Activation of NK-1 receptors increases the excitability of guinea pig dorsal root ganglion cells.

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Running Title: NK-1 receptors in guinea pig DRG neurons

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Number of text Pages: 37
Tables: 2
Figures: 7
References: 46
Words in Abstract: 248
Words in Introduction: 700
Words in Discussion: 1448

Section: Gastrointestinal, Hepatic, Pulmonary, and Renal

Abbreviations: DRG, dorsal root ganglion; OAB, overactive bladder; AHP, after hyperpolarization. AP, action potential; NK, neurokinin; APD, action potential duration; Sar-Met-SP, [Sar(9), Met(O2)(11)]-substance P; SP, substance P; NTP, Netupitant (2-[3,5-Bis(trifluoromethyl)phenyl]-N-[6-(4-methylpiperazin-1-yl)-4-(o-tolyl) pyridin-3-yl]-N-methylisobutyramide);
Abstract

The suppression of overactive bladder symptoms in patients and overactive bladder reflexes in animal models by NK-1 receptor antagonists raises the possibility that these drugs target sensory neurons. This mechanism was evaluated by examining the interactions between a specific NK-1 agonist, [Sar(9), Met(O2)(11)]-substance P (Sar-Met-SP) and a potent NK-1 antagonist, netupitant (NTP) on small size (20-30 μm) dissociated L6 and S1 dorsal root ganglion (DRG) neurons from female guinea pigs. Current clamp recording revealed that Sar-Met-SP (1μM) elicited a membrane depolarization (average 8.05±1.38 mV) in 27% (18/65) of DRG neurons. In 74% of the remaining neurons (35/47) Sar-Met-SP decreased the rheobase for action potential (AP) generation and increased the response to a suprathreshold stimulus (3 times rheobase) without changing the membrane potential. Sar-Met-SP also induced changes in AP waveform, including: (1) an increase in overshoot (average 5 mV, n=35 neurons), (2) a prolongation of AP duration (from 4.64 to 5.29 ms, n=34) and (3) a reduction in the maximal rate of AP repolarization. NTP (200 nM) reversed the Sar-Met-SP induced changes. Ca²⁺ imaging showed that application of Sar-Met-SP (1 μM) decreased the tachyphylaxis induced by repeated application of capsaicin (0.5 μM), an effect blocked by pretreatment with NTP (200 nM). These results raise the possibility that activation of NK-1 receptors in primary sensory neurons plays a role in the generation of overactive bladder and that block of NK-1 receptors in these neurons may contribute to efficacy of NK-1 antagonists in the treatment of overactive bladder symptoms.
Introduction

Considerable attention has been focused on the role of substance P (SP) and related neuropeptides in the neural control of the urinary bladder (Lecci & Maggi 2001, Candenas et al. 2005). These substances are expressed in bladder afferent neurons and are released in the spinal cord or in the bladder by capsaicin-induced stimulation of nociceptive afferent nerves (Lecci & Maggi 2001). SP can modulate bladder activity by acting on three subtypes of neurokinin receptors (NK-1, NK-2 and NK-3) expressed by bladder smooth muscle cells, urothelial cells, afferent nerves or neurons in the spinal cord (Morrison 1999, de Groat & Yoshimura 2001, Lecci & Maggi 2001, Morrison et al. 2002, Candenas et al. 2005).

The contribution of NK-1 receptors to the pathophysiology of bladder dysfunction, has attracted the most interest because clinical studies revealed that NK-1 receptor antagonists, aprepitant, (Green et al. 2006) and serlopitant (Frenkl et al. 2010) reduce overactive bladder (OAB) symptoms. These agents also reduce normal bladder reflexes in guinea pig, rat or cat (Doi et al. 1999, Doi et al. 2000, Kamo & Doi 2001) as well as hyperactive bladder reflexes induced by spinal cord injury (Doi et al. 2000), systemic administration of L-DOPA (Ishizuka et al. 1995) or application of irritant chemicals to the bladder in the rat (Abdel-Gawad et al. 2001) or guinea pig (Doi et al. 1999, Doi et al. 2000). Various animal studies indicate that these effects are due at least in part to actions on peripheral or central bladder sensory pathways (Lecci & Maggi 1995, Lecci & Maggi 2001, Seki et al. 2005).

In the bladder SP released from afferent nerves can influence afferent nerve
activity indirectly by inducing smooth muscle contractions (Lecci & Maggi 2001) or
directly by acting in an auto-feedback manner to regulate afferent nerve excitability
(Morrison et al. 2002, Sculptoreanu & de Groat 2007, Sculptoreanu et al. 2008,
Sculptoreanu et al. 2009). Different neurokinin receptor subtypes contribute to the
regulation of bladder activity depending on the species and site of action (Lecci &
Maggi 2001, Candenás et al. 2005). In the rat all three receptor subtypes are
involved in the SP induced bladder contractions; however, NK-2 receptors are
primarily responsible for the facilitatory effect of intravesically administered SP on
bladder afferent nerve firing (Morrison 1999). Similarly, activation of NK-2 receptors
by SP in rat dissociated dorsal root ganglion (DRG) neurons enhances firing to
depolarizing current pulses (Sculptoreanu & de Groat 2007), enhances L- and N-type
Ca\(^{2+}\) currents (Sculptoreanu & de Groat 2003) and the excitatory effect of capsaicin
on TRPV1 channels (Sculptoreanu et al. 2008) and reduces A-type K\(^{+}\) currents
(Sculptoreanu et al. 2009). On the other hand, activation of NK-1 receptors on
dissociated DRG neurons in rat induces an inward current (Li & Zhao 1998), and
enhances N-methyl-D-aspartic acid (Wu et al. 2004), Nav1.8 (Cang et al. 2009) and
TRPV1 currents (Zhang et al. 2007). Thus, the rat may not be a good model for
testing NK-1 receptor antagonists because neurokinin mechanisms in rat sensory
neurons are complicated and neurokinin receptors have a different pharmacology in
rats and humans. The guinea pig may be a better model (Palea et al. 2010a) because
the pharmacology of NK-1 receptors in guinea pig and human is very similar (Saria
1999). Unfortunately, the effect of neurokinin receptor activation in DRGs neurons in
the guinea pig has not been studied.

In the present experiments we examined the interactions between a selective NK-1 agonist (Sar-Met-SP) and a selective NK-1 antagonist, netupitant (NTP) (Hoffmann et al. 2006) on dissociated L6 and S1 dorsal root ganglion (DRG) cells from female guinea pigs to determine if the depressant effects of NTP on reflex bladder overactivity observed in anesthetized guinea pigs (Palea et al. 2010a, Palea et al. 2010b) might be due to action on sensory neurons. Our experiments revealed that NTP blocks the various excitatory effects elicited by activation of NK-1 receptors in primary sensory neurons, raising the possibility that NK-1 receptors have a role in the generation of OAB symptoms and that block of these receptors may contribute to efficacy of NK-1 antagonists in the treatment of OAB. A preliminary report of some of these observations has appeared in an abstract (Zhang et al. 2011).
Materials and Methods

Animals. Adult female guinea pigs (300–450 g; Harlan, Indianapolis, IN) were used in this study. All experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were consistent with the guidelines of the National Institutes of Health and the International Association for the Study of Pain.

DRG neuron culture. L₆ and S₁ DRG which contain sensory neurons that innervate the urinary bladder were removed bilaterally after laminectomy under isoflurane anesthesia. DRGs were enzymatically treated (collagenase type 4 and trypsin; Worthington Biochemical, Lakewood, NJ) and mechanically dissociated as described elsewhere (Dang et al. 2005). The cells were plated on poly-l-lysine-coated (Sigma, St. Louis, MO) glass cover slips and incubated at 37°C in 5% CO₂ and 90% humidity for at least 2–3 h to allow recovery from the dissociation procedure before Ca²⁺ imaging or electrophysiological studies. Cells were studied within 2 to 3 days after dissociation.

Patch clamp recording. Current-clamp recordings were performed using an Axopatch 200B (Molecular Devices, Sunnyvale, CA) controlled with pClamp (version 8.2). Data were low-pass filtered at 5–10 kHz with a four-pole Bessel filter and digitally sampled at 25–100 Hz. Hank's balanced salt solution (HBSS) containing (in mM): 138 NaCl, 5 KCl, 0.3 KH₂PO₄, 4 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 5.6 glucose, pH 7.4, 320 mosm/l was used as bath solution. Electrode solution contained (in mM): 140 KCl, 5 NaCl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES, 2
Mg-ATP, and 1 Li-GTP; pH was adjusted to 7.2 with Tris-base, and osmolality was adjusted to 310 mosm/l with sucrose. All salts were obtained from Sigma. After formation of a tight seal (>3 GΩ) and compensation of pipette capacitance with amplifier circuitry, whole cell access was established, and a -60 mV holding potential was applied. Cell capacitance was determined with four hyperpolarizing pulses (10 ms duration) ranging from –60 to –80 mV. Then recording was switched to current clamp configuration. A single action potential was evoked with depolarizing current injection (4 ms duration rectangular pulse) (Figure 1A) to assess properties of the action potential waveform (duration, overshoot, rate of rise and decay, and the afterhyperpolarization magnitude). A 750 ms depolarizing current injection was used to assess excitability (action potential threshold, rheobase and suprathreshold responses) (Figure 1B and C). After establishing the baseline action potential waveform, action potential threshold, rheobase, and the response to suprathreshold stimuli, a depolarizing stimulus with rheobase intensity (750 ms) was applied every 10 sec to monitor the changes of membrane potential and firing. After a stable baseline (6 to 8 sweeps) was recorded, 1 µM Sar-Met-SP was bath applied for 30 sec to 1 min and then action potential waveform and excitability data were collected 2 to 3 min after Sar-Met-SP application. In some neurons membrane potential was recorded without a depolarizing stimulus (as shown in Figure 6A). The effects of Sar-Met-SP on excitability and action potential waveform were only assessed in neurons demonstrating stable membrane potential. A neuron was considered excited by Sar-Met-SP if there was membrane depolarization, a decrease in threshold or
rheobase or an increase in spike number of at least 2 SD from baseline mean.

**Ca\textsuperscript{2+} imaging.** Fura 2 Ca\textsuperscript{2+} imaging was performed as described previously (Zhang et al. 2010). Briefly, DRG cells were loaded with Fura 2-AM (2 \(\mu\)M; Molecular Probes, Eugene, OR) for 30 min at 37°C in an atmosphere of 5% CO\textsubscript{2}. Fura 2-AM was dissolved in HBSS solution to which BSA was added (5 mg/ml; Sigma) to promote loading. Cover slips were placed on an upright epifluorescence microscope (Olympus IX70) and continuously superfused (3–4 ml/min) with HBSS. Fura 2 was excited alternately with ultraviolet light at 340 and 380 nm; and the fluorescence emission was detected at 510 nm using a computer-controlled monochromator. Image pairs were acquired every 1–30 s using illumination periods between 20 and 50 ms. Wavelength selection, timing of excitation, and the acquisition of images were controlled using the program C-Imaging (Compix, Cranberry Township, PA) running on a personal computer. Digital images were stored on hard disk for off-line analysis. One coverslip usually contained 20–40 DRG neurons/microscopic field. On each experimental day, we first collected the control data from 4 to 5 coverslips and then collected data from another 4 coverslips after Sar-Met-SP or substance P (SP) application and followed by another 4 coverslips to test the effect of NTP on agonist responses.

**Data analysis.** In Ca\textsuperscript{2+} imaging studies, data were analyzed using program C-Imaging (Compix). Background was subtracted to minimize camera dark noise and tissue autofluorescence. An area of interest was drawn around each cell, and the average value of all pixels included in this area was taken as one measurement. The
ratio of fluorescence signal measured at 340 nm divided by the fluorescence signal measured at 380 nm was used to measure the increase in intracellular Ca^{2+}.

Action potential duration (APD) was determined at 0 mV (Figure 1A). Action potential overshoot was measured as the maximal deflection above 0 mV (Figure 1A). Maximum rates of rise and fall of the action potentials were determined by taking the first derivative (dV/dt) of the action potential waveform. Magnitude of the afterhyperpolarization (AHP) was determined relative to the resting membrane potential at the largest potential change following the action potential (Figure 1A). The AHP was fitted with a single exponential equation to estimate the decay rate (Figure 1A inset). Action potential threshold was defined as the maximum depolarization obtained in the absence of an action potential (Figure 1B). Rheobase was defined as the minimum depolarizing current injection necessary to evoke an action potential with a long depolarizing current pulse (750ms) (Figure 1B). The response to suprathreshold stimuli was assessed by stimulating neurons with depolarizing current injection equal to 1, 2, and 3 times rheobase (Figure 1C) or with a maximum intensity (420 pA). A paired t-test was used to assess the statistical significance of Sar-Met-SP-induced changes in action potential waveform and excitability. One- way ANOVAs followed by Dunnett's post hoc test were used to assess the blocking effect of NTP on Sar-Met-SP induced enhancement of capsaicin response and the increase in excitability. All data are expressed as means ± SE.

**Drugs.** Sar-Met-SP ([Sar(9), Met(O2)(11)]-substance P ) and SP which were purchased from Tocris were prepared in 1mM stocks in HBSS solutions and stored at
-20°C. NTP

(2-[3,5-Bis(trifluoromethyl)phenyl]-N-[6-(4-methylpiperazin-1-yl)-4-(o-tolyl)pyridin-3-yl]-N-methylisobutyramide) provided by the Helsinn Company, was freshly prepared in a 1 mM solution in DMSO on the day of the experiment and applied to the external bathing solution in a volume so that the DMSO concentration was less than 1%. Capsaicin (from Sigma) was prepared as 1 mM stock solution in 100% ethanol. All drugs were delivered via bath application using a gravity-driven application system.
Results

General electrophysiological properties of DRG neurons. Because NK-1 receptors are mainly expressed in small size DRG neurons (Li & Zhao 1998, Segond von Banchet et al. 1999, Szucs et al. 1999), L6 and S1 DRG neurons ranging from 20 to 30 µm in diameter were selected for study using current clamp recording. The neurons (n=65) had an average resting membrane potential of -57.7±0.6 mV, cell capacitance of 26.4±0.7 pF and action potential duration (APD) at 0 mV of 4.6±0.2 ms. A 750 ms duration, 420 pA (near maximal stimulus intensity) depolarizing current pulse evoked an average of 1.44±0.11 action potentials indicating that these neurons are phasic neurons according to our previous criteria (Sculptoreanu & de Groat 2007).

Sar-Met-SP induced membrane depolarization. Sar-Met-SP applied for 30 to 60 sec at 1 µM concentration which was used in our previous experiments (Sculptoreanu & de Groat 2007), did not change membrane potential (Figure 2A upper figure) in 47 of 65 neurons. These neurons are classified as Type I neurons in this paper. In the remaining 18 neurons, Sar-Met-SP induced a membrane depolarization (Figure 2B and 2C) after a delay ranging from 10 sec to 2 min (average 40±6 sec). The amplitude of the depolarization ranged from 4 to 25 mV (average 8.05±1.38 mV, n=18). Four of these neurons (Type II) exhibited a transient depolarization lasting less than 1 min even during continuous exposure to Sar-Met-SP (Figure 2B). In the other 14 neurons (Type III), Sar-Met-SP induced a transient membrane depolarization followed by a low amplitude, prolonged long lasting depolarization which lasted 5 to 20 min after washout of Sar-Met-SP (Figure 2C). The depolarization
induced by Sar-Met-SP was repeatable in both types neurons 5 min after recovery from the first response but the amplitude was usually smaller in Type III neurons compared with the first depolarization (data not shown). The Sar-Met-SP induced depolarizations did not induce action potentials (AP). Resting membrane potential and the threshold for eliciting AP with depolarizing current pulses were not different in the three types of neurons.

**Sar-Met-SP induced increase in excitability.** In Type I neurons, even though Sar-Met-SP did not elicit a membrane depolarization, it still produced a change in the excitability in 35 of 47 neurons (74%) (Figure 3) and/or a change in action potential waveform (Fig. 4) within 2-3 min after application. Although Sar-Met-SP did not alter the firing to stimuli at rheobase intensity (Figure 2A bottom figures) it significantly decreased the rheobase (Figure 4C), increased the number of action potentials elicited by stimuli 3 times the rheobase current (Figure 3B) or by a maximum stimulus intensity (420 pA, Figure 3C and table 1), but did not induce a significant change in the voltage threshold for triggering an action potential (Figure 4B and Table 1). In Type I neurons Sar-Met-SP also increased the overshoot and the duration of the action potential (APD) (Figure 4A and Table1), and reduced the decay rate of the action potential (Table 1). However, it did not change the amplitude of AHP or the rate of AHP decay (Figure 4A and Table1). The effect of Sar-Met-SP on excitability lasted for over 10 min (3 neurons tested).

In Type II and Type III neurons a depolarizing current injection at rheobase intensity elicited more firing during the Sar-Met-SP induced depolarization (Figure
NTP reverses Sar-Met-SP induced changes in membrane potential, excitability and AP waveform. NTP which is a selective and potent NK-1 receptor antagonist with an EC$_{50}$ of approximately 10 nM (Hoffmann et al. 2006, Campi et al. 2010, Palea et al. 2010a, Palea et al. 2010b) was tested at 200 nM and 500 nM concentrations. In bladder smooth muscle preparations NTP in concentrations between 10 and 100 nM selectively suppresses the contractions evoked by an NK-1 agonist but a high concentration (1 μM) reduces the responses evoked by carbachol or KCl (Palea et al. 2010a, Palea et al. 2010b) In our experiments, NTP at 200 nM did not affect neuronal excitability of untreated neurons (Figure 5D); however at 500 nM it enhanced the firing induced by depolarizing current pulses (n=3 neurons) (Figure 5B). Thus, in the remaining experiments 200 nM NTP was used as an antagonist against Sar-Met-SP. In Type III neurons (n=3), NTP (200 nM) rapidly reversed the membrane depolarization evoked by Sar-Met-SP (Figure 6A). In Type I neurons the reduction in the rheobase (Figure 6B and Table 2), and the increased firing to suprathreshold stimuli (Table 2) elicited by Sar-Met-SP was rapidly reversed by NTP (200 nM) (Table 2, Figure 6B and 6C). NTP also reversed the Sar-Met-SP induced changes in action potential waveform (Fig 6D and Table 2).

Pretreatment with 200 nM NTP also blocked the Sar-Met-SP induced excitation in Type I neurons. In 5 Type I neurons pretreated with 200 nM NTP, Sar-Met-SP did not induce changes in rheobase, suprathreshold responses or action potential waveform (data not shown). Based on our control data, 3 to 4 neurons among these 5
neurons (74%) would have been expected to exhibit changes in these properties following application of Sar-Met-SP.

**Sar-Met-SP enhancement of capsaicin induced Ca$^{2+}$ responses in DRG neurons is blocked by NTP.** Activation of NK-1 or NK-2 receptors by SP or Sar-Met-SP has been reported to enhance TRPV1 activation by capsaicin and decrease TRPV1 desensitization in rat DRG neurons (Zhang et al. 2007, Sculptoreanu et al. 2008). To determine if NK-1 receptors mediate a similar interaction between capsaicin and Sar-Met-SP in guinea pig DRG neurons, the Ca$^{2+}$ imaging method was used to record the increase in intracellular Ca$^{2+}$ elicited by repeated applications of capsaicin (0.5 µM, 15 sec duration, at 15 min intervals) that induce tachyphylaxis. Capsaicin elicited Ca$^{2+}$ increases in 21% of small to medium size, (286/1339) DRG neurons obtained from four guinea pigs. This percentage of capsaicin responsive neurons was much lower than that reported in rat DRG neurons (60-70%) (Sculptoreanu et al. 2010a). Using this protocol the second capsaicin response was significantly smaller in amplitude than the first response (Figure 7A). However, when 1µM Sar-Met-SP was applied in the bath 3 min before second capsaicin application (as shown in Figure 7B), the amplitude of the second capsaicin response was significantly larger than the second response in control experiments (Figure 7A) indicating a facilitation of the capsaicin response or a reduction of tachyphylaxis. SP (1µM) applied in the same manner also enhanced the second capsaicin response (the ratio of second to the first response peak was 0.98±0.15, n=28 cells from 2 guinea pigs.). Neither SP or Sar-Met-SP alone evoked a detectable...
increase in intracellular Ca$^{2+}$.

When neurons were pretreated with NTP (200 nM) 2 min before Sar-Met-SP application (Figure 7C), the second capsaicin responses were comparable in amplitude with the responses in the control untreated group ($p>0.05$). The pooled data obtained from DRG neurons from 4 guinea pigs revealed that NTP completely blocked the enhancement of capsaicin responses induced by Sar-Met-SP (Figure 7D). NTP (200 nM) alone did not change the amplitude of capsaicin response (F340/F380 ratios: $1.43\pm0.05$ in control group, $n=22$ cells and $1.38\pm0.04$ in the presence of NTP, $n=20$ cells) or the extent of tachyphylaxis (the ratio of second to the first response peak: $0.52\pm0.03$ in control, $n=25$ cells and $0.50\pm0.03$ in the presence of NTP, $n=17$ cells). We also compared the effect of NTP with another selective NK-1 antagonist, aprepitant (Green et al. 2006). Aprepitant (1µM) blocked the Sar-Met-SP enhancement of capsaicin responses (the ratio of second to the first response peak: $0.56\pm0.04$ in control, $n=14$ cells and $0.82\pm0.05$, $n=12$ cells after Sar-Met-SP and $0.58\pm0.03$, $n=12$ cells, after Sar-Met-SP when pretreated with 1µM aprepirant).
Discussion

This study revealed that Sar-Met-SP, a selective NK-1 receptor agonist increases the excitability of a large percentage of small to medium size DRG neurons in the guinea pig and reduces the tachyphylaxis of TRPV1 receptors induced by repeated application of capsaicin. NTP, a selective and potent NK-1 receptor antagonist (Hoffmann et al. 2006) suppressed these effects of Sar-Met-SP. The results suggest that NK-1 receptors in sensory nerves in addition to those at other sites such as the spinal cord and bladder smooth muscle may play important role in the generation of overactive bladder and that block of these receptors may contribute to efficacy of NK-1 antagonists in the treatment of OAB.

Since the first report that SP depolarizes rat DRG neurons (Dray & Pinnock 1982), many studies have focused on the actions of neurokinins on sensory neurons (Spigelman & Puil 1990, Inoue et al. 1995, Akasu et al. 1996, Li & Zhao 1998, Abdulla et al. 2001) and on firing in bladder afferent nerves (Morrison 1999). Generally, SP elicits excitatory actions including membrane depolarization (Spigelman & Puil 1990, Li & Zhao 1998), inward current (Inoue et al. 1995, Akasu et al. 1996, Yang et al. 2003) or enhanced excitability in the absence of a membrane potential change (Abdulla et al. 2001). However, the effects have been variable. In some experiments SP induced a membrane depolarization in rat DRG neurons (Li & Zhao 1998), while in others studies it did not change membrane potential (Abdulla et al. 2001). Some papers described transient depolarizations (Spigelman & Puil 1990, Yang et al. 2003); while others reported long lasting depolarizations (Li & Zhao
1998), which seem to be mediated by an opening of non-selective cation channels or by an inhibition of K\(^+\) channels (Spigelman & Puil 1990, Inoue et al. 1995). These inconsistencies may depend on many factors including: (1) different actions of the three tachykinin receptors (NK-1, NK-2 and NK-3); (2) different species (rat vs guinea pig); (3) different recording conditions (intracellular versus whole cell patch clamp).

Our study indicates that a large percentage (74%) of small-medium size L6-S1 DRG neurons from female guinea pigs express functional NK-1 receptors and that activation of these receptors can increase electrical excitability evident as: (1) decreased rheobase, (2) enhanced responses to suprathreshold stimuli, (3) prolonged AP duration and increased AP overshoot and (4) enhanced capsaicin induced increase in intracellular Ca\(^{2+}\). However, these effects were associated with membrane depolarization in only a subpopulation (<30%) of the neurons. This is different than in guinea pig TG neurons where 75% of the neurons exhibit a SP induced depolarization (Spigelman & Puil 1990). While the Sar-Met-SP induced depolarization would be expected to enhance the firing of afferent nerves elicited by other stimuli, it alone was not of sufficient magnitude to induce firing or a detectable increase in intracellular Ca\(^{2+}\) suggesting that the depolarization is induced by an unusual ionic mechanism such as the opening of a non-selective cation channel as proposed for the SP induced depolarization in rat DRG neurons (Inoue et al. 1995).

The enhancement of DRG neuron excitability that occurs in the absence of depolarization must be mediated by another mechanism. In rat DRG neurons voltage
and current clamp recording revealed that SP increases the number of action potentials evoked by depolarizing current pulses (i.e., conversion of phasic firing to tonic firing) and that this effect is mediated by a suppression A-type K⁺ channels (Sculptoreanu & de Groat 2007). Similar increases in firing were elicited by traditional A-type K⁺ channel blockers (4-aminopyridine or heteropodotoxin) and reversed by an A-type K⁺ channel opener (KW-7178) (Sculptoreanu et al. 2008, Sculptoreanu et al. 2009). However in the rat DRGs this effect was dependent on the activation of NK-2 receptors (Sculptoreanu et al. 2009). Activation of NK-2 receptors also enhances the firing of mechano-sensitive bladder afferent nerves in the rat (Morrison 1999) indicating that NK receptors present in dissociated DRG cells are also expressed in peripheral afferent terminals. A reduction in AP threshold and an increase in firing to suprathreshold current pulses has been detected in sensory neurons innervating the bladder (Yoshimura & de Groat 1999), ileum (Moore et al. 2002) or masseter muscle (Harriott et al. 2006) following inflammation of the target organ. Reductions in A-type K⁺ currents contribute to these pathology induced increases in afferent excitability.

It seems likely that an inhibition of A-type K⁺ channels also underlies the Sar-Met-SP induced increase in excitability in the guinea pig DRG, but the receptors are different in rat and guinea pig. The increased duration of the action potential could be mediated by a similar suppression of A-type K⁺ channels or by inhibition of Ca²⁺ activated K⁺ channels (Spigelman & Puil 1990, Sculptoreanu et al. 2009). However because Sar-Met-SP did not change the amplitude or the decay of the AHP both of
which depend on Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, it seems likely that inhibition of A-type K\textsuperscript{+} channels plays an important role in the effects of NK-1 receptors on the action potential in guinea pig DRG neurons.

The increase in action potential overshoot by Sar-Met-SP may be due to a third mechanism, i.e., enhancement of voltage gated Na\textsuperscript{+} channels. Consistent with this idea, it has been shown in small DRG neurons of the rat that activation of NK-1 receptors increases the amplitude of the tetrodotoxin resistant sodium current, the main contributor to the upstroke of AP (Cang et al. 2009). In addition our preliminary studies using whole cell voltage clamp methods in dissociated small to medium size guinea pig DRG neurons have revealed that Sar-Met-SP also increases the amplitude of tetrodotoxin resistant sodium currents and that this effect is blocked by NTP (Sculptoreanu et al. 2010b).

Enhancement of capsaicin induced opening of TRPV1 channels is a fourth mechanism that might underlie the facilitating effect of Sar-Met-SP on guinea pig DRG neurons. TRPV1 are expressed on C-fiber afferent nerve terminals in the bladder wall and activation of these channels with capsaicin or acidic conditions (such as intravesical acetic acid infusion in in vivo experiments (Palea et al. 2010a) can increase afferent firing and induce bladder overactivity (de Groat & Yoshimura 2009). Thus activation of NK receptors on primary afferent terminals in the bladder should enhance TRPV1 channel activity. Previous studies in rat DRG have shown that pretreatment with SP enhances capsaicin induced currents and that this effect is mediated by NK-2 receptors and an intracellular signaling mechanism involving
protein kinase C (PKC) (Sculporeanu et al. 2008). On the other hand, in rat DRG neurons PKC has not been linked with the SP suppression of K⁺ channels and increased firing evoked by depolarizing current pulses, indicating that multiple intracellular signalling pathways as well as multiple receptors are involved in the actions of neurokinins (Sculporeanu et al. 2009). Further experiments are needed to determine if PKC signalling mechanisms are also involved in the NK-1 facilitation of TRPV1 channels in guinea pig DRG.

Application of NTP suppresses bladder hyperactivity induced by intravesical infusion of acetic acid in guinea pig (Palea et al. 2010a), indicating that NK-1 receptors either in the peripheral or central nervous system play a role in the increased reflex voiding frequency. Because NTP reduces voiding frequency without changing the amplitude of voiding contractions (Palea et al. 2010a) it is likely that the drug acts on the afferent rather than the efferent limb of the micturition reflex. These findings are consistent with previous reports that NK-1 antagonists decrease reflex bladder activity in anesthetized guinea pigs (Yamamoto et al. 2003); and increase bladder capacity without changing voiding efficiency in decerebrate cats (Kamo & Doi 2001).

NK-1 receptors on bladder afferent terminals may participate in complex sensory mechanisms that regulate bladder activity. The receptors could be activated in an autofeedback manner by SP or other neurokinins released from the afferent terminals but also by neurokinins released from urothelial cells (Birder et al. 2008). Because activation of the receptors induces membrane depolarization and hyperexcitability the responses of the afferent terminals to mechanical (i.e., bladder stretch) or chemical
stimuli (ATP, low pH, TRPV1 or TRPA1 agonists) would be enhanced. In addition, NK-1 induced facilitation of the opening of voltage gated Ca\textsuperscript{2+} channels by protein kinase induced phosphorylation (Sculptoreanu & de Groat 2003) or by increased overshoot and duration of action potentials would enhance Ca\textsuperscript{2+} influx and further increase neurokinin release from the afferent terminals. Thus NK-1 receptors could play a key role in a positive feedback, amplification mechanism that enhances sensory input from the bladder and contributes to abnormal reflex bladder activity in guinea pigs. Clinical studies showing that NK-1 antagonists (aprepitant and serlopitant) are effective in treating overactive bladder symptoms (Green et al. 2006, Frenkl et al. 2010) raise the possibility that similar mechanisms exist in humans.
Acknowledgement

We thank Stephanie L Daugherty for her kind assistance editing the manuscript
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Reference


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Footnote

This work was supported by National Institute of Health [Grant DK07783]; and Helsinn HealthCare.

Portions of this work were presented previously in poster form at annual meeting of Neuroscience, Washington DC, and November 2011.
Figure legends

Figure 1. Analysis of the electrophysiological properties of guinea pig DRG neurons using whole cell current clamp recording. (A) Action potential (AP) was evoked with 4 ms depolarizing current injection through the recording electrode. AP overshoot above 0 mV, AP duration (APD) at 0 mV and amplitude of after hyperpolarization (AHP) below resting membrane potential were measured. Rate of decay of AHP was determined with a single exponential fitted to the voltage trace (shown in inset). (B) AP was evoked with a prolonged depolarizing current injection (750ms). AP threshold was the largest membrane depolarization prior to AP generation (e.g., -24 mV), rheobase (R) was defined as the least amount of depolarizing current needed to evoke a single AP (e.g., 80 pA). (C) Once R was determined; responses to 2 and 3 times R stimuli were measured using the same current injection protocol as in B.

Figure 2. Variability of Sar-Met-SP (1 μM) induced changes in membrane potential and excitability in three guinea pig DRG neurons. A prolonged depolarizing current pulse (750 ms duration) was applied at rheobase intensity every 10 sec to evoke APs (bottom figures) before (a), during (b) application of Sar-Met-SP and after washout (c). Membrane potential (indicated by dots in top figures) was measured every 10 sec just before each depolarizing current pulse. Sar-Met-SP was applied for 30 to 60 sec. (A) In this neuron application of Sar-Met-SP (1 μM) did not change membrane potential or the number of evoked APs (a, b and c). (B) In this neuron Sar-Met-SP induced a transient 15 mV membrane depolarization and a marked increase in evoked firing (b). (C) In this neuron Sar-Met-SP induced a transient 20 mV depolarization followed by a
long lasting (over 10 min) depolarization. More firing was elicited during depolarizations (b and c). The number above each graph indicates the number of neurons of each type. Resting membrane potentials were -60 mV (A), -58 mV (B) and -62 mV (C).

Figure 3. Sar-Met-SP (1μM) increased the response to suprathreshold stimuli in guinea pig Type 1 DRG neurons. (A) APs were evoked by 1, 2 and 3 times rheobase (R) before (left traces) and after (right traces) Sar-Met-SP application. (B) Pooled data from 33 DRG neurons demonstrating increased response to suprathreshold stimuli (3 times R stimuli) after Sar-Met-SP application ($p<0.05$). Note that the rheobase intensity was reduced after Sar-Met-SP. (C). Pooled data from 15 DRG neurons showing greater firing was evoked in response to a maximum depolarizing current pulse (420 pA) after Sar-Met-SP application.

Figure 4. Sar-Met-SP (1μM) changed action potential waveform and reduced the rheobase in guinea pig Type 1 DRG neurons. (A) Sar-Met-SP increased AP overshoot and AP duration (dashed trace) compared with control (solid trace). However, Sar-Met-SP did not change the AHP amplitude or decay rate. (B) Pooled data from 35 DRG neurons indicate that Sar-Met-SP did not significantly change AP threshold ($p>0.05$). (C) Pooled data from 35 DRG neurons indicate that Sar-Met-SP reduced rheobase ($p<0.001$).

Figure 5. Firing induced by a prolonged stimulus pulse (750 ms) at rheobase intensity in a guinea pig Type 1 DRG neuron was increased by a high concentration of NTP (500 nM) but not by a lower concentration (200 nM). (A) Control before NTP, (B)
during application of NTP (500 nM), (C) after washout, (D) NTP (200 nM) had no effect in the same neuron.

Figure 6. NTP (200 nM) reversed the Sar-Met-SP induced depolarization, action potential waveform changes and increased excitability in Type 1 DRG neurons. (A) The reduction in the resting membrane potential after application of Sar-Met-SP was reversed by 200 nM NTP (// indicates 4 min gap in the recording). (B) The Sar-Met-SP reduction in the rheobase (n=10, ** $p<0.01$.) is reversed by NTP. NTP reversed the Sar-Met-SP increased firing at 3 times R stimulus (n=10, * $p<0.05$) (C) as well as the Sar-Met-SP induced changes in AP shape (D).

Figure 7. Sar-Met-SP enhanced the capsaicin (Cap) induced increase in intracellular Ca$^{2+}$ responses and this effect was blocked by NTP (200 nM). (A) Cap increases F340/F380 ratio indicating an increase in intracellular Ca$^{2+}$ in a DRG neuron. A second Cap application (0.5 µM for 15sec) (15 min after the first) elicited a reduced response (tachyphylaxis). (B) Application of Sar-Met-SP (1µM) 3 min before the second Cap application reduced tachyphylaxis. (C) Pretreatment with NTP (200 nM) 2 min before the application of Sar-Met-SP suppressed the Sar-Met-SP facilitating effect. (D) Pooled data from 4 guinea pigs. The magnitude of tachyphylaxis is indicated by the ratio of second Ca$^{2+}$ peak vs first peak, Sar-Met-SP application reduced tachyphylaxis ($p<0.01$), and NTP pretreatment suppressed Sar-Met-SP effect ($p>0.05$). n is the number of DRG neurons in each group.
**Tables**

Table 1. Effect of Sar-Met-SP (1 μM) on action potential (AP) waveform and excitability in Type I neurons.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sar-Met-SP 1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP Overshoot (mV)</td>
<td>34.7±0.96</td>
<td>39.4±1.2 (35)   ***</td>
</tr>
<tr>
<td>AP duration (ms)</td>
<td>4.65±0.19</td>
<td>5.29±0.17 (34)  ***</td>
</tr>
<tr>
<td>AP rate of rise (mV/ms)</td>
<td>41.9±0.68</td>
<td>39.7±0.18 (35)</td>
</tr>
<tr>
<td>AP rate of fall (mV/ms)</td>
<td>26.3±0.47</td>
<td>24.6±0.48 (35)  ***</td>
</tr>
<tr>
<td>Amplitude of AHP (mV)</td>
<td>8.69±0.61</td>
<td>8.24±0.66 (33)</td>
</tr>
<tr>
<td>τ_{AHP} (ms)</td>
<td>131.7±12.9</td>
<td>150±45 (28)</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>-22.5±0.7</td>
<td>-22.3±0.9 (35)</td>
</tr>
<tr>
<td>Rheobase (R) (pA)</td>
<td>113.2±10.2</td>
<td>94.6±8.8 (35)   ***</td>
</tr>
<tr>
<td>Number of AP (1x R)</td>
<td>1.03±0.02</td>
<td>1.03±0.02 (34)</td>
</tr>
<tr>
<td>Number of AP (2x R)</td>
<td>1.06±0.04</td>
<td>1.12±0.05 (33)</td>
</tr>
<tr>
<td>Number of AP (3 x R)</td>
<td>1.3±0.17</td>
<td>2.08±0.34 (14)  **</td>
</tr>
<tr>
<td>Number of AP (420 pA)</td>
<td>2.15±0.40</td>
<td>2.84±0.45 (15)  ***</td>
</tr>
</tbody>
</table>

AP overshoot is amplitude above 0 mV; AP duration is at 0 mV; AP rate of rise and fall are maximal velocities. After hyperpolarization (AHP) amplitude is measured from resting potential; τ_{AHP} is time constant of decay of the AHP. Rheobase (R) is the minimum depolarizing current injection (pA) to evoke an AP. Comparisons between control and during Sar-Met-SP application were performed with paired t tests. ** p<0.01, *** p<0.001. Number of neurons for each comparison are indicated in parentheses.
Table 2 Reversal of Sar-Met-SP (1 uM) effect on action potential (AP) waveform and excitability by NTP (200 nM) in Type I neurons.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Sar-Met-SP</th>
<th>NTP 200 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP Overshoot (mV)</td>
<td>38.6±1.4</td>
<td>46.3±1.7**</td>
<td>40.2±1.5</td>
</tr>
<tr>
<td>AP duration (ms)</td>
<td>4.6±0.2</td>
<td>5.9±0.3**</td>
<td>4.82±0.25</td>
</tr>
<tr>
<td>AP rate of rise (mV/ms)</td>
<td>45.8±1.0</td>
<td>42.8±1.7</td>
<td>44.7±0.9</td>
</tr>
<tr>
<td>AP rate of fall (mV/ms)</td>
<td>26.7±0.5</td>
<td>23.3±0.9**</td>
<td>26.6±0.8</td>
</tr>
<tr>
<td>AHP amplitude (mV)</td>
<td>6.4±1.0</td>
<td>5.8±1.1</td>
<td>7.9±1.3</td>
</tr>
<tr>
<td>(\tau_{\text{AHP}}) (ms)</td>
<td>135±21</td>
<td>127.4±24</td>
<td>142.6±28</td>
</tr>
<tr>
<td>AP threshold (mV)</td>
<td>-24.3±0.8</td>
<td>-24.2±1.1</td>
<td>-22.7±0.4</td>
</tr>
<tr>
<td>Rheobase (R) (pA)</td>
<td>79±12</td>
<td>64±1*</td>
<td>87±11.7</td>
</tr>
<tr>
<td>number of AP (1x R)</td>
<td>1.0±0</td>
<td>1.0±0</td>
<td>1.0±0</td>
</tr>
<tr>
<td>number of AP (2x R)</td>
<td>1.0±0</td>
<td>1.3±0.2</td>
<td>1.16±0.16</td>
</tr>
<tr>
<td>number of AP (3x R)</td>
<td>1.7±0.3</td>
<td>3.0±0.5*</td>
<td>1.67±0.49</td>
</tr>
<tr>
<td>number of AP (420 pA)</td>
<td>2.3±0.4</td>
<td>3.16±0.4*</td>
<td>2.5±0.9</td>
</tr>
</tbody>
</table>

Summary of data from 10 DRG neurons. All parameters were recorded before, during Sar-Met-SP and after NTP in the presence of Sar-Met-SP. One- way ANOVAs followed by Dunnett's post hoc test were used to assess effect of Sar-Met-SP and NTP. Differences between the Sar-Met-SP group and control are indicated by * \(p<0.05\), ** \(p<0.01\). Data in the NTP and control groups were not different.