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Pharmacodynamic evaluation of Lys⁵, MeLeu⁹, Nle¹⁰-NKA_(4–10) prokinetic effects on bladder and colon activity in acute spinal cord transected and spinally intact rats

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Abstract

Purpose—To determine feasibility of a novel therapeutic approach to drug-induced voiding after spinal cord injury (SCI) using a well-characterized, peptide, neurokinin 2 receptor (NK₂ receptor) agonist, Lys⁵, MeLeu⁹, Nle¹⁰-NKA_(4–10) (LMN-NKA).

Methods—Cystometry and colorectal pressure measurements were performed in urethane anesthetized, intact and acutely spinalized, female rats. Bladder pressure and voiding were monitored in response to intravenous LMN-NKA given with the bladder filled to 70% capacity.

Results—LMN-NKA (0.1–300 µg/kg) produced dose dependent, rapid (< 60 s), short duration (< 15 min) increases in bladder pressure. In intact rats, doses above 0.3–1 µg/kg induced urine release (voiding efficiency of ~ 70% at 1 µg/kg). In spinalized rats, urine release required higher doses (> 10 µg/kg) and was less efficient (30–50%). LMN-NKA (0.1–100 µg/kg) also produced dose dependent increases in colorectal pressure. No tachyphylaxis was observed, and the responses were blocked by an NK₂ receptor antagonist (GR159897, 1 mg/kg i.v.). No obvious cardiorespiratory effects were noted.

Conclusions—These results suggest that rapid-onset, short duration, drug-induced voiding is possible in acute spinal and intact rats with intravenous administration of an NK₂ receptor agonist. Future challenges remain in regards to finding alternative routes of administration that produce clinically significant voiding, multiple times per day, in animal models of chronic SCI.

Keywords

spinal cord injury; cystometry; neurokinin 2 agonist; colorectal pressure; bladder

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Integrated Laboratory Systems animal care and use committee and followed the NIH Guidelines for the Care and Use of Laboratory Animals. This article does not contain any studies with human participants performed by any of the authors.

Introduction

Spinal cord injury (SCI) is a traumatic condition that results in irreversible loss of sensation and voluntary control of bladder and bowel function. Acute SCI results in paralyzed or 'areflexive' bladder and bowel and inability to void. With time, individuals with injuries above the sacral level develop weak, brief, involuntary, bladder contractions, often along with bladder-sphincter dyssynergia (also known as detrusor-sphincter dyssynergia or DSD), leading to urinary retention. Injuries involving the sacral cord result in a permanently areflexive bladder with predominant incontinence (de Groat and Yoshimura 2012; Panicker et al. 2015). For bowel, injuries above the sacral level result in poor colonic motility and tightening of the sphincters and pelvic floor muscles, which leads to constipation. Injuries involving the sacral cord result in weak sphincters and pelvic floor muscles, which leads to stool incontinence (Fajardo et al. 2003; Hughes 2014). The current standard therapy for urinary retention consists primarily of clean intermittent catheterization. While this is an effective treatment for complete bladder emptying, significant catheter related problems occur. These include urinary tract infections, bleeding, strictures, and stones, which pose a significant health risk to the individual and account for high costs of healthcare (Cardenas et al. 2004; Gregory et al. 1984; Singh et al. 2011; Yilmaz et al. 2014). Voiding of stools are managed by digital rectal stimulation with digital removal of stools, or with enemas, all of which are time consuming and greatly reduce the quality of life of affected individuals and caregivers (Hughes 2014). Therefore, efficient and effective pharmacological treatments to elicit bladder and bowel emptying after SCI are imperative.

Neurokinin A (NKA), an endogenous peptide agonist for the NK₂ receptor, and NK₂ receptors themselves are expressed in the gastrointestinal and urinary bladder pathways. *In vitro* binding studies demonstrated the expression of NK₂ receptors in human and rodent bladder and colon smooth muscle (Burcher et al. 2000; Matuszek et al. 1998; Warner et al. 2003). In human tissue, receptor density seems slightly higher in the colon compared to the bladder (Burcher et al. 2000; Warner et al. 2003). *In vitro* contractility studies in several species (human, rat, hamster) demonstrated NK₂ receptor mediated contraction of the bladder (Giuliani et al. 1993; Palea et al. 1996; Quinn et al. 2004; Tramontana et al. 1998; Warner et al. 2003) and gastrointestinal tract (Carini et al. 2001; Lecci et al. 2006; Lecci et al. 2004; Mule et al. 2000). Moreover, the NK₂ receptor mediated bladder contractions in human SCI-induced neurogenic overactive detrusor strips were similar to those from detrusor tissue taken from normal individuals (Sellers et al. 2006). *In vivo* experiments in anesthetized animals (rat, hamster, guinea pig) report that NK₂ receptor agonists (NKA, ([β-Ala⁸]NKA₍₄₋₁₀₎, Lys⁵, MeLeu⁹, Nle¹⁰-NKA₍₄₋₁₀₎) produce an increase in bladder contractility, baseline tone, and distension evoked responses (Kullmann et al. 2013; Maggi et al. 1987; Maggi et al. 1986; Tramontana et al. 1998). Enhancement of neuronally evoked contractions in response to NK₂ receptor stimulation was also reported, suggesting that efferent neuronal activity may be augmented (Maggi et al. 1986; Tramontana et al. 1998). Additionally, NK₂ receptor activation may lower the threshold for triggering micturition by increasing the excitability of primary afferent neurons (primarily C-fibers) innervating the bladder (Maggi et al. 1987; Maggi et al. 1986; Morrison et al. 1999; Sculptoreanu et al. 2009; Sculptoreanu et al. 2008; Sculptoreanu and de Groat 2003).

Animal studies investigating *in vivo* gastrointestinal motility have shown increased activity after stimulation of NK₂ receptors (Carini et al. 2001; Lecci et al. 2006; Lecci et al. 2004; Lordal et al. 2001; Lordal et al. 1997; Mule et al. 2000; Warner et al. 2003). These effects are thought to be mediated by a direct action on circular smooth muscle with a possible modulatory effect on cholinergic nerves (Lecci et al. 2006; Lecci et al. 2004). Most importantly, human volunteer studies demonstrated that intravenous infusion of exogenous NKA (6, 12, 25, and 50 pm/kg/min for 4 hours) produced dose dependent increases in gastrointestinal motility mediated by NK₂ receptors, with a good tolerability and safety profile (Lordal et al. 2001; Lordal et al. 1997; Schmidt et al. 2003). Together, these studies suggest that activation of NK₂ receptors has the potential to trigger contractions of the bladder and colon and could induce voiding after SCI.

The present study explores *in vivo* pharmacodynamic characteristics of NK₂ receptor-induced activation of the bladder and colon using Lys⁵, MeLeu⁹, Nle¹⁰-NKA₍₄₋₁₀₎ (LMN-NKA), as a tool to determine feasibility of this novel therapeutic approach to drug-induced voiding.

Materials and methods

Animals

Adult, female, Sprague-Dawley rats (230–300 g, n= 67, Charles River, Raleigh, NC) were maintained under standard laboratory housing conditions with free access to water and food. All experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Integrated Laboratory Systems animal care and use committee. All efforts were made to minimize animal stress and to reduce the number of animals used.

Surgical procedures

Rats were anesthetized with urethane (1.2–1.4 g/kg urethane s.c.) and body temperature was maintained at 37°C by a heated blanket. The carotid artery and jugular vein were cannulated to allow blood pressure measurements and intravenous (i.v.) drug delivery, respectively. The abdomen was opened, and the ureters were ligated and cut distally. Saline-filled polyethylene tubing with a flared tip (PE 50) was inserted through a small incision in the dome of the bladder and tied in place. Colorectal pressure was measured in a separate group of animals. The lower gastrointestinal tract and rectum were cleared of feces and a balloon catheter (2.5 cm length, made using the tip of a Trojan ENZ non-lubricated condom attached to PE50 tubing and a 20G blunt needle) was placed approximately 3–4 cm into the rectum, through the anus. The bladder or colorectal cannulas were connected to a T-tube, for saline infusion (room temperature, PHD2000 infusion pump, Harvard Apparatus, Holliston, MA, USA) and measurement of bladder or colorectal pressure (CDXIII Transducer). The signal was amplified using a bridge amplifier (Transbridge 4M, World Precision Instruments). Animals were either spinally intact or, 1 hour prior to study, received a complete spinal cord transection at the T9–T10 level after exposure of the vertebrae and a laminectomy (Tanahashi et al. 2012).

Drugs

All drugs were purchased from Tocris (Bristol, UK). NKA (NK receptor agonist), Lys⁵, MeLeu⁹, Nle¹⁰-NKA₍₄₋₁₀₎ (NK₂ receptor agonist, LMN-NKA), and spantide I (NK₁ receptor antagonist) were dissolved in saline, [bAla⁸]-Neurokinin A(4–10) (NK₂ receptor agonist) was solubilized in water or saline. GR159897 (NK₂ receptor antagonist) was solubilized at 50 mM in DMSO and diluted with saline to a concentration of 1 mg/ml (2.4 mM). Final DMSO concentration was 4.8%. All drugs were administered intravenously and followed by 0.3 ml saline flush. In antagonists studies, LMN-NKA (10 or 30 µg/kg) was given before and 5 min after each antagonist (1 mg/kg) and responses compared.

Isovolumetric cystometry

was conducted in acute SCI rats. To determine functional bladder capacity (Fig 1), the bladder was emptied and a cystometrogram (CMG) performed with the urethra opened (infusion rate 0.06 – 0.1 ml/min). The volume necessary to fill the bladder from an empty state to the threshold volume for triggering leaking in spinalized animals, was measured. This process was repeated 3–6 times per rat; the volumes were averaged and taken as the functional bladder capacity. For drug testing, the urethra was clamped externally at the meatus, and the bladder filled to 70% of functional bladder capacity where it remained throughout the experimental procedure. Each rat was given saline vehicle and then ascending doses of LMN-NKA (0.01 – 300 µg/kg). The time between dosing was 15–45 min to allow the bladder to return to a stable pressure.

Voiding cystometry

(Kullmann et al. 2014) was performed (Fig 1) as described above to determine each rat's bladder capacity and bladder pressure during voiding (intact rats) or the leak point pressure (acute SCI rats). The bladder infusion rate (0.04–0.1 ml/min) was adjusted for each animal to reach bladder capacity within ~10–15 min and maintained constant throughout the experiment. For drug testing, the bladder was filled to 70% of functional bladder capacity, the infusion pump turned off, and the rat dosed with the vehicle. After 5 min, in which the vehicle's effects were monitored, the bladder was emptied and voided volume (if any) and residual volume measured. The infusion pump was restarted and distension induced contractions were observed for 20–60 min to allow the bladder to return to control voiding/leaking characteristics. This cycle was repeated with ascending doses of LMN-NKA (0.1–300 µg/kg), according to the protocol outlined in figure 1.

Parameters measured

Maximal bladder pressure; bladder pressure response (maximal bladder pressure minus baseline pressure); time from injection to peak bladder pressure (time to peak); duration of action (time from injection to return to within 5 mmHg of pre-treatment bladder pressure); voided volume (VV); residual volume (RV); and voiding efficiency (VE), calculated as $((VV / (VV + RV)) * 100\%)$, were measured. In addition, the voiding responder rate was calculated as the percentage of rats voiding > 0.03 ml divided by total number of rats/group * 100%.

Colorectal activity

A colorectal balloon catheter was inflated by incrementally adding 0.1 ml saline until a steady pressure reading of 15–20 mmHg was obtained (total volume 0.3–0.7 ml). Once the colorectal pressure was stable, dosing of vehicle, followed by ascending log doses of LMN-NKA (0.1–100 µg/kg i.v.) were administered. The time between drug doses was approximately 20–45 minutes.

Parameters measured

As the gastrointestinal tract presents intrinsic activity that is tonic in nature and drug effects are superimposed on this tonic activity, two measurements were chosen: a) area under the curve (AUC), which reflects tonic and drug induced activity, and b) duration of action (min) following drug administration. This measure is a close approximation of drug induced activity. The threshold amplitude of the colonic activity was chosen at 5 mmHg, which was the average amplitude of spontaneous pressure fluctuations during control periods.

Selectivity of the NK₂ receptor agonist

To explore the effects of spantide I, an NK₁ receptor antagonist, and GR159897, an NK₂ receptor antagonist, on the LMN-NKA induced increase in bladder pressure and colorectal activity, the animals received a 10 or 30 µg/kg i.v. dose of LMN-NKA before, and 5 minutes after, each antagonist (1 mg/kg for each antagonist). Responses to LMN-NKA (10 or 30 µg/kg, i.v.) were compared before and after antagonist treatment.

Data analysis and statistics

Data were recorded using LabChart software (version 7; ADInstruments, Australia) and analyzed using Excel (Microsoft, Redmond, WA) and Prism 6 (GraphPad Software Inc., San Diego, CA). Data are presented as mean and standard deviation. One way ANOVA with Dunnett's Multiple Comparison post-test or paired Student's t-test was used, as appropriate; $p < 0.05$ was considered statistically significant.

Results

Onset and duration of action, tachyphylaxis examination, and involvement of the NK₂ receptor in LMN-NKA effects on bladder function (isovolumetric cystometry) in acute spinal rats

To determine the effects of LMN-NKA on bladder contractility, acute SCI rats were dosed with ascending doses of the drug, 0.1–100 µg/kg i.v. ($n = 4$ –16 rats per group). Under isovolumetric conditions with the bladder filled to 70% capacity, LMN-NKA produced dose-dependent bladder contractions (Fig 2a, 2b). At doses < 1 µg/kg, contractions were monophasic with a rapid rise to peak at ~40 s and a gradual return to baseline within 15 minutes. At higher doses, a biphasic response was observed that consisted of 1 or 2 immediate, rapid spikes in pressure that seemed to be superimposed on the gradual contraction seen at lower doses (Fig 2a). Drug induced bladder contractions returned to baseline levels within 15–20 minutes depending on the dose (Fig 2c). Similar effects were

observed with the NK₂ receptor specific agonist, [β Ala⁸]-NKA_(4–10), and with NKA (data not shown).

The selectivity of the effects of LMN-NKA for NK₂ receptors was examined by using the specific NK₂ receptor antagonist, GR159897. GR159897 (1 mg/kg i.v.) significantly inhibited the maximal bladder pressure induced by LMN-NKA (Table 1). Bladder contractions were not affected by the NK₁ receptor antagonist, spantide I (1 mg/kg), but were significantly reduced by the NK₂ receptor antagonist, GR159897 (1 mg/kg, Table 1). Repeated administration of LMN-NKA (10 or 30 μ g/kg, every ~30 – 70 min) in SCI rats produced consistent bladder contractions (Fig 2d), suggesting no tachyphylaxis.

Drug-induced urine release in acute SCI rats (voiding cystometry)

To determine whether LMN-NKA can induce urine release in acute SCI rats, cystometry was performed while giving ascending doses of the drug, 0.1–300 μ g/kg i.v., according to the protocol outlined in Fig 1. Acute SCI rats did not void with bladder filling, but instead began to leak when the maximal bladder pressure reached ~35 mmHg (Fig 3a white bar indicates the bladder pressure response at leak point pressure). At 70% bladder capacity, saline produced no pressure change or urine release, while LMN-NKA induced dose-dependent increases in bladder pressure (Fig 3a) and urine release (Table 2) beginning at 1 μ g/kg. Voiding efficiency also increased dose-dependently, reaching ~ 50% at 300 μ g/kg (Fig 3b). A separate, small group of rats (n=4) were pretreated with the NK₂ receptor antagonist GR159897 (1 mg/kg) before a single dose of 30 μ g/kg LMN-NKA. GR159897 (1 mg/kg) virtually abolished voiding in acute SCI rats (reducing voiding efficiency from $18.5 \pm 17.8\%$ to $0.75 \pm 1.5\%$, n=4; $p < 0.05$ paired Student t-test). Control voiding efficiency in response to 30 μ g/kg LMN-NKA was lower in this small group than that observed in the dose-response group (~35%; figure 3b), presumably reflecting variability between animals and the small sample size (n=4) for the antagonist experiments. Nevertheless, GR159897 (1 mg/kg) virtually abolished voiding in acute SCI rats.

Drug-induced voiding in intact rats at bladder volumes below micturition threshold

To determine whether LMN-NKA can induce voiding in intact rats, voiding cystometry was performed with ascending doses of the drug, 0.1–300 μ g/kg i.v. according to the protocol outlined in Fig 1. With the bladder filled to 70% capacity, LMN-NKA induced dose-dependent increases in bladder pressure in all animals, up to 50–60 mmHg at a dose of 3 μ g/kg, with no further increase at higher doses (Fig 4a). About half of animals voided when given 0.3 μ g/kg LMN-NKA, and all animals voided at doses > 10 μ g/kg (Table 2). The onset of voiding occurred about 30 s after drug administration. Voiding efficiency was greater than 70% at 10 μ g/kg, with no further increase at higher doses (Fig 4b). Voiding efficiency during physiologically-induced voiding, measured prior to drug dosing, was $72.00 \pm 3.50\%$ (n=19 rats; white bar in Fig 4b). In a separate group of rats, GR159897 (1 mg/kg) abolished LMN-NKA (30 μ g/kg) -induced VE in 5 out of 6 rats (from $84.86 \pm 7.9\%$ to $0 \pm 0\%$ n=5; $p < 0.05$ paired Student t-test) and had no effect in n=1 rat.

Effects on colonic activity

To determine whether LMN-NKA increases colonic activity, the effects of LMN-NKA on colorectal contractility were investigated in spinally intact and acute spinalized rats (Fig 5). Vehicle (i.v.) did not alter colorectal pressure. LMN-NKA (0.1–100 µg/kg i.v.) produced a dose related increase in colorectal activity that occurred within 60 s of drug administration. No significant differences were seen between the intact and SCI rats, thus the data were pooled for analysis. The AUC significantly increased with ascending doses, with doses of 1–100 µg/kg being significantly different compared to vehicle (one way ANOVA followed by Dunnett's multiple comparison post-test, $p < 0.05$; Fig 5; $n=5-8$ rats). Pretreatment with the NK₁ receptor antagonist, spantide I (1 mg/kg i.v.), did not reduce the effects of LMN-NKA (10 µg/kg i.v.) on colorectal activity. In contrast, pretreatment with the NK₂ receptor antagonist, GR159897 (1 mg/kg i.v.), virtually abolished tonic activity and the effects of LMN-NKA (10 µg/kg i.v.) on colonic activity (Fig 5c; $n=3$ rats), consistent with the well documented expression of NK₂ receptors in the circular smooth muscle of the gastrointestinal tract and their involvement in colonic activity (Carini et al. 2001; Lecci et al. 2006; Lecci et al. 2004; Lordal et al. 2001; Lordal et al. 1997; Mule et al. 2000; Warner et al. 2003).

Other effects

Tachykinins have been involved in regulation of vascular tone and blood flow as well as modulation of blood pressure and heart rate, with most effects being attributed to the NK₁ receptors (Couture et al. 1989; Hoover et al. 2000; Longmore et al. 1997; Walsh and D 2006). There are numerous species differences in the expression of NK receptors, and in animal studies the depth and type of anesthesia influence cardiovascular parameters, such that effects on blood pressure and heart rate measured under anesthesia are not necessarily reflective of the awake state. Therefore, we only assessed cardiovascular effects qualitatively. In these studies doses 30 µg/kg produced inconsistent, small drops in blood pressure (10% change) with little change in heart rate. Further studies should be performed to determine any changes in blood pressure and heart rate in the conscious rat.

Tachykinins have been involved in bronchoconstriction, especially under inflammatory (asthma) conditions and the NK₂ receptor agonists have been shown to contract bronchiolar smooth muscle in various species (Advenier et al. 1992; Cohen et al. 2005; Floch et al. 1994; Joos et al. 2000; Naline et al. 1989). Thus, particular attention was focused on respiratory effects following *in vivo* administration of NK₂ receptor agonists. No significant drug related changes in respiration and no labored breathing were observed.

Dose related flushing of the extremities (ears, paws) was observed in both intact and acute SCI rats with doses greater than 3 µg/kg. Minor to moderate salivation and lacrimation were only observed with high doses (100–300 µg/kg). At doses of 3 and 10 µg/kg, which induced efficient voiding and/or urine release, these effects were absent or very mild.

Discussion

The results from these pharmacodynamic studies demonstrate that intravenous administration of a peptidergic NK₂ receptor agonist produces rapid-onset (< 60 s), short-duration (< 15 min) bladder contractions of sufficient power to induce voiding in acute spinalized and spinal intact urethane-anesthetized rats. In acutely spinalized rats, doses of 10 µg/kg and above produced urine release with a voiding efficiency of 30–50% at bladder pressures slightly higher than the leak point pressure. Although bladder pressure remained slightly elevated, urine voiding only lasted 1–3 min after drug administration. In rats with an intact spinal cord, LMN-NKA administration produced voiding that was as efficient as physiological voiding (~70% efficiency under urethane anesthesia) but with a higher micturition pressure, which may reflect small increases in urethral resistance (see below). The duration of action was relatively short (peak bladder pressure and voiding of urine decreased rapidly within 2 minutes post administration), and no tachyphylaxis was observed. Similar doses increased colorectal activity in acute spinalized and intact rats. Together, these results indicate that an agonist for the NK₂ receptor has the potential to trigger voiding and defecation after spinal cord injury.

The NK₂ receptors are expressed at several sites in the micturition pathways, including the detrusor smooth muscle (Burcher et al. 2000; Giuliani et al. 1993; Palea et al. 1996; Tramontana et al. 1998; Warner et al. 2003), urethral smooth muscle (Palea et al. 1996), afferent neurons (Maggi et al. 1987; Maggi et al. 1986; Morrison et al. 1999; Sculptoreanu et al. 2009; Sculptoreanu et al. 2008; Sculptoreanu and de Groat 2003) and possibly efferent neurons (Maggi et al. 1986; Tramontana et al. 1998). Our data support the expression of functional receptors at these sites. The elevation in voiding pressure and leak point pressure in spinally intact and acute spinal animals, respectively, suggests that LMN-NKA is stimulating NK₂ receptors on both the detrusor and urethral smooth muscle. Indeed, urethral smooth muscles express NK₂ receptors that mediate contraction, but the potency and maximal efficacy of NK₂ receptor agonists to induce contraction is about 10 times less in human urethra compared to human bladder (Palea et al. 1996). It is relevant to note that, in spinally intact rats, the dose-response curves for bladder voiding pressure plateaued at 3 µg/kg, while bladder voiding efficiency continued to increase at 10 µg/kg (Fig 4). This suggests that bladder force continued to increase at 10 µg/kg, while urethral resistance did not. In the acute spinal rat, doses above 3 µg/kg produced both higher voiding efficiency and higher leak point pressures (Fig 3). This suggests that in the absence of neural control, increased drug doses are correlated with increases in both bladder and urethra contractions and that in the presence of neural control, reflex mechanisms may suppress urethral contractions (probably through nitrergic mechanisms) at higher doses.

Our data also support the conclusion that administration of an NK₂ receptor agonist triggers micturition reflexes in the spinally-intact animal, by showing that LMN-NKA-induced voiding of urine at doses (0.3 µg/kg) that were below those that produced significant increases in bladder pressure in the acute spinal rat (1 µg/kg) (compare Figs 3, 4). Because voiding efficiency was constant with increasing doses, it is tempting to speculate that LMN-NKA triggered activation of the supraspinal micturition, which then emptied the bladder to the same degree (~70%) as “natural” voiding that results from distension of the bladder

sufficient to trigger the micturition reflex. Furthermore, our experimental observations (data not shown) show that voiding at these doses was accompanied by high frequency oscillations (HFOs) of bladder pressure, reflecting urethral rhabdosphincter contractions that accompany physiological voiding (i.e. activation of supraspinal micturition reflex pathways to the lower urinary tract). Thus, NK₂ receptor activation may “trigger” micturition reflexes that reinforce bladder contractions, activate rhabdosphincter HFOs, and reduce urethral resistance. This triggering may result from stimulation of NK₂ receptors on afferent (and/or efferent) neurons or due to indirect activation of afferent terminals during bladder contractions subsequent to direct stimulation of NK₂ receptors on smooth muscle cells. The exact site of action and mechanisms remain to be further investigated. HFOs were absent from acute spinal rats, consistent with abolishment of micturition reflexes. Thus, in acute spinal rats, LMN-NKA-induced voiding appears to be due to direct stimulation of NK₂ receptors on detrusor smooth muscle, which was powerful enough to overcome urethral resistance.

LMN-NKA also increased colonic activity (Fig 5), consistent with the expression of NK₂ receptors in the smooth muscle of the colon and/or myenteric neurons, as reported in previous rodent and human studies (Carini et al. 2001; Lecci et al. 2006; Lecci et al. 2004; Lordal et al. 2001; Lordal et al. 1997; Mule et al. 2000; Warner et al. 2003). Similar to the urethra, the NK₂ receptors are likely present in the internal anal sphincter (Tichenor et al. 2002), where their activation may keep the sphincter tight, thus providing resistance to defecation. Future studies would need to investigate the role of these receptors in decreasing defecation efficiency and whether sphincter relaxants (such as phosphodiesterase inhibitors, topical diltiazem, a calcium channel blocker, botulinum toxin A injection and other) would provide additional benefits (Ballester et al. 2007; Gandomkar et al. 2015; Jones et al. 2002; Vaithianathan and Panneerselvam 2015). While we did not assess defecation in this study, pilot studies in chronic SCI rats showed that LMN-NKA reliably induces defecation (in progress L. Marson), suggesting that if stools were present in the rectum, both urine and stools would be excreted (sitting on a toilet to urinate would be advisable).

Tachykinins have been implicated in the function and regulation of the cardiovascular and respiratory systems (Advenier et al. 1992; Cohen et al. 2005; Couture et al. 1989; Floch et al. 1994; Hoover et al. 2000; Joos et al. 2000; Longmore et al. 1997; Naline et al. 1989; Walsh and D 2006), thus cardiorespiratory effects of an NK₂ receptor agonists will need to be carefully investigated. In human volunteers, prolonged i.v. infusion of NKA (6, 12, 25, 50 pm/kg/min for 4 hours) produced dose dependent increases in gastrointestinal (GI) motility mediated by the NK₂ receptors with no significant changes in blood pressure or respiration in these subjects (Lordal et al. 2001; Lordal et al. 1997; Schmidt et al. 2003). A small but significant change in heart rate was observed at 50 pm/kg/min for 4 hr (Lordal et al. 1997) and 64 pm/kg/min for 5 min (Evans et al. 1988). Thus, even at maximal doses tested, intravenous NKA (which activates all NK receptors and is more selective for the NK₁ versus the NK₂ receptors) produced no significant changes in blood pressure or respiration in these subjects. Consistent with these studies, in anesthetized rats, non-significant fluctuations in arterial pressure were observed after dosing with LMN-NKA, indicating a cardiovascular study should be conducted in unanesthetized rats.

In the present study no respiratory effects were observed following LMN-NKA administration. Furthermore, a recently completed GLP safety study conducted in awake rats, also showed no effects of LMN-NKA on respiration at doses below 100 µg/kg i.v. (unpublished results). One may be surprised at the absence of respiratory effects following systemic administration of NK₂ receptor agonists in rats and humans (Lordal et al. 2001; Lordal et al. 1997; Schmidt et al. 2003) since previous studies reported in vitro effects of NK₂ receptor agonists on respiratory smooth muscle in most species tested. However, even aerosol administration of NKA directly into the lungs produced no increase in bronchoconstriction in healthy subjects, and only mild, asymptomatic bronchoconstriction was produced in asthmatic patients (Advenier et al. 1992; Cohen et al. 2005; Evans et al. 1988; Floch et al. 1994; Joos et al. 2000; Naline et al. 1989). This difference in sensitivity for asthmatic patients indicates that safety studies in this population patients are warranted, but for most patients, respiratory effects do not seem to be a concern. In summary, it is unlikely that NK₂ receptor agonists will produce significant respiratory and cardiovascular effects at doses that will produce urination and defecation. However, these possible side effects remain to be carefully investigated in human.

Though future preclinical studies in animal models of chronic spinal cord injury are still needed to assess doses and routes of delivery, our results and previous studies indicate that a drug like LMN-NKA might offer a convenient method for inducing on-demand micturition and defecation in individuals with SCI.

Conclusion

Systemic administration of a peptidergic NK₂ receptor agonist triggered bladder contractions and urine release in both spinalized and intact rats when the bladder was filled to a sub-threshold voiding volume and induced powerful contractions of the colorectum. These results suggest that rapid-onset, short duration, drug-induced voiding is possible in spinal and control rats with intravenous administration of an NK₂ receptor agonist. Further studies will extend these results to chronic SCI animal models as well as to translational studies focused on finding doses and routes of administration (e.g. sublingual or intranasal) that are convenient, safe, and efficacious. The development of pharmacological compounds that achieve efficient and effective bladder and colon emptying in individuals with SCI would greatly reduce medical complications associated with bladder and bowel management after SCI and greatly improve the quality of life of patients.

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Figure 1. Schematic of experimental design

a. The experiment is divided into three main blocks: establishment of baseline voiding parameters, determination of functional bladder capacity and investigation of drug effects. First, cystometry is performed for 20–60 minutes to establish baseline voiding parameters. Next, functional bladder capacity is determined as follows (b): the bladder is emptied and a cystometrograph (CMG) is started. When the rats void (intact) or start leaking (acutely spinalized) the infusion pump is stopped and the infused, voided and residual volumes are measured. The process is repeated 3 times and the infused volume necessary to trigger voiding or leaking is averaged and termed 100% functional bladder capacity. c. The last block determines drug effects. For this, the bladder is filled to 70% capacity, the infusion pump is stopped and vehicle or drug are given i.v. Five minutes following vehicle/drug dosing, voided (VV) and residual (RV) volumes are measured. The infusion pump is then turned on and a period of at least 20 minutes is allowed for recovery prior to next drug dose.

Figure 2. Onset, duration of action and reproducibility of the NK₂ receptor agonist, LMN-NKA

a. Example of bladder contractions induced by increasing doses of LMN-NKA in acute spinalized rats under isovolumetric conditions. b. LMN-NKA produced a dose dependent increase in the bladder pressure in acute spinalized rats under isovolumetric conditions. Number of rats in each group = 16 (V), 2 (0.01 $\mu\text{g/kg}$), and 4–15 for 0.1–100 $\mu\text{g/kg}$. (*) indicates values significantly different from the vehicle (V) ($p < 0.05$, one-way ANOVA ($F(5,43) = 222$) followed by Dunnett's Multiple Comparison post-test). c. Duration of bladder activity induced by LMN-NKA in acute spinalized rats under isovolumetric conditions. Time from injection to return to baseline for 0.01 and 0.1 $\mu\text{g/kg}$ doses are not shown as no significant increase in bladder pressure was seen. d. Repeated dosing of LMN-NKA in acute SCI rats produced similar bladder pressure responses. Graph shows an example of 4 doses (10 $\mu\text{g/kg i.v.}$) given to the same rat at 45–75 min intervals.

Figure 3. Drug-induced urine release in acute SCI rats

a. LMN-NKA produced a dose dependent increase in the bladder pressure at which urine release was triggered. b. LMN-NKA increased voiding efficiency in a dose dependent manner. (*) indicates values significantly different from the vehicle (V) ($p < 0.05$, one-way ANOVA followed by Dunnett's Multiple Comparison post-test). $F(8, 54) = 18.98$ for bladder pressure and $F(8, 64) = 7.661$ for voiding efficiency. LPP stands for 'leak point pressure' and indicates parameters measured during bladder distention to full capacity (100%), when urine leaks occur in the absence of any drugs. Drugs were given when the bladder was at 70% capacity. Number of rats in each group = 12 (LPP), 9 (V), 4 (0.1 $\mu\text{g/kg}$), and 6–10 for 0.3–300 $\mu\text{g/kg}$.

Figure 4. Drug-induced voiding at sub-threshold voiding volumes in intact rats

a. LMN-NKA produced a dose dependent increase in voiding bladder pressure. b. LMN-NKA increased voiding efficiency in a dose dependent manner. (*) indicates values significantly different from the vehicle (V) ($p < 0.05$, one-way ANOVA followed by Dunnett's Multiple Comparison post-test). ANOVA $F(8,72) = 25.88$ for bladder pressure and $F(8,62) = 14.26$ for voiding efficiency. P stands for 'physiological' and indicates parameters measured during bladder distention to full capacity (100%) in the absence of any drugs. Drugs were given when the bladder was at 70% capacity. Number of rats in each group = 19 (P), 12 (V), 4–8 for 0.1 – 300 $\mu\text{g/kg}$.

Figure 5. NK₂ receptor-mediated prokinetic effect of LMN-NKA on colorectal contractility
a–b. LMN-NKA produced a dose dependent increase in the AUC and duration of action of colorectal contractions. (*) indicates values significantly different from the vehicle (V) ($p < 0.05$, one-way ANOVA $F(4,27) = 10.15$ for AUC and $F(4,26) = 8.9$ for duration, followed by Dunnett's Multiple Comparison post-test). Number of rats in each group = 8 (V), 5 (0.1 $\mu\text{g/kg}$), 6 (1 $\mu\text{g/kg}$), 8 (10 $\mu\text{g/kg}$), 5 (100 $\mu\text{g/kg}$). V - represents the baseline response in the absence of any drugs. c. LMN-NKA-induced increase in colorectal activity is mediated via NK₂ receptors. Upper trace illustrates a control response to LMN-NKA (10 $\mu\text{g/kg}$; i.v.) given at the arrow. Lower trace illustrates lack of response to LMN-NKA (10

µg/kg; i.v.) when given 5 min after the NK₂ receptor antagonist, GR159897 (1 mg/kg, i.v.; n=3 rats).

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Table 1

Reproducibility and specificity of LMN-NKA for the NK₂ receptors in the bladder.

Drug/dose	Pressure response (mmHg)	n (rats)	Condition
LMN-NKA (30 µg/kg) 1 st dose	40.01 ± 4.78	5	Acute SCI
LMN-NKA (30 µg/kg) last dose	31.43 ± 4.83		
LMN-NKA (30 µg/kg)	34.90 ± 2.98	5	Acute SCI
LMN-NKA+ GR159897 (1 mg/kg)	16.58 ± 3.99 *		
LMN-NKA (30 µg/kg) 1 st dose	47.64 ± 10.85	7	Intact
LMN-NKA (30 µg/kg) last dose	45.24 ± 7.59		
LMN-NKA (30 µg/kg)	48.82	2	Intact
LMN-NKA+ spantide I (1 mg/kg)	64.53		

Data are mean ± SD.

* indicates statistically different values tested with student t-test, p<0.05.

Table 2
Percentage of rats displaying drug induced voiding (responders) after LMN-NKA.

		LMN-NKA µg/kg i.v.							
	saline	0.1	0.3	1	3	10	30	100	300
Intact									
Responder rate (%)	5 (1/20)	14.3 (1/7)	57.1 (4/7)	77.8 (7/9)	87.5 (7/8)	100 (9/9)	100 (12/12)	100 (7/7)	100 (5/5)
Acute SCI									
Responder rate (%)	10.5 (2/19)	0 (0/3)	0 (0/5)	71.4 (5/7)	50 (3/6)	85.7 (6/7)	83.3 (10/12)	88.9 (8/9)	100 (3/3)