Published in final edited form as:

J Neurotrauma. 2008 March ; 25(3): 214–224. doi:10.1089/neu.2007.0328.

Functional Reinnervation of the Canine Bladder after Spinal Root Transection and Immediate Somatic Nerve Transfer

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Abstract

This study was performed to determine whether nerve transfer immediately after spinal root transection would lead to bladder reinnervation in a canine model. In one animal, the left T12 intercostal nerve was mobilized, cut and attached to the severed ends of sacral roots inducing bladder contraction using a graft from the T11 intercostal nerve. On the right side and bilaterally in two other dogs, coccygeal roots innervating tail musculature were cut and attached to the severed bladder sacral roots (coccygeal nerve transfer [CG NT]). In four other dogs, bladder sacral roots were transected in the vertebral column, and the genitofemoral nerve was transferred within the abdomen to the pelvic nerve (genitofemoral nerve transfer [GF NT]). After 14 months for CG NT and 4.5 months for GF NT, electrical stimulation of the pelvic nerve induced bladder pressure and urethral fluid flow on the intercostal nerve transfer side, in each of the five CG NT sites and bilaterally in three of the four GF NT animals. Reinnervation was further shown by retrograde labeling of spinal cord neurons following fluorogold injections into the bladder wall and by histological examination of the root/nerve suture sites. In all CG NT animals, labeled neuronal cell bodies were located in ventral horns in lamina IX of coccygeal cord segments. In the three GF NT animals in which pelvic nerve stimulation induced bladder contraction, abundant labeled cell bodies were observed in lamina IX and lateral zona intermedia of upper lumbar cord. These results clearly demonstrate that bladder reinnervation can be accomplished by immediate nerve transfer of intercostal nerves or coccygeal spinal roots to severed bladder sacral roots, or by transfer of peripheral genitofemoral nerves (L1,2 origin) to pelvic nerves.

Keywords

animal studies; axonal injury; neuroplasticity; peripheral nerve injury; regeneration

INTRODUCTION

One of the nearly universal sequelae of spinal cord or sacral root injury is urinary tract dysfunction. Although in developed countries such as the United States, improved urologic care has significantly decreased renal disease as the major cause of death in this group of patients, their quality of life would be substantially improved if restoration of urinary bladder emptying function could be accomplished. Bladder stimulation strategies with neuroprosthetic implants that can achieve bladder emptying have been developed for patients with spastic bladders resulting from “upper motor neuron” lesions which spare the cell bodies in the S2-4 spinal cord segments that innervate the bladder (Brindley et al., 1986). Unfortunately, these strategies are ineffective in patients with flaccid bladders resulting from “lower motor neuron” lesions in which the sacral cord cell bodies innervating the bladder are damaged or their axons are severed. The goal of this line of investigation is to develop surgical strategies to bypass the denervation by nerve transfer (NT) using unaffected nerves from above or near a spinal root lesion site.

Using a canine model, we have previously shown that transection of spinal roots innervating the bladder followed immediately by homotopic end-on-end repair results in functional reinnervation as evidenced by increased bladder pressure upon functional electrical stimulation up to 1 year after spinal root transection and repair. Retrograde fluorogold tracing along with postmortem lipophilic dye tracing studies confirmed regrowth of axons through the repair site to the bladder (Ruggieri et al., 2006). This current study was performed to determine whether heterotopic nerve or root transfer immediately after bladder spinal root transection would lead to similar reinnervation. Nerve transfer was accomplished using either a thoracic intercostal nerve or coccygeal (CG) ventral spinal roots in the spinal column as well as genitofemoral (GF) nerves (which are of lumbar origin) in the lower abdomen.

METHODS

All studies were approved by the Temple University Institutional Animal Care and Use Committee in accordance with the laboratory animal care guidelines of both the U.S. Department of Agriculture and the Association for Assessment and Accreditation of Laboratory Animal Care. The study subjects were fully conditioned female mongrel hounds 6–12 months of age and 18–22 kg body weight (Marshall BioResources, North Rose, NY). A total of 10 dogs were used: three nerve transection with CG NT, four nerve transection with GF NT, and three sham operated, nerve intact controls.

Surgical Preparation

Animals were fasted the day prior to surgery and covered with antibiotics (30 mg/kg trimethoprim and 6 mg/kg sulfadiazine p.o.). A fentanyl patch (75–100 mg/h for a 20-kg dog) was adhered to the shaved skin of the inner thigh and left in place for 3 days. Perioperative pain management included morphine (10 mg/L) in the intravenous Ringers lactate delivered at 60–100 mL/h. Postoperative pain management also included 2 mg/kg ketoprofen i.m. for 2 days beginning on the second day post-surgery. Propofol (6 mg/kg iv) was administered to allow insertion of an endotracheal tube for isoflurane anesthesia (0.5–4% mean alveolar concentration) using oxygen as the carrier gas. For postoperative management of the neurogenic bladder, an abdominal vesicostomy was created as previously described (Ruggieri et al., 2006).

Bladder denervation was also performed as previously described (Ruggieri et al., 2006). Briefly, with the animal in the prone position, a 30 degree V-laminectomy of the L7 vertebral body and a partial laminectomy of the L6 and S1 vertebral bodies was done (Fig.

1), so that the S1 and S2 ventral roots innervating the bladder could be stimulated with a unipolar probe electrode. The two bilateral ventral sacral roots that induced increased bladder pressure upon intraoperative electrical stimulation were transected (post mortem examination revealed that these roots were S1,2). In the initial animals, completeness of bladder denervation after root transection was confirmed by the disappearance of bladder contractions upon stimulation of the entire conus medullaris with an epidural electrode placed in the midline under the L5 vertebral body.

Coccygeal Root and Intercostal Nerve Transfer

For CG NT, spinal roots inducing only tail movement upon electrical stimulation were transected and the proximal ends were sutured to the distal ends of the transected bladder spinal roots (two per side, four total) by end-on-end anastomosis using 10-0 nylon sutures at three to four sites around the nerve circumference (Fig. 1, left side). In one of the three animals, nerve transfer was performed on the left side using intercostal nerves. In order to reach the severed sacral spinal roots located at the level of the L7 vertebral body, the left T12 intercostal nerve was harvested and attached by end-to-end anastomosis to distal cut end of the mobilized left T11 intercostal nerve. This T11–T12 complex was then repositioned along the vertebral column and attached to distal severed ends of the sacral roots mediating bladder contraction on the left side. A self-conforming, spiral, tripolar nerve cuff electrode (Axon Engineering, Cleveland, OH) was placed around the two root bundles on each side, proximal to the anastomosis site, and the leads were coiled into a silicone pouch which was inserted subcutaneously lateral to the lumbosacral incision. In the first animal, the leads were tunneled subcutaneously to exit the skin on the back of the animal between the scapulae and secured to the skin with silk sutures.

Genitofemoral Nerve Transfer

For GF NT, approximately 15-mm lengths of the transected bladder roots in the lumbosacral spine were removed to prevent spontaneous orthotopic reinnervation (Fig. 1, right side). The lumbosacral incision was then closed and the animal was repositioned to the supine position. After surgically prepping and sterile draping of the lower abdomen, the GF nerves were located along the psoas muscle after a lower abdominal midline incision. The distalmost 3 cm of the GF nerves up to the point at which they exit the abdominal wall were mobilized. The pelvic nerves were transected as they emerged from the pelvic plexus towards the urinary bladder and the GF nerves were attached bilaterally by end-to-end anastomosis using 10-0 nylon sutures (Fig. 1, right side). In two of the four animals, the self conforming tripolar nerve cuff electrodes were placed around the GF nerves at a point approximately 3 cm proximal to the nerve transfer site and secured to the psoas muscle using 8-0 silk. The electrode leads were also coiled into a silicone pouch, which was inserted subcutaneously in the lower abdomen lateral to the incision. The other two GF NT animals did not receive implanted nerve cuff electrodes.

Sham-operated controls received the lumbosacral laminectomy, intraoperative identification of bladder nerve roots with electrical stimulation and the abdominal vesicostomy but did not receive root transection, nerve transfer nor implantation of nerve cuff electrodes.

Electrical Stimulation

Beginning approximately 4 months postoperatively, the silicone pouch containing the implanted electrode leads was retrieved under isoflurane anesthesia and stimulated (1.2-mA, 20-Hz, 0.5-msec quasi trapezoidal wave trains of 20-sec duration) while monitoring bladder pressure to determine return of bladder function. Changes in the pressure inside the urinary bladder was measured with an external pressure transducer interfaced with a data acquisition system (AD Instruments, Colorado Springs, CO). A Foley catheter was passed into the

vesicostomy and after inflating the balloon to 10 mL, gentle tension was placed on the catheter to prevent leakage. The catheter was connected via a three-way valve to the pressure recording system and a syringe infusion pump which was used to fill the bladder with normal saline solution to approximately 30 mL. Gentle manual pressure on the lower abdomen was used to ensure that the measurement system was functional and capable of detecting pressure changes inside the urinary bladder. During electrical stimulation, the abdomen was palpated to ensure that stimulation of the rectus abdominalis and thus increasing the intra-abdominal pressure was not being induced. Immediately before the animals were euthanized, in addition to stimulation of the implanted electrodes as described above, the pelvic nerves leading to the bladder were also evaluated for their ability to induce bladder pressure and urine flow by intraoperative electrical stimulation (15-V, 20-Hz, 1-msec square wave trains) using a unipolar probe electrode. Observation of increased bladder pressure following direct stimulation of the pelvic nerve but not by stimulation of the implanted electrodes was taken as evidence for successful bladder reinnervation but failure of the implanted electrodes. For each functional electrical stimulation (FES) session, bladder pressure was recorded for three to eight separate stimulations and results are presented as means $\pm$ standard deviations. For these stimulations, a catheter was not passed into the urethra so that electrical stimulation induced flow of fluid from the bladder out the urethra could be observed.

Retrograde Neuronal Tracing

Fluorogold retrograde neuronal tracing from the urinary bladder to the spinal cord was performed on all animals as previously described (Ruggieri et al., 2006). Briefly, 3 weeks before animals were euthanized, four injections of 4% (w/v) fluorogold were made lateral to each ureteral orifice (50 μ L/injection, 400 μ L total per animal). For the CG NT animals, a distance of 25–30 cm was estimated from x-ray images for the length that coccygeal nerve must regrow from the cell bodies in the spinal cord, through S2 and S3 sacral foramen, to innervate the urinary bladder. Thus, at a growth rate of 1 mm per day, we estimated that the nerve regrowth might take 250–300 days to reach the urinary bladder if regrowth from the cell body in the spinal cord was required. If the cut axons could regrow from the site of transection then the estimated distance would be 12–18 cm, a distance requiring 120–180 days to traverse. Since additional time may be required for these regrowing nerves to form functional synaptic connections with the bladder, a post nerve transfer period of approximately 14 months was allowed for the CG NT animals and a post nerve transfer period of 4–5 months was allowed for the GF NT animals before the animals were euthanized.

The dogs were euthanized with an intravenous injection of 360 mg/kg sodium pentobarbital. The spinal column with intact spinal cord, roots and spinal nerves was removed and postfixed *en bloc* by immersion in freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 3–5 days at 4°C. The spinal cord from the lumbar through the coccygeal regions was removed from the vertebral column, the right side marked with a permanent tissue marker for later identification purposes, cryoprotected in 30% sucrose in 0.1M phosphate buffer (pH 7.4) for 2 days, and frozen-sectioned into 18- μ m coronal plane sections mounted immediately onto coated slides (Fisher Plus). The slides were coverslipped using 80% glycerol in 0.1M phosphate buffer, and used to measure mean number and diameter of fluorogold retrogradely labeled neuronal cell bodies (measurement methods described below). Cresyl violet staining of adjacent slides was used to further confirm segmental location.

Quantitative Analysis of Fluorogold Labeling

Spinal cord sections were analyzed quantitatively for the presence of fluorogold retrogradely labeled motor neuronal cell bodies using a Nikon fluorescence microscope interfaced with a quantitation system (Bioquant OsteoII, Bioquant, TN) and an X,Y motorized stage. Three sections per cord segment (lumbar, sacral or coccygeal) were analyzed bilaterally for each animal using an independent-random sampling approach as described in Mouton et al. (2002). To meet the needs of this sampling approach, every 10th serial section was collected at the sites of dorsal roots for the generation of at least six sets of slides (with three sections of a cord segment per slide) per cord segment, and for a total of at least 150 sections per cord. Then, with the gray matter of each section analyzed, four regions of the intermediate and ventral cord was examined at 20× magnification in order to determine the mean number of fluorogold labeled neurons per section, per side, and per segment for each surgical group. For the sham-operated animals this was performed on the left side only because the right side of the cord was used for a different and separate experimental paradigm. Also, eight regions of the intermediate and ventral cord was examined at 40× magnification in order to quantify the mean cross-sectional diameter of the fluorogold labeled neurons per section, per side, and per segment for each surgical group. To avoid bias in estimating the number of neurons, only fluorogold-labeled cells in which the nucleus (unlabeled) was visible were measured. Also, the smallest cross-sectional diameter of these neurons was measured in order to assure that these measurements were made near the middle of cell bodies. The representative ventral horn cartoons shown in Figure 3 were generated using the Bioquant program topography, landmark, and region of interest tools (ROI) at 4× magnification, and then by topographically mapping the fluorogold labeled neurons at 20× using an object count array and the motorized stage within the larger ROI defined at the lower magnification in a manner similar to that used to count the labeled neurons.

Statistical Analysis

Means and standard deviations (SD) are presented. Two-way analyses of variances were used to compare the mean number of fluorogold-labeled neurons per section using the factors cord region (lumbar, sacral, and coccygeal) and surgical group (sham, GF NT, and CG NT). Bonferroni post hoc analysis was used to compare the differences between individual groups. The differences in the mean diameter of fluorogold-labeled neurons were analyzed using a one-way analysis of variance (ANOVA). There were no labeled neurons in the lumbar or coccygeal regions in the sham group and very few in the lumbar cord in the CG NT group or in the coccygeal cord of the GF NT group, thus eliminating these groups from this particular analysis. A Tukey post hoc analysis was used to compare the differences between individual groups. Probability values of 0.05 were considered statistically significant.

RESULTS

For the first animal in which the intercostal nerves were used for nerve transfer on the left side, the immediate postoperative recovery was arduous and the animal required resuscitation from hypovolumic shock with a blood transfusion. During the surgical procedure in this animal, a decision was made to reinnervate the bladder nerves on the right side with coccygeal spinal roots mediating tail movement. Because of these surgical complications in this first animal, in lieu of the intercostal nerve transfer, in two additional dogs, the coccygeal spinal roots innervating tail musculature were transferred to the transected bladder spinal roots (CG NT) to provide proof of concept that a somatic nerve innervating striated muscle may be capable of reinnervating the urinary bladder. We could then compare these results to somatic nerve transfer using the genitofemoral nerve, a nerve with sensory axons to lower abdominal genital skin regions and motor axons to the

cremasteric muscle (Luria and Laufer, 2007; Zempoalteca et al., 2002). A representative trace of the increased bladder pressure resulting from FES is shown in Figure 2. Results of all animals for the FES and retrograde neuro tracing of fluorogold injected into the bladder wall are shown in Table 1.

Functional Electrical Stimulation

FES of the implanted nerve cuff electrodes—For the first animal in which the electrode leads were tunneled subcutaneously to exit the skin on the back, these leads became increasingly damaged over time which led to implanting the leads in a subcutaneous silicone pouch in the subsequent animals. Exterior access to the leads in the first dog allowed for more frequent evaluation of possible restoration bladder function and these leads were tested with FES at post-operative days 134, 163, 191, 265, and 290, and on day 450 immediately before euthanasia. No increased bladder pressure was induced by FES of the implanted electrodes at any time periods (dog 1, Table 1). In the other two animals with CG NT, FES at day 178 induced increased bladder pressure and urethral fluid flow in one animal (dog 2, Table 1) but not on post-operative day 142 in the other (dog 3, Table 1). In the two GF NT animals that had implanted nerve cuff electrodes, increased bladder pressure and urethral fluid flow was induced by FES on post-operative day 96 in one animal (dog 5, Table 1) but not on post-operative day 98 in the other (dog 4, Table 1).

Intraoperative stimulation of pelvic nerve prior to euthanasia—Pelvic nerve stimulation on the left intercostal nerve transferred side of the first dog produced the largest increase in bladder pressure (Table 1). Stimulation of the pelvic nerve with the CG root transferred on right side of this dog as well as in the other two CG NT animals also produced significant increased bladder pressure: mean = 6.2 ± 2.7 cm H₂O, with 21 stimulations of five pelvic nerves with CG NT in three animals. Pelvic nerve stimulation in the animals receiving GF NT also produced a similar increase in bladder pressure in three of four animals: mean = 6.4 ± 3.3 cm H₂O, with 27 stimulations in six pelvic nerves with GF NT. The results for each individual animal are shown in Table 1. Fluid flow from the urethra was observed during all stimulations that produced increased bladder pressure.

Evidence of Regrowth of Axons from Cord to Bladder—In the three sham-operated control animals, the majority of labeled cord neurons were located in the lateral zona intermedia and a few in lamina IX of the ventral horns of sacral (S) 1 and S2 cord segments (Fig. 3A,B). There were 12 ± 3 labeled neuronal cell bodies per section. The cell diameters of these fluorogold-labeled cell bodies that normally innervate the urinary bladder were 18 ± 10 μ m (Table 1). Also, in these sham-operated neurally intact animals, no fluorogold-labeled neurons were observed in any lumbar or coccygeal cord segments (Table 1).

In the first animal in which intercostal nerve transfer was performed on the left side and coccygeal root transfer on the right side, there were no fluorogold-labeled cell bodies detected on the left side in either the lumbar, sacral, or coccygeal cord segments (dog 1, Table 1). On the right side, a considerable number of fluorogold-labeled cell bodies were observed in L5 and coccygeal cord segments (10.5 and 14.5 cells per section, respectively; Table 1). The majority of the fluorogold-labeled neurons in all of the CG NT animals were located in the ventral horns in lamina IX of the coccygeal spinal cord segments (Table 1 and Fig. 3D). An average of 8.50 ± 4.20 fluorogold-labeled cell bodies per section were counted in the coccygeal cord of the CG NT animals. However, in one of the CG NT animals, a low number of fluorogold-labeled cell bodies were also observed bilaterally in the S3 cord segment (dog 2, Table 1), a result suggestive of an underlying anatomical variation in this dog in which the bladder is innervated by not just S1,2 segments but also S3.

In the four GF nerve transfer animals, an average of 20 ± 7 fluorogold-labeled cell bodies per section were counted in the lumbar cord compared to 3 ± 1 cells per section in the sacral cord (Table 1). In all but one of the four animals that received surgical transection of ventral and dorsal roots followed by GF NT to the pelvic nerve leading to the bladder, fluorogold-labeled neuronal cell bodies were observed in primarily the lateral zona intermedia (with a few also in lamina IX) of upper lumbar spinal segments (Fig. 3F), indicating regrowth of motor axons from the lumbar cord to the bladder, and thus reinnervation of the bladder. Bladder pressure could not be induced by pelvic nerve stimulation in the one GF NT animal that showed no retrogradely labeled cell bodies in the lumbar cord (dog 6, Table 1). A few additional fluorogold-labeled cells were also observed in S3 cord segments and a very few in the coccygeal cord on the right side in one animal (dog 4, Table 1). Examples of the fluorogold-labeled neurons and a drawing showing their general locations in the different nerve/root transfer paradigms are shown in Figure 3.

Two-way ANOVA analysis of the number of fluorogold-labeled cells per cord region revealed significant differences between cord regions ($p = 0.014$) and in the interaction of cord region by group ($p < 0.001$). There was not a statistically significant difference in the total number of fluorogold-labeled cells between the CG NT, GF NT, and sham-operated groups ($p = 0.068$). Post-hoc analyses revealed that the sham operated animals contained a significantly greater number of fluorogold-labeled neurons in the sacral cord region compared to the other two groups ($p < 0.001$). The CG NT animals contained a significantly greater number of fluorogold-labeled neurons in the coccygeal cord segments compared to the other two groups ($p < 0.05$). Finally, the GF NT animals contained a significantly greater number of fluorogold-labeled neurons in the lumbar cord compared to the other two groups ($p < 0.05$). This last result suggests that different types of motor neurons reinnervate the bladder from the coccygeal cord via the transferred coccygeal nerves than with transfer of the GF nerve or in the normal innervation of the sham-operated animals.

Regarding the diameter of the retrogradely labeled neurons, the fluorogold-labeled cell bodies in the coccygeal cord segments of the right side of dog 1 and in the other two animals with bilateral CG NT were of larger diameter ($35 \pm 11 \mu\text{m}$) than the sacral cord labeled cells in the sham-operated controls ($18 \pm 10 \mu\text{m}$) or the labeled neurons in lumbar ($25 \pm 9 \mu\text{m}$) or sacral cord ($26 \pm 11 \mu\text{m}$) in the GF NT animals ($p < 0.001$; Table 1 and Fig. 3A,C,E). The larger diameter neurons in the GF NT animals were located in lamina IX of the lumbar cord, while the smaller diameter neurons were located in the lateral zona intermedia of lumbar cord.

We also examined the coccygeal root suture site as well as the genitofemoral-pelvic nerve suture site using hematoxylin and eosin. We found healthy roots (CG NT site) and nerves (GF NT site) with successful regrowth across the repair site (data not shown), further confirming the fluorogold retrograde labeling from the bladder to the spinal cord.

DISCUSSION

It has previously been shown that cell bodies in the ventral horns and the lateral zona intermedia of the sacral cord (S1,2), but not other regions of the spinal cord, are labeled by retrograde neuronal tracing of horseradish peroxidase injected into the canine bladder dome, bladder neck, urethra or pelvic nerve (Kokotas et al., 1978; Petras and Cummings, 1978; Shefchyk, 2002). This documents that the normal motor innervation of the canine bladder and urethra, similar to other mammals (Sundin and Carlsson, 1972), including humans (Bradley et al., 1974), emanates from the upper sacral spinal cord via the pelvic nerve. The results of our study clearly demonstrate that after transection of the spinal roots innervating the urinary bladder, reinnervation can be accomplished by immediate nerve transfer using

either coccygeal spinal roots in the lumbosacral spine or peripheral genitofemoral nerves in the lower abdomen. Evidence of reinnervation includes (1) bladder pressure and urine flow during FES of implanted electrodes proximal to the nerve transfer site in two of five CG NT sites and in two of four GF NT sites; (2) bilateral pelvic nerve FES induced bladder pressure and urine flow in all nerve transfers except one animal with GF NT; (3) in all but the one GF NT animal, retrograde transport of fluorogold injected into the bladder that predominately labeled cell bodies in spinal cord segments and spinal lamina appropriate for the transferred CG roots and GF nerves; and (4) histochemical evidence of root and nerve regrowth across the site of suture.

Of the 10 nerve cuff electrodes implanted bilaterally in five animals (dogs 1–5, Table 1), FES of these implanted electrodes induced increased bladder pressure and urethral fluid flow in four electrodes bilaterally in two animals (dogs 2 and 5, Table 1). Because stimulation of the pelvic nerves to the bladder just prior to euthanasia induced increases in bladder pressure and urethral fluid flow in all 10 sides of these same five animals, either electrode failure or incomplete initial bladder denervation could account for these findings. However, incomplete denervation cannot explain these findings in two animals because no fluorogold-labeled cell bodies were observed in the sacral cord, which indicates that initial bladder denervation was complete in these animals (dogs 1 and 3, Table 1). Thus, for these two animals, the most likely explanation for the inability to induce bladder contraction and emptying with FES of the implanted electrodes is electrode failure at some site between the end of the leads and the nerve cuff.

In one animal in which intercostal nerve transfer was performed on the left side, complete initial bladder denervation by transection of S1 and S2 roots was verified by the absence of fluorogold labeled cell bodies in the sacral cord. FES of the reinnervated pelvic nerve on the intercostal nerve transfer side induced substantial bladder pressure and urine flow. Although the thoracic spinal cord was not available for examination of fluorogold labeling in this animal, these results suggest that bladder reinnervation occurred on this side via the T11 intercostal nerve axons growing through the T12 intercostal nerve graft into S1 and S2 nerve roots to which they were sutured and on to the bladder during the 450-day postoperative period. This finding indicates a potential for bladder reinnervation using nerve transfer, although additional experiments are needed to confirm this finding. CG NT was performed on the right side, and we observed considerable fluorogold cell body labeling in both the coccygeal and L7 cord segments on the right side of the spinal cord in this dog. Although FES of the implanted electrodes failed to induce bladder contractions, FES of the pelvic nerves just before the animal was euthanized did induce bladder contraction and urethral fluid flow which indicates failure of the implanted electrodes. The nerve roots used for nerve transfer on the right side of this animal were chosen as those that induced primarily tail movement without significant leg involvement upon intraoperative electrical stimulation. The L7 cord cell body labeling on the right side of this animal is unclear. It may be due to aberrant anatomy in which the L7 cord gave rise to motor axons innervating the bladder (in addition to the transected S1 and S2 roots). Since only the S1,S2 axons were transected, any axons in adjacent cord segments that might innervate the bladder would have been spared. Also, it may be that a lumbar root was inadvertently cut and sutured to one of the sacral roots. Nevertheless, the fluorogold results show that coccygeal cord motor neurons are able to innervate the bladder.

In each of the GF NT dogs (dogs 4–7, Table 1), we may have had incomplete denervation of sacral roots innervating the bladder. This is because fluorogold-labeled cell bodies retrogradely labeled from the bladder were observed in not only upper lumbar cord segments of all but one dog (dog 6, Table 1), but also in upper sacral cord segments in varying numbers. The number of fluorogold-labeled cell bodies in the sacral cord were much lower

than the sham-operated animals, indicating that most of the sacral motor axons to the bladder had been transected. In dogs 4, 5, and 7, it is possible to make a reasonably confident conclusion that bladder reinnervation by the transferred GF nerves was successful, bilaterally, since abundant cell labeling was observed in the lumbar cords and the animals showed bladder contraction and urethral fluid flow with FES of the pelvic nerve and one even had successful FES of the implanted electrodes (dog 5). This is also the case for one of the CG NT animals (dog 2, Table 1). A very small number of labeled S3 cell bodies was also observed in the one GF NT animal in which pelvic nerve FES did not induce bladder contraction (dog 6, Table 1); in this animal, no labeled cell bodies were observed in the lumbar region. The conclusion must be made for this animal that the GF NT was not successful in reinnervating the urinary bladder. However, in two of the GF NT animals (dogs 4 and 5 Table 1) and one of the CG NT animals (dog 2, table 1), incomplete initial bladder denervation remains a possible explanation for the successful pelvic nerve stimulation results.

We also observed a very small number of coccygeal cord labeled neurons on the right side in one of the GF NT animals (dog 4, Table 1). In the GF NT group of animals, there was no intentional reinnervation procedures performed in the lumbo-sacral spine and a 15-mm section of the transected ventral roots that induced bladder contraction were removed in an attempt to prevent this. One possibility is that a coccygeal root was partially severed in this animal as a result of the denervation procedure, and that this coccygeal root sprouted axons that grew into the end of the transected sacral nerve root and partially reinnervated the bladder in this manner. In the rat sciatic nerve model, it has been suggested that misdirected axonal growth can occur during stump-end nerve repair (Amara et al., 2000).

In our previous report (Ruggieri et al., 2006) and in the present study, in all animals only two nerve roots on each side of the spinal cord (S1 and S2) could be identified that induced increases in bladder pressure upon intraoperative electrical stimulation during the denervation procedure. In our previous study as well as in the initial animals in this present study, stimulation of the entire conus medullaris with an epidural spinal electrode was capable of inducing bladder contraction before transection of these nerve roots but did not induce contraction after the roots were severed. Although this finding demonstrates functional denervation of the bladder, it is possible that in some animals a few motor fibers from adjacent lumbar segment or S3 also innervate that bladder, but are insufficient in number to induce a measurable increase in bladder pressure upon conus medullaris stimulation. The possibility exists that these few fibers may gain functional effectiveness during the long recovery period such that they may be capable of inducing bladder contraction.

The larger diameter of cell bodies that reinnervated the urinary bladder from the CG NT procedure is consistent with bladder reinnervation by alpha motoneurons (Ishihara et al., 2003) that normally innervate the skeletal musculature of the tail (Kitzman, 2005). The diameter of the cell bodies that reinnervated the bladder following GF NT were, on average, intermediate between the diameter of the coccygeal labeled cell bodies in the GF NT animals and the smaller diameter labeled cell bodies in the intermediate zone of the sacral cord of the sham-operated animals. Because these intermediate diameter cell bodies in the lumbar cord of the GF NT animals were located in lamina IX of the ventral horn, it is likely that these represent smaller motoneurons running in the GF nerve that formally innervated the cremasteric muscle (Luria and Laufer, 2007; Zempoalteca et al., 2002). The smaller diameter labeled cell bodies in the lateral zona intermedia of the lumbar cord in the GF NT animals are most likely sympathetic motor neurons of the GF nerve that were formerly involved in innervating smooth muscle of blood vessels for vasomotor control.

Others have previously shown in cats that return of the micturition reflex occurs following intradural S1 or S2 transection and reanastomosis or L7 to S1, L7 to S2, or L6 to S1 nerve transfer after periods of regeneration ranging between 4 and 7 months (Carlsson and Sundin, 1968). It has also been shown in pigs that after transection and reanastomosis of S2 or S3 roots, return of the micturition reflex occurred in four of six animals after a 7-month nerve regeneration period (Conzen and Sollmann, 1982). Similar studies in rats failed to demonstrate a return of the micturition reflex, although evoked potentials from the nerve graft to the bladder demonstrated that regenerated axons had indeed reached the urinary bladder (Conzen and Sollmann, 1982). Another more recent study in rats found that, after bilateral avulsion of L5-S2 ventral roots, reimplantation of the L6 and S1 trimmed roots into the lateral funiculus of the L6 and S1 spinal cord segments led to functional reinnervation of the bladder and external urethral sphincter at 12 weeks after implantation (Hoang et al., 2006). Our study confirms previous findings that nearby motor roots can provide a functioning substitute for the parasympathetic fibers that normally provide motor innervation of the urinary bladder in a different species (canines). This work also extends these findings to show that new and different nerve cell bodies in the spinal cord (i.e., lumbar in this study) can reinnervate the bladder following heterotopic nerve transfer to a site distant from the transected roots.

The findings of this study provide proof of concept that nerve transfer using somatic motor nerves that normally innervate skeletal muscle such as the coccygeal nerve innervating the tail musculature or the intercostal nerve innervating thoracic wall musculature can be used as a source for bladder reinnervation. In addition, a nerve that serves both sensory and motor functions to skin and muscle in the genital area, the genitofemoral nerve, can also be used for nerve transfer to the pelvic nerve in the lower abdomen for bladder reinnervation. The last finding paves the way for potential clinical application of this surgical approach in patients with lower motor nerve lesions of the urinary bladder but intact L1 spinal cord function. Because the genitofemoral nerve is involved in the sensory and motor limbs of the cremasteric reflex, the presence of the cremasteric reflex and/or sensation in the abdominal-inguinal region could be used preoperatively to determine whether patients could benefit from this nerve transfer approach to bladder reinnervation.

Acknowledgments

We would like to acknowledge the expert perioperative and postoperative veterinary care provided by Bernadette Simpkins and Lewis Bright. Mamta Anim, Shreya Amin, and Phylliss Beaton are also acknowledged for their expert technical assistance in the histologic studies. This work was supported by a research grant from the Shriners Hospitals (to M.R.R.).

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FIG. 1.

Diagram of the surgical nerve transfer and repair methodology. A spinal laminectomy was performed, removing lumbar 7 (L7) and part of L6 and sacral 1 (S1) lamina and spinous processes. On the left side of the diagram, the site of sacral root transection and transfer of coccygeal (CG) roots to this transection site and subsequent surgical repair (indicated by arrowheads) is shown. The right side of the diagram illustrates the denervation procedure used for the genitofemoral nerve transfer in which 15 mm of sacral roots were removed (S1,2). The genitofemoral nerve (L1,2 origin) is mobilized within the abdomen, transferred medially, and sutured to the cut pelvic nerve (PN) prior to its innervation of the bladder (GF NT). The placement of the stimulating electrode sheath on the coccygeal roots in the sacral

spine is shown on the left and on the genitofemoral nerve within the abdomen on the right. DRG, dorsal root ganglion; tr pr, transfer processes.

FIG. 2. Functional electrical stimulation. Representative *in vivo* bladder pressure recordings (dog 2, Table 1) during electrical stimulation. (**Top trace**) Stimulation of the implanted tripolar nerve cuff electrodes surrounding the coccygeal nerve ventral and dorsal root bundles in the sacral spine proximal to the nerve transfer site on postoperative day 178. (**Bottom trace**) Intraoperative pelvic nerve stimulation with unipolar probe electrode immediately before the animal was euthanized on postoperative day 373. Stimulation of the implanted electrodes at this time had no effect indicating failure of the electrode implants. Fluid flow out the urethra was noted during each electrical stimulation that induced increased bladder pressure.

FIG. 3.

Neuronal cell bodies in the spinal cord retrogradely labeled with fluorogold injected into the urinary bladder. (A) Cells located in the sacral cord of a sham-operated animal demonstrating the autonomic nerve cell bodies that normally innervate the urinary bladder. (B) A 4× magnification photo of a sacral cord from a sham-operated animal with an overlay showing the location of fluorogold-labeled neurons. (C) Cell bodies in a coccygeal cord segment in an animal in which the coccygeal nerves innervating the tail were transferred to the nerves mediating bladder contraction (CG NT). Note that the cell diameter of these somatic nerves that reinnervated the urinary bladder are noticeable larger than those

normally innervating the bladder shown in panel A. **(D)** A 4× magnification photo of a coccygeal cord from a CG NT animal with an overlay showing the location of fluorogold-labeled neurons. **(E)** Neurons located in the lumbar region of the spinal cord in an animal in which the genitofemoral nerve was transferred to the pelvic nerve in the lower abdomen after bladder denervation in the lumbosacral spine (GF NT). With GF NT, the bladder becomes reinnervated with both large-diameter somatic nerves as well as smaller diameter autonomic nerves. **(F)** A 4× magnification photo of a lumbar cord from a GF NT animal with an overlay showing the location of fluorogold-labeled neurons. Scale bar = 50 μ m.

Summary of Functional Electrical Stimulation (FES) and Neuro Tracing Results

Table 1

Implanted electrode FES			Pelvic nerve FES				No. of fluorogold-labeled neurons						Diameter of labeled neurons						
Dog	Surgery	Postoperative day	Bladder pressure		Post-operative day	Bladder pressure		Lumbar		Sacral		Coccygeal		Lumbar		Sacral		Coccygeal	
			Left	Right		Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
1	L-IC NT R-CG NT	290	0.0	0.0	450	22.1 ± 5.6	8.1 ± 1.2	0.0	10.5	0.0	0.0	0.0	14.5	—	35 ± 10	—	—	—	37 ± 9
2	CG NT	178	5.3 ± 0.2	4.8 ± 0.1	373	7.0 ± 2.1	6.9 ± 2.6	0.0	0.0	2.0	3.5	6.5	8.5	—	—	21 ± 30	23 ± 80	27 ± 10	37 ± 16
3	CG NT	142	0.0	0.0	426	6.5 ± 3.0	2.9 ± 1.2	0.0	0.0	0.0	0.0	11.0	2.0	—	—	—	—	35 ± 70	37 ± 20
4	GF NT	98	0.0	0.0	32	4.6 ± 0.9	5.2 ± 1.8	28.5	10.0	4.5	4.0	0.0	2.0	26 ± 10	25 ± 10	18 ± 70	26 ± 90	—	18 ± 70
5	GF NT	96	1.7 ± 0.4	3.1 ± 0.5	138	2.7 ± 1.0	5.9 ± 1.0	16.5	19.0	4.0	4.0	0.0	0.0	30 ± 10	30 ± 49	33 ± 11	27 ± 13	—	—
6	GF NT	—	—	—	146	0.0	0.0	0.0	0.0	1.5	0.5	0.0	0.0	—	—	29 ± 50	32	—	—
7	GF NT	—	—	—	138	8.3 ± 2.7	12.1 ± 2.4	26.0	21.5	0.0	0.0	0.0	21 ± 6	21 ± 60	23 ± 4	—	—	—	—
8	SHAM	—	—	—	—	—	—	0.0	—	16.0	—	0.0	—	—	—	18 ± 13	—	—	—
9	SHAM	—	—	—	—	—	—	0.0	—	10.0	—	0.0	—	—	—	14 ± 60	—	—	—
10	SHAM	—	—	—	—	—	—	0.0	—	11.5	—	0.0	—	—	—	20 ± 40	—	—	—

Bladder pressure refers to the change in pressure inside the urinary bladder that is induced by electrical stimulation of the nerve. In dogs 1, 3, and 4, FES of the implanted electrodes failed to increase bladder pressure; however, this was induced by direct pelvic nerve stimulation immediately prior to euthanasia indicating electrode failure in these animals. The fluorogold-labeled lumbar neurons on the right side of dog 1 is unexpected and may indicate either aberrant anatomy or that a lumbar root was inadvertently used for root transfer to one of the sacral roots that induced bladder contractions in this animal. The low number of sacral neurons labeled in dogs 2 and 4-7 is also unexpected and may indicate that either bilateral transection of the two sacral roots inducing bladder contraction in these animals did not cause absolutely complete denervation or that a small number of these nerve fibers were able to reinnervate the bladder through the nerve transection. Only dog 6 showed no evidence of bladder reinnervation.