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Colocalization of neurokinin-1, NMDA, and AMPA receptors on neurons of the rat nucleus tractus solitarii

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Abstract

Substance P (SP) and glutamate are implicated in cardiovascular regulation by the nucleus tractus solitarii (NTS). Our earlier studies suggest that SP, which acts at neurokinin 1 (NK1) receptors, is not a baroreflex transmitter while glutamate is. On the other hand, our recent studies showed that loss of NTS neurons expressing NK1 receptors leads to loss of baroreflex responses and increased blood pressure lability. Furthermore, studies have suggested that SP may interact with glutamate in the NTS. In this study, we sought to test the hypothesis that NK1 receptors colocalize with glutamate receptors, either N-methyl-D-aspartate (NMDA) receptors or alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors or both in the NTS. We performed double-label immunofluorescent staining for NK1 receptors and either NMDA receptor subunit 1 (NMDAR1) or AMPA receptor subunit (GluR2) in the rat NTS. Because vesicular glutamate transporter 2 (VGLUT2) containing fibers are prominent in portions of the NTS where cardiovascular afferent fibers terminate, we also performed double-label immunofluorescent staining for NK1 receptors and VGLUT2. Confocal microscopic images showed that NK1 receptors-immunoreactivity (IR) and NMDAR1-IR colocalized in the same neurons in many NTS subnuclei. Almost all NTS neurons positive for NK1 receptor-IR also contained NMDAR1-IR, but only 53.4% to 74.8% of NMDAR1-IR positive neurons contained NK1 receptors-IR. NK1 receptor-IR and GluR2-IR also colocalized in many neurons in NTS subnuclei. A majority of NK1 receptor-IR positive NTS neurons also contained GluR2-IR, but only 45.8% to 73.9% of GluR2-IR positive NTS neurons contained NK1 receptors-IR. Our results also showed that fibers labeled for VGLUT2-IR were in close apposition to fibers and neurons labeled for NK1 receptor-IR. The data support our hypothesis, provide an anatomical framework for glutamate and SP interactions, and may explain the loss of baroreflexes when NTS neurons, which could respond to glutamate as well as SP, are killed.

Keywords

Neurokinin 1 receptor; substance P; N-methyl-D-aspartate; AMPA; vesicular glutamate transporter 2; nucleus tractus solitarii

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INTRODUCTION

The nucleus tractus solitarii (NTS) is the primary site of termination of cardiovascular and visceral afferent fibers of the vagus and glossopharyngeal nerve and plays important roles in circulatory, ventilatory, gastric, and gustatory control (Bradley et al., 1996; Kalia et al., 1980). Of the numerous neurotransmitters found in the NTS, glutamate is the principal excitatory neurotransmitter and has been recognized to play a pivotal role in regulating cardiovascular functions in the NTS (Seagard et al., 2001; Talman et al., 1980; Talman, 1997). Studies have also suggested that substance P (SP), acting at neurokinin-1 (NK1) receptors (Feldman, 1995; Zhang et al., 2000), plays a role in neuromodulation of cardiovascular control in the NTS (Boscan et al., 2002; Gillis et al., 1980; Potts, 2006), though a role for the peptide in transmission of baroreflex signals has been controversial. For example, administration into the NTS of SP or agonists for NK1 receptors evoked contradictory arterial baroreceptor responses (Bauman et al., 2002; Carter and Lightman, 1985; Feldman, 1995; Kubo and Kihara, 1987; Nagashima et al., 1989; Talman and Reis, 1981). In addition, injection of SP into the NTS has been shown to potentiate the baroreceptor reflex vaso-depressor response in one study (Seagard et al., 2000), but was reported to inhibit the carotid sinus nerve evoked response and attenuate the baroreceptor reflex bradycardia gain in other studies (Boscan et al., 2002; Perez et al., 1992). Supporting a role in SP in transmission of baroreflex, recent studies have shown that a loss of NK1 receptors expressing neurons in the NTS leads to attenuated baroreflex sensitivity (Abdala et al., 2006; Potts et al., 2007; Riley et al., 2002). A potential explanation for this finding would be colocalization of NK1 receptors with receptors for glutamate transmission on the same neurons in NTS. Thus, ablation of neurons with NK1 receptors would also affect glutamate transmission and thus transmission of baroreflex signals.

While SP appears to play some role in the NTS in modulating cardiovascular responses, mechanisms responsible for cardiovascular modulation by SP in the NTS are not clear. Possible involvement in somatosensory inhibition of baroreflex neurons in the NTS by SP has been suggested in studies showing that its interaction with γ -aminobutyric acid (GABA) mediated central cardiovascular control (Pickering et al., 2003; Potts et al., 2007). However, other evidence also supports its having an excitatory role in NTS. Depressor and bradycardic responses induced by microinjection of the amino-terminal fragment of SP were significantly reduced by prior injection of glutamate antagonists at the same site (Hall et al., 1993). Those glutamate agonists in the NTS may mediate their actions through activation of N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) or metabotropic receptors (Andresen and Yang, 1994; Aylwin et al., 1997; Ohta and Talman, 1994; Zhang and Mifflin, 1998), but SP in the NTS may also potentiate AMPA receptor agonist-induced depressor responses (Cowan et al., 2000). Integration of SP and glutamate receptor actions is further supported by the observation that application of either NMDA agonists or AMPA agonists into the NTS induces SP release as suggested by internalization of NK1 receptors (Colin et al., 2002). Thus, these pharmacological data suggest that interactions between glutamate and SP at their receptors in the NTS may influence cardiovascular regulation through the NTS. There is precedence for interactions between SP and glutamate. For example, in spinal cord (Liu et al., 1997; Marvizon et al., 1997; Randic et al., 1990) SP may also interact with glutamate to exert its effects (Bailey et al., 2004; Colin et al., 2002; Cowan et al., 2000; Hall et al., 1993). However, anatomical data supporting such interactions in NTS are sparse. The only anatomical study published to date is a limited ultrastructural study that found glutamate in most SP-containing terminals in the NTS (Saha et al., 1995). NK1, NMDA and AMPA receptors have been found in the NTS (Ambalavanar et al., 1998; Davis and Smith, 1999; Dixon et al., 1998; Lacassagne and Kessler, 2000; Lin and Talman, 2002; Sato et al., 1993), but it is not known if glutamate receptors and NK1 receptors are found in the same NTS neurons. Therefore, we sought to test the hypothesis that NK1 and NMDA receptors and that NK1 and AMPA receptors colocalize in NTS neurons. We performed

double-label immunofluorescent staining for NK1 and NMDA receptor subunit 1, the fundamental subunit of NMDA receptor (Hollmann and Heinemann, 1994), and for NK1 receptors and AMPA receptor subunit 2 (GluR2), the main AMPA receptor subunit expressed by NTS neurons (Lacassagne and Kessler, 2000; Sato et al., 1993) in the rat NTS. Because VGLUT2 expressing fibers have been reported to be abundant in areas of the NTS that are implicated in cardiovascular regulation (Lachamp et al., 2006; Lin et al., 2004), we also performed double-label immunofluorescent staining for NK1 receptors and vesicular glutamate transporter 2 (VGLUT2). Labeled sections were then examined by a confocal microscope.

EXPERIMENTAL PROCEDURES

All procedures conformed to standards established in the *Guide for Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C. 1996). The Institutional Animal Care and Use Committees of the University of Iowa and Department of Veterans Affairs Medical Center, Iowa City reviewed and approved all protocols. Both institutions are AAALAC accredited. All efforts were made to minimize the number of animals used and to avoid their sustaining pain or distress.

Animals and tissue preparation

Adult male Sprague Dawley rats (275–340 g, $n = 10$) were deeply anesthetized (with no response to painful stimuli) with pentobarbital (50 mg/kg) and perfused through the heart, first with chilled (4°C) phosphate buffered saline (PBS, pH 7.4) for 2 min at a perfusion rate of 20 ml/min, then with freshly prepared chilled 4% paraformaldehyde in PBS for 20 min at the same perfusion rate. The brain stem was then removed, postfixed in 4% paraformaldehyde for 2 hr and then cryoprotected for 2 days in 30% sucrose in PBS at 4°C. Frozen 20 μ m coronal sections were cut with a cryostat and processed for double-label immunofluorescent staining for NK1 receptors and for NMDAR1, GluR2, or VGLUT2.

Double-label immunofluorescent staining

Brain stem sections were washed with PBS and then blocked with 10% donkey normal serum (Jackson ImmunoResearch, PA) in PBS at 25°C for 1 hr. For double-label immunofluorescent staining of NK1 receptors and NMDAR1, brain stem sections were incubated in a mixture of Guinea pig anti-NK1 receptor antibody (1:50, Chemicon, CA) and rabbit anti-NMDAR1 antibody (1:10, Chemicon) in 10% donkey normal serum for 24 hr in a humid chamber at 25°C. The sensitivity and specificity of the Guinea pig antibody to the NK1 receptor has been confirmed (Mounir and Parent, 2002) and the antibody used by other investigators (Liu et al., 2005a; Mounir and Parent, 2002; Setou et al., 2000; Stornetta et al., 2003). The specificity of the NMDAR1 antibody has also been confirmed (Petrulia et al., 1994) and the antibody has been used in previously published studies (Gracy and Pickel, 1995; Lin and Talman, 2000; Liu et al., 2005b). After being washed with PBS, sections were incubated with a mixture of rhodamine red X (RRX)-conjugated donkey anti-Guinea pig (1:200, Jackson ImmunoResearch, PA) and fluorescein-5-isothiocyanate (FITC)-conjugated donkey anti-rabbit (1:200, Jackson ImmunoResearch) in PBS for 20–24 hr at 4°C. Sections were then washed, air-dried, and mounted with Prolong Gold Antifade Reagents (Invitrogen-Molecular Probes, CA). Similar procedures were used for double-label immunofluorescent staining for NK1 receptors and GluR2, except that mouse anti-GluR2 (1:50, Chemicon) replaced NMDAR1 antibody and FITC-conjugated donkey anti-mouse antibody (1:200, Jackson ImmunoResearch) replaced FITC-conjugated donkey anti-rabbit antibody. The anti-GluR2 antibody is also specific (Vissavajhala et al., 1996) and has been used successfully by others (King et al., 2006; Steinberg et al., 2006). Similarly, for double immunofluorescent labeling of NK1 receptors and VGLUT2, a rabbit anti-VGLUT2 antibody (1:4000, a generous gift from

Drs. R. T. J. Fremeau and R. H. Edwards) replaced NMDAR1 antibody. This antibody's specificity has also been documented (Fremeau et al., 2002) and the antibody used in other studies (Fremeau et al., 2002; Fremeau et al., 2004; Lin et al., 2004). Negative controls consisted of tissue processed a) in the absence of primary antibodies or b) after addition of only one of the primary antibodies.

Confocal laser scanning microscopy

Labeled sections were analyzed with a Zeiss LSM 510 confocal laser scanning microscope that was equipped with an inverted Axiovert 100M microscope, an argon laser (458/488/514 nm, 25 mW), a green helium/neon laser (543 nm, 1 mW) and a red helium/neon laser (633 nm, 5 mW) for excitation of FITC, TRD and Cy5, respectively. The LSM was controlled through a standard high-end Pentium® 4 PC (32 bit Microsoft Windows XP operating system) linking with the electronic control system via an ultrafast SCSI interface. We scanned specimens sequentially by two channels to separate labels. For optimal visualization of the relationship between RRX and FITC-labeled elements, images from 2 channels were assigned the pseudocolor red (for RRX) or green (for FITC) and were superimposed. The pinhole size was set at 100 (at a scale of 0 – 1000) to give an effective optical section thickness of approximately 0.5–1.0 μm . Confocal images were obtained at 100X and 400X magnifications, with or without zooming functions (1X – 3X), and were processed with software provided with the Zeiss LSM 510. Adobe Photoshop image editing software (Adobe Photoshop CS2) was used to examine if a structure was single-labeled for one of the antigens or double-labeled for two antigens by switching between red and green channels on the monitor. Another image editing program, Microsoft PowerPoint (2003), was used to create montages. Contrast and brightness of images were the only variables we adjusted digitally.

We counted stained cells in a subnucleus seen on 2–3 sections selected from the same level of brain stem in each rat and included both sides of the NTS in our analysis. Sections chosen for this analysis were those that best demonstrated that subnucleus, i.e. showed the subnucleus in its largest dimensions, at any given level. For example, sections from Bregma –13.47 mm to Bregma –13.18 mm were used for studies of the central subnucleus, and sections from Bregma –14.00 mm to –13.64 mm were used for studies of the dorsolateral subnucleus. Sequential sections were avoided so that sections used for counting were at least 20 μm (the thickness of the sections) apart in order to minimize the possibility of counting the same stained neurons twice. Stained cells in each section were counted three times, first using the red channel for the number of NK1 receptors-IR positive cells, then the green channel for NMDAR1-IR or GluR2-IR positive neurons, and finally the combined channel for double-stained neurons for each NTS subnucleus. The percentage of single- to double-labeled cells in each section was calculated after each section had been counted. Percentages of single- to double-labeled cells in NTS subnuclei were expressed as mean $\pm$ standard deviation (STD). Nomenclature used for subnuclei of NTS was based on publications of Kalia and Sullivan (Kalia and Sullivan, 1982) and Paxinos et al. (Paxinos et al., 1999).

RESULTS

In our control experiments, we found no apparent FITC (for NMDAR1, Fig. 1A, GluR2, Fig. 1E, VGLUT2, not shown) or RRX (for NK1 receptors, Fig. 1C) staining when the respective antibody had been omitted. When NK1 antibody was included, we observed numerous cells and processes in many areas of the brain stem, including the NTS, contained NK1 receptor-immunoreactivity (IR). We considered a stained structure a cell when its general shape was oval or round and its diameter was 4 μm or greater regardless of there being processes on the structure. A stained structure was considered a process when its outside dimension was less than 3 μm in cross section or when it was a linear structure of any length when cut tangentially.

Although the antiserum recognizes NK1 receptors located on the cell surface, we observed intense staining outlining the soma (Fig. 2D) as well as staining within the soma (Fig. 2A). That finding was consistent with observations made in another study (Dixon et al., 1998). The distribution of cells and fibers positive for NK1 receptor-IR in rat brain stem in the present study was very similar to that described earlier (Dixon et al., 1998; Riley et al., 2002). Briefly, almost all NTS subnuclei contained NK1 receptor-IR positive cells and processes. The medial (Fig. 3A), intermediate (Fig. 3B) and ventral subnuclei contained a high to moderate level of NK1 receptor-IR, while the dorsolateral (Fig. 3C), central (Fig. 3D), and interstitial subnuclei contained moderate to low level of NK1 receptor-IR. Here we use the terms “intensity” and “levels” to describe the relative amount of immunoreactive labeling in positive structures when they were compared with others in the same section. These descriptors do not imply quantification of the respective protein. We also observed a high level of NK1 receptor-IR in the dorsal motor nucleus of vagus (DMV) (Fig. 3E) and the nucleus ambiguus (NA) (Fig. 3F) when compared with nearby structures.

NK1 receptor-IR and NMDAR1-IR

The distribution of NMDAR1-IR positive cells and processes in the rat brain stem was similar to that reported previously (Ambalavanar et al., 1998; Lin and Talman, 2002). Briefly, numerous cells positive for NMDAR1-IR were observed in all NTS subnuclei with the medial (Fig. 3A) and the ventral subnuclei being most intensely stained for NMDAR1-IR. When confocal images of NK1 receptors-IR (RRX labeled, pseudo-colored red) and NMDAR1-IR (FITC labeled, pseudo-colored green) were merged, cells and processes stained for both NK1 receptors-IR and NMDAR1-IR (Fig. 2C) were noted in the NTS. These double-stained cells and fibers were found in almost all NTS subnuclei, including the medial subnucleus (Fig. 3A), the intermediate subnucleus (Fig. 3B), the dorsolateral subnucleus (Fig. 3C) and the central subnucleus (Fig. 3D). Cells stained for NMDAR1-IR alone were also noted in all NTS subnuclei (Fig. 3, A–D). However, we rarely observed cells that stained for NK1 receptor-IR alone because almost all NK1 receptor-IR positive cells also contained NMDAR1-IR.

We counted cells that stained for NMDAR1 alone and cells stained for both NMDAR1 and NK1 receptor-IR, and noted that the percentage of NMDAR1-IR positive cells that were also NK1 receptor-IR positive varied slightly among NTS subnuclei (Table 1). Because NK1 receptor-IR containing cells and fibers were sparse in the NTS caudal to the area postrema, we only included the level of the NTS that was rostral to the caudal pole of the area postrema in this analysis. The dorsolateral subnucleus contained the highest percentage of NMDAR1-IR positive cells that were also NK1 receptor-IR positive. The DMV and the NA also contained numerous cells and fibers double-labeled for NMDAR1-IR and NK1 receptor-IR (Fig. 3E and 3F, respectively). Similar to the NTS, almost all NK1 receptor-IR positive neurons in the DMV and the NA were NMDAR1-IR positive. As shown in Table 1, the percentages of NMDAR1-IR positive cells that also contained NK1 receptor-IR in the NA and the DMV were higher than those of NTS subnuclei. In the NA, almost all NMDAR1-IR expressing neurons also expressed NK1 receptor-IR.

NK1 receptor-IR and GluR2-IR

The distribution of GluR2-IR positive cells and processes in the rat brain stem was similar to that of an earlier report (Lacassagne and Kessler, 2000). We also observed medium to high levels of GluR2-IR in many NTS subnuclei, including the medial (Fig. 4A), intermediate (Fig. 4B), dorsolateral (this subnucleus was called the dorsal subnucleus in the Lacassagne and Kessler paper) (Fig. 4D), and interstitial subnuclei. Prominent GluR2-IR positive cells and processes were also noted in the DMV (Fig. 4E) and the NA (Fig. 4F). When confocal images of NK1 receptor-IR and GluR2-IR were merged, we observed cells and processes double-labeled for NK1 receptor-IR and GluR2-IR in almost all NTS subnuclei, including the medial

(Fig. 4A), the intermediate (Fig. 4B), the dorsolateral (Fig. 4C) and the central (Fig. 4D) subnuclei. Cells labeled for NK1 receptor-IR alone or GluR2-IR alone were observed in all NTS subnuclei (Fig. 4A–D). We counted cells labeled for NK1 receptor-IR alone, GluR2-IR alone and cells double-labeled for both and calculated the percentages of NK1 receptor-IR positive cells and the percentage of GluR2-IR positive cells that were double-stained. Similar to the analysis of NK1 receptor-IR positive cells and NMDAR1-IR positive cells, this analysis included only the levels of NTS rostral to the caudal pole of the area postrema. As shown in Table 2, very high percentages (ranging from 87.4% to 95.8%) of NK1 receptor-IR positive cells in NTS subnuclei also contained GluR2-IR. The percentages of GluR2-IR positive cells also containing NK1 receptor-IR ranged from 45.8% to 73.9% in NTS subnuclei. Colocalization of NMDAR1-IR and GluR2-IR was also noted in the DMV and NA. Almost all NK1 receptor-IR positive cells in the DMV and the NA also contained GluR2-IR. The percentages of GluR2-IR positive cells that also contained NK1 receptor-IR in the NA and the DMV were higher than those of NTS subnuclei.

NK1 receptor-IR and VGLUT2-IR

The distribution of VGLUT2-IR in the NTS was similar to that of our earlier report (Lin et al., 2004). VGLUT2-IR exhibited a punctate pattern of fluorescent staining; indicating staining in neuronal processes (Fujiyama et al., 2001). We found a moderate (10 – 40 processes per 400 μ m confocal imaging area) to high density (more than 40 processes per 400 μ m confocal image area) of VGLUT2-IR fibers in the gelatinosus subnucleus, subpostremal area, dorsolateral, central, lateral and medial subnuclei of the NTS, as well as in the DMV and the NA. When confocal images of NK1 receptor-IR and VGLUT2-IR were merged, we observed numerous VGLUT2-IR fibers apposed to NK1 receptor-IR positive fibers and cell bodies in many NTS subnuclei. Examples of such apposition in the dorsolateral subnucleus and the medial subnucleus were shown in Fig. 5. NK1 receptor-IR never colocalized with VGLUT2-IR in cells or fibers in the NTS, the DMV or the NA.

DISCUSSION

The present study supports our hypothesis that SP receptors and glutamate receptors colocalize in the rat NTS. Our data show that 1) NK1 receptor-IR and NMDA-IR colocalized in cells and fibers in almost all NTS subnuclei; 2) All NK1 receptor-IR positive cells contained NMDAR1-IR, but only a portion of NMDAR1-IR positive cells contained NK1 receptors in NTS subnuclei; 3) NK1 receptor-IR and GluR2-IR colocalized in cells and fibers in rat NTS subnuclei; 4) Most NK1 receptor-IR positive cells contained GluR2-IR, but only a portion of GluR2-IR positive cells contained NK1 receptor-IR in NTS subnuclei; 5) VGLUT2-IR positive fibers were apposed to NK1 receptor-IR containing fibers and cell bodies in the NTS.

Our findings that NK1 receptor-IR colocalized with NMDAR1-IR and GluR2-IR in NTS neurons and fibers are consistent with a previous anatomical study that showed colocalization of SP and glutamate in NTS terminals (Saha et al., 1995). These anatomical data support the hypothesis that SP and glutamate may interact in the NTS to modulate heart rate and blood pressure regulation (Bailey et al., 2004; Colin et al., 2002; Cowan et al., 2000; Hall et al., 1993). The precise mechanism for an interaction between SP and glutamate in the NTS is not known. Because SP was shown to enhance electrical activity induced by glutamate, it was suggested that SP might modulate the excitatory effects of glutamate from primary afferent terminals in the spinal cord (Zieglansberger and Tulloch, 1979). On the other hand, it was suggested that glutamate neurons in the NTS are activated downstream to SP release (Saha et al., 1995) in that glutamate antagonists can block the effect of SP, but SP(1-7) antagonists are not able to block the effect of glutamate (Hall et al., 1993). Because NK1 receptors are present mostly in dendrites and cell bodies (Aicher et al., 2000; Klooster et al., 2000; Pickel et al.,

2000), our data indicate that the interaction between glutamate and SP might happen at the same postsynaptic site, although we do not exclude the possibility that the interaction might happen at other sites as well.

Colocalization of NK1 receptors with NMDAR1 and with GluR2 types of glutamate receptors in the majority of NTS neurons suggests that activation of NK1 receptors may also affect responses to glutamate receptor activation. This suggestion is consistent with studies in which microinjection into the NTS of SP, which would activate NK1 receptors, potentiated AMPA receptor-induced depressor responses (Cowan et al., 2000). The interaction between SP and glutamate may be more than unidirectional. Colocalization of NK1 receptors and NMDAR1 and GluR2 in NTS neurons may also suggest that activation of glutamate receptors could affect results of NK1 receptor activation. This suggestion is supported by pharmacological studies showing that activation of either NMDA or AMPA receptors in the NTS caused internalization of NK1 receptors, which would facilitate release of SP from primary afferents (Colin et al., 2002). In addition, our observation that VGLUT2-IR positive fibers often were apposed to NK1 receptor-IR cells and fibers in the NTS is also consistent with this suggestion. This observation may suggest that glutamate release from neurons that express VGLUT2 may activate NMDA and AMPA receptors that, in turn, may lead to internalization of NK1 receptors facilitated release of SP. Of note, we never observed colocalization of VGLUT2-IR and NK1 receptor-IR in any of NTS subnuclei and other brain stem areas. This is consistent with published studies in other areas of the brain that showed exclusive location of VGLUT2-IR in axon or axonal terminal (Fujiyama et al., 2001) and preferential location of NK1 receptors-IR in dendrite or soma (Aicher et al., 2000; Klooster et al., 2000; Pickel et al., 2000).

Although NK1 receptors are mainly found in dendrites and somas, they are occasionally found in axons and axonal terminals (Pickel et al., 2000). A role for presynaptic NK1 receptors in the NTS has been suggested by a study that used whole-cell patch-clamp recording to show increased glutamate release after NK1 receptor activation (Bailey et al., 2004). Therefore, it is possible that the interaction between NK1 receptors and glutamate receptors may happen at a presynaptic site. However, this possibility is limited by the aforementioned preferential localization of NK1 receptor protein in dendrites and cell bodies and is not supported by the current study. Thus, the interaction between glutamate and SP in the NTS may be reciprocal, and may occur both at pre- and postsynaptic sites. Future studies are needed to delineate the ways in which SP and glutamate interact in the NTS and how those interactions exert effects on cardiovascular regulation.

Ablation of NTS neurons that express NK1 receptors after injection of SP conjugated with saporin (SP-SAP) leads to attenuated baroreflex sensitivity (Abdala et al., 2006; Potts et al., 2007; Riley et al., 2002). The reduced baroreflex sensitivity has been attributed to a loss of glutamatergic neurons (Potts et al., 2007) despite the absence of supportive anatomical data. Our finding that almost all NK1 receptor-IR positive cells also contained NMDAR1-IR in the NTS provides anatomical support for this suggestion. Because all NK1 receptor-IR positive cells contained NMDAR1-IR, a loss of cells that contain NK1 receptors would result in a loss of the same number of NMDAR1 containing cells. The killed NMDAR1-IR positive cell would belong to a subpopulation of NMDAR1 containing cells in the NTS. Depending on the NTS subnucleus under consideration that subpopulation represents 53.4% to 77.8% of total NMDAR1 containing neurons. Because NMDA receptors play a role in mediating glutamatergic neurotransmission in the NTS (Kubo and Kihara, 1988; Neff et al., 1998; Ohta and Talman, 1994), a loss of substantial percentage of NMDA containing cells in the NTS would be expected to impair the baroreceptor reflex. Furthermore, we also observed a high percentage (ranging from 87.4% to 95.8%, Table 2) of NK1 receptors-IR positive cells that also contained GluR2-IR in NTS subnuclei. This means that 87.4% to 95.8% of all NK1 receptors-IR positive cells killed were GluR2-IR positive cells. These killed GluR2 cells

represented 45.8% to 73.9% of GluR2-IR positive cells (Table 2) in the NTS. Although it is not known what percentage of the total AMPA receptor expressing neurons these GluR2 positive neurons represent in the NTS, it is known that GluR2 is expressed abundantly in the NTS and is known to be the major AMPA receptor subunit expressed by NTS neurons (Sato et al., 1993). Because glutamatergic baroreflex transmission in the NTS is also mediated by AMPA receptors (Kubo and Kihara, 1991; Neff et al., 1998; Ohta and Talman, 1994; Yen et al., 1999), a loss of both NMDAR1-IR and GluR2-IR containing neurons in the NTS could contribute to the impairment of baroreflex following loss of neurons that express NK1 receptors. Of note, a previous study from our laboratory showed that NMDA receptors and AMPA receptors colocalize in the NTS very frequently (Lin and Talman, 2002). This suggests that some NK1 receptors containing cells would contain both NMDA and AMPA receptors. Although we did not include triple-label immunofluorescent staining of NK1 receptors, NMDA receptors and AMPA receptors in this report, we did observe triple-stained neurons in a preliminary study. Therefore, the population of neurons that contained NMDA receptors that was killed by SP conjugated with saporin is likely to overlap with the population of neurons that contained AMPA receptors that was killed by the toxic conjugate. Furthermore, the effect of SP conjugated saporin that targeted at NK1 receptors containing neurons in the NTS may not be limited to NMDAR1 and GluR2 containing neurons in the NTS. SP conjugated saporin may bind to presynaptic NK1 receptors and may be retrogradely transported to afferent cell populations projecting to the NTS. If that were the case saporin could cause additional damage as did dopamine β -hydroxylase conjugated saporin in killing distant projecting neurons (Schreihofer et al., 2000). However, the possibility of SP conjugated saporin killing afferent cell populations to the NTS is not high because, as discussed previously, there are very few NK1 receptors on axons, and there has been no convincing evidence that SP conjugated saporin is transported retrogradely (Wiley and Kline IV, 2000).

In addition to the NTS, colocalization of NK1 receptor-IR with NMDAR1-IR or GluR2-IR was also observed in very high percentage of NA and DMV neurons. Although both SP and glutamate are implicated in cardiovascular regulation in the NA (Agarwal and Calaresu, 1991; Massari et al., 1996) and in modulating gastric motility in the DMV (Cruz et al., 2007), it has not been shown that they may interact with each in regulating these functions. Therefore, the significance of colocalization of NK1 receptors with NMDAR1 or GluR2 awaits future investigation. The observation that colocalization of NK1 receptors and NMDA or AMPA receptors was found not only in the NTS, but also in the NA and DMV indicates that the potential for interactions between SP and glutamate may be ubiquitous. If this is the case, the interaction of SP and glutamate may not be limited to cardiovascular regulation but instead it may also contribute to regulation of gustatory and respiratory function and to modulation of pain perception.

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Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ce	central subnucleus
dl	dorsolateral subnucleus

DMV	dorsal motor nucleus of vagus
FITC	fluorescein-5-isothiocyanate
GluR2	AMPA specific glutamate receptor subunit 2
im	intermediate subnucleus
is	interstitial subnucleus
me	medial subnucleus
NA	nucleus ambiguus
NK1	neurokinin-1
NMDA	N-methyl-D-aspartate
NMDAR1	NMDA receptor subunit 1
NTS	nucleus tractus solitarii
PBS	phosphate buffered saline
RRX	rhodamine red X
SP	substance P
VGLUT2	vesicular glutamate transporter 2
vt	ventral subnucleus

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Fig. 1.

Pseudo-colored confocal images of negative controls. No FITC (for NMDAR1-IR and GluR2-IR, panel A and panel E, respectively) or RRX (for NK1 receptor-IR) staining was apparent on sections when the primary antibody was omitted. Panels B, D, F show NMDAR1-IR, NK1 receptor (NK1R)-IR and GluR2-IR, respectively, in the medial subnucleus (me) of the NTS when the respective primary antibody was included in the incubation solution. Inset in E shows the location of the me subnucleus in the NTS (the shaded area) at the level of the area postrema (AP). Scale bar = 20 μm.

Fig. 2.

Pseudo-colored confocal images of NTS cells and fibers double-labeled for NK1 receptors and NMDAR1 (A–C), and for NK1 receptors and GluR2 (D–F). Panels A and D show NK1 receptor (NK1R)-IR, RRX labeled and pseudo-colored red. Panel B shows NMDAR1-IR, FITC labeled and pseudo-colored green. Panel E shows GluR2-IR, FITC labeled and pseudo-colored green. Panel C is a merged image of panel A and B. Panel F is a merged image of D and E. In all figures double-labeled structures are pseudo-colored yellow. White arrows in A–C indicate a cell, appearing yellow, which contains both NK1 receptor-IR and NMDAR1-IR. White arrows in E–F indicate a cell that contains both NK1 receptor-IR and GluR2-IR. Arrowheads indicate a fiber double-stained for NK1 receptor-IR and NMDAR1-IR in A–C and NK1 receptor-IR and GluR2-IR in E–F. White arrows indicate a cell that contains NMDAR1-IR alone in A–C and GluR2-IR alone in E–F. Scale bar = 10 μ m.

NK1R-IR + NMDAR1-IR

Fig. 3.

Merged confocal image of the medial (me, panel A), the intermediate (im, panel B), the dorsolateral (dl, panel C), and the central (ce, panel D) subnuclei of the NTS, the dorsal motor nucleus of vagus (DMV, panel E) and the nucleus ambiguus (NA, panel F) stained for NK1 receptors (NK1R) and for NMDAR1. Inset in B shows the location of the im, dl and ce subnuclei in one side of the NTS (the shaded area) at the level of the area postrema (AP). The location of the me subnucleus is shown in Fig. 1. Numerous double-labeled cells (white block arrows) and fibers (arrow heads) are observed in all NTS subnuclei, the DMV and the NA. White arrows indicate cells labeled for NMDAR1-IR alone. Almost all cells containing NK1

receptor-IR also contain NMDAR1-IR, but only a portion of NMDAR1-IR containing cells contain NK1 receptor-IR. Scale bar = 20 μ m.

Figure 4.

Merged confocal image of the medial (me, panel A), the intermediate (im, panel B), the dorsolateral (dl, panel C), and the central (ce, panel D) subnuclei of the NTS, the dorsal motor nucleus of vagus (DMV, panel E) and the nucleus ambiguus (NA, panel F) stained for NK1 receptors (NK1R, red, RRX-labeled) and GluR2. Many double-labeled cells (white block arrows) and fibers (arrowheads) are observed in all NTS subnuclei, the DMV and the NA. White arrows indicate cells labeled for GluR2-IR alone. A high percentage of cells containing NK1 receptor-IR also contain GluR2-IR, a lower percentage of GluR2-IR containing cells contain NK1 receptor-IR. Scale bar = 20 μ m.

Fig. 5.

Merged confocal image of double-label immunofluorescent staining of NK1 receptors (NK1R, red, RRX labeled) and VGLUT2 (green, FITC labeled) in the dorsolateral subnucleus (dl, panel A) and the medial subnucleus (me, panel B) of the rat NTS. Fibers labeled for VGLUT2-IR are seen in close apposition to fibers labeled for NK1 receptor-IR (indicated by white arrows). VGLUT2-IR labeled fibers are also in close apposition to cell bodies that are NK1 receptor-IR positive, indicated by open arrows. Scale bar = 10 μ m.

Table 1
Colocalization of NK1 receptor-IR and NMDAR1-IR in subnuclei of NTS.

NTS subnuclei	
Dorsolateral subnucleus	74.8 ± 5.2 % (n = 493)
Ventral subnucleus	57.9 ± 10.4 % (n = 1390)
Medial subnucleus	56.2 ± 7.3 % (n = 1977)
Intermediate subnucleus	57.8 ± 7.8 % (n = 589)
Interstitial subnucleus	58.4 ± 11.6 % (n = 683)
Central subnucleus	53.4 ± 10.5 % (n = 635)
DMV	70.3 ± 9.5% (n = 914)
NA	97.3 ± 2.9% (n = 194)

Values are percentages (mean ± STD) of NMDAR1-IR positive cells that were also positive for NK1 receptor-IR in NTS subnuclei, the dorsolateral motor nucleus of vagus (DMV) and nucleus ambiguus (NA). Numbers in parentheses represent numbers of cells counted. Because almost all NK1 receptor-IR positive cells contained NMDAR1-IR that comparison is not shown here.

Table 2
Colocalization of NK1 receptor-IR and GluR2-IR positive cells in subnuclei of NTS

	% of double-labeled NK1 receptor-IR positive cells	% of double-labeled GluR2-IR positive cells
NTS subnuclei		
Dorsolateral	92.3 ± 7.4 % (n=233)	45.8 ± 6.8 % (n=466)
Ventral	87.4 ± 7.8 % (n=401)	64.3 ± 8.4 % (n=563)
Medial	91.8 ± 6.1 % (n=792)	66.6 ± 13.7 % (n=734)
Intermediate	91.5 ± 7.7 % (n=463)	66.5 ± 9.0 % (n=635)
Interstitial	91.4 ± 5.1 % (n=569)	73.9 ± 6.2 % (n=701)
Central	95.8 ± 6.4 % (n=244)	49.2 ± 11.2 % (n=454)
DMV	99.7 ± 2.7 % (n=318)	86.4 ± 9.1 % (n=375)
NA	99.7 ± 2.6 % (n=306)	87.7 ± 5.8 % (n=348)

Values are percentages (mean ± STD) of NK1 receptor-IR and GluR2-IR positive cells which were double-labeled in NTS subnuclei, the dorsal motor nucleus of vagus (DMV) and nucleus ambiguus (NA). Numbers in parentheses represent numbers of cells counted.