight.

Transplantation: On days 9 and 10 after SCI, rats were administered cyclosporin (Bedford Laboratories, Bedford, OH, USA; 5mg/kg/day IP) as described by Ibarra et al. (1996 a,b). On day 9 after SCI animals were anesthetized with inhaled isoflurane (2-3%). A pre-operative dose of buprenorphine hydrochloride (Buprenex, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, USA; 0.05mg/kg SQ) was administered. The dorsal midline incision was re-opened, the injured spinal cord was re-exposed, and the rostral half lamina of T11 was removed. The transplant procedure was carried out using a 10μl bevel-tip (diameter = 180 μm) FlexiFil microsyringe (World Precision Instruments Inc., Sarasota, FL, USA) assembled in a micromanipulator. The first 3.3μl of either a GRP cell suspension (2-3x10^6 cells) or PBS was injected over 3 min in the lesion center. The second and third 3.3μl aliquots were injected into the lesion region at 3.5 ± 0.1 mm rostral and distal to the first injection site. Depth of injections was approximately 1 mm. Using a similar 10μl FlexiFil syringe assembled in the micromanipulator, 0.25μl db-cAMP (Sigma, St. Louis, MO, USA; cAMP control and GRP cAMP groups) or NaCl (OP control and GRP control groups) was administered on either side (0.7mm) of midline approximately 1mm rostral and caudal to the rostral and caudal injections sites, respectively. Depth of injections was 1 mm. The site of injections is shown in Figure 5.2. The muscle layers were
sutured and the skin was apposed with skin staples. The animal was allowed to recover from anesthesia.

On day 11 after SCI the maple syrup water was replaced by 20ml/day of 1.2% liquid vitamin C (Vedco, Inc., St. Joseph, MO, USA) prepared in maple syrup water (60ml/L). Also, on day 11 after SCI cyclosporin was added to the drinking water. Our goal was to deliver 10mg/kg/day by mouth of cyclosporin throughout the remainder of the study duration (Ibarra et al., 1996a; Ibarra et al., 1996b). For this we assumed that our rats drank 120-144ml/day.

Figure 5.2: Site of injections made into the dorsal spinal cord during the transplant procedure. Thick arrows indicate the site of the 3 GRP or PBS injections. Thin arrows indicate the site of the 4 db-cAMP or NaCl injections.
**Osmotic pump removal:** On day 14 after SCI rats were anesthetized with inhaled isoflurane. The skin at the dorsal midline incision was re-opened over a length of 1cm and the osmotic pump was removed. The skin was apposed with skin staples and the animal was allowed to recover from anesthesia.

**Sacrifice:** At 12 weeks post SCI animals were anesthetized with xylazine (TranquiVed™, Vedco Inc., St. Joseph, MO, USA; 10 mg/kg IP) and ketamine (ketamine HCl, Abbott Laboratories, N.Chicago, IL, USA; 80 mg/kg IP). This was followed by transcardiac perfusion with 0.9% NaCl and 4% paraformaldehyde in PBS. The lesion region and the L4-S2 segment of the spinal cord were isolated and kept in 4% paraformaldehyde for 24 hours and cryoprotected in 30% sucrose in PBS for 24-48 hours. The tissue was frozen at –80°C until further analysis.

**Glial Restricted Precursor Cells**

Transgenic E13.5 rat embryos were used that harbored the heat-stable human placental alkaline phosphatase (hPLAP) gene. In these transgenic rats, the ROSA26 gene promoter directs ubiquitous expression of the hPLAP marker gene (Kisseberth *et al.*, 1999). Glial restricted precursor (GRP) cells were isolated as described previously (Hill *et al.*, 2004; Rao *et al.*, 1998). Trunk segments including the last 10 somites were dissected and titrated to remove the neural tube from the somites. The neural tube was dissociated using papain digestion. E-NCAM-positive cells, which have been shown to be neuron-restricted precursor cells (Mayer-Proschel *et al.*, 1997), were removed by plating the suspension onto E-NCAM-coated dishes for 20 min at 37°C. The supernatant
was then removed and A2B5-positive GRP cells were isolated by positive immunopanning. Cells were plated onto A2B5 antibody-coated dishes for 20 min at 37°C. The supernatant was removed, the plate washed with culture medium, and the bound A2B5-positive cells were placed into 75 cm² tissue culture flasks, coated with fibronectin/laminin solution (FN/LN) containing DMEM-F-12-BS with 10μg/ml of bFGF (10ml total/75 cm² flask). Cells were grown in an incubator at 37°C, 6-7.5% CO₂. After 5-7 days, when cultures reached 50-70% confluence, cells were harvested and transported overnight for transplantation. This procedure yields >98% A2B5-positive cells (Hill et al., 2004). Previous clonal studies have confirmed that these A2B5-positive cells are all GRP cells and can differentiate into oligodendrocytes and astrocytes but not neurons (Mayer-Proschel et al., 1997; Rao et al., 1998).

On arrival, prior to transplantation, viability and number of GRP cells were assessed. The suspension (15 ml total volume) was centrifuged (Centra CL3R, International Equipment Company, Needham Heights, MA, USA) at 300g (1000rpm) for 5 min at room temperature. The pellet containing the GRP cells was then re-suspended in 10μl PBS and used for transplantation as described above.

**Behavioral Tests**

*Locomotor:* Open-field walking was evaluated before SCI (Baseline, BL) and at 24 hours, 48 hours, and 7, 10, 16, 22, 30, 37, 44, 51, 58, 65, 72, 79, and 86 days
following SCI using the 21-point BBB Locomotor Rating Scale (Basso et al., 1995). Furthermore, activity in the cage was determined by telemetry. Movement of the transducer over different detection fields of the receiver was recorded and using Dataquest ART software (Data Sciences International, St. Paul, MN, USA) a moving average in counts per minute was acquired. Data were recorded on days 1, 5-8, 10-15, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 after SCI.

**Micturition and erectile function:** Telemetric data were collected on 7 out of 11 OP control rats, 7 out of 11 GRP control rats, 7 out of 12 cAMP control rats, 9 out of 11 GRP cAMP rats, and 5 out of 6 AM control rats (Table 1). Micturition and erectile characteristics were evaluated as previously described (Nout et al., 2005). Briefly, physiological telemetry data (CSP pressure) were recorded on a PC computer using Dataquest ART software. Data were subsequently analyzed with Spike2 data acquisition and analysis software (version 3.1, Cambridge Electronic Design Limited, Science Park, Milton Road, Cambridge CB40FE, U.K.). All recording occurred while animals were housed in their regular cages. Animals underwent 2x24h CSP pressure baseline (BL) recording prior to SCI. After SCI, CSP pressure was recorded (24 hours/day) on days 1 – 15, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 and analyzed for days 2, 5, 14, and 84.

CSP pressure data were analyzed for the total number of micturitions, full erectile events (tumescence pressure of at least 30 mmHg above baseline on top of which at least 1 suprasystolic pressure peak of 100 mmHg above the tumescence pressure occurs), and partial erectile events (similar to full erectile
events with the exception that peaks never reach 100 mmHg over the
tumescence pressure) in a 24 hour period. Furthermore, a detailed waveform
analysis was performed for 4 days (BL, day 5, 15, 84). For micturitions and
partial erectile events this was performed on the complete 24 hour period and for
full erectile events this was performed on an 8 hour window (9pm – 5am) within
the 24 hour recording period. Data analyzed were duration of the events, area
under the curve (AUC), total number of pressure peaks, peak frequency and
mean and maximum pressures. Furthermore, a peak analysis was performed.
For micturition events this included the number of afterpeaks, and afterpeak
duration, AUC, and mean and maximum pressures. For erectile events this
included the duration, AUC, and mean and maximum pressures of the peaks that
occurred during those events.

Reflex erection tests: Ex copulatory reflex erection tests were performed as
previously described (Hart, 1968; Nout et al., 2005; Schmidt, M. H. et al., 1995).
Briefly, rats were placed on a board in dorsal recumbency with the head and
anterior torso in a loose-fitting restraining cylinder. The abdomen was secured to
the board with masking tape. The head and anterior torso could move freely in
the cylinder. The telemetric receiver unit was placed directly under the board.
The preputial sheath was retracted, which is the stimulus to elicit reflex erections,
and maintained in retracted position by placing the glans penis through a hole in
a small piece of masking tape fastened to the abdomen. Once the sheath was
retracted, the reflex test lasted for 20 min. Rats were habituated to this procedure
prior to surgery for 2x10 min and 2x20 min and the animals quickly adapt to the testing situation. Events were visually scored according to previously described criteria (Schmidt, M. H. et al., 1995): E1, weak glans engorgement; E2, moderate glans engorgement involving some dilation of the distal glans; E3, intense flaring or cup of the distal glans; F1, dorsiflexion of the penile body; F2, dorsiflexion or flip of the penile body greater than 90° with respect to the body of the rat. These reflexes were scored with a numerical keypad as events 1 – 5, respectively.

Reflex testing was performed twice prior to SCI and on days 2, 7, 16, 22, 30, 44, 58, 72, and 86 following SCI. The 6 animals in the AM control group were tested twice, once prior to and once after transducer implantation.

**Histopathology and Analysis – Lesion Center**

Lesion regions (10mm) were sectioned at 20 μm on a cryostat transversally (5 out of 10 OP control animals, 5 out of 10 GRP control animals, 6 out of 11 cAMP control animals, and 5 out of 9 GRP cAMP animals) or horizontally (5 out of 10 OP control animals, 5 out of 10 GRP control animals, 5 out of 11 cAMP control animals, and 4 out of 9 GRP cAMP animals) (Table 5.1). Lesion regions were randomly assigned to each of the 2 groups.

First, we verified that the immunohistochemical staining protocol using the human placental alkaline phosphatase (hPLAP) antibody described below was useful in detecting the transplanted GRP cells *in vivo*. One animal was sacrificed 48 hours after receiving an injection of 3x10^6 GRP cells into the spinal cord at T9. The technique used for injecting GRP cells was similar to that described for the
transplantation surgery. The injection area was cut transversally at 20 μm on a cryostat. Every other section was stained for hPLAP using both the enzymatic alkaline phosphatase (AP) staining method and the immunohistochemical protocol described below and in Table 5.2.

Endogenous AP was denatured by heating the slides to 60ºC for 1 hour before reacting the tissue with Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, toluidine salt; Roche Diagnostics Corporation, Indianapolis, IN, USA; NBT/BCIP stock solution diluted 1/20) for 1.5 hours in AP buffer (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) with 5mM levamisole to block any remaining endogenous AP activity. The other sections were stained using the immunohistochemistry procedure described below.

**Immunohistochemistry:** Every sixth transverse section was stained for hPLAP and glial fibrillary acidic protein (GFAP, for astrocytes), hPLAP and CC-1 (for oligodendrocytes), and hPLAP and nestin (for undifferentiated cells). Every third horizontal section was stained for hPLAP and serotonin. Immunohistochemistry was performed at room temperature. Slides were first rinsed with PBS for 2x10 min and subsequently incubated with blocking solution containing 10% Goat Serum (Sigma, St. Louis, MO, USA) and 0.2% triton in PBS for 60 min. Incubation with primary and secondary antibodies followed as specified in Table 5.2 after which slides were rinsed with PBS for 2x10 min. Slides were then coverslipped with Vectashield with DAPI (nuclear counterstain) hard-set mounting media (Vector’s Laboratory Inc., Burlingame, LA, USA and stored at 4ºC.
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Inc. time</td>
<td>Isotype</td>
</tr>
<tr>
<td>hPLAP</td>
<td>1:200</td>
<td>O/N</td>
</tr>
<tr>
<td>CC-1</td>
<td>1:100</td>
<td>O/N</td>
</tr>
<tr>
<td>GFAP</td>
<td>1:1000</td>
<td>O/N</td>
</tr>
<tr>
<td>Nestin</td>
<td>1:10</td>
<td>O/N</td>
</tr>
<tr>
<td>5-Ht</td>
<td>1:500</td>
<td>O/N</td>
</tr>
<tr>
<td>Syn.</td>
<td>1:200</td>
<td>O/N</td>
</tr>
</tbody>
</table>

Table 5.2: List of antibodies used for immunohistochemistry. Source, concentration, and incubation time is shown for each antibody. Inc. time = incubation time, The Nestin Rat-401 hybridoma was developed by Susan Hockfield and obtained from the Developmental Systems Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. [ ] = concentration, Inc. time = incubation time, hPLAP = human placental alkaline phosphatase, O/N = overnight, CC-1 = APC antibody CC-1, GFAP = glial fibrillary acidic protein, 5-Ht = serotonin, Syn. = synaptophysin, GAM = goat-anti-mouse, GAR = goat-anti-rabbit, AF = Alexa-Fluor, PBS = phosphate buffered saline (ph=7.4).
**Analysis:** For the 21 animals from which the lesion region was cut transversally, the area of tissue at the lesion center was determined using Adobe Photoshop vs.5 (Adobe Systems Incorporated, San Jose, CA, USA). For this, one section per animal taken at the lesion center was used. These sections were stained for hPLAP and GFAP. Slides were examined and images taken under standard fluorescence at 10x (Zeiss Axioplan2 microscope, Carl Zeiss, Hallergmoos, Germany). Dimensions were normalized to a more distal section to correct for tissue loss. We determined GFAP and hPLAP area in one section at the lesion center that was identified by containing the smallest amount of tissue.

For the 21 animals that received a GRP cell transplant, an approximation of the volume of GRP cells that occupied the lesion region was determined. In the transversally cut sections this was done by first imaging every other section on slides stained for hPLAP and GFAP. Images were then analyzed using MetaMorph software. The outline of the section, lesion cavity, and hPLAP-positive area were traced. Area of lesion cavity was subtracted from the section area and the percentage of hPLAP-positive area was calculated for each animal. The area of GRP cells in horizontal sections was determined in sections stained for hPLAP and serotonin using PhotoShop. For the 9 animals in this group 3 images of each lesion region were made. Area of tissue, hPLAP-positive area, and area of lesion cavities were determined. The percentage hPLAP-positive area in the lesion region was determined.

To determine GRP cell survival and differentiation we examined sections for the presence of double-labeled cells expressing hPLAP and nestin, hPLAP
and GFAP, or hPLAP and CC-1. Slides from 10 animals were examined and images were taken on a Zeiss 510 META confocal laser scanning microscope (Hallergmoos, Germany) at 63x. Stacks of images were collected with a distance of 0.37 μm between consecutive slices. These stacks were processed using AutoDeblur & AutoVisualize vs.9.3 software (AutoQuant Imaging Inc., Watervliet, NY, USA) and analyzed using MetaMorph vs.6.3 software (Molecular Devices Corporation, Downingtown, PA, USA). A rostral, middle, and caudal section were used for this analysis. Images were made from the area where the transplant integrated with the host tissue. Three images were made from 3 different parts of the transplant in that section and while choosing the area to be imaged, the slide was examined for hPLAP only. This ensured our sampling technique was not based on double-labeling. The slice showing most co-localization was determined using MetaMorph software and cells were manually counted. The number of cells that expressed hPLAP was determined and the number of cells that expressed both hPLAP and nestin, hPLAP and CC-1, or hPLAP and GFAP was determined.

**Histopathology and Analysis – Lumbosacral Spinal Cord**

Lumbosacral spinal cord blocks for all available animals (n = 44) were sectioned transversally at 20 μm on a cryostat. In order to evaluate the serotonergic input to the lumbosacral spinal cord, immunohistochemistry for synaptophysin and serotonin was performed on every other section. The immunohistochemical protocol was similar to what is described above and in
Table 5.2 for the lesion region, with the exception that TOTO-3 cyanine dimer nucleic acid stain (Invitrogen Corporation, Carlsbad, CA, USA; 2mM) was added to the solution containing the secondary antibodies. Slides were then cover-slipped with Vectashield hard-set mounting media (Vector’s Laboratory Inc., Burlinghame, LA, USA and stored at 4°C.

Analysis: Slides from 44 animals were examined and images taken on a Zeiss 510 META confocal laser scanning microscope (Hallergmoos, Germany) at 63x with 2x zoom. For each animal 12 motor neurons located in the DL nuclei (6 left side, 6 right side) and 8 motor neurons located in the DM nuclei (4 left side, 4 right side) were imaged. Images were made based on synaptophysin and TOTO-3 staining. Serotonin staining was not examined prior to collecting the images. Stacks of images were collected with a distance of 0.37 μm between consecutive slices. These stacks were processed as described above using AutoDeblur & AutoVisualize vs.9.3 software and analyzed using MetaMorph vs.6.3 software. The amount of serotonin within the stack was determined. This enabled us to quantify the amount of serotonin in close proximity to motor neurons in the DM and DL nuclei.

Statistics

Data are presented as means ± standard error of the mean (SE). A factorial repeated measures analysis of variance (ANOVA) was used to analyze
all behavioral data. Furthermore, a factorial repeated measures analysis of covariance (ANCOVA) with the covariate factor being baseline data was used to analyze behavioral data. The null hypothesis was rejected at $\alpha = 0.05$. A one-way ANOVA was used to compare BL and end point data of the OP control, GRP control, cAMP control, and GRP cAMP groups to that of the AM control group. A one-way repeated measures ANOVA was used to determine significant differences between BL and endpoint data. Significant differences identified by the ANOVA were isolated using the Holm-Sidak procedure for pairwise multiple comparison post-hoc test.

A t-test was used to analyze the area of tissue occupied by GRP cells and the differentiation of the GRP cells. Other histopathological data were analyzed using a one-way ANOVA. Significant differences identified by the ANOVA were isolated using the Holm-Sidak procedure for pairwise multiple comparison post-hoc test. The statistical computations were performed with software packages (Sigmastat 3.0, SPSS, Chicago, IL and SPSS 12.0, SPSS, Chicago, IL).

5.3 Results

Rats were 71 ± 2 days and weighed 293 ± 1 g for transducer implantation. Rats were 77 ± 2 days and weighed 318 ± 2 g at the time of SCI. At the time of transplant and sacrifice rats weighed 289 ± 3 g and 385 ± 6, respectively. At the time of sacrifice average weights of animals were similar in all groups (OP control: 392 ± 11 g; GRP control: 383 ± 14 g; cAMP control: 380 ± 13 g; GRP cAMP: 391 ± 12 g). Five animals died unexpectedly during the time-course of this
study; 1 animal from the OP control group died on day 9 following SCI, 1 animal in the cAMP control group died on day 11 following SCI, 1 animal in the GRP control group died on day 22 following SCI, and 2 animals in the GRP cAMP group died on days 53 and 79 following SCI.

The moderate to severe SCI that rats were subjected to in this study was a very consistent lesion based on the parameters recorded by the impactor device (Figure 5.3).

![Impact parameters](image)

**Figure 5.3:** Impact parameters. Subset of impact parameters shown for the 4 groups of rats injured in this study.
Behavioral Outcomes

**Locomotor:** The 25 g-cm SCI resulted in an initial profound paraplegia in all rats characterized by BBB locomotor scores of 0.6 ± 0.3 (OP control) 0.5 ± 0.3 (GRP control) 0.4 ± 0.3 (cAMP control), and 0.6 ± 0.1 (GRP cAMP) at 24 hours after SCI. Gradually, rats showed some recovery of hind limb locomotor function (Figure 5.4), however, no significant differences were found between groups. BBB locomotor scores were 8.2 ± 0.4 (OP control) 8.0 ± 0.6 (GRP control) 8.0 ± 0.5 (cAMP control), and 8.6 ± 0.4 (GRP cAMP) at the time of sacrifice (Figure 5.4). This remained significantly different from BL (p<0.001; F=404). A BBB score of 8 indicates animals are able to extensively move all 3 joints of the hind limbs, and in some cases animals are able to place their paws in a plantar position, however, there is no weight support.

The level of activity of the rats when housed in their cages is shown in Figure 5.5. The immediate reduction of activity following SCI was followed by a steady recovery during the first 3 weeks after SCI. The transplantation surgery at day 9 resulted in a second drop in level of activity. This is seen in Figure 5.5 as a set-back during the recovery phase after SCI. In our animals the level of activity reached a plateau between 3 – 5 weeks following SCI. The level of activity of all rats at the end point of this study was similar to that of the AM control group, which was significantly less than activity recorded for rats at BL for the OP control (p=0.03; F=9), cAMP control (p<0.001; F=36), and GRP cAMP (p=0.009; F=14) groups. No significant differences were found between groups.
Figure 5.4:  Recovery of locomotion function. For the 4 groups of rats the BBB locomotor score (AVG ± SE) is shown for baseline (BL) and for the duration of the study. Black arrow indicates time of transplantation surgery (day 9).
★ All groups significantly different from BL.

Figure 5.5:  Activity of all rats in their cages. The level of activity is shown in arbitrary units. Black arrow indicates time of transplantation surgery (day 9).
★ OP control, cAMP control, GRP cAMP, and AM control significantly different from BL.
**Micturition function:** Baseline data showed an average number of micturition events per 24 hours per rat of 22 ± 2 (OP control), 20 ± 2 (GRP control), 29 ± 2 (cAMP control), and 27 ± 1 (GRP cAMP). First spontaneous micturition events were detected by telemetry 2 – 4 days following SCI. Recovery of the total number of micturition events is shown for BL and days 2, 5, 14, and 84 after SCI in Figure 5.6A. Total number of micturition events on day 84 following SCI was 12 ± 3 (OP control), 10 ± 2 (GRP control), 15 ± 3 (cAMP control), and 19 ± 1 (GRP cAMP). No statistically significant differences were present between groups. To determine the effect of baseline variation an ANCOVA was performed. Results are plotted (Figure 5.6B).

Although in most animals recovery of micturition was sufficiently effective that manual bladder expression was no longer required, the number of micturitions per 24 hour period at the end point of the study remained significantly lower than the number of micturitions recorded at baseline for all groups (OP control: p=0.03; F=12; GRP control: p=0.05; F=11; cAMP control: p=0.02; F=11; GRP cAMP: p=0.008; F=18). Furthermore, the number of micturitions at the end point of the study was lower than the number of micturitions recorded from the AM control group (23 ± 2) as shown in Figure 5.6A; however, this was only significant for the OP control and GRP control groups (p=0.008; F=4.6).
Figure 5.6 A,B: Recovery of micturition function shown for all groups. The number of micturitions per 24 hours recorded by telemetry is shown for baseline (BL) and days 2, 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 25.3 micturitions/24h. ★ significantly different from BL; + significantly different from AM control.
Performing detailed pressure waveform analysis revealed increased duration of micturition events over time (Figure 5.7A), increased frequency of micturition pressure peaks (Figure 5.8A), and increased mean pressures during micturition events (Figure 5.9A). To determine the effect of baseline variation ANCOVA’s were performed on each of these parameters; results are plotted in Figures 5.7b, 5.8B, and 5.9B.

Micturition peak frequency is a robust parameter as demonstrated by small standard errors and little variability between groups. Furthermore, the micturition peak frequency recorded in the AM control group was not significantly different from BL data of the other 4 groups. Comparing day 84 to BL revealed significant increase of micturition peak frequency for the OP control and cAMP control groups (p=0.03; F=11.6 and p=0.01; F=12.7, respectively). At day 84 the micturition peak frequency of the cAMP control and the GRP cAMP groups were significantly larger compared to the AM control group (p=0.02; F=3.6). No significant effect of group over time was seen.
Figure 5.7 A,B: Duration of micturition events. The duration of micturition events is shown for baseline (BL) and days 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 3.4s.
Figure 5.8 A,B: Frequency of micturition pressure peaks. The frequency of micturition pressure peaks is shown for baseline (BL) and days 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 8.6 Hz.

★ significantly different from BL; + significantly different from AM control.
Figure 5.9 A,B: Mean pressure during micturition events. The mean pressure of micturition events is shown for baseline (BL) and days 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 32.5 mmHg.
Erectile function: Baseline data resulted in an average number of full erectile events per 24 hours per rat of 52 ± 3 (OP control), 63 ± 4 (GRP control), 47 ± 6 (cAMP control), and 63 ± 9 (GRP cAMP). Figure 5.10A shows the change in number of events on days 2, 5, 14, and 84 after SCI. The total number of full erectile events on day 84 following SCI was 32 ± 2 (OP control), 40 ± 10 (GRP control), 30 ± 4 (cAMP control), and 37 ± 7 (GRP cAMP). This reduction in number of erectile events per 24 hours was only significant in the OP control and cAMP control groups (p=0.006; F=28 and p=0.03; F=8.7, respectively). No statistically significant differences were present between groups or between the injured groups and the AM control group (45 ± 5 erectile events per 24h). To determine the effect of baseline variation an ANCOVA was performed; results are plotted (Figure 5.10B). Performing detailed pressure waveform analysis revealed slight increases in waveform characteristics such as erectile event duration (Figure 5.11A) and erectile event maximum pressures (Figure 5.12A); however the only significant effects were an increase in maximum pressure on day 84 after SCI compared to BL in the OP control and cAMP control groups (p=0.02; F=9.1). To determine the effect of baseline variation ANCOVA’s were performed on each of these parameters; results are plotted in Figures 5.11B and 5.12B.
Figure 5.10 A,B: Number of full erectile events. The number of full erectile events per 24 hours recorded by telemetry is shown for baseline (BL) and days 2, 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 54 erectile events per 24h.
★ significantly different from BL.
Figure 5.11 A,B: Duration of full erectile events. The duration of full erectile events is shown for baseline (BL) and days 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 22.4s.
Figure 5.12 A,B: Maximum pressure during full erectile events. The maximum pressure during full erectile events is shown for baseline (BL) and days 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 394 mmHg. ★ significantly different from BL.
Baseline data resulted in an average number of partial erectile events per 24 hours per rat of 8 ± 2 (OP control), 8 ± 2 (GRP control), 7 ± 2 (cAMP control), and 13 ± 3 (GRP cAMP). Figure 5.13A shows the change in number of events on days 2, 5, 14, and 84 after SCI. The total number of partial erectile events on day 84 following SCI was 3 ± 1 (OP control), 3 ± 2 (GRP control), 3 ± 1 (cAMP control), and 3 ± 1 (GRP cAMP). Although these numbers were significantly lower than those recorded on BL for the OP control (p=0.002, F=54), cAMP control (p=0.005; F=19), and GRP cAMP (p=0.007; F=19) animals, no statistically significant differences were present between groups. Furthermore, the number of partial erectile events per 24 hours at 84 days after SCI was lower in all injured groups compared to those recorded for the AM control group (6 ± 3), but this was not statistically significant. To determine the effect of baseline variation an ANCOVA was performed; results are plotted (Figure 5.13B).
Figure 5.13 A,B: Number of partial erectile events. The number of partial erectile events per 24 hours recorded by telemetry is shown for baseline (BL) and days 2, 5, 14, and 84 after SCI (A). In B the estimated marginal means are shown determined by ANCOVA set at BL = 10.6 partial erectile events per 24h. ★ significantly different from BL.
*Reflex erection tests:* Following SCI, latency (time of first erectile event following sheath retraction) decreased at all time points (Figure 5.14). Although some recovery occurred, latency remained significantly lower than BL for all groups (OP control: p<0.001; F=28; GRP control: p<0.001; F=29; cAMP control: p=0.002; F=16; GRP cAMP: p<0.001; F=61). Furthermore, latency recorded in the AM group was significantly lower than latency measured at BL in the injured groups (p<0.001; F=14). At day 86 after SCI, latency in the GRP control and GRP cAMP groups remained significantly lower than that of the AM group (p<0.001; F=14).

![Reflex erection test - latency](image)

**Figure 5.14:** Latency during the reflex erection test. For all groups of rats the latency (time to first erection after sheath retraction) during the ex copulatory reflex erection test is shown for baseline (BL) and for the duration of the study. ★ all groups significantly different from BL. ✫ GRP control and GRP cAMP groups significantly different from AM control.
The number of suprasystolic pressure peaks that occurred during erectile events increased from BL at all time points following SCI (Figure 5.15). At day 86 after SCI the number of pressure peaks per event was significantly higher than at BL for the GRP control (p=0.002; F=28) and cAMP control (p=0.016; F=92) groups. Furthermore, the number of pressure peaks at day 86 following SCI remained significantly greater than that recorded in the AM control group for all groups (p<0.001; F=7.6).

Figure 5.15: Number of peaks per erectile event. For all groups of rats the number of suprasystolic pressure peaks per event during the ex copulatory reflex erection test is shown for baseline (BL) and for the duration of the study. ★ GRP control and cAMP control significantly different from BL. ● all groups significantly different from AM control.
Histopathology – Lesion Center

Specificity of the hPLAP antibody we used in this study is demonstrated in Figure 5.16. In Figure 5.16 two transverse sections of spinal cord are shown of a rat that had GRP cells injected into its spinal cord 48 hours prior to sacrifice. The section in Figure 5.16A is stained using the enzymatic AP method and in Figure 5.16B GRP cells are identified by immunohistochemistry.

Figure 5.16 A,B: Thoracic spinal cord stained for GRP cells 48 hours after cell transplant. Transverse sections (20 μm) of spinal cord showing the area of the dorsal funiculi just dorsal to the central canal. In A GRP cells are identified by the enzymatic alkaline phosphatase method and appear dark brown – black. In B GRP cells are identified using immunohistochemistry and appear green. Cell nuclei appear blue due to the DAPI nuclear counterstain.
**Tissue at the lesion center:** No residual gray matter was observed at the lesion center, but a thin layer of white matter remained. Tissue (GFAP positive) present at the lesion center was determined in transverse sections of the spinal cord at the level of the lesion center (Figure 5.18 and 5.19). Figure 5.18 shows examples of lesion centers for all 4 groups. In Figure 5.19A the percentage of tissue present at the lesion center is shown as compared to tissue in a more distal section of normal appearing thoracic spinal cord. In Figure 5.19B the hPLAP positive area in the lesion center is shown as a percentage of tissue in a more distal section of normal appearing thoracic spinal cord.

**Figure 5.17:** Illustrations of lesion center and lesion region. The lesion region typically spanned a length of 10mm, whereas the lesion center was a 20µm thick section.
Figure 5.18 A-D: Representative lesion centers for all groups. A = OP control, B = GRP control, C = cAMP control, D = GRP cAMP. Sections are 20 μm thick and stained for GFAP, hPLAP, and DAPI by immunohistochemistry. Green represents astrocytes (GFAP), red / yellow represents GRP cells (hPLAP), and blue represents nuclei (DAPI). Scale bar = 20mm.
Figure 5.19A,B: Tissue at lesion center. In A the area of GFAP positive tissue at lesion center is shown as a percentage of GFAP positive tissue in a distal section of thoracic spinal cord. In B the area of hPLAP positive tissue at lesion center is shown as a percentage of GFAP positive tissue in a more distal section of thoracic spinal cord. ★ significantly different from GRP control.
Although there appears to be more tissue at the lesion center in the 2 groups of animals that received GRP cell transplants, these differences were not statistically significant. The hPLAP area at the lesion center was significantly larger in the GRP control group compared to the GRP cAMP group (p<0.001; F=15.6).

**Volume of tissue and GRP cells in lesion region:** GRP cells were present at 84 days after SCI in all animals that had received transplants. In the Appendix representative images are shown of the transplants of each of the 19 animals that received GRP cell transplants. Images are shown from an area in the lesion region, adjacent but not at lesion center. To determine the area occupied by tissue (GFAP positive) and GRP cells (hPLAP positive) in the transversally cut spinal cords from 10 animals, 35 – 45 (range) sections were imaged per animal. The distance between consecutively imaged sections was approximately 200 μm. Figure 5.20 shows that there was significantly more tissue present throughout the lesion region in animals that received a GRP cell transplant (p=0.003; F=6.9).

Figure 5.21A shows the hPLAP positive area throughout the lesion region determined for the 2 groups. Although the hPLAP area in the GRP control group appears larger than in the GRP cAMP group this difference was not statistically significant. For 9 animals the hPLAP positive area was determined in horizontal sections. The distance between the 3 horizontal sections that were imaged was approximately 280 μm. The hPLAP positive area is shown for both groups in Figure 5.21B.
Figure 5.20: Tissue in lesion region. Area of GFAP positive tissue in lesion region corrected for tissue loss. ★ significantly different from OP control and cAMP control.

Figure 5.21 A,B: Area of hPLAP positive cells in the lesion region. Percentage of tissue in the lesion region occupied by hPLAP positive cells is shown for transverse sections (A) and for horizontal sections (B).
**GRP cell survival and differentiation:** GRP cells were present at 84 days after SCI in all animals that had received transplants and were found to express nestin, GFAP, and CC-1 (Figure 5.22 A-C and 5.23). The quantification method used in this study allowed determination of cell differentiation of a total of 16,046 hPLAP positive cells (8,744 GRP control and 7,302 GRP cAMP); 5,356 were examined for nestin expression, 5,018 for GFAP expression, and 5,672 for CC-1 expression. The proportion of hPLAP positive cells that expressed nestin was 12 ± 4% for GRP control and 10 ± 2% for GRP cAMP. The proportion of hPLAP positive cells that expressed GFAP was 60 ± 7% for GRP control and 42 ± 6% for GRP cAMP (p=0.07; F=4.4). The proportion of hPLAP positive cells that expressed CC-1 was 33 ± 5% for GRP control and 41 ± 5% for GRP cAMP. These results are shown in Figure 5.23.
Figure 5.22 A-C: Differentiation of hPLAP positive GRP cells. Each confocal image represents one optical slice from a 20 μm section. In A GRP cells are shown that express both hPLAP (red) and nestin (green). In B GRP cells are shown that express both hPLAP (red) and GFAP (green). In C GRP cells are shown that express both hPLAP (red) and CC-1 (green). Cell nuclei are stained with DAPI (blue). Scale bar = 50μm.
Figure 5.23: Quantification of differentiation of hPLAP positive GRP cells. The percentage of hPLAP positive cells expressing nestin, GFAP, or CC-1 is shown.

Analysis of Lumbosacral Spinal Cord

For 44 animals 20 cells from the DM and DL nuclei in the lumbosacral spinal cord were imaged. The location of the DM and DL nuclei is shown in Figure 5.24 A and B, respectively. Quantification of the amount of serotonin in close proximity to these motor neurons was significantly less following SCI when compared to the AM control group (p<0.001; F=5.4), however, no differences between groups were found (Figure 5.25). Figure 5.26 shows examples of cells imaged in these nuclei.
Figure 5.24 A,B: Dorsomedial and dorsolateral nuclei in the lumbosacral spinal cord. Both sections are stained for serotonin (green) and cell nuclei (blue) by immunohistochemistry. In A the 2 dorsomedial nuclei are shown left and right from midline just ventral to the central canal. B shows the right dorsolateral nucleus in the ventral horn of the lumbosacral spinal cord.

Figure 5.25: Serotonergic input to motor neurons in the lumbosacral spinal cord. Quantification of serotonin in close proximity to motor neurons shown in arbitrary units for all groups of rats. + significantly different from AM control.
Figure 5.26 A,B: Motor neurons found in the dorsomedial and dorsolateral nuclei. Two representative images of motor neurons located in the dorsomedial or dorsolateral nuclei. Sections are 20 μm thick and stained for serotonin (green), synaptophysin (red), and DNA/RNA (TOTO-3) by immunohistochemistry. The extensive dendritic field with synapses in these nuclei and the serotonergic input (green) to the motor neurons is shown in this figure. Scale bar = 50 μm.

5.4 Discussion

The transplantation technique used in this study did not have a significant impact on locomotor or autonomic behavioral outcomes; however GRP cells did alter the cellular composition of the lesion region. The treatment strategy used here resulted in a significant increase of tissue throughout the lesion region. Moreover, GRP cells appeared to increase the amount of tissue at the lesion center. The GRP cells survived, differentiated, and formed extensive transplants that were well integrated with host tissue in all animals that received a GRP cell
transplant. Furthermore, in the face of elevated cAMP concentrations these GRP
cells appeared to differentiate more into oligodendrocytes and less into
astrocytes than when cAMP concentrations were not altered. Elevated cAMP
concentrations, however, did reduce the area occupied by GRP cells at the
lesion center and appeared to reduce the volume of GRP cells throughout the
lesion region. The histopathological alterations we found in the lesion region did
not appear to affect serotonergic input to the DM and DL nuclei in the
lumbosacral spinal cord.

Impact parameters produced by the MASCIS-NYU device were similar to
previous reports such as Basso et al., 1996. Our moderate contusion injury
produced a very consistent lesion with the area of remaining tissue at the lesion
center in the OP control group (12.4 ± 2.7%) in the same range as described
previously for this level of injury (9.9 ± 4.8%; Basso et al., 1996). The treatment
interventions studied in this experiment, transplantation of GRP cells and
elevation of cAMP concentrations, did not negatively impact animal health based
on similar weight gain in all experimental groups. However, final locomotor
scores in this study were lower than previously reported for this level of injury at 6
weeks after SCI. (BBB score OP control: 8.2 ± 0.4 vs. 10.3 – 10.6; Basso et al.,
1995 and 1996). Although Basso et al. (1996) examined recovery of males and
females from this level of injury and did not find a significant difference between
the 2 sexes, group numbers were low and weight differences between groups
were not reported. More recently it has been shown that gender differences do
exist during recovery from central nervous system trauma in humans (Stein,
2001) and rats (O'Connor et al., 2003; Roof and Hall, 2000). Furthermore, a recent study demonstrated improved recovery from SCI in female rats and mice when compared to males (Hauben et al., 2002). The exact mechanism for this is unknown but likely is a result of multiple factors, such as differences in hormones, immune responses, and size/weight. One group has suggested that smaller animals recover faster from SCI than larger ones (Bambakidis and Miller, 2004). When comparing our results with other similar studies, one must keep in mind that we examined relatively heavy male rats unlike most other studies that have looked at recovery in lighter female rats.

Prior to the start of this experiment, the transplantation technique using the same FlexiFil syringe was examined in 2 animals with intact spinal cords. No detrimental effect on hind limb motor function was noticed in these animals when examined using the BBB locomotor rating scale. However, the impact of this procedure may be larger in injured animals that are just recovering from a moderate contusion injury. When the BBB locomotor scores are examined closely one is able to distinguish a short period in which the recovery appears blunted between 7 and 10 days after SCI. Perhaps the damage from our transplantation procedure that is negligible in healthy rats was sufficient to negatively affect recovery during a critical phase after SCI in the rats used in this study. From the graph that depicts the movement of animals in their cages, one recognizes a negative impact on activity of the transplantation procedure. In fact it takes approximately 7 days for rats to regain their pre-transplantation surgery level of activity. It has been well established that exercise improves locomotor
recovery after SCI (Behrman and Harkema, 2000; Engesser-Cesar et al., 2005; Field-Fote and Tepavac, 2002; Van Meeteren et al., 2003); however it remains to be determined whether a transient decrease of activity during this sub-acute phase after SCI would have a substantial effect on long-term outcome.

Interestingly, a negative effect from the transplantation procedure may also contribute to what is seen in another study (Mitsui et al., 2005b) in which rats underwent a similar protocol of SCI followed by transplantation of precursor cells in 3 sites of the lesion at 9 days following SCI. In this study transplant injections in the dorsal midline of the spinal cord were performed using a Hamilton syringe with a tip of 310μm which is significantly larger than what we used (180μm). This study demonstrated a beneficial effect of transplanting a combination of neuronal precursor (NRP) and GRP cells that resulted in improvement of the BBB locomotor score from 7.1 (operated control group) to 9.4 (NRP GRP group). The operated control group not only had a low final BBB score, but also showed a drop in locomotor recovery at 1 week after transplant. The apparent negative impact of the surgical procedure seen in the operated control group, which is more dramatic than what we have seen in our study, was not discussed. Mitsui et al. (2005b) attributed the comparatively low BBB score seen in their operated control group to the modified-moderate contusion injury they use, in which the impactor rod is left on the spinal cord for an additional 5 sec following a 25 g-cm contusion. However, review of the paper that demonstrates their modified-moderate contusion model (Mitsui et al., 2005a) indicates that BBB scores are expected to be 3.5 in operated control animals at 8
weeks after transplant. This latter study (Mitsui et al., 2005a) demonstrated a positive effect of transplanting fibroblasts expressing brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) on recovery of locomotor function following SCI in a similar experimental paradigm as described above for their NRP GRP study. Transplantation of fibroblasts expressing BDNF and NT-3 improved BBB locomotor scores from 3.5 (operated control) to 8.2. From the results of that study no negative impact of the surgical procedure was seen on locomotor recovery.

Another study (Pearse et al., 2004) demonstrated positive effects on locomotor outcome of a combination of transplanting Schwann cells 7 days after a moderate SCI and elevating cAMP concentrations. This group used the same methodology as we did to perform the transplant and elevate cAMP concentrations. Although at 8 weeks after transplant the BBB score of the injured-only group was 10.4 vs. 15.0 in the treated group, at 1 week after transplant the injured-only group had a BBB score of 7 vs 10.5 in the treated group. No BBB scores are shown prior to 1 week after transplant. Unfortunately it is unknown whether pre-existing differences in BBB scores were present. Furthermore, this does not allow interpretation of acute effects of the transplantation surgery.

Transplantation of multineurotrophin-expressing GRP cells had positive effects on locomotor recovery (Cao et al., 2005) as demonstrated by a BBB score of 15.6 in treated animals vs. 13.1 in operated control animals. The experimental paradigm was similar to our study and the studies conducted by
Mitsui et al. (2000a and 2000b), in that transplantation was performed 9 days after SCI. However, the level of injury (150 kdyn; Infinite Horizon device) was much less severe and resulted in BBB scores of 10 – 12 prior to transplant and 12.2 – 15.6 (operated control and multitrophin-expressing GRP group, respectively) at 6 weeks after SCI. This study used a transplantation technique that involved a total of 4 injections; 2 bilaterally rostral to the lesion region 0.6 mm off midline and 2 bilaterally distal to the lesion region 0.6 mm off midline with a glass micropipette with an outer diameter of 50-70μm. No negative effects of the surgical procedure were apparent from the locomotor recovery graphs.

Other studies examining the effects of different glial progenitor cells such as the human embryonic oligodendrocyte precursor cells (OPCs) (Keirstead et al., 2005) and oligodendrocyte precursors (Bambakidis and Miller, 2004) have demonstrated positive effects on locomotor outcome however these studies were conducted in less severe SCI models (Bambakidis and Miller, 2004; Keirstead et al., 2005). One elegantly performed study in male rats of similar weight as ours, demonstrated a positive effect of transplanting oligodendrocyte-type 2 astrocytes (O-2A) into the lesion center on locomotor recovery after a 25 g-cm SCI (Lee et al., 2005). Although a significant difference was found in BBB score at 6 weeks after transplantation (9.5 in operated control animals vs. 12.3 in treated animals), few significant effects of transplantation were seen during electrophysiological assessment of these animals. The O-2A cells used in this study differentiated into oligodendrocytes that had the ability to promote remyelination and regeneration of axons. Differences between GRP cells and O-2A/OPC cells
include different chemokine response patterns, different responses to inducers of differentiation, and different behaviors of the 2 precursor cell populations following transplantation (Rao et al., 1998). The latter is most striking and reflects the ability of GRP cells to readily generate astrocytes whereas the O-2A/OPC cells only generate oligodendrocytes (Noble et al., 2004). Differences in characteristics of these progenitor cells undoubtedly affect eventual functional outcomes.

Although number of micturitions per 24 hours, micturition duration, and erectile event duration appeared to have recovered more in the GRP cAMP treatment groups, none of these outcomes were unequivocally positively affected by our intervention. We did not find significant beneficial effects of our transplantation strategy on micturition or sexual outcome measurements. Few studies have examined effects of cell replacement strategies on micturition function of which the most interesting are the 2 studies by Mitsui et al. (2005a and 2005b) of which the locomotor recovery data have been discussed above.

Fibroblasts engineered to secrete BDNF and NT-3 (Mitsui et al., 2005a) and the combined application of NRP and GRP cells (Mitsui et al., 2005b) appeared to accelerate recovery of micturition from the period of bladder areflexia in rats observed in metabolic cages. Treatment differences were no longer apparent by 4 weeks after transplantation. Both of these treatments also appeared to reduce signs of detrusor sphincter dyssynergia and detrusor hyperreflexia as determined by cystometry and determination of bladder weights. Considering the enormous difference in BBB locomotor scores between the 2
groups (3.5 vs 8.2) after transplant of genetically modified fibroblasts, it is not surprising other behavioral outcomes improved as well. Interestingly, development of thermal hypersensitivity was not affected by this treatment. The combined NRP GRP transplant improved BBB scores from 7.1 to 9.4 and also diminished thermal hypersensitivity. Not only did both of these studies report beneficial effects from their transplantation strategies, investigators also determined increased serotonergic input to the DL nucleus in the lumbosacral spinal cord; however review of data shown (Mitsui et al., 2005a) questions location of nucleus examined for serotonin. An earlier report (Mitsui et al., 2003) demonstrated beneficial effects from transplanted immortalized neural stem cells on micturition function, however, the experimental paradigm in that study was significantly different and the report did not include locomotor recovery data.

Previously it has been shown that when neurons grown on inhibiting substrates are exposed to BNDF and NT-3 their regenerating capability is improved in vitro (Bregman et al., 1998). Subsequent studies showed that this was mainly due to downstream effects of the cAMP-dependent protein kinase (PKA) (Cai et al., 1999). Activation of PKA induces transcription of regeneration-related genes and production of polyamine products. Polyamines have been shown to be involved in regulation of microtubule assembly and modulation of expression of cytoskeleton proteins. It was suggested that neurotrophin treatment resulted in elevated cAMP concentrations that consequently reduced responsiveness of neurons to inhibitory cues provided by myelin. Preventing changes induced by the myelin-associated inhibitors occur potentially through
stabilization and rearrangement of cytoskeletal structures through effects of polyamines (Spencer and Filbin, 2004). Pearse et al. (2004) demonstrated that administration of the PDE-IV inhibitor rolipram and db-cAMP, using the same methodology as we did in our study, prevented the SCI-induced reduction of spinal cord cAMP concentrations. This study demonstrated that the combination of Schwann cell transplants and elevation of cAMP concentrations following SCI resulted in significant improvement of locomotor function and promoted supraspinal and propriospinal axon sparing and myelination. Also, serotonergic fiber growth into and beyond the graft was promoted by this treatment. This study did not quantify extent of transplant grafts within the injury lesion region and whether or not this was affected by cAMP elevation. Anti-inflammatory effects such as prevention of immune cell activation and pro-inflammatory cytokine production have been reported for cAMP. In the study described above, TNF-α production was inhibited but no effect was seen on IL-1β concentrations (Pearse et al., 2004). Another study by this group showed that treatment with rolipram improved functional recovery, promoted axonal growth, and attenuated astrogliosis (Nikulina et al., 2004). Density of sections stained with GFAP was quantitated and in rolipram-treated animals this density was one third less compared to vehicle-treated animals. This is particularly interesting in light of our findings of reduced differentiation of GRP cells into astrocytes. Perhaps reduced activation of endogenous astrocytes and reduced glial scarring is produced through similar mechanisms of cAMP that alter lineage differentiation of GRP cells. In the present study significantly less double labeled hPLAP-GFAP positive
cells were seen in the animals with elevated cAMP concentrations (GRP cAMP group). Although more hPLAP-CC-1 positive cells were seen in the GRP cAMP group this had no beneficial effect on functional outcome. With increased availability of oligodendrocytes, one would expect to see enhanced remyelination and potentially improved functional outcome. However, it is possible that the total number of oligodendrocytes (hPLAP-positive) was not increased in our GRP cAMP group since cAMP appeared to have a negative effect on GRP cell survival.

As far as the author is aware, this is the only study that has quantified extent of transplant within the lesioned spinal cord. Certainly there are no other reports on the effect of cAMP on survival of transplanted cells within an injured spinal cord. Our study suggests that elevation of cAMP is not necessarily beneficial to survival of GRP cells. This is unlikely to be due to the altered immune response that is caused by cAMP elevation, since it has been well established that cAMP has predominantly anti-inflammatory effects that are described above. Macrophages failed to differentiate into activated macrophages when exogenous cAMP was added in an in vitro model (Peters et al., 1990) and phagocytotic function of macrophages is inhibited by cAMP (Aronoff et al., 2005; Peters et al., 1990). Numerous reports have shown that cAMP is involved in regulation of cell survival, in particular of neoplastic and progenitor cells.

In B-cell chronic lymphocytic leukemia (B-CLL) PDE-IV is the predominant PDE expressed in the neoplastic cells. Rolipram induces apoptosis in B-CLL cells through a cAMP dependent and caspase dependent manner. Furthermore,
rolipram was found to suppress the anti-apoptotic members of the Bcl-2 family and induced the pro-apoptotic protein Bax. Combining these mechanisms resulted in a shift of the balance between pro- and anti-apoptotic members of the Bcl-2 family towards a pro-apoptotic direction (Siegmund et al., 2001). In acute lymphoblastic leukemia (ALL) cells, elevation of cAMP through rolipram administration resulted in G1 and G2/M cell cycle arrest and increased apoptosis (Ogawa, R. et al., 2002a). Chronic lymphocytic leukemia (CLL) cells undergo apoptosis following administration of rolipram. Contributing mechanisms included mitochondrial depolarization, release of cytochrome c into the cytosol, and caspase-9 and -3 activation (Moon and Lerner, 2003). The most recent study determined that rolipram increased the efficacy of glucocorticoid-mediated apoptosis in B-CLL. PKA was required for this effect and enhanced apoptosis by modulating glucocorticoid receptor signal transduction (Tiwari et al., 2005).

Elevation of cAMP concentration has also been shown to inhibit cell growth and induce apoptosis in other cancer cell lines such as retinoblastoma cells (Fassina et al., 1997), papilloma cells (Marko et al., 1998), glioma cells (Chen et al., 2002), neuroblastoma cells (Kumar et al., 2004), and esophageal cancer cells (Wang, H. M. et al., 2005a). In cancer cell lines this effect of PDE-IV inhibition is an obvious area of interest since halting growth of these cells is a much desired therapeutic goal.

In the treatment of bronchial asthma, PDE inhibition is thought to be an important mechanism of the anti-inflammatory actions of theophylline. Theophylline and rolipram have been shown to increase eosinophil intracellular
cAMP concentrations and inhibit eosinophil survival (Momose et al., 1998; Takeuchi et al., 2002; Wang, W. et al., 2005b). Recently it has been demonstrated that cAMP elevation resulted in significant inhibition of colony growth and induced apoptosis of progenitor cells in asthmatics, but not in normal subjects. These effects were not limited to the eosinophil lineage alone (Wang, C. H. et al., 2003). A number of possible mechanisms for this exist including internucleosomal DNA cleavage, G1 cell arrest, and effects through CREB gene regulation. Another potential mechanism for the pro-apoptotic effect of cAMP is given in a recent study of pulmonary hypertension. Pulmonary arterial hypertension is a proliferative vascular disease characterized by aberrant regulation of smooth muscle cell proliferation and apoptosis in distal pulmonary arteries. Elevating cAMP concentrations through administration of PDE inhibitors suppressed proliferation and matrix metalloproteinase activity and promoted apoptosis in these cells. One of the important mechanisms appeared to be attenuation of DNA synthesis (Growcott et al., 2006).

In conclusion, this study showed no beneficial effects on functional outcome measurements of transplanting GRP cells and elevating cAMP concentrations after SCI. Although elevation of cAMP has been reported to have beneficial effects in neural regeneration and may have beneficial effects on lineage differentiation of GRP cells, from our study it appears that cAMP elevation may reduce survival of GRP cells following transplantation into the injured spinal cord. Continued research should be undertaken into the effects of these combined treatment strategies for SCI. Transplant strategies appear
promising; however it seems necessary to combine this with other therapies such as treatments that induce differentiation of these progenitors into a cell population with desired functional characteristics, treatments that affect regeneration, and/or treatments that alter the spinal cord milieu. It is important that investigators critically examine effects of all these treatments, not only on functional outcome measurements but also on histopathological features. Particularly in combination therapies it is important to also examine interactions of the therapeutics used. Effects of the complimentary treatments such as administered neurotrophins or cAMP elevation may have different effects in normal adult cells vs. in progenitor such as used in current transplantation strategies.

5.5 References


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CHAPTER 6

DISCUSSION

Urination and sexual function are compromised after SCI. The resulting dysfunction represents a combination of recovery of reflexes and plasticity that occurs after loss of descending input, and that subsequently produces hyperactive reflexes. Treatments aimed at ameliorating secondary injury and enhancing sprouting or regeneration may have positive effects on lumbosacral autonomic function, but more information regarding the normal and pathophysiological eliminative and sexual functions and their responses following reparative therapies is needed to plan treatment strategies. This should be especially true since spinal cord injured people consider recovery of these functions to be of highest priority (Anderson, 2004).

In this sequence of investigations we demonstrate the value of a novel method to assess autonomic behavior in freely moving rats and apply that technique to investigate the use of a cell transplantation technique in experimental SCI. Using a telemetric technique to measure CSP pressure we demonstrate the ability to assess details of micturition and erectile function in conscious male rats. Using this technique we found that partial recovery of
micturition function occurs after mild SCI but that micturition characteristics remain altered such as demonstrated by a 2-fold increased duration and increased mean pressures of micturition events 3 weeks after injury. Similar changes were seen after analysis of number and characteristics of the erectile events. We found a decreased number of both full and partial erectile events following mild SCI and we found significant trends towards increased duration, area under the curve, and maximum pressures of the events as well as trends towards increased peak duration, peak area under the curve, and peak maximum pressures. We suggest this is a result from development of bulbospongiosus muscle spasticity or hyperreflexia after SCI.

Finally, using this technique to assess recovery of micturition and erectile function after SCI, we evaluated these autonomic outcomes in rats that were subjected to moderate SCI and received a treatment intervention that included a combination of transplantation of GRP cells and elevation of cAMP concentrations. However, no beneficial effects on functional outcome measurements of this transplantation strategy were observed in this study. We used a more severe level of injury in this latter study since other investigators have shown that less severe injuries have minimal impact on bladder function (Mitsui et al., 2005; Pikov and Wrathall, 2001). We elected to ensure substantial damage in order to show improvement of autonomic parameters after transplantation of GRP cells on day 9 after SCI. However, in our moderately injured animals we detected more variation within a number of our measurements than was apparent in our mildly injured animals. This may have
contributed to the lack of finding a significant effect of treatment, although, even in some of the more robust measurements no significant effect of our therapy was seen. Since we did observe long-term changes in waveform characteristics after a mild SCI (Nout, Y. S. et al., 2005b) investigators should consider to perform future studies of treatment interventions in milder models of SCI. Furthermore, it appears that beneficial effects from transplantation strategies may be more easily identified following mild SCI. Unless animals recover spontaneously from deficits incurred by mild SCI, less severe models of SCI may prove to be valuable in future investigations of transplant strategies.

Two ways of limiting variability of CSP measurements are increasing the number of subjects and/or limit the events analyzed. One way to reduce some of the variability seen in measurements of erectile function would be to only examine specific erectile events. From our study that examined CSP recording in uninjured rats, it was apparent that erectile events are context specific as has been shown before (Schmidt and Schmidt, 2004; Schmidt et al., 1994). For example, erections that occur during ex-copulatory reflex erection tests are very different from those that are seen in mating tests. In order to specifically assess whether the spinal cord is intact, one could limit analysis of erectile events to those that are non-reflex mediated, in other words, to erectile events that are produced by supraspinal regulatory areas of the brain. In future research one could do this by investigating only sleep-related erections. This would require continuous electroencephalogram recordings of rats in order to only examine erections that occur during rapid eye movement sleep of the rat.
Although we did not find behavioral benefits of our treatment we did find that transplanting GRP cells increased the amount of tissue present at the lesion center and we found interesting effects of elevation of cAMP on GRP cell survival and differentiation. From our study it appears that cAMP elevation may reduce survival of GRP cells following transplantation into the injured spinal cord. Continued research should be undertaken into the effects of these combined treatment strategies for SCI. Current modern imaging and analytical technologies enable scientists to perform detailed histopathological investigations into the roles that these treatments play. Furthermore, continued development of magnetic resonance imaging (MRI) will potentially allow us to examine properties of transplanted cells \textit{in vivo}.

Up to now, most pathophysiological studies in the field of SCI research have been based on postmortem evaluation of the lesions but recent progresses in the use of MRI provide new tools to examine the rodent central nervous system \textit{in vivo}, and, eventually monitor progression of lesions. The essential aspect of utilization of MRI in neurotrauma consists in monitoring acute-to-chronic posttraumatic physicochemical changes and correlating them with biochemical and morphological changes \textit{ex vivo}. We have recently demonstrated the utilization of MRI in a thoracic SCI model in which we compared MRI to histopathological data (Nout, Y.S. \textit{et al.}, 2005a). Moreover, use of MRI may assist in the progress of developing transplant repair strategies for nervous system damage because MRI will permit investigations of these therapies through tracking the fate and time-course of migration of the transplanted cells in
the host organism *in vivo*. Following transplanted stem cells by MRI requires a labeling method that will make grafted cells distinguishable from host cells. The contrast produced by the label must be sufficient to permit detection of small cell clusters using high-resolution MRI. Studies have examined the use of gadolinium rhodamine dextran (GRID), which is detectable by both MRI and fluorescence microscopy (Modo et al., 2002), and superparamagnetic iron oxide nanoparticles (magnetodendimers) (Dunning et al., 2004; Jendelova et al., 2004; Lee et al., 2004). Although these investigations provide evidence for maintenance of functional properties of graft cells and their migration in spinal cord injury, further development of these techniques remains necessary to improve image quality. Technical advances to date include imaging techniques such as diffusion tensor MRI (Lin et al., 2001), use of implantable coils (Bilgen et al., 2001; Silver et al., 2001), and respiratory gating software (Bonny et al., 2004).

Transplant strategies appear promising, but it is important that investigators critically examine effects of all these treatments, not only on functional outcome measurements but also on histopathological features. There are currently a number of injury models available in rats and mice and laboratories use different behavioral tests and analytical techniques to evaluate their therapeutic strategies. This broad approach to SCI research has the benefit of investigating many different things at the same time; however, the downside is that comparing results between laboratories becomes more difficult. Prior to recommending treatments for clinical trials, it is important that these treatments are evaluated by more than one group of investigators. Rosenzweig and
McDonald (2004) state that the needs of individuals with SCI dictate that we must improve the comparability of our studies to allow more balanced decisions about the possibilities for translation to human therapies.

In conclusion, the experiments performed here are a start to answer to the request of the spinal cord injured population that more research should be focused on quality of life issues secondary to disruption of autonomic nervous system pathways after injury. The experimental model that we have developed here allows detailed study of micturition and erectile function in rats and should prove valuable in future research focused on these bodily functions after SCI. None of our behavioral outcomes were affected by the transplantation technique used here, but other valuable information was obtained from this study; the detrimental effect of cAMP on GRP cell survival that we showed here must be further studied. Continued research into the capabilities of precursor transplantation techniques is essential in this road towards finding a cure for SCI.
APPENDIX

IMAGES FROM SPINAL CORD SECTIONS

Figure A.1 A-J: Representative images of animals that received GRP cell transplants – transverse sections lesion region. Images A – E are from each of the 5 animals in the GRP control group and images F – J are from each of the 5 animals in the GRP cAMP group. Sections are 20 μm thick and immunohistochemically stained for astrocytes (GFAP = green), GRP cells (hPLAP = red), and cell nuclei (DAPI = blue).
Figure A.1 continued

C

D

E

F

Continued
Figure A.1 continued
Figure A.2 A-I: Representative images of animals that received GRP cell transplants – horizontal sections lesion region. Images A – E are from each of the 5 animals in the GRP control group and images F – I are from each of the 4 animals in the GRP cAMP group. Sections are 20 μm thick and immunohistochemically stained for serotonin (green), GRP cells (hPLAP = red), and cell nuclei (DAPI = blue).
Figure A.2 continued
Figure A.2 continued
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