Published in final edited form as:

J Chem Neuroanat. 2012 March ; 43(2): 141–148. doi:10.1016/j.jchemneu.2012.02.001.

Collateral damage and compensatory changes after injection of a toxin targeting neurons with the neurokinin-1 receptor in the nucleus tractus solitarii of rat

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Abstract

Injection into the nucleus tractus solitarii (NTS) of toxins that target substance P (SP) receptors ablates neurons that express neurokinin-1 (NK1) receptors, attenuates baroreflexes, and results in increased lability of arterial pressure. We and others have shown that the toxin leads to loss of neurons containing SP receptors and loss of GABAergic neurons in the NTS; but given that neither type neuron is thought to be integral to baroreflex transmission in NTS, mechanisms responsible for the cardiovascular changes remained unclear. Because NK1 receptors colocalize with N-methyl-D-aspartate (NMDA) receptors and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in NTS and because glutamate transmission may be integral to baroreflex transmission in the NTS we hypothesized that the toxic lesions may interrupt mechanisms for glutamate transmission. Interruption of those mechanisms could be responsible for the cardiovascular effects. We tested the hypothesis by performing fluorescent immunohistochemistry, confocal microscopy and image analysis after injecting stabilized SP-SAP (SSP-SAP) unilaterally into the NTS. We assessed changes in immunoreactivity (IR) of NMDA receptor subunit 1 (NMDAR1), AMPA receptor subunit 2 (GluR2), and 3 types of vesicular glutamate transporters (VGluT) as well as IR of gamma-aminobutyric acid receptors type b (GABAb), neuronal nitric oxide synthase (nNOS), tyrosine hydroxylase (TH), and protein gene product 9.5 (PGP 9.5), a neuronal marker, in the NTS. When compared to that of the same section of the un-injected NTS, IR decreased significantly in the injected side for NMDAR1 ($p < 0.01$), GluR2 ($p < 0.01$), VGluT3 ($p < 0.01$), GABAb ($p < 0.001$), and PGP9.5 ($p < 0.001$). In contrast, IR for VGluT1 ($p < 0.001$), VGluT2 ($p < 0.001$), nNOS ($p < 0.001$), and TH ($p < 0.001$) increased significantly. We conclude that pathologic effects following ablation of neurons with NK1 receptors in NTS may result from interruption of neurotransmission through other neurochemical systems associated with NK1 receptors-containing neurons.

Keywords

Confocal microscopy; glutamate; fluorescent immunohistochemistry; neurokinin-1 receptor; nucleus tractus solitarii; substance P conjugated saporin

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1. Introduction

Previous studies have suggested that substance P (SP) is integral to the baroreflex and is released from baroreceptor afferent nerve fibers in the NTS (Helke and Seagard, 2004). Our own studies showing that injection of SP into NTS did not produce cardiovascular effects (Talman et al., 1980) supported our view that SP was not integral to baroreflex transmission in NTS. However, in a recent report we showed that injection of a conjugate of substance P or stabilized substance P and the ribosome-inactivating protein saporin (SP-SAP or SSP-SAP respectively) into the rat nucleus tractus solitarius (NTS) to kill cells that express neurokinin 1 (NK1) receptors attenuated baroreflex responses, decreased baseline baroreflex sensitivity, and increased lability of arterial pressure (Riley et al., 2002; Abdala et al., 2006; Potts et al., 2007; Nayate et al., 2009). Mechanisms for these effects are not clear, but some have suggested glutamate receptors that colocalize with NK1 receptors in NTS neurons play a role (Potts et al., 2007; Nayate et al., 2009). Previously, we showed that NK1 receptors were present in 53% to 75% of neurons with N-methyl-D-aspartic acid (NMDA) receptors and 46% to 74% of neurons with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in different NTS subnuclei (Lin et al., 2008). Therefore, we hypothesized that ablation of NK1 receptor-containing cells would similarly decrease NMDA receptor-containing neurons and AMPA receptor-containing neurons in the NTS and thus could interrupt glutamatergic mediation of cardiovascular baroreflexes. To test this hypothesis, we performed fluorescent immunohistochemistry for NMDA and AMPA receptors in brain stem sections after injection of SSP-SAP into rat NTS. Because vesicular glutamate transporters (VGluTs) have been shown to be markers for glutamatergic system, we also performed fluorescent immunostaining for vesicular glutamate transporters (VGluT) type 1, type 2 and type 3 (VGluT1, VGluT2, VGluT3) to see if they are affected by SSP-SAP injection. In addition, because NK1 receptors are expressed in GABAergic NTS neurons (Bailey et al., 2004) and that a decrease in glutamic acid decarboxylase in the NTS was demonstrated after SP-SAP injection (Potts et al., 2007), we also examined receptor type b (GABAb) in the NTS after SSP-SAP injection. Neuronal nitric oxide synthase (nNOS) is an important enzyme that synthesizes nitric oxide and plays an important role in cardiovascular control in the NTS (Ruggiero et al., 1996; Ogawa et al., 1995). Our previous studies showed that it colocalizes with both NMDA and AMPA receptors (Lin and Talman, 2000; Lin and Talman, 2001; Lin and Talman, 2002). Therefore, it would be interesting to see if it is affected by SSP-SAP injected into the NTS. We also examined tyrosine hydroxylase (TH) in the NTS because it was decreased in the locus coeruleus after SP-SAP has been injected to this area (de Carvalho et al., 2010). Finally, we also performed immunostaining for protein gene product 9.5 (PGP9.5, a neuronal marker) to if overall neuronal staining is affected by killing NK1 receptors containing neurons with SSP-SAP. We examined fluorescent immuno-labeled NTS sections with a confocal microscope and performed image analyses to quantify changes in immunoreactivity of the neurochemical markers after SSP-SAP injection.

2. Material and methods

2.1. Animals and injections

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 experiencing pain or distress.

Adult male Sprague Dawley rats (275 – 340g, n = 19) were anesthetized with isoflurane (5% induction and 1.5–2.0 % maintenance) delivered in 100% O₂ (2 L/min) by a nasal mask. The dorsal surface of the brain stem was exposed as previously described (Talman, 1989), and a glass micropipette filled with SSP-SAP was stereotactically placed (0.4 mm rostral to the calamus scriptorius, 0.5 mm from the midline, and 0.5 mm below the surface of the brain stem) unilaterally into the dorsolateral and medial subnuclear regions of the NTS at the level of the area postrema. The diameter of the glass micropipette was 20 – 25 microns. Injections (individual increments of 25 – 50 nl to a combined total 9 ng SSP-SAP in 200 nl) were made over 15 minutes. The pipette was left in place for 15 additional minutes to limit efflux of injectate from the pipette track. Surgical wounds were closed, hemostasis assured, the animal treated with buprenorphine (0.05 mg/kg), and anesthesia stopped. After recovery from anesthesia the animal was returned to the animal care facility until it was brought to the laboratory to be euthanized 7 days later. Procedures for euthanasia and perfusion fixation of tissues have been described in our earlier publications (Lin and Talman, 2005; Lin and Talman, 2006; Lin et al., 2007). After euthanasia, the brain was removed, postfixed in 4% paraformaldehyde for 2 hr and then cryoprotected for 2 days in 30% sucrose in PBS at 4° C. Frozen 20 µm coronal sections were cut with a cryostat and processed for immunofluorescent staining as described below.

2.2. Immunofluorescent staining

Procedures similar to those described in our previous publications (Lin and Talman, 2005; Lin and Talman, 2006; Lin et al., 2007) were used for immunofluorescent staining of brain stem sections, which were incubated in a primary antibody (see Table 1 for sources and dilutions of antibodies) in 10% donkey normal serum for 24 hr in a humid chamber at 25° C. We then washed the sections with PBS followed by incubation with fluorophore conjugated secondary antibody made in donkey (1:200, Jackson ImmunoResearch Labs, USA) and carried in PBS for 20–24 hr at 4°C. Stained sections were washed and mounted with Prolong Gold Antifade Reagents (Invitrogen-Molecular Probes, USA). In some cases, to reduce the number of animals needed, immunofluorescent staining for multiple antibodies was performed in the same sections according to methods described in our previous publications (Lin and Talman, 2000; Lin and Talman, 2002; Lin et al., 2008). In these cases, primary antibodies that were raised in different species were mixed in incubation medium and appropriate fluorescent secondary antibodies made in donkey against respective primary antibodies were used. The specificity of NMDAR1 (for NMDA receptors), GluR2 (for AMPA receptors), nNOS, VGluT1, VGluT2, VGluT3, nNOS, PGP9.5 antibodies have been tested (see Table 1 for references). Previous studies have demonstrated minimal cross reactivity with other receptors. We have used these antibodies and reported satisfactory results previously (Lin et al., 2004; Lin and Talman, 2005; Lin et al., 2007; Lin et al., 2008; Lin et al., 2010). The staining pattern obtained with GABAb receptor antibody has been reported to correspond to the pattern described using *in situ* hybridization with probes to rat GABAb receptor mRNA (information provided by Millipore). Western blot analysis showed that TH antibody labeled a single band at approximately 62kDa, corresponding to the molecular weight of TH (Nunez et al., 2007). In addition, we included negative controls which consisted of tissue processed in the absence of primary antibodies. We saw no staining with any of the negative controls. Images for negative controls for nNOS (Lin et al., 2000), NMDAR1 (Lin and Talman, 2000), GluR2 (Lin et al., 2008), VGluT3 (Lin and Talman, 2005) can be found in our previous publications. Images for negative controls for VGluT1, VGluT2, PGP9.5, GABAb and TH were like those for nNOS, NMDAR1, GluR2 and VGluT3, they showed no staining.

2.3. Confocal laser scanning microscopy

As previously described (Lin and Talman, 2006; Lin et al., 2007) we analyzed labeled sections with a Zeiss LSM 510 or a Zeiss LSM 710 confocal laser scanning microscope. Confocal images were obtained and processed with software provided with each microscope. An image editing program, Microsoft PowerPoint (2003), was used to create montages; however, we digitally adjusted no variables other than image contrast and brightness.

2.4. Data analysis

We quantified immunoreactivity in the NTS with the NIH ImageJ software, a public domain program available from the National Institutes of Health (<http://rsb.info.nih.gov/ij/>). The IR of each neurochemical marker we analyzed included all IR observed in cells and processes in the NTS and normalized by the NTS area selected for analysis and was measured as gray value. The un-injected side of each NTS section served as an intra-animal and intra-section control for the side injected with SSP-SAP. IR of all neurochemical markers in the un-injected NTS did not differ from that in the NTS from unoperated normal rats or PBS injected rats. We used 2–3 sections of the NTS within 200 μ m of the center of the injection site from 5–7 rats for each type of immunostaining analysis. Data were converted to percentage of control and were expressed as mean $\pm$ standard deviation. Two tailed T-test was used to determine if IR of a neurochemical marker was significantly altered by SSP-SAP injection for each neurochemical.

3. Results

We observed a decrease in IR for some neurochemical markers and an increase in IR for other neurochemical markers after SSP-SAP injection in the NTS as described below.

3.1. Neurochemical markers that showed decreased IR

3.1.1. NMDAR1—As we reported previously (Lin and Talman, 2002) NMDAR1-IR was seen in both fibers and cell bodies in the NTS (Lin and Talman, 2002). Injection of SSP-SAP caused a significant decrease ($p < 0.01$, Fig. 1 and 3) in NMDAR1-IR from a gray value of 61.4 ± 3.5 in the control side to 50.3 ± 6.0 in the injected NTS. We also noted a decrease in NMDAR1-IR in the DMV and dorsal part of the hypoglossal nucleus in some animals (Fig. 1). Because it was not the focus of this study we did not quantify this change in the DMV and hypoglossal nucleus.

3.1.2. GluR2—GluR2-IR was present both in cells and fibers in the NTS, as reported earlier (Lin et al., 2008). GluR2-IR in the control side NTS had a gray value of 52.8 ± 21.1 , while SSP-SAP injected side had a gray value of 26.7 ± 8.7 . SSP-SAP injection caused a significant decrease in GluR2-IR in the NTS ($p < 0.001$) when compared to that of the control side (Fig. 1 and 3). We also noted a decrease in GluR2-IR in the DMV and dorsal part of the hypoglossal nucleus in some animals but again we did not perform quantitative image analysis (Fig. 1).

3.1.3. VGluT3—VGluT3-IR was seen both in cells and fibers in the NTS, but the majority of VGluT3-IR represented staining of cell bodies as we reported previously (Lin and Talman, 2005). The injected NTS showed a noticeable decrease in VGluT3-IR when compared to that of the control side (Fig. 1 and 3). Image analysis showed that VGluT3-IR of the injection of SSP-SAP significantly decreased ($p < 0.01$) from the gray value of 79.2 ± 7.2 in the control side to 55.4 ± 13.8 in the injected side. A decrease in VGluT3-IR was also seen in the DMV and the dorsal part of the hypoglossal nucleus in some animals (Fig. 1).

3.1.4. PGP9.5—PGP9.5-IR in the NTS was observed in cells and fibers. In the NTS that had been injected with SSP-SAP, we noted a decrease in PGP9.5-IR when compared to that in the control side (Fig. 1 and 3). Image analysis showed that PGP9.5-IR of injected NTS decreased significantly ($p < 0.001$) from the gray value of 85.3 ± 5.7 in the control side to 66.0 ± 4.3 in the injected side. We also observed, but did not quantify, a reduction in PGP9.5-IR in the dorsal motor vagus nucleus and the dorsal part of the hypoglossal nucleus in some animals.

3.1.5. GABAb receptor—We observed IR for GABAb receptors in cells and fibers in the NTS, DMV and hypoglossal nucleus (Fig. 1). After SSP-SAP injection, we noted a significant ($p < 0.001$) decrease in GABAb receptor-IR in the injected NTS (gray value = 52.2 ± 7.5) when compared to that of the control side (gray value = 73.6 ± 5.1) (Fig. 1 and 3). A decrease in GABAb receptor-IR was also seen in the DMV and dorsal part of the hypoglossal nucleus in some animals (Fig. 1).

3.2. Neurochemical markers that showed increased IR

3.2.1. TH—We observed numerous neurons and fibers that were positive for TH in the NTS. After SSP-SAP injection, there was a marked increase in TH-IR in the NTS when compared with that of the un-injected control side (Fig. 2 and 3). The increase in TH-IR appeared to be due to an increase in numbers of immunoreactive neurons and fibers; however, the increase in density of TH-IR fibers often made it difficult to identify boundaries of neuronal structures. Image analysis showed that TH-IR increased significantly ($p < 0.001$) from a gray value of 18.9 ± 3.9 in the control side to 39.1 ± 9.0 in the injected side. We also noted an increase in cells and fibers positive for TH-IR in the DMV (Fig. 2), but we did not appreciate an increase in TH-IR in the hypoglossal nucleus.

3.2.2. nNOS—The patterns of nNOS-IR in cells and fibers in the NTS were similar to those in our previous publications (Lin and Talman, 2002; Lin et al., 2007; Lin, 2009). We noted an increase in nNOS-IR in the NTS after SSP-SAP injection (Fig. 2). This increase was mostly due to an increase in fibers that were positive for nNOS-IR. There appeared to be a decrease in number of cells positive for nNOS-IR, but the high density of nNOS-IR positive fibers after SSP-SAP injection often made identification of neuronal boundaries difficult and thus precluded accurate analysis of changes in cell numbers. Image analysis showed that nNOS-IR in the NTS increased significantly ($p < 0.001$, Fig. 3) from a gray value of 55.9 ± 14.4 in the control side to 81.9 ± 22.0 in the injected side. An increase in number of nNOS-IR positive fibers was observed in the DMV ipsilateral to the injection in some rats, but a decrease was found in others. A decrease in nNOS-positive neurons was noted in the ipsilateral DMV in most rats. We did not observe any changes in nNOS-IR in the hypoglossal nucleus ipsilateral to the injection.

3.2.3. VGluT2—As reported in our previous publication (Lin et al., 2004), VGluT2-IR was present only in fibers in the NTS. Following injection of SSP-SAP we observed a dramatic increase in VGluT2-IR in the NTS (Fig. 1), DMV and dorsal portion of hypoglossal nucleus (Fig. 1). An increase in fibers that were positive for VGluT2-IR accounted entirely for the increase in IR. VGluT2-IR of the injected NTS increased significantly ($p < 0.001$, Fig. 3) from a gray value of 41.4 ± 6.2 in the control side to 104.3 ± 14.2 in the SSP-SAP injected side.

3.2.4. VGluT1—Like VGluT2-IR, VGluT1-IR was present only in fibers in the NTS with a distribution that differed from that of VGluT2-IR (Lin et al., 2004). The majority of fibers containing VGluT1-IR were found in the lateral NTS. Following SSP-SAP injection, there was an increase in VGluT1-IR (Fig. 2) in the NTS when compared with that of the control

side. This increase in VGluT1-IR was entirely due to an increase in numbers of fibers that were positive for VGluT1. When we examined the middle part of the NTS with higher magnification, we noted that this part of the NTS which normally contains few fibers that are positive for VGluT1-IR, showed many fibers that were positive for VGluT1-IR (Fig. 2). Similarly, we noted an increase in VGluT1-IR positive fibers in the DMV and the dorsal portion of the hypoglossal nucleus in some animals when examined at high magnifications. Image analysis of the NTS showed that SSP-SAP injection increased VGluT1-IR significantly ($p < 0.001$, Fig. 3) from a gray value of 32.1 ± 11.6 in the control side to 71.1 ± 21.3 in the control side.

4. Discussion

The results of the present study support our hypothesis that ablation of neurons expressing NK1 receptors likewise decreases neurons expressing glutamate receptors in the NTS. Thus, SSP-SAP injection decreased IR both for NMDA receptors and AMPA receptors in the NTS. The study also shows that SSP-SAP in the NTS causes differential changes in VGluTs with a decrease in VGluT3-IR but increases in VGluT1-IR and VGluT2-IR, both found in fibers in the NTS. Injection of SSP-SAP also resulted in decreased GABA_B receptor-IR and PGP9.5-IR in the NTS while causing an increase in TH-IR and nNOS-IR in the NTS.

SAP conjugates that bind to SP receptors have been used to selectively kill neurons that express NK1 receptors in many studies in brain and spinal cord (Mantyh et al., 1997; Wiley and Kline IV, 2000; Wang et al., 2002; Wiley et al., 2007; Wilkinson et al., 2011). Injection of SP-SAP interferes with nociception in the spinal cord where SP plays a pivotal role in transmission of signals produced by highly noxious stimuli and in maintenance of hyperalgesia (Mantyh et al., 1997). In the NTS, it blocks baroreflex responses, increases lability of blood pressure and causes sudden death (Riley et al., 2002; Abdala et al., 2006; Potts et al., 2007; Nayate et al., 2009). These effects of SP-SAP have been attributed largely to the ability of SP-SAP to bind specifically to NK1 receptors containing neurons and eliminate them (Wiley and Lappi, 1997) while sparing other types of neurons. However, because NK1 receptors colocalize with other neurochemicals in some NTS neurons (Bailey et al., 2004; Lin et al., 2008), killing neurons containing NK1 receptors would also result in a decrease in those neurons that contain other neurochemicals. Indeed, this is the case of GABAergic neurons in the NTS. Following SP-SAP injection, a notable reduction in the number of glutamic acid decarboxylase immunoreactive soma and terminals was found in the NTS (Potts et al., 2007). Consistent with results of that study, we observed a decrease in IR of GABA_B receptors in the NTS after SSP-SAP injection, supporting there being a disturbance of GABAergic transmission as a result of SSP-SAP administration. Furthermore, as predicted, we found a significant decrease in NMDA receptors and AMPA receptors after SP-SAP injection. Therefore, Simultaneous loss of neurokininergic neurons/ GABAergic neurons and/or neurokininergic/glutamatergic neurons may contribute to baroreflex impairment and labile blood pressure after SP-SAP injection into the NTS, as suggested from results of earlier studies (Potts et al., 2007; Lin et al., 2008; Nayate et al., 2009). However, though a link between GABA and substance P has been implicated as a mechanism for modulating baroreflex activity seen with somatosensory afferent stimulation (Potts et al., 2007), evidence suggests that GABA is not integral to transmission of baroreflex signals from baroreceptor axon terminals in NTS (Talman et al., 1980). Similarly, although SSP-SAP induced impairment of baroreflex is largely attributed to a loss of neurons that express NK1 receptors (Riley et al., 2002; Abdala et al., 2006; Potts et al., 2007; Nayate et al., 2009), it has been suggested substance P may not be an integral part of baroreflex (Talman and Reis, 1981) and it solely serves a modulatory role in primary baroreflex neurons or in interneurons that modulate baroreflex function under behavioral conditions such as the defense response (Comet et al., 2005).

The extent of disturbance in the glutamatergic system in the NTS by SSP-SAP may not be limited to NMDA receptors and AMPA receptors. We also noticed a significant decrease in VGluT3 containing neurons and fibers in the NTS. Unlike VGluT1 and VGluT2, which are found only in neuronal processes in the NTS (Lin et al., 2004), VGluT3 is present in cells and processes in the NTS, although its function in the NTS is not clear (Lin and Talman, 2005). We do not know if a decrease in VGluT3-IR by SSP-SAP is due to colocalization of VGluT3 and NK1 receptors in the NTS. However, our earlier studies have shown that more than 80% of nNOS containing neurons in the NTS contained VGluT3 (Lin and Talman, 2005) and that all nNOS containing neurons contain NMDA receptors and AMPA receptors (Lin and Talman, 2000; Lin and Talman, 2002). Therefore, it is probable that VGluT3 colocalizes with NMDA receptors and AMPA receptors in some NTS neurons. Hence, the observed decrease in VGluT3-IR positive cells after SSP-SAP injection could correlate with the decrease in NTS neurons containing NMDA receptors and AMPA receptors. Furthermore, VGluT3 has been found to be expressed in cells containing glutamic acid decarboxylase in the stratum radiatum of the hippocampus (Freneau et al., 2002). It is not known if VGluT3 and glutamic acid decarboxylase colocalize in NTS neurons, however, if they do, then a decrease in VGluT3-IR could also be related to SSP-SAP induced reduction in glutamic acid decarboxylase containing neurons.

VGluT1 and VGluT2 are two known markers of terminals that use glutamate as a transmitter (Kaneko and Fujiyama, 2002) and are present in the NTS in a complementary manner (Lin et al., 2004). In this study, we found that both VGluT1-IR and VGluT2-IR were increased after SSP-SAP injection. Increased expression of VGluTs has been demonstrated in a number of disease models associated with excitotoxicity. VGluT1 was increased in hippocampus of patients with temporal lobe epilepsy (van der Hel et al., 2009) and in rat hippocampus after hyperthermic seizures (Kwak et al., 2008), and increased expression of VGluT2 was noted in the cortex in a rat absence seizure model (Touret et al., 2007). We do not know what causes the increased expression of VGluT1 and VGluT2 in the SSP-SAP model reported here, but it is possible that the increased expression represented plasticity and compensatory responses in the axonal terminals as a result of decreases in NMDA and AMPA receptor-containing neurons in the NTS. In fact, others have shown that central axotomy leads to increased VGluT2 expression in axon terminals (Aymerich et al., 2006). The increased levels of VGluT protein may augment glutamate release (Wojcik et al., 2004; Moechars et al., 2006), an effect that could lead to excitotoxic neurodegeneration (Daniels et al., 2011). Thus, both direct and indirect effects of SSP-SAP in NTS could further contribute to impaired glutamatergic transmission and result in altered baroreflex function independent of peptidergic transmission associated with SP.

Previous studies reported that SSP-SAP injection did not change the total number of cells in the NTS when sections were examined with non-specific cell or nuclear stains (Riley et al., 2002; Abdala et al., 2006; Wilkinson et al., 2011). Our results showed that IR of PGP9.5, a neuron-specific marker, in the injected NTS decreased to $77.6 \pm 5.6\%$ of the control NTS. The difference between our findings and those made by others could be explained by our having directly assessed neuronal loss in a region with many other non-neuronal cell types. Thus, by assessing the loss of cells from all cell groups, one might well miss changes in a given subpopulation of cells within the group. Our results would be consistent with a reduction of the total number of neurons without a significant reduction in the total number of cells as a result of killing NK1 receptor-containing neurons.

As mentioned above, almost all nNOS containing neurons in the NTS contain NMDA receptors or AMPA receptors (Lin and Talman, 2000; Lin and Talman, 2002). Because there was a decrease in NMDAR1-IR receptor-containing neurons and a decrease in GluR2-IR receptor-containing neurons in the NTS after SSP-SAP injection, we would have expected

decreased nNOS-IR in the NTS. To our surprise, we saw a significant increase in nNOS-IR after SSP-SAP injection in the NTS. However, we found that this increase was due to an increase in fibers that were positive for nNOS-IR while there was the anticipated decrease in the number of nNOS-IR positive cells. Many studies suggest an interaction between nitric oxide (NO) and glutamate in cardiovascular regulation in the NTS (for review: see (Talman and Lin, 2006; Lin, 2009)). Thus, our finding increased nNOS-IR may be fully consistent with our also finding increased VGluT1-IR and VGluT2-IR in NTS. Because NO release causes glutamate receptor activation in the NTS (Di Paola et al., 1991), the increase in nNOS-IR positive fibers, like the increase in VGluT2, could be the result of a compensatory effect in axonal terminals in response to the decreases in NMDA receptors and AMPA receptors after SSP-SAP injection. In that both nNOS and VGluT's are found in baroreceptor nodose ganglion neurons that project to NTS (Lin and Talman, 2005), the simultaneous increase in both markers in NTS nerve fibers is consistent with compensatory effects in those afferent terminals. The increase in nNOS-IR could also be a compensatory effect of an SSP-SAP induced decrease in GABA_B receptors in that NO may potentiate GABA release in the NTS (Wang et al., 2006; Pajolla et al., 2009). On the other hand, the increase in nNOS-IR may be associated with the pathogenesis of neuronal degenerations of NK1 receptor-containing cells, as suggested by studies that showed an association between NOS expression and nerve cell death (Dawson et al., 1992; Clowry, 1993). In that NO or nitrosothiols may play a role in cardiovascular regulation in the NTS (Lewis et al., 1991; Machado and Bonagamba, 1992), the increase in nNOS-IR in the NTS may participate in the physiological and pathological changes caused by SSP-SAP.

This study further showed that there was a marked increase in TH-IR positive cells and fibers in the NTS after SSP-SAP injection. A reduction of TH-IR positive neurons was reported when SP-SAP was injected to the rat locus coeruleus (de Carvalho et al., 2010) and no change was noted when SSP-SAP was injected to the rat striatum (Wiley and Lappi, 1999). Although a decrease in TH-IR in the locus coeruleus by SSP-SAP can be explained by there being NK1 receptors on all TH-IR neurons in the locus coeruleus (Chen et al., 2000), it is hard to explain the increase in TH-IR in the NTS in that none of the neurons positive for NK1 receptor IR colocalize TH-IR (Le, I et al., 2008). Increased TH-IR in the NTS suggests that the catecholaminergic system in NTS was activated after SSP-SAP injection. Further studies are needed to understand the correlation between an increase in TH in the NTS and SSP-SAP injection. However, it is unlikely that changes in NTS catecholamine transmission account for baroreflex changes after toxic destruction of NK1 neurons in that baroreflex function may be preserved after ablation of TH positive neurons in the region (Itoh et al., 1992).

5. Conclusions

The current study demonstrates that application of SSP-SAP in NTS leads to changes in several neuronal markers that would accompany the loss of NK1 neurons as we have previously shown (Riley et al., 2002; Nayate et al., 2009). Baroreflex impairment and increased blood pressure lability induced by SP-SAP could be explained by simultaneous loss of neurokininergic/GABAergic neurons and/or neurokininergic/glutamatergic neurons. However, those changes associated with diminished glutamate transmission (loss of glutamate receptors) likely contribute substantially to baroreflex dysfunction.

Acknowledgments

This work was funded in part by NIH RO1 HL 088090 (to L.H. Lin and W. T. Talman) and by a VA Merit Review (to W. T. Talman).

Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	area postrema
DMV	dorsal motor nucleus of vagus
GluR2	AMPA receptor subunit 2
GABA_b	gamma-aminobutyric acid receptor type b
Gr	gracilus nucleus, neuronal nitric oxide synthase
NK1	neurokinin-1
NMDA	N-methyl-D-aspartic acid
NMDAR1	NMDA receptor subunit 1
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NTS	nucleus tractus solitarii
PBS	phosphate buffered saline
PGP9.5	protein gene product 9.5
SAP	saporin
SP	substance P
SSP-SAP	stabilized substance P-saporin
TH	tyrosine hydroxylase
tr	tractus solitarius
VGluT	vesicular glutamate transporters (VGluT)
VGluT1	vesicular glutamate transporters type 1
VGluT2	vesicular glutamate transporters type 2
VGluT3	vesicular glutamate transporters 3

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Highlights

In addition to decreasing neurons with the NK1 receptor

- SSP-SAP decreases NMDA receptors and AMPA receptors in the NTS.
- SSP-SAP decreases VGluT3 but increases VGluT1 and VGluT2 in the NTS.
- SSP-SAP decreases GABAB receptors and neuronal marker PGP9.5 in the NTS.
- SSP-SAP increases TH and nNOS in the NTS.

Fig. 1.

Pseudo-colored confocal images of the NTS show IR of the neurochemical markers that decrease (downward arrow) after injection of SSP-SAP when compared to that of the control side in the same section. Panel F is the schematic sketch of the NTS at the same rostral-caudal level. AP, area postrema; DMV, dorsal motor nucleus of vagus; tr, tractus solitarius. Scale bar = 100 μ m.

Fig. 2. Pseudo-colored confocal images of the NTS show IR of the neurochemical markers that increase (upward arrow) after injection of SSP-SAP when compared to that of the control side in the same section. Panel E is the schematic sketch of the NTS at the same rostral-caudal level. Panels F and G are higher magnification images of boxed areas in Panel D. Abbreviations are as in Fig. 1. Scale bar = 100 μm.

Fig. 3.

Bar graphs show results of image analysis of IR change in the neurochemical markers in the NTS after SSP-SAP injection. Upper graph shows those that decreased; lower graph shows those that increased after the injection. Data are expressed as percentage of control side. * $p < 0.01$, ** $p < 0.001$.

Table 1

Sources, dilutions and references of primary antibodies

Antibody Name	Host species	Source	Dilution	Reference
TH	Rabbit	Millipore	1:100	(Nunez et al., 2007)
nNOS	Sheep	Dr. P. C. Emson	1:1000	(Herbison et al., 1996)
VGluT1	Rabbit	Dr. R. Edwards	1:1000	(Fremeau et al., 2001)
VGluT2	Rabbit	Dr. R. Edwards	1:1000	(Fremeau et al., 2001)
NMDAR1	Mouse	Millipore	1:100	(Lin and Talman, 2002)
PGP9.5	Rabbit	Millipore	1:100	(Lin et al., 2010)
VGluT3	Guinea pig	Millipore	1:500	(Lin and Talman, 2005)
GluR2	Rabbit	Millipore	1:10	(Lin et al., 2008)
GABAb	Guinea pig	Millipore	1:200	(Waldvogel et al., 2004)