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Neuromuscular nicotinic receptors mediate bladder contractions following bladder reinnervation with somatic to autonomic nerve transfer after decentralization by spinal root transection

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Abstract

This study investigates whether the reinnervated neuronal pathway mediates contraction via the same neurotransmitter and receptor mechanisms as the original pathway. Following bladder decentralization by transection of sacral roots, peripheral nerve transfer was performed with bilateral genitofemoral to pelvic nerve transfer (GFNT) and unilateral (left) femoral nerve to bilateral pelvic nerve transfer (FNT). Reinnervation was assessed 7.5 months post operatively by monitoring bladder pressure during electrical stimulation of the transferred nerves, spinal ventral roots, and spinal cord. Of the 17 animals with GFNT, 14 (82%) demonstrated functional bladder reinnervation as evidenced by increased bladder pressure during stimulation of the transferred GFN or the L3 or L4 spinal ventral roots. Lumbar spinal cord stimulation caused increased bladder pressure in 9 of 10 (90%) animals with FNT. Succinylcholine (SCh) virtually eliminated bladder pressure increases induced by electrical stimulation of the transferred somatic nerves or the lumbar spinal segments that contribute axons to these donor nerves. In control animals (either un-operated or sham-operated) SCh had no effect on nerve evoked bladder pressure increases but substantially reduced urethra and anal sphincter pressure induced by stimulation of either the lumbosacral spinal cord or the S2-3 spinal ventral roots. The reinnervated detrusor muscles of GFNT and FNT animals also contained increased alpha1 nicotinic receptor subunit immunoreactivity in punctate dots on detrusor muscle fascicles and in neuronal cell bodies, staining not observed in control animals.

Keywords

neurosurgical procedures; nerve transfer; nerve regeneration; spinal cord injuries; nicotinic receptors

INTRODUCTION

Our previous studies in a canine model showed that after bladder decentralization by transection of sacral roots (S1-3), reinnervation can be achieved by several means. This includes repair of sacral roots to the bladder, transfer of coccygeal roots to the sacral roots to the bladder and after both immediate and delayed transfer of somatic nerves to vesicle branches of pelvic nerves.¹⁻⁴ Both the genitofemoral nerve (GFN) and branches of the femoral (FN) nerves, originating from upper lumbar (L) spinal cord segments, can successfully be used to reinnervate the bladder after decentralization by sacral root transection. While both GFN and FN contain motor and sensory components, the branches of the FN contain primarily motor fibers and more effectively restores nerve evoked bladder contraction than using the GFN.⁵

In these studies, succinylcholine (suxamethonium chloride; SCh) was routinely used to produce neuromuscular blockade of external urethra and anal sphincter skeletal muscle. SCh is a skeletal neuromuscular junction nicotinic acetylcholine receptor (nAChR) agonist, which produces sustained depolarization, thereby blocking further activation of the receptor. We investigated whether the reinnervated neuronal pathway mediates contraction via the same neurotransmitter and receptor mechanisms as the original pathway. We also investigated the location of $\alpha 1$ nicotinic receptor subunit immunoexpression in detrusor smooth muscle fascicles and intramural ganglia. The present study details a SCh-sensitive component of bladder contraction and $\alpha 1$ nicotinic receptor subunit immunostaining that appears following nerve transfer and bladder reinnervation, which is not present in control dogs.

MATERIALS AND METHODS

Animals

Studies were approved by the Temple University IACUC, compliant with NIH, USDA and AAALAC guidelines. Thirty-seven female mongrel hound dogs, 6 to 8 months of age, 16 to 20 kg (Marshall BioResources, North Rose, NY) were used. These were part of bladder reinnervation studies⁵ and were divided into the following experimental groups: 17 genitofemoral nerve transfer (GFNT) animals, 12 with abdominal vesicostomies (GFNTV) and 5 without vesicostomies (GFNTNV); 10 femoral nerve transfer (FNT) animals, 5 with abdominal vesicostomies (FNTV) and 5 without vesicostomies (FNTNV); and 8 control animals, 3 sham-operated and 5 un-operated normal controls (3 of the latter were used only for immunohistochemical assays).

Nerve Transfer Surgeries

Surgical preparation, extradural bladder decentralization, GFNT and FNT were as described.¹⁻⁵ Briefly, after bladder decentralization by S1-3 spinal root transection, 17 of the 37 animals underwent GFN identification and nerve coaptation of the GFN distal end to the proximal end of the anterior vesical branch of the pelvic nerve, bilaterally. In 12 GFNTV dogs, radiofrequency (RF) micro-stimulators (Alfred Mann Foundation, Valencia, CA) interfaced with nerve cuff electrodes were placed on the transferred GFNs bilaterally.

Insufficient numbers of micro-stimulators were available for implantation in all animals. Only 2/5 GFNTNV dogs received a nerve cuff, unilaterally, on the right. Another 10 of the 37 animals, underwent bladder decentralization followed by identification of femoral branches innervating the gracilis muscle in the left pelvic hind limb (a femoral nerve innervation pattern present in dogs), then the branches were mobilized subcutaneously and tunneled into the pelvic cavity through the abdominal wall muscles, followed by coaptation of the distal ends of the femoral branches to the proximal ends of the anterior vesical branches of the pelvic nerve, bilaterally.¹ Twelve of the 17 GFNT animals and 5 of the 10 FNT animals received an abdominal vesicostomy to empty the bladder during the time of recovery. In the remaining 5 GFNT and 5 FNT animals, bladder emptying was achieved by continuous catheterization during the first week post-surgery and then by use of the Credé maneuver when necessary. The 3 sham-operated controls underwent lumbosacral laminectomy, bladder nerve root identification via electrical stimulation and abdominal vesicostomy, but no root transection or nerve transfer.

Functional Electrical Stimulation (FES)

On the day of euthanasia, 7.8 ± 0.4 months after nerve transfer, the implanted electrodes were activated, the FNT transferred nerves were stimulated transcutaneously and the spinal cord segments and roots were activated by direct stimulation using mono- or bipolar electrodes. Detrusor pressure was obtained as described previously.⁵ Briefly, a Foley catheter was passed into the vesicostomy or through the urethra in dogs without vesicostomy. Bladder capacity was determined with 3 successive filling cystometrograms using normal saline at 30 mL/minute. Preliminary studies in control animals revealed that filling rates slower than 30 mL/minute did not result in different calculated bladder capacity so this rate was chosen for convenience. The bladder was then emptied and filled to half of this measured bladder capacity (approximately 30 mL). Immediately prior to euthanasia, intraoperative electrical stimulation (2-21 mAmp, 20-Hz, 0.2 msec square wave trains) of the L1-L7 spinal cord segments, bilateral spinal ventral roots, the PN, the transferred GFN and FN was used to evaluate return of nerve-evoked detrusor contractile function. For the nerve transfer animals, only some of the dogs that showed return of nerve-evoked detrusor contractile function after GFNT (N=9) and FNT (N=3)⁵ received an intravenous (i.v.) dose of SCh (1.5mg/Kg). The SCh effect on nerve evoked bladder pressure was tested in 12 dogs immediately prior to bladder decentralization, and then immediately prior to euthanasia in GFNT or FNT animals, as well as in the 3 sham-operated controls and 2 un-operated controls. The mean and SEM of the highest detrusor pressure obtained as result of nerve stimulation pre and post-SCh are presented.

Pressures were monitored with external pressure transducers interfaced with a multichannel PowerLab® data acquisition system and LabChart® software (ADInstruments, Colorado Springs, CO.) for continuous electronic capture of the pressure data at 4Hz. Rate of pressure increase following nerve stimulation was determined by measuring the pressure change during the linear portion of the upswing of the pressure curve and dividing this by the time between the start and the end of that linear portion of the curve.

Alpha1 nicotinic receptor subunit immunohistochemical staining and quantification

Bladders from the GFNT (N=17), FNT (N=10), bladder decentralization (N=5), sham-operated (N=1) and un-operated control animals (N=4), were collected post-euthanasia and fixed by immersion in phosphate buffered 4% paraformaldehyde for 4-6 weeks at 4°C. A full thickness section of bladder wall approximately 5-7 mm² in size, was dissected away from the rest of the bladder from between the ureters. Bladder tissue was equilibrated in 30% sucrose for 3 days, and then cryo-sectioned into 15 micrometer sections through the depth of the bladder wall. Sections were mounted immediately onto coated slides (Fisher Plus), and kept frozen at -80°C until use. Sections on slides were washed in phosphate buffered saline (PBS) for 1 minute, incubated in 0.1% Triton in PBS for 20 min at room temperature, washed three times for 5 min each in PBS, and then with goat serum (10%) in PBS for 30 min at room temperature. Without washing again, sections were then incubated with cocktail of a specific antibodies against the nAChR $\alpha 1$ subunit (#ANC-001, Alomone Labs, Jerusalem, Israel; 1:200 dilution in 10% goat serum in PBS) and mouse anti-pgp9.5 (protein gene product 9.5, a pan neuronal marker; #ab10404, Abcam, Cambridge, MA; 1:50 dilution), overnight at room temperature. After washing, sections were incubated with appropriate secondary antibodies conjugated to Dylight 488 (anti mouse green fluorescent tag, 1:100 dilution in PBS, Jackson Immuno) for pgp9.5 and Cy3 (anti rabbit red fluorescent tag, Jackson Immuno) for $\alpha 1$ nicotinic subunit, for 2 hours at room temperature. Negative controls were performed by pre-absorption of the primary antibody with excess immunizing antigen. Sections were counterstained with DAPI, a blue fluorescent nuclear stain (6-diamidine-2-phenylindole dihydrochloride; # Life technologies, Cat# D1306, diluted 1:1000 in PBS), before cover slipping with 80% glycerol in PBS for visualization.

The number of $\alpha 1$ nicotinic receptor subunit immunopositive punctate dots on detrusor muscle fascicles, and neuronal cell bodies in and around detrusor muscle fascicles were counted, separately, in 10 fields per section, per sample, at 600 $\times$ magnification and the number per mm² was determined. All microscope fields per section were analyzed systematically using an XY motorized microscope stage and were measured using a Nikon E800 microscope interfaced with a computerized quantification system (Bioquant, Nashville, TN). Intramural ganglia cells were identified based on morphology: a group of 4-5 neuronal cell bodies with DAPI stained nuclei, 20-30 micrometers in diameter and pgp9.5 immunopositive. Other neuronal cell bodies that were not associated with the ganglia were identified as pgp9.5 immunopositive and having a DAPI stained nucleus. Axons were identified by morphology and were pgp9.5 immunopositive. Bladder detrusor muscle cells were identified based on morphology.

Statistical analysis

Statistical analysis was performed by ANOVA with post hoc Student-Newman-Keuls pairwise comparisons, or Student's t-tests, where appropriate. In the event that the Bartlett test for homogeneity of variances was statistically significant, a non-parametric Kruskal-Wallis H test followed by pairwise Mann-Whitney U tests were used. A p value of < 0.05 was considered statistically different. All data are expressed as mean and standard error (SEM).

RESULTS

Functional Electrical stimulation (FES)

Figure 1 shows representative urodynamic traces of the nerve evoked pressure responses during electrical stimulation. In sham-operated and un-operated control animals, stimulation of the anterior vesical branch of the pelvic nerve between the pelvic plexus and the bladder dome as shown in Figure 1A and 1B produced a slowly increased detrusor pressure. This slow increase in pressure with nerve stimulation was also seen in control animals with stimulation of the sacral roots or the sacral spinal cord segment. In animals with bladders reinnervated by GFNT or FNT, the nerve evoked detrusor pressure increases are seen to be much more abrupt (Figure 1C and 1E). The rate of pressure increase before SCh administration is shown in the bar graphs at the bottom of figure 1, which demonstrates a 4-5 fold increased rate in the reinnervated bladders.

Figure 2 shows the mean nerve evoked detrusor pressure increases before and then 5-10 minutes after i.v. administration of 1.5 mg/kg SCh. In control animals, increased urethral and anal sphincter pressures induced by electrical stimulation of sacral cord segments or sacral spinal ventral roots were blocked by SCh. There was no effect of SCh on detrusor pressure induced by FES of the anterior vesical branch of the pelvic nerve, sacral cord or S1-3 ventral root, in sham (n= 3), pre-decentralized (n= 10), and normal control dogs (n= 2); therefore, these data were combined into a single control group (N=15). In contrast, SCh virtually eliminated the nerve stimulation induced increases in detrusor pressures in 9 GFNT (8.3 ± 2.1 versus 0.5 ± 0.2 cm H₂O) and 3 FNT (7.7 ± 0.7 vs. 0 cm H₂O) animals (Figure 2). The pre-SCh nerve evoked detrusor pressures in the GFNT and FNT animals were statistically significantly lower than the pre-SCh detrusor pressures in the controls.

Nicotinic acetylcholine receptor alpha1 staining of bladder wall

Representative images of immunostaining for $\alpha 1$ nicotinic receptor subunits is shown in Figure 3 and 4, and the quantification is shown in Figure 5. Antigen pre-absorption of both pgp9.5 and $\alpha 1$ nicotinic receptor subunit antibodies completely eliminated fluorescence (data not shown). No difference was observed between sham-operated and un-operated controls; therefore, these data were combined. Control animals contained only a small number of punctate profiles on detrusor muscle fascicles that were $\alpha 1$ nicotinic receptor subunit immunopositive (Figures 3A-C and 5). In contrast, GFNT and FNT animals showed increased punctate profiles on detrusor muscle fascicles that were $\alpha 1$ nicotinic receptor subunit immunopositive (Figure 3D,G, indicated with black arrows; Figure 5). Many of these $\alpha 1$ nicotinic receptor subunit punctate profiles were in close proximity with pgp9.5 labeled axon endings on detrusor muscle fibers (Figure 3D-I and insets, indicated with black arrows). Insets in Figure 3 G and H show close proximity but not complete overlap of $\alpha 1$ nicotinic receptor subunit and pgp9.5 immunoexpression on muscle fascicles. There were also muscle fascicle areas with $\alpha 1$ nicotinic receptor subunit immunoexpression but no pgp9.5 immunostaining (Figure 3H-I, indicated with white arrows). Axons of passage, a term that excludes axon terminals, were not immunopositive for $\alpha 1$ nicotinic receptor subunits (indicated with arrowheads in Figure 3B,E,H; compare to Figures 3A,D,G).

GFNT and FNT animals also had neuronal cell bodies in intramural ganglia with low to high levels of $\alpha 1$ nicotinic receptor subunits immunostaining (Figure 4 A-C and G-I). GFNT and FNT animals also contained small cell bodies near muscle fascicles, and not within intramural ganglia, that were immunopositive for both $\alpha 1$ nicotinic receptor subunits and pgp-9.5, indicating that they were small neuronal cell bodies (Figure D-F, J-L, indicated by black arrows). Numbers of neuronal cells bodies (within intramural ganglia or near muscle fascicles) that were $\alpha 1$ nicotinic receptor subunit immunopositive were significantly greater in GFNT and FNT dogs, than in control dogs (Figure 5).

DISCUSSION

It is well established that the major mechanism of nerve evoked urinary bladder contraction involves neuronal release of acetylcholine acting on muscarinic receptors, whereas neuronally released ATP acting on purinergic receptors plays a minor role. Although pre-junctional nicotinic receptors that facilitate acetylcholine release have been identified in the rat bladder,^{6, 7} nicotinic receptors are not thought to directly mediate contraction of the normal bladder muscle.

SCh has been used for over half a century and remains one of the most widely used muscle relaxants in both veterinary and human clinical medicine. Because SCh consists of two acetylcholine molecules linked at the acetyl side, it has the potential to activate many acetylcholine receptors. However, it is known not to act on muscarinic receptors⁸ and causes selective depolarization blockade of muscle type nicotinic receptors composed of $\alpha 1\beta 1\delta\epsilon$ subunits and not neuronal nicotinic receptors composed of $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$ or $\alpha 7$ subunits.⁹ We chose to use a relative high, 1.5 mg/kg dose of SCh for this study to assure complete neuromuscular blockade and found no inhibitory effect on nerve evoked bladder pressure increase in control animals but a virtual complete blockade in bladders reinnervated by GFNT or FNT (Figure 2). This is evidence that bladder reinnervation after decentralization induces plasticity in either bladder nerves or smooth muscle such that skeletal muscle type neuromuscular junction nicotinic receptors ($\alpha 1$, $\beta 1$, δ , γ or ϵ subunit composition) participate in the contractile process. This new somatic-to-bladder pathway following GFNT and FNT was found to induce much less nerve evoked bladder pressure than the normal pathway. It is possible that this could increase with additional post-operative recovery beyond the approximate 8 month post-operative recovery used in this investigation.

Nicotinic receptors are ligand gated cation channel receptors and activation causes rapid cell depolarization whereas muscarinic receptors are metabotropic G-protein coupled receptors. We showed in rats that there are several sequential and parallel intracellular signal transduction mechanisms that mediate muscarinic receptor stimulated bladder smooth muscle contraction¹⁰ and that these change following denervation.¹¹ Activation of nicotinic receptors on smooth muscle cells would be expected to lead to rapid depolarization, increased intracellular calcium through voltage gated calcium channels, calcium-calmodulin activation of myosin light chain kinase, actin-myosin crossbridge cycling and smooth muscle contraction. This would be expected to cause a much more rapid rate of increased bladder pressure than activation of muscarinic receptors. We found that the rate of nerve evoked bladder pressure increase in the reinnervated animals is 4-5 fold higher than in the

controls (Figure 2B), further evidence of reinnervation induced smooth muscle plasticity. This is further supported by immunohistochemical localization of $\alpha 1$ nicotinic receptor subunits in punctate profiles on smooth muscle in the reinnervated bladder (Figures 3 and 4).

Whenever examined, autonomic ganglia, including bladder intramural parasympathetic ganglion cells, express multiple types of nicotinic receptor subunits, and mice deficient in $\alpha 3$, $\beta 2$ and $\beta 4$ subunits have enlarged dysfunctional bladders.¹² To our knowledge, this is the first time that anyone has examined bladder intramural parasympathetic ganglia for the presence of $\alpha 1$ nAChR subunits. Its expression in these autonomic motor cell types only after decentralization and reinnervation by somatic motor neurons is likely the result of a peripheral neuroplastic change. Neuronal type nAChR subunits (i.e. $\alpha 3$, $\alpha 4$ or $\alpha 7$ receptors) have been identified on a variety of non-neuronal tissues including: skin keratinocytes,¹³ bronchial epithelial cells,¹⁴ colonic epithelial cells,¹⁵ vascular endothelial cells,¹⁶ and immune cells.¹⁷

The type of nAChR subunits expressed in various types of skeletal muscle is affected by interactions between neural factors and paracrine muscle signals, exposure to transient imprinting neural factors during postnatal development, and continuously acting signals from neurons.¹⁸ Skeletal muscle denervation alters the interactions of these regulatory factors. In adult innervated skeletal muscle nAChRs are concentrated in the post neuromuscular junction membrane and nearly absent from the remainder of the muscle plasma membrane. Denervation increases the gene expression of myogenic regulatory factors (MRFs) and the expression of $\alpha 1$, $\beta 1$, δ and ϵ subunit genes, as well as the embryonic γ subunit, throughout the muscle fiber plasma membrane.¹⁹⁻²² Nerve transection, such as used in this study, leads to Wallerian degeneration of a distal nerve stump. In skeletal muscle, this leads to a loss of the integrity of the neuromuscular junctions and then often permanent destabilization with reduced ability for functional reinnervation.²¹ In bladder smooth muscle the presence of intramural ganglion cells appears to create a different scenario. The observed increase in $\alpha 1$ nicotinic receptor subunit in the detrusor muscle fascicles, small neurons within these fascicles, and intramural ganglia may be the result of reinnervation by somatic motor axons rather than parasympathetic axons. It is well known that in skeletal muscle nicotinic receptors are pentameric agonist activated cation channels with a stoichiometry of $(\alpha 1)_2\beta 1\delta\epsilon$. In embryonic and adult denervated muscle, the γ subunit replaces the ϵ subunit.²³ This indicates a neuronal influence on nicotinic receptor subunit stoichiometry at the neuromuscular junction. It is possible that bladder decentralization induces expression of neuromuscular nicotinic receptor subunit including embryonic forms. These findings suggest reinnervation induced plasticity in the smooth muscle or intramural ganglion neurons. Future studies will focus on this question.

CONCLUSIONS

In the normal bladder, all axons in the pelvic nerve innervate intramural postganglionic (parasympathetic) ganglia cells;²⁴ this pathway is not blocked by SCh. However, the new neuronal pathway, achieved by either GFNT or FNT, is blocked by SCh. These data suggest that succinylcholine-sensitive nicotinic receptors that normally mediate only skeletal muscle neuromuscular junction neurotransmission appear in the new neuronal pathway after GFNT

and FNT, suggesting neuroplasticity in the end organ after reinnervation by somatic motor axons.

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Figure 1.

Top: Urodynamic traces before and after SCh. A&B: Control responses to stimulation of left (Lt) or right (Rt) anterior vesical branch of the pelvic nerve. C&D: Implanted RF micro-stimulator responses in a GFNT animal. E&F: Responses in a FNT animal to stimulation of the left transferred FN. ASphP = anal sphincter pressure; IVsP = intravesical pressure, UP = urethral pressure, RP = rectal pressure, DP = detrusor pressure (IVsP minus RP). Bottom: Rate of pressure generation in response to FES in control (CON), GFNT and FNT. *= Significantly different from control (p<0.01) Mann-Whitney U test.

Figure 2.

FES induced (nerve evoked) detrusor pressure increases before and after SCh in GFNT, FNT and control animals and sphincter pressures in control urethra and anal sphincters. * = statistically significantly different from pre-SCh $p < 0.01$ Student's *t* test, # = Statistically significantly different from control detrusor pre-SCh $p < 0.01$ Mann-Whitney U test.

Figure 3.

Immunohistochemistry of $\alpha 1$ nicotinic receptor subunits (red) in detrusor muscle in sham GFNT and FNT. DAPI (blue) A-C: Arrowheads indicate axons (pgp9.5 immunopositive, green). D-F: Arrowheads-asterisks indicate axons, higher power in insets. Black arrows illustrate small punctate immunopositive profiles on muscle fascicles, enlarged in insets. G-I: Arrowheads and black arrows as above. White arrow-asterisk and insets show $\alpha 1$ subunit immunopositive non-neuronal structure on muscle fascicle surface. m=muscle fascicle; ct = connective tissue.

Figure 4.

Photomicrographs of $\alpha 1$ nicotinic receptor subunits in intramural ganglia and muscularis. Colors are as indicated in Figure 3. A-C: Intramural ganglion neurons with low level of $\alpha 1$ nicotinic receptor subunit immunostaining. D-F: Black arrow indicates a small double-labeled neuronal cell body at edge of muscle fascicle. Insets show enlargement of asterisk indicated neuron. G-L: FNT dog. G-I: Intramural ganglion neurons with $\alpha 1$ nicotinic receptor subunit immunostaining. J-L: Black arrow indicates a small double-labeled neuronal cell body at edge of muscle fascicle. Insets show enlargement of asterisk indicated neuron.

Figure 5.

Mean number of punctate profiles on muscles and nerve cell bodies co-immunostained with anti-α1 nicotinic receptor subunit and anti-pgp9.5 antibodies. * = Statistically significantly different from Sham/un-operated controls by ANOVA with post hoc Student-Newman-Keuls tests, $p < 0.05$.