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TARGETED DELETION OF NEUROKININ-1 RECEPTOR EXPRESSING NUCLEUS TRACTUS SOLITARIII NEURONS PRECLUDES SOMATOSENSORY DEPRESSION OF ARTERIAL BARORECEPTOR–HEART RATE REFLEX

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Abstract

Neurokinin-1 receptor (NK1-R) expressing neurons are densely distributed throughout the nucleus tractus solitarii (NTS). However, their fundamental role in arterial baroreflex function remains debated. Previously, our group has shown that activation of contraction-sensitive somatic afferents evoke substance P (SP) release in the NTS and resets the arterial baroreflex via activation of a GABAergic NTS circuit. Based on these findings, we hypothesized that modulation of arterial baroreflex function by somatic afferents is mediated by NK1-R dependent inhibition of barosensitive NTS circuits. In the present study, SP-conjugated saporin toxin (SP-SAP) was used to ablate NK1-R expressing NTS neurons. Contraction-sensitive somatic afferents were activated by electrically-evoked muscle contraction and the arterial baroreceptor–heart rate reflex was assessed by constructing reflex curves using a decerebrate, arterially-perfused preparation. Baseline baroreflex sensitivity was significantly attenuated in SP-SAP-treated rats compared with control rats receiving either unconjugated SAP or vehicle. Muscle contraction significantly attenuated baroslope in SAP and vehicle-treated animals and shifted the baroreflex curves to higher systemic pressure. In contrast, somatic afferent stimulation failed to alter baroslope or shift the baroreflex curves in SP-SAP-treated animals. Moreover, when reflex sensitivity was partially restored in SP-SAP animals, somatic stimulation failed to attenuate baroreflex bradycardia. In contrast, SP-SAP and somatic stimulation failed to blunt the reflex bradycardia evoked by the peripheral chemoreflex. Immunohistochemistry revealed that pretreatment with SP-SAP significantly reduced the number of NK1-R expressing neurons in the caudal NTS, while sparing NK1-R expressing neurons rostral to the injection site. This was accompanied by a significant reduction in the number of glutamic acid decarboxylase (GAD67) expressing neurons at equivalent levels of the NTS. These findings indicate that immunoablation of NK1-R expressing NTS neurons selectively abolishes the depressive effect of somatosensory input on arterial baroreceptor–heart rate reflex function.

Keywords

nucleus tractus solitarii; neurokinin receptors; substance P; GABA; arterial baroreflex; exercise

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The nucleus tractus solitarius (NTS) receives afferents from visceral, somatic and cardiopulmonary structures. Recently, considerable attention has been placed on the functional role of intrinsic excitatory and inhibitory NTS inter-neurons in the processing of primary baroreceptor afferent inputs (Andresen et al., 2004; Chen and Bonham, 2005; Kawai and Senba, 1996; Paton, 1999). However, the precise role for NTS neurons in modulating arterial baroreflex function during physiological perturbations, such as exercise, remains unresolved.

Over a decade ago, we showed that the operational properties of the carotid baroreflex were reset during volitionally exercise in humans, while retaining overall reflex sensitivity (Potts et al., 1993). Subsequently, these findings have been confirmed in humans and animals by many other laboratories (Boscan et al., 2002; Burger et al., 1998; Fadel et al., 2001; McIlveen et al., 2001; Norton et al., 1999; Papelier et al., 1994; Raven et al., 1997). In addition, neural feedback from skeletal muscle has been shown to reset the arterial baroreflex in a manner similar to the changes that occur during exercise (Carrington et al., 2003; Gallagher et al., 2001; Ichinose et al., 2002; Iellamo et al., 1997; McIlveen et al., 2001; McWilliam et al., 1991; Potts, 2002; Potts et al., 1998; Potts and Li, 1998; Potts and Mitchell, 1998). Collectively, these findings suggest that an alteration in the central neurotransmission of arterial baroreceptor afferent signals may be involved in baroreflex resetting during exercise. Although the precise mechanism(s) mediating this process remains unknown, there are several lines of evidence suggesting that GABAergic and tachykinergic mechanisms may be involved. First, it has been shown that substance P (SP) influences the activity of cardiovascular and respiratory circuits within the medulla (Helke and Seagard, 2004; McKay et al., 2005; Nattie and Li, 2002; Riley et al., 2002; Wang et al., 2001). SP exerts its effect by acting on a diverse class of neurokinin receptors (NK1, NK2 and NK3) that share 65% amino acid homology (Mantyh, 2002; Regoli et al., 1994). Medullary regions, including the NTS, nucleus ambiguus (NA), hypoglossal motor nucleus (XII) and the ventral respiratory group (VRG), express high levels of NK1-R (Dixon et al., 1998; Guyenet et al., 2002; Lawrence and Jarrott, 1996; Wang et al., 2003).

Previous studies have reported that stimulation of somatic and arterial baroreceptor afferents release SP in the NTS (Potts and Fuchs, 2001; Potts et al., 1999; Williams and Fowler, 1997; Williams et al., 2002) and that neural feedback from skeletal muscle depresses arterial baroreceptor signaling, in part, through activation of a local GABAergic NTS circuit (Boscan et al., 2002; Potts et al., 2003). Since depression of the arterial baroreflex can be prevented by pharmacological blockade of NK1-R in the NTS (Boscan et al., 2002), we propose that somatosensory input blunts baroreflex function by targeting a population of NK1-R expressing GABAergic neurons in the NTS.

The purpose of the present study is to determine whether somatosensory evoked depression of the arterial baroreceptor–heart rate (HR) reflex could be prevented by selective loss of NK1-R expressing NTS neurons. Recent development of the ribosomal inactivating protein saporin (SAP) has provided an effective and efficient means to selectively eliminate NK1-R expressing neurons (Wiley and Lappi, 1997). This approach has previously been used to successfully ablate NK1-R expressing neurons in the NTS (Riley et al., 2002) and ventrolateral medulla (Nattie and Li, 2002; Wang et al., 2002). Using SP-conjugated SAP toxin (SP-SAP), we hypothesized that elimination of NK1-R expressing NTS neurons would abolish the depressive effect of somatic afferents on arterial baroreflex function.

EXPERIMENTAL PROCEDURES

Experiments were performed on male Sprague–Dawley rats (70–100 g) in accordance with the regulations stipulated in the US Department of Health and Human Services and were approved by the Animal Investigation Committee at the University of Missouri–Columbia and Wayne State University School of Medicine. Every attempt was made to minimize the number of

animals used in these studies and to limit their distress and potential suffering. Rats ($n = 26$) were anesthetized with ketamine (60–80 mg/kg, i.p.) and xylazine (6–7 mg/kg, i.p.). Adequacy of anesthesia was periodically determined by lack of a withdrawal response to firm hindlimb compression and absence of corneal reflex. When a response was evoked supplemental anesthesia (ketamine, 35 mg/kg, i.m.; xylazine 3.5 mg/kg, i.m.) was administered. The skull was shaved, the skin sterilized with Betadine and alcohol and the animal was positioned in a stereotaxic head frame (Kopf, Tujunga, CA, USA). A limited craniotomy was performed and the cerebellum retracted to expose the dorsal surface of the brainstem.

Immunolesioning of NK1-R expressing neurons

A single-barrel glass micropipette (tip diameter $<10\ \mu\text{m}$, o.d.) was stereotaxically positioned in the caudal NTS (250 μm lateral of midline, 350 μm ventral of dorsal surface) at the level of calamus scriptorius. Rats received bilateral microinjection (1.5 ng/100 nl per side; Advanced Targeting Systems, San Diego, CA, USA) of either SP-SAP (experimental group, $n = 5$), free SAP (toxin control; $n = 5$) or phosphate buffered saline (PBS, vehicle control, $n = 5$) via a pneumatic picopump (model PV 820; WPI, Sarasota, FL, USA). The dose of SP-SAP was based on preliminary experiments using several SP-SAP doses (0.5–10 ng in 100 nl). We found that 1–3 ng of SP-SAP produced lesions that were confined to the medial and commissural subdivisions of the NTS caudal to calamus scriptorius. Smaller doses produced inconsistent or partial lesions, while larger doses (5–10 ng) lesioned regions outside the NTS, including the dorsal motor nucleus of the vagus (X), dorsal column nuclei and the XII. All injection volumes were measured directly using a surgical microscope (Zeiss Stemi SV11; Zeiss Surgical, Thornhill, NY, USA) equipped with a calibrated eyepiece reticule and were performed over 3–5 min to limit potential damage to NTS circuits. Following the first microinjection, the pipette remained in place for 10 min prior to repositioning it into the contralateral region of the NTS. Following completion of the second injection, the wound was closed with sterile suture and buprenorphine (0.5 mg/kg, i.m.) was administered for analgesia. Animals were permitted to recover for 5–7 days. This was based on preliminary time-course experiments performed in our laboratory, as well as findings reported from a previous study (Riley et al., 2002).

Assessment of NK1-R ablation on arterial baroreflex and chemoreflex function

Experiments were performed using the *in situ* arterially-perfused, decerebrate working heart–brainstem preparation (WHBP) (Paton, 1996). Briefly, rats were deeply anesthetized via inhalation with halothane and bisected sub-diaphragmatically. The rostral portion (head, forelimbs and thorax) was immediately submerged in ice-cold Ringer solution bubbled with 95% gas O_2 –5% CO_2 mixture. A precollicular decerebration was performed and a portion of the posterior thorax was removed to expose the heart and lungs. The thoracic aorta was isolated by blunt dissection and the left phrenic nerve was identified visually and sectioned at the level of the diaphragm. Finally, the cerebellum was removed to expose the dorsal surface of the brainstem. The preparation was transferred to an acrylic chamber where the thoracic aorta was cannulated with a double lumen catheter (16 ga/18 ga; Braintree Scientific, Braintree, MA, USA) and perfused with Ringer solution containing an oncotic agent (Ficoll, 70 kDa, 1.25%; Sigma, St. Louis, MO, USA) by a roller pump (model 505S pump; model 314DW2 pump head; Watson-Marlow, Chicago, IL, USA). The perfusate was gassed with 95% O_2 –5% CO_2 , warmed to 32–34 °C and passed through two in-line bubble traps and a filter (polypropylene mesh, pore size: 40 μm). The time to the beginning of perfusion following induction of anesthesia was <10 min. Pump flow rate was monitored via an in-line volumetric flow probe (model 2N; Transonic Systems, Ithaca, NY, USA) and perfusion pressure (PP) was measured using a strain-gauge pressure transducer (model P23; Statham, Oxnard, CA, USA). At flow rates used in the present study (25–35 ml/min), systemic PP averaged 85 ± 8 mm Hg. Phrenic nerve discharge (PND) was recorded with a glass suction electrode (tip diameter: 0.2–0.3 mm).

The raw phrenic neurogram was amplified 10,000–20,000 (Neurolog NL104; Digitimer, Letchworth Garden City, UK), integrated using a time constant of 100 ms (Neurolog NL703), filtered (Neurolog NL126; 500 Hz–5 kHz) and displayed on an analog oscilloscope (model 7603, Tektronic). The electrocardiogram (EKG) was amplified (Grass P511-Telefactor, West Warwick, RI, USA), filtered (100 Hz–3 kHz) and displayed on an oscilloscope. A discriminator circuit (Neurolog NL 201) generated a TTL pulse to threshold crossing of the upstroke of R-wave and instantaneous HR was derived from the measured inter-beat interval. Neuromuscular paralysis was produced by addition of vecuronium bromide (50 μ g) directly to the perfusate.

Arterial baroreceptor afferents were activated using rapid pressure ramps (25–30 mm Hg/s, 1–2 s) generated by a digital pump (model 505S, Watson-Marlow). A series of pressor ramps of varying amplitudes (10–50 mm Hg) was performed in random order and peak systemic pressure and reflex bradycardiac responses to each pressure ramp were used to plot the arterial baroreceptor–HR reflex function curve. Sixty seconds was permitted to reestablish baseline HR and PP between pressure ramps.

Peripheral chemoreceptors were activated by an intra-aortic injection of NaCN (0.03%, 10–20 μ l) via a side port of the aortic catheter. A range of sodium cyanide (NaCN) doses was performed and a supra-threshold, but submaximal dose, was used for the remainder of the experiment. The HR nadir evoked by NaCN injection was used to assess the sensitivity of the peripheral chemoreflex to control HR.

Somatic afferents innervating the forelimb skeletal muscle were activated by electrical stimulation of forelimb flexors. To evoke a muscle twitch, fine wire (0.7 mm o.d.) stimulating electrodes were positioned into forelimb flexors and current pulses (1 ms pulse duration, 250 μ A–1 mA, 0.5 Hz) were applied. Every effort was made to minimize the amplitude of the current pulse used to evoke contraction. Previously, we reported that contraction-evoked reflex cardiorespiratory responses were prevented by neuromuscular paralysis and transection of the brachial plexus (Potts et al., 2000). Tension development was measured using an isometric force-displacement transducer (FT-100; CB Science, Dover, NH, USA) mounted in a micromanipulator. The forelimb was pre-loaded to 5–10 g of tension by displacing the forelimb using the micromanipulator (maximal displacement did not exceed 2 mm).

Experimental protocols

Arterial baroreceptor–HR reflex function curves were constructed before, during and immediately following electrically-evoked fore-limb contraction in animals that received vehicle, SAP or SP-SAP microinjections into the NTS. All current intensities and stimulation frequencies were chosen such that the stimulus was sub-threshold for evoking cardiovascular responses; thereby, minimizing changes in baseline PP and HR. Based on previous work, this approach is an effective means to indirectly activate contraction-sensitive skeletal muscle afferents as evidenced by reflex-evoked tachypnea in the absence of reflex cardiovascular responses (Potts et al., 2000,2003). The onset of twitch contraction preceded the pressure ramp by 3–5 s.

In a subset of SP-SAP-treated animals, bicuculline (BIC) (50 nl, 10 μ M) was microinjected bilaterally into the NTS at the level of the caudal pole of area postrema (500 μ m lateral to midline, 500 μ m ventral of the dorsal surface). Bilateral injections were completed within 45–60 s. Immediately following the second injection graded pressor ramps were performed.

Immunohistochemistry and tissue staining

At the end of each experiment, the brain was removed and fixed in 10% buffered formalin at 4 °C for 24 – 48 h. This was followed by cryoprotection for 12–18 h at 4 °C using 30% sucrose

or until the brain equilibrated. The brain was sectioned (30 μm thick) using a freezing microtome (MicroM, Walldorf, Germany; mode D6900) and standard immunohistochemical procedures were used to visualize NK1-R protein. Briefly, sections were washed 4 $\times$ 10 min in PBS (pH 7.4) at room temperature and were blocked with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in 0.3% Triton X-100 in PBS. Sections were then incubated with rabbit polyclonal anti-NK1-R antibody (1:1000, Novus Biologicals, Littleton, CO, USA) overnight on a shaker at room temperature. Following washing (4 $\times$ 10 min) with PBS, sections were incubated for 2 h in donkey anti-rabbit NK1-R secondary antibody conjugated with Cy-3 (1:300, Jackson ImmunoResearch). Finally, sections were washed (3 $\times$ 10 min) in PBS, transferred onto plus-coated slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). Tissue processed without the primary NK1-R antibody served as a negative control. Adjacent sections were mounted onto plus-coated slides and stained using Neutral Red (0.5%, Sigma), washed through a series of increasing alcohol concentration, cleared with xylene and coverslipped with Permount (Fisher Scientific, Hampton, NH, USA).

In an additional series of experiments, glutamic acid decarboxylase (GAD67) was visualized in the brainstem following a recently developed protocol (Fong et al., 2005). Briefly, animals received bilateral microinjections of either SAP (1.5 ng/100 nl, $n = 3$) or SP-SAP (1.5 ng/100 nl, $n = 3$) in the NTS and recovered for 5–7 days. Rats were then anesthetized, transcardially perfused and brains were recovered and immunohistochemically stained. Control experiments included incubation of sections in the absence of primary antisera or secondary antisera. In addition, we immunohistochemically stained brain tissue from a control, untreated rat for comparison of GAD67-ir labeling.

Mapping and imaging

A set of 30 μm thick sections was examined using an Olympus BX51 microscope equipped with epifluorescence and the appropriate Cy3 filter set (excitation, 545 nm; emission, 610 nm; dichrotic, 570 nm). A series of three sections containing the NTS was obtained: 1) level of the caudal-most pole of area postrema (Bregma -14.08); 2) mid-AP level (Bregma -13.68); and 3) level of 4th ventricle (Bregma -13.30). For descriptive purposes, the terms caudal NTS (Bregma -14.08), subpostremal NTS (Bregma -13.68) and intermediate NTS (Bregma -13.30) will be used in the remainder of the text. Images were captured using a cooled monochrome CCD camera (Orca; Hamamatsu, Hamamatsu City, Japan). NK1-R immunoreactivity was observed by immunofluorescent labeling of neuronal membranes and soma. NK1-R immunoreactive profiles were counted by two individuals who were blind to the experimental conditions. Criterion for a positively-labeled NK1-R neuron was that each cell must have a soma diameter of $>10 \mu\text{m}$ diameter with one or more processes. The likelihood of possible errors arising from double-counting was minimized by the fact that sections counted were separated by $>150 \mu\text{m}$. GAD67 immunoreactivity was analyzed at the same three levels as NK1-R and sections reconstructed using a commercially-available software package (NeuroLucida; MicroBright-field, Williston, VT, USA) and a motorized stage (Ludl, Hawthorne, NY, USA). Some section outlines were obtained from Paxinos et al. (1985).

Solutions

The Ringer solution contained 125 mM NaCl, 24 mM NaHCO_3 , 5 mM KCl, 2.5 mM CaCl_2 , 1.25 mM MgSO_4 , 1.25 mM KH_2PO_4 and 10 mM D-glucose. In addition, a high molecular weight (70 kDa) compound was added to the perfusate contained as an oncotic agent (1.25%, Ficoll, type 70, Sigma). Oncotic pressure of the perfusate was $\sim 310 \text{ mOsm/kg H}_2\text{O}$ measured using a micro-osmometer (model 3300; Advanced Instruments, Norwood, MA, USA). SP-SAP and SAP (Advanced Targeting Systems), vecuronium bromide (0.04 $\mu\text{g/ml}$, Norcuron;

Organon, West Orange, NJ, USA) and BIC (free base, 10 μ M, Sigma) were reconstituted in fresh PBS. NaCN (0.03%, Sigma) was reconstituted in distilled water.

Statistical and data analyses

Physiological data were collected by computer equipped with a 16-bit analog-to-digital interface (1401 Plus, Cambridge Electronic Design [CED], Cambridge, UK) using Spike 2 software (ver. 6, CED) and data were analyzed off-line using custom-written script files. Baroreflex function curves were constructed data fit using a curvilinear function (Sigma Plot, ver. 8.02; SPSS Inc., Chicago, IL, USA). Linear regression fits were applied to Δ HR and Δ PP data and the slope of this relationship was calculated as a measure of baroreflex sensitivity (Linear Regression Model, SAS, ver. 9.3; SAS, Cary, NC, USA). HR and PP data obtained from pressor ramps were analyzed using a three-way repeated-measures ANOVA (three levels of GROUP-SP-SAP, SAP, vehicle; 2 levels of CONDITION-control, contraction; five levels of PRESSURE; SAS). The Bonferroni multiple comparisons adjustment was applied to these contrasts. The effects of muscle contraction on baroslope and chemoreflex responses were assessed using a two-way ANOVA (three levels of GROUP-SP-SAP, SAP, vehicle; two levels of CONDITION-control, somatic stimulation). NK1 receptor and GAD67 positive neurons in the NTS were summed per section (three sections) and compared using a two-way ANOVA (three levels of GROUP-SP-SAP, SAP, vehicle; three levels of SECTION-Bregma -14.08, -13.68, -13.30). The Bonferroni multiple comparisons adjustment was applied to these contrasts. All data are presented as means $\pm$ S.E.M. Significance level was set at 0.05.

RESULTS

Baseline arterial baroreceptor–HR reflex function

SP-SAP lesions had no significant effect on baseline HR and PP (Table 1). However, SP-SAP-treated rats exhibited a marked reduction in baseline baroreflex regulation of HR (Fig. 1). Compared with SAP- and vehicle-treated rats, the magnitude of the reflex bradycardia (with respect to a given pressor stimulus) was significantly attenuated in SP-SAP-treated rats (see Fig. 1A). Analysis of baroreflex function curves showed that the baroslope (-0.6 ± 0.2 vs. $-2.1\pm 0.$ and -2.1 ± 0.1 beats/min mm Hg $^{-1}$, SP-SAP vs. SAP and vehicle, $P<0.05$) was also significantly attenuated in SP-SAP-treated rats compared with SAP- and vehicle-treated animals (see Fig. 1B, inset and Table 1). Importantly, despite the reduction in baroslope in SP-SAP-treated animals there remained a significant baroreflex bradycardia.

Effect of somatic stimulation on arterial baroreflex and chemoreflex function

To evaluate the effect of NK1-R expressing NTS neurons on somatic afferent evoked depression of the arterial baroreceptor–HR reflex, reflex function curves were assessed in the presence and absence of electrically-evoked muscle contraction (Fig. 2). In SAP- and vehicle-treated animals, peak reflex bradycardia to a given pressor stimulus was blunted by muscle contraction (see Fig. 2A). In contrast, somatosensory input failed to blunt the peak reflex bradycardia in SP-SAP-treated rats. Somatosensory activation significantly attenuated baroslope in SAP (-2.1 ± 0.1 vs. -1.0 ± 0.3 beats/min mm Hg $^{-1}$, baseline vs. somatic stimulation, $P<0.05$) and vehicle-treated rats (-2.1 ± 0.1 vs. -1.3 ± 0.2 beats/min mm Hg $^{-1}$, baseline vs. somatic stimulation, $P<0.05$) and appeared to shift the reflex function curves rightward to higher systemic pressure. However, muscle contraction failed to alter baroslope or shift the baroreflex curve in SP-SAP-treated rats (-0.6 ± 0.2 vs. -0.6 ± 0.1 beats/min mm Hg $^{-1}$, baseline vs. somatic stimulation, $P=0.9568$) (Fig. 2B and Table 1).

To determine whether the reduction in basal baroreflex sensitivity by SP-SAP treatment may have limited somatic-evoked depression of arterial baroreceptor–HR reflex, BIC was microinjected bilaterally into the NTS to increase baroreflex sensitivity ($n = 4$). Prior to BIC

injection, muscle contraction had no effect on baroreflex HR responses in SP-SAP-treated animals (Fig. 3A). This confirmed findings reported earlier in this study. Bilateral microinjection of BIC into the NTS produced a 40% increase in basal baroreflex sensitivity (-0.6 ± 0.1 vs. -0.9 ± 0.2 beats/min mm Hg⁻¹, $P < 0.05$, pre vs. post BIC, $P < 0.05$) (Fig. 3B). However, despite the partial restoration of reflex sensitivity, muscle contraction remained ineffective in depressing baroreflex function (Fig. 3C).

To assess whether ablation of NK1-R expressing neurons disrupted the transmission of other sensory inputs in the NTS, we examined the reflex HR responses evoked by activation of peripheral chemoreceptors (Fig. 4A). Activation of peripheral chemoreceptors by intra-aortic injection of NaCN (10–20 μ l, 0.03%) evoked a brisk increase in the frequency of PND and an immediate reflex bradycardia. HR nadir typically occurred 1–2 s following intra-aortic injection. Irrespective of whether animals were pre-treated with SP-SAP, SAP or vehicle baseline chemoreflex, as well as chemoreflex-evoked responses during somatic activation, was unaltered (see Fig. 4B). Chemoreflex activation produced variable, non-significant changes in systemic pressure (either small increases or decreases). Together, these findings suggest that ablation of NK1-R expressing neurons selectively attenuated baroreceptor signaling, while sparing peripheral chemoreceptor signaling in the NTS.

Immunolesioning of NK1-R expressing NTS neurons

In SP-SAP-treated rats, there was a substantial reduction in the number of NK1-R expressing neurons in the medial and commissural subdivisions of the caudal NTS (Fig. 5A) compared with SAP treatment (Fig. 5B). This difference was especially apparent when comparing NK1 immunoreactivity in the medial subdivision of the NTS (compare Fig. 5A2 and Fig. 5B2). In the medial subdivision there was a marked reduction in the number of NK1-R-labeled soma in the SP-SAP-treated animal, as well as a decrease in the density of immunoreactive fibers and processes. SP-SAP-induced reduction in NK1-R immunoreactivity appeared to be specific since it was not associated with an obvious decrease in the total number of neurons as determined from adjacent sections stained with Neutral Red (Fig. 5A3 and Fig. 5B3). In addition, there was no evidence of necrotic damage in the immediate region surrounding the injection site. In two cases, we noted a slight reduction in NK1-R immunoreactivity in the X (compare Fig. 5A1 with Fig. 5B1). Overall, however, the predominant regions of immunolesioning were confined to the medial and commissural subdivisions of the NTS. At the level of the 4th ventricle (~ 700 μ m rostral of caudal pole of area postrema) there was no difference in the extent of NK1-R immunoreactivity (Fig. 5C1 and Fig. 5C2). NK1 immunoreactivity in other medullary regions, such as the NA and the rostral ventrolateral medulla was unaffected by the immunotoxin (Fig. 5D1 and Fig. 5D2). The specificity of NK1-R labeling was confirmed by an absence of immunoreactivity when the primary antibody was omitted (Fig. 5E). An example of an SP-SAP injection site is shown in Fig. 5F. The core of the injection was located in the medial NTS subdivision.

The distribution of NK1-R immunoreactive soma from animals treated with SAP, vehicle or SP-SAP toxin at three rostral–caudal levels of the medulla is shown in Fig. 6A and group data are presented in Fig. 6B. At the caudal NTS (Bregma -14.08) and subpostremal NTS (Bregma -13.68), the number of NK1-R expressing cells was significantly reduced in SP-SAP-treated rats. However, no significant difference was found between groups in the intermediate NTS (Bregma -13.30). No differences in the number of NK1-R immunoreactive cells were found between SAP- and vehicle-treated animals. These data demonstrate that NTS injections of SP-SAP successfully eliminated NK1-R expressing neurons in the medial NTS subdivision in a rostral–caudal pattern extending from the caudal pole of area postrema (AP) to mid-AP level.

Reduction in GAD67 immunoreactivity

In a subset of animals, we determined the effect of NK1-R immunolesioning on the number of GAD67 expressing neurons in the NTS (Fig. 7). In SAP-treated animals ($n = 3$), GAD67 immunoreactive soma and terminals were found throughout the NTS (Fig. 7A1–A3). However, there was a notable reduction in the number of GAD67 immunoreactive soma and terminals in SP-SAP-treated animals ($n = 3$) in the caudal and subpostremal NTS, especially medial to the solitary tract (Fig. 7B1–7B2). For comparison, GAD67 staining was also performed in an untreated animal (Fig. 7C). There was no difference in the number of GAD67 neurons in the NTS between control and SAP-treated animals. The specificity of GAD67 labeling was evident when an adjacent section was processed in the absence of the primary anti-GAD67 antibody (Fig. 7D). Reconstruction of the NTS revealed that SP-SAP treatment significantly decreased the number of GAD67 expressing neurons at the caudal and subpostremal levels of the NTS (Fig. 8). However, there was no difference at the level of the intermediate NTS. The loss of GAD67 expressing neurons by SP-SAP treatment appeared qualitatively similar to the reduction in NK1-R expressing neurons (compare Fig. 8B with Fig. 6B).

DISCUSSION

The focus of this study was to determine whether neural feedback from somatosensory afferent fibers modulates arterial baroreflex function via a SPergic signaling mechanism in the NTS. We reported that destruction of NK1-R expressing NTS neurons using the ribosomal inactivating toxin SP-SAP completely abolished the depressive effect of somatic afferent activation on arterial baroreflex function. This was accompanied by a significant reduction in the number of NK1-R and GAD67 expressing NTS neurons. Muscle contraction remained ineffective in depressing baroreflex function in SP-SAP-treated animals when reflex sensitivity was partially restored. This suggests that somatosensory-evoked depression of arterial baroreflex function was independent of basal reflex sensitivity. Our results also confirm earlier findings that SP-SAP lesions in the caudal NTS significantly decreased basal baroreflex sensitivity (Abdala et al., 2006). In contrast, we reported that SP-SAP failed to alter the reflex bradycardia mediated by the peripheral chemoreflex. Therefore, these findings in conjunction with previous reports showing that somatic-evoked attenuation of baroreflex function was abolished by blockade of either GABA_A receptors or NK1-R (Boscan et al., 2002; Potts et al., 2003) suggest that a population of NK1-R expressing GABA neurons driven by input from spinal dorsal horn projecting cells selectively inhibits barosensitive NTS neurons.

Previous studies have employed SAP toxin to destroy NK1-R expressing neurons as a means to evaluate their role in cardiorespiratory regulation (Nattie and Li, 2002; Riley et al., 2002; Wang et al., 2002; Wiley and Lappi, 1997). Riley et al. (2002) directly evaluated the effect of SP-SAP lesions on basal arterial baroreflex function in anesthetized rats. They reported that immunolesioning of NK1-R expressing NTS neurons significantly blunted baroreflex-evoked bradycardia and tachycardia to hypertensive and hypotensive challenges, respectively. In the present study, we also reported that basal baroreflex function was significantly attenuated by SP-SAP. This may be explained by a loss of NK1-Rs expressing glutamatergic neurons since NK1-Rs are expressed at pre- and postsynaptic sites on both glutamatergic, as well as GABAergic, NTS neurons (Bailey et al., 2004). Therefore, in the present study it is likely that a portion of the lesioned neurons was glutamatergic. Although a reduction in excitatory NTS neurotransmission may have contributed to the decrease in basal baroreflex sensitivity following SP-SAP treatment, it cannot fully explain the inability of somatosensory stimulation to depress the arterial baroreceptor–HR reflex. A tenable explanation for this may be a decrease in the number of GABAergic NTS neurons. We found that the number of NK1-Rs and GAD67-expressing neurons was significantly reduced in the caudal and subpostremal levels of the NTS, while sparing neurons expressing both proteins at the level of the intermediate NTS (compare

Fig. 6 and Fig. 8). Our findings are supported by those from Boscan et al. (2002) showing that nociceptive afferents activate NK1-R expressing NTS neurons, which in turn excite GABAergic interneurons impinging on cells mediating the cardiac component of the baroreceptor reflex. Collectively, these results suggest that inhibition of arterial baroreceptor signaling at the level of the NTS requires activation of NK1-R expressing GABA neurons.

We have used the above findings to propose that feedback from somatosensory afferents activate a distinct population of GABA neurons that, in turn, inhibit barosensitive NTS neurons. Indirect evidence supporting this comes from a recent study that characterized the synaptic responses of somatosensitive NTS neurons (Potts and Waldrop, 2005). This study found that somatosensitive NTS neurons rarely responded to increases in systemic blood pressure; thus, rendering them baroreceptor-insensitive. Others have reported similar findings (Boscan et al., 2002; Person, 1989; Toney and Mifflin, 1994, 1995, 2000). Although the neurochemical phenotype of this NTS population has not yet been identified, it may indeed be GABAergic. However, this requires future experimentation.

Proposed microcircuitry in the NTS complex

A hypothetical model of the interaction between baroreceptor, chemoreceptor and somatosensory receptor afferents in the NTS is illustrated in Fig. 9. In this model, glutamatergic and GABAergic neurons, some which express NK1-Rs, form a network that receives and processes arterial baroreceptor input. Unlike primary baroreceptor afferents that project to the caudal and intermediate levels of the NTS (Ciriello, 1994), dorsal horn projections are restricted to the caudal levels (Gamboa-Esteves et al., 2001; Guan et al., 1998; Potts et al., 2002). Since baroreceptor and somatosensory receptors rarely converge within the NTS (Boscan et al., 2002; Person, 1989; Potts and Waldrop, 2005; Toney and Mifflin, 1994, 1995), it is likely that the inhibitory interaction between baroreceptor and somatosensory inputs requires an intervening synapse involving GABA. Therefore, we propose that somatic stimulation excites NK1-R expressing GABA neurons residing in the caudal NTS. These neurons, in turn, will inhibit barosensitive neurons and reduce the level of NTS output directed to the heart leading to a reduction in baroreflex bradycardia. We have placed these GABAergic neurons outside the “baro- and chemo-” NTS circuits since we have previously shown that this GABA population is autonomous of baro- and chemosensitive NTS circuitry (Potts et al., 2003). Results from the present study also indicate that somatic stimulation had no effect on HR responses evoked by the peripheral chemoreflex. This is in accordance with our earlier study (Potts et al., 2003). In our model, we propose that this may be due to a lack of synaptic input from contraction-sensitive somatic afferents.

Following SP-SAP treatment, NK1-R expressing neurons are selectively lesioned and destroyed. This will result in the following: 1) a significant reduction in the number of NK1-R expressing GABA neurons in the caudal and intermediate NTS; 2) a reduction in basal baroreflex sensitivity [likely caused by a shift in the balance between excitatory and inhibitory barosensitive neurons]; and 3) a lack of effect of somatic stimulation on baroreflex and chemoreflex bradycardia. Finally, the model predicts that disinhibition of the NTS will partially restore baroreflex sensitivity (as reported in the present study).

We speculate that regional inhibition of barosensitive neurons will reduce the overall output from the NTS directed to cardiac vagal motoneurons. This, in turn, will require a greater level of blood pressure (i.e. greater baroreceptor activation) to excite cardiac vagal motoneurons. From a functional standpoint, this would reset the baroreflex to higher systemic pressure. It is also possible that other neural pathways that inhibit baroreceptor signaling in the NTS, such as descending drive from the hypothalamic defense area (Mifflin et al., 1988; Spyer, 1994), may activate this population of NK1-R expressing GABA neurons. Whether these neurons

receive direct or indirect synaptic input from spinal dorsal horn neurons must be confirmed by additional studies.

Technical considerations

Several technical issues require attention. First, the specificity, location and spread of SP-SAP lesions should be addressed. We feel that the SP-SAP lesions were specific for the following reasons. First, there was no detectable difference in the number of NTS neurons between SP-SAP- and SAP-treated animals based on Neutral Red staining. Second, there was no evidence of necrosis in regions immediately adjacent to the core of the injection site. And finally, there was no difference in baroreflex function when comparing animals treated with SAP and those treated with vehicle. These findings suggest that the dose of SP-SAP toxin failed to produce non-specific effects. With respect to toxin spread, we found that the decrease in NK1-R expression and GAD67 expressing neurons was restricted to the medial and commissural NTS subdivisions. However, in two animals we found a slight reduction of NK1-R immunoreactivity in the X. We doubt, however, that this altered the baroreflex chronotropic responses since the majority of cardiac vagal motoneurons that mediate rapid reflex changes in HR are located in the NA (Mendelowitz, 1999) which was unaffected by toxin injection (see Fig. 4D). Finally, due to the high affinity of NK1-Rs for SP (Regoli et al., 1994) and their preferential expression in the NTS (Lawrence and Jarrott, 1996), it is unlikely that other NKs mediated the neurotoxic effect of SP-SAP.

Since the SP-SAP lesions significantly reduced basal baroreflex sensitivity it could be argued that reflex gain was at a minimum, and thus, could not be reduced further by somatic input. To address this possibility, we performed experiments to restore basal baroreflex sensitivity in SP-SAP-lesioned animals (see Fig. 3). Results from these experiments clearly demonstrate that despite a significant increase and partial restoration of reflex sensitivity, muscle contraction failed to depress the arterial baroreceptor–HR reflex, thus, indicating the depressive effect of somatosensory input was independent of basal baroreflex sensitivity.

Our results suggest that SP-SAP lesions selectively attenuated NTS sensory neurotransmission in a sensory modality-specific manner. This may have been a consequence of the location of SP-SAP lesion relative to the projection targets of primary baroreceptor and chemoreceptor afferents. However, we reported that the greatest loss of NK1-R immunoreactivity was restricted to the caudal and intermediate regions of the NTS where both arterial baroreceptor and peripheral chemoreceptor afferents project (Ciriello, 1994). Therefore, it is unlikely that the location of SP-SAP injections biased our results. Instead, it may be possible that chemosensitive NTS neurons do not express NK1-Rs and, thus, were not lesioned. Indirect evidence supporting this idea comes from our previous study showing that activation of contraction-sensitive somatic afferents selectively attenuated arterial baroreflex, but not peripheral chemoreflex, HR responses (Potts et al., 2003). However, some caution should be taken since compensatory changes in the remaining NTS neurons may have occurred following SP-SAP-induced cell death.

Finally, previous studies have reported that SP microinjections into the NTS produce cardiovascular responses that resemble those evoked by activation of barosensitive neurons (i.e. bradycardia and hypotension). This appears to be at odds with the hypothesis put forward by the present study, namely, that release of SP inhibits barosensitive NTS neurons. We contend that depression of baroreflex function by somatosensory input is likely mediated by a subpopulation of GABA neurons that are “anatomically-restricted” to the caudal portions of the NTS. Although barosensitive neurons (both glutamatergic and GABAergic) are located throughout the caudal and intermediate regions, somatosensory-evoked inhibition of barosensitive neurons was most likely limited to the caudal NTS where the majority of NTS-projecting dorsal horn neurons project (Gamboa-Esteves et al., 2001; Potts et al., 2002).

Therefore, the cardiovascular effects of SP depend directly on the site of endogenous SP release and its spread to local NTS circuit neurons.

Summary

Results from the present study provide new information indicating that NK1-R expressing GABA neurons in the NTS appear to be a synaptic target for somatosensory input and function to depress arterial baroreceptor–HR reflex function. Support for this came from the finding that somatosensory stimulation failed to depress arterial baroreceptor reflex function following selective elimination of NK1-R expressing NTS cells (which included a significant reduction in GABAergic NTS neurons). The population of NK1-R expressing GABA neurons represents a potential “neural substrate” that modulates arterial baroreflex function during muscular activity (Carrington et al., 2003; Gallagher et al., 2001; Ichinose et al., 2002; McIlveen et al., 2001; McWilliam et al., 1991; Potts et al., 1998; Potts and Li, 1998; Potts and Mitchell, 1998; Smith et al., 2000).

CONCLUSION

In conclusion, these findings provide new evidence suggesting that a restricted population of NK1-R expressing GABA neurons participates in rapid and reversible changes of arterial baroreflex function, presumably by decreasing the excitability of barosensitive NTS neurons. In this manner, a reduction in NTS excitability may contribute to resetting of the arterial baroreceptor–HR reflex during exercise by limiting the degree of excitatory drive to cardiac vagal motoneurons.

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Abbreviations

BIC	bicuculline
CED	Cambridge Electronic Design
GAD67	glutamic acid decarboxylase
HR	heart rate
NA	nucleus ambiguus
NaCN	sodium cyanide
NK	neurokinin receptor
NTS	nucleus tractus solitarii
PBS	phosphate-buffered saline
PND	phrenic nerve discharge
PP	perfusion pressure
SAP	saporin
SP	substance P
X	dorsal motor nucleus of the vagus
XII	

hypoglossal motor nucleus

Fig 1.

The SP-SAP immunotoxin significantly attenuated the baseline arterial baroreceptor–HR reflex. (A) Sequence of pressure ramps used to evaluate baroreflex evoked HR responses in SAP ($n = 5$)-, vehicle ($n = 5$)- and SP-SAP ($n = 5$)-treated animals. (B) Mean data for arterial baroreflex function curves. Data represent peak HR and PP responses obtained during each pressor challenge. Insets show changes in HR as a function of changes in PP. * Significant difference from baseline HR ($P < 0.05$); ** Significant difference between SP-SAP and vehicle/SAP ($P < 0.05$).

Fig 2.

SP-SAP abolished the depressive effect of somatic afferent activation on arterial baroreflex function. (A) Sequence of pressor ramps used to activate the arterial baroreflex in the absence (Baseline) and presence (Somatic stim.) of somatic stimulation in SAP-, vehicle- and SP-SAP-treated animals. (B) Mean arterial baroreceptor-HR reflex function curves obtained before (Baseline) and during somatic stimulation. Insets plot changes in HR as a function of changes in PP. * Significant difference from baseline HR ($P < 0.05$); ** significant difference between baseline and Somatic stim ($P < 0.05$), NS, non-significant.

Fig 3.

Effect of disinhibiting NTS on somatosensory-evoked depression of baroreflex function. (A) Prior to BIC microinjection, somatic stimulation failed to depress arterial baroreceptor–HR reflex in SP-SAP-lesioned rats. (B) Following bilateral microinjection of BIC into NTS, basal reflex sensitivity was increased significantly. However, somatic stimulation failed to have an effect on the arterial baroreflex. (C) Group data ($n = 4$) showing lack of effect of somatic stimulation on baroreflex control of HR despite BIC-induced increase in reflex sensitivity in SP-SAP-treated rats. * Significant difference from baseline HR ($P < 0.05$); ** significant difference between baseline and stimulation conditions ($P < 0.05$), NS, non-significant.

Fig 4.

SP-SAP failed to alter the reflex HR response evoked by the arterial chemoreflex. (A) Activation of arterial chemoreceptors (arrowhead) produced a brisk increase in frequency of PND and an immediate reflex bradycardia. However, neither SAP ($n = 4$), vehicle ($n = 3$), SP-SAP ($n = 5$) nor somatic stimulation (somatic stim) altered chemoreflex evoked bradycardia. (B) Mean data illustrating HR responses during control (Baseline) and somatic stimulation (Somatic stim). * Significant difference between pre-stimulus HR (pre) and the peak decrease in HR by NACN injection (Nadir) ($P < 0.05$).

Fig 5.

Neurokinin-1 receptor immunoreactivity (NK1-ir) in the subpostrema level of the NTS in SP-SAP- (A) or SAP- (B) treated animals. The region of the NTS outlined in A1 and B1 is illustrated in A2 and B2 at higher magnification. Note, sparse NK1-ir in the SP-SAP animal compared with positive labeling of soma and processes in the SAP animal. A3 and B3 are the adjacent section stained with Neutral Red. Note the lack of a detectable difference in the number of NTS neurons in this region. (C, D) NK1-ir in the NTS at the level of the 4V (C) or in the rostral ventrolateral medulla (D). Note the similar labeling pattern for NK1-ir in SP-SAP- and SAP-treated animals. (E) Control immunohistochemical staining in the absence of primary NK1-R antibody. (F) Example of a SP-SAP injection site in NTS. * Staining artifact. Scale bar = 50 μ m. AP, area postrema; cc, central canal; ts, solitary tract; 4V, 4th ventricle.

Fig 6.

Immunolesioning significantly reduced the number of NK1 receptor (NK1-R) expressing neurons in the NTS. (A) Pattern of NK1 immunore-activity in a SP-SAP-, vehicle- and SAP-treated animal at three rostral-caudal levels (Bregma -14.08, -13.68, -13.30). (B) Mean number of NK1-R positive neurons per level of the NTS in SAP- ($n = 4$), SP-SAP- ($n = 5$) and vehicle- ($n = 3$) treated animals. In caudal NTS (Bregma -14.08 and -13.68), the number of NK1-R neurons in SP-SAP animals was significantly reduced compared with vehicle and SAP animals. However, no significant difference was found at the rostral level (Bregma -13.30). Section outlines were obtained from Paxinos et al. (1985); pyr, pyramidal tract; LRN, lateral reticular nucleus; sp5, spinal tract of the trigeminal nerve.

Fig 7.

Staining for GAD67 immunoreactivity (GAD67-ir) in the NTS of a SAP- and SP-SAP-treated animal. (A, B) Positive GAD67-ir labeling was found in both SAP and SP-SAP animal. Inset shows outlined area at a higher magnification. There was a noticeable reduction in positive immunolabeling in the SP-SAP-treated animal at Bregma -14.08 and -13.68. (C) GAD67-ir in an untreated control animal is presented for comparison. (D) Complete lack of immunolabeling in the absence of primary antibody. Scale bar=40 μm. AP, area postrema; cc, central canal; ts, solitary tract; 4V, 4th ventricle.

Fig 8.

Reconstruction of NTS section presented in Fig. 7 showing the location and number of GAD67-expressing neurons. (A) SP-SAP decreased the number of GAD67-expressing neurons in the NTS compared with SAP animal. (B) Group data showing the average number of GAD67-expressing neurons per level of NTS in SAP ($n = 3$)- and SP-SAP ($n = 3$)-treated animals. Note, there was a significant reduction in the number of GAD67 neurons in caudal two levels of the NTS. * Significant difference between SAP and SP-SAP ($P < 0.05$).

Fig 9.

Hypothetical model depicting the interaction between baroreceptor (BARO), chemoreceptor (CHEMO) and somatosensory (SOMATO) receptor afferents in the NTS. Left panel: Control condition mimicking SAP injection. Right panel: Experimental condition following treatment with SP-SAP toxin. Please see text for additional discussion. AP, area postrema.

Table 1

Linear regression analysis of arterial–cardiac baroreflex function curves

	SAP treated (<i>n</i> = 5)	Vehicle treated (<i>n</i> = 5)	SP-SAP treated (<i>n</i> = 5)
Control			
HR, bpm	350±4	355±10	350±4
PP, mm Hg	70±6	82±5	70±16
Slope, bpm mm Hg ⁻¹	-2.05±0.12 [*]	-2.10±0.10 [*]	-0.59±0.16
Somatic stimulation			
HR, bpm	361±8	361±25	361±6
PP, mm Hg	84±10	86±8	73±6
Slope, bpm mm Hg ⁻¹	-1.02±0.34, ^{*,§}	-1.29±0.16, ^{*,§}	-0.58±0.12

Values are means±S.E.M.; *n*, number of rats;^{*} significantly different from SP-SAP (*P*<0.05);[§] significant difference between baseline and somatic stimulation (*P*<0.05).