Comparison of baroreceptive to other afferent synaptic transmission to the medial solitary tract nucleus

Michael C. Andresen and James H. Peters
Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, Oregon

Submitted 28 May 2008; accepted in final form 9 September 2008

Andresen MC, Peters JH. Comparison of baroreceptive to other afferent synaptic transmission to the medial solitary tract nucleus. Am J Physiol Heart Circ Physiol 295: H2032–H2042, 2008. First published September 12, 2008; doi:10.1152/ajpheart.00568.2008.—Cranial nerve visceral afferents enter the brain stem to synapse on neurons within the solitary tract nucleus (NTS). The broad heterogeneity of both visceral afferents and NTS neurons makes understanding afferent synaptic transmission particularly challenging. To study a specific subgroup of second-order neurons in NTS, we anterogradely labeled arterial baroreceptor afferents of the aortic depressor nerve (ADN) with lipophilic fluorescent tracer (i.e., ADN/H11001) and measured synaptic responses to solitary tract (ST) activation recorded from dye-identified neurons in medial NTS in horizontal brain stem slices. Every ADN+ NTS neuron received constant-latency ST-evoked excitation from baroreceptor cell bodies in the nodose ganglion, as well as their central terminations of the solitary tract (ST) within caudal NTS (21, 33, 53). These ADN-associated NTS second-order neurons lie within the medial subnucleus and are the first central neurons within the arterial baroreflex pathway that autonomically regulate heart rate on a beat-to-beat basis (5, 62).

Among cranial afferents, the aortic depressor nerve (ADN) has several unique properties that provide an anatomical basis for identification of a specific subset of NTS neurons connected to arterial baroreceptors. The ADN contains only axons from stretch-sensitive primary afferents (aortic baroreceptors) of the aortic arch (35, 68, 69). The nerve trunk of the ADN contains only one functional modality and is thus distinct from other peripheral nerve trunks that contain both mixtures of various afferent modality axons, as well as intermingled efferent axons. Tracer applied to ADN identifies both baroreceptor cell bodies in the nodose ganglion, as well as their central terminations of the solitary tract (ST) within caudal NTS. These ADN-associated NTS second-order neurons lie within the medial subnucleus and are the first central neurons within the arterial baroreflex pathway that autonomically regulate heart rate on a beat-to-beat basis (5, 62).

With the use of tracer-identified NTS neurons (ADN+) in horizontal brain stem slices, our studies found that all ADN+ NTS neurons received ST-evoked excitation from baroreceptor cell bodies and second-order nerve trunk of the ADN, as well as their central terminations of the solitary tract (ST) within caudal NTS. These ADN-associated NTS second-order neurons lie within the medial subnucleus and are the first central neurons within the arterial baroreflex pathway that autonomically regulate heart rate on a beat-to-beat basis (5, 62).

VISERAL AFFERENTS. The IX and Xth cranial nerves, enter the brain at the solitary tract nucleus (NTS) (5). These afferents provide information for vital homeostatic reflexes that coordinate systemic control of cardiovascular, respiratory, and gastrointestinal function, as well as visceral aspects of integrated satiety, body temperature, neuroendocrine, and stress responses (19, 24, 41, 67). These diverse afferents belong to two broad classes, those with myelinated or unmyelinated axons, and each class distinctively expresses characteristic ion channels and receptors (32, 43, 48). Patterns of the distribution of afferent fibers outline a loose viscerotopy (46, 52). However, cellular heterogeneity, even within NTS subregions, is substantial and includes varied afferent sources (e.g., heart, airways, gastrointestinal, etc.), neurotransmitters, interconnections, cellular phenotypes, and projection targets (4, 11, 16, 62, 78). This heterogeneity challenges experimental approaches to better understand central transmission from specific afferents and the cellular basis of viscerosensory integration within the NTS.
Labeling of Central Terminals of Aortic Baroreceptors

For the anatomical identification of NTS neurons receiving ADN central synaptic contacts, we used our procedure for dye labeling and quality control, as described previously (20, 33, 53). Rats were prepared in an initial surgery that placed tracer dye onto a cervical segment of the ADN trunk. Young rats (20–30 days old) were anesthetized with anesthetic cocktail administered intramuscularly (1 ml/kg body wt, 56 mg/ml ketamine, 6 mg/ml xylazine, and 1 mg/ml acepromazine) (27). Using a dissection microscope, the ADN was located and separated from the surrounding tissue 1 cm peripheral to joining the superior laryngeal nerve and the nodose ganglion. We used the lipophilic fluorescent dye fast DiI (Molecular Probes, Eugene, OR). Dye containment was essential to minimize contamination of adjacent nerves and accomplished using a premolded shell that is then sealed in place with fresh dental molding compound (Coltene, Mahwah, NJ) (31, 33). In all cases, a minimum of 5 wk was allowed for transport of dye-containing lysosomes to NTS (42). This in vivo endocytotic mechanism contrasts distinctly from the fates of such dyes in fixed tissue in which diffusion within the plasma membrane slowly disperses the dye (40). Retrograde transport of carboxy cyanines (25) fills the soma and proximal dendrites with fluorescent puncta, and such label can be recognized as intracellular by comparing focal planes within the neuron. Following removal of the NTS slice for electrophysiology, the remaining brain stem was surveyed at the dorsal motor nucleus of the vagus (DMNV) for retrogradely transported dye. NTS slices were rejected from animals in which DMNV showed filled cell bodies. Such retrograde dye indicates dye contamination that reached the peripheral vagal nerve trunk and subsequent retrograde transport along efferent axons. Retrograde vagal labeling meant that anterograde transport along vagal afferents would contaminate ST staining and include a mixture of vagal as well as ADN afferents, so that such slices were rejected from study. An additional group of animals that were equivalent in age (8–16 wk old at the time of death) but without dye surgery were used as naive rats for electrophysiological studies.

Neuron Slice Preparation, Identification, and Recordings

Brains were removed from deeply anesthetized (4% isoflurane) rats and placed in cold (0–2°C) artificial cerebral spinal fluid (aCSF) composed of the following (in mM): 125 NaCl, 3 KCl, 1.2 K2HPO4, 1.2 MgSO4, 25 NaHCO3, 10 dextrose, 2 CaCl2, and bubbled with 95% O2–5% CO2 (pH 7.4). Slices (250 μm) were micromute cut (Leica VT-1000S Leica Microsystems, Bannockburn, IL) in a quasi-horizontal orientation using a sapphire knife (Delaware Diamond Knives, Wilmington, DE). Electrodes (1.8–3.5 MΩ) were filled with a solution composed of the following (in mM): 10 NaCl, 130 potassium-glucanote, 11 EGTA, 1 CaCl2, 2 MgCl2, 10 HEPES, 1.0 Na2ATP, 0.1 NaGTP; pH 7.3, 295 mosM. aCSF used for recording was identical to that used for brain slicing procedures in aCSF. In experiments focused on glutamate release probability, these solutions were modified as described below.

For recording, slices were mounted in the perfusion chamber, and cell bodies medial to the ST and <250 μm from obex were visualized using infrared differential interference contrast (IR DIC) optics (Axioskop FS2+, Zeiss, Oberkochen, Germany). Next, fluorescent images of ADN contacts within NTS neurons at ×400 were captured (Axioskop FS2+, Zeiss) and compared with digitally superimposed real-time IR DIC images. By focusing through the cell and back, only neurons in which dye puncta clearly corresponded to the DIC image of the neuron soma surface or dendrite were considered anatomically identified as AND-contacted neurons (ADN+). In electrophysiological experiments, results were compared between ADN− neurons found in close proximity to ADN+ neurons within NTS. A group of second-order NTS neurons from the equivalent medial subregion was studied with identical methods from slices of naive rats. After locating such neurons, voltage-clamp recordings were made at 32–34°C using a Multiclamp 700B or Axoclamp 2A/B amplifier and pClamp 9 software (Molecular Devices, Union City, CA). No leak subtractions, liquid junction potentials, or series resistance compensations were performed. Input resistance was monitored throughout recordings. Cell recordings were halted if series or input resistance was not stable.

Remote Activation of ST Afferents

A concentric bipolar stimulating electrode (50 μm inner core diameter, 200 μm outer diameter, F. Haer, Bowdoinham, ME) was placed on the visible ST as it coursed rostrally at a distance of up to 5 mm but no less than 1 mm from the recorded neurons. Patterned bursts of five stimulus shocks (0.1-ms duration spaced at 20-ms intervals) were generated with a duty cycle of 3–6 s (Master-8, AMPI, Jerusalem, Israel). After determining the response threshold (see below), most studies were conducted at an intensity set at twice the event threshold. Responses to the burst of five such shocks were designated EPSC1, EPSC2, etc., to indicate the shock position within the sequence.

Latency Variability-Synaptic Jitter

The latency to event onset and its variation across trials are fundamental indicators of the synaptic transmission process. Analysis of latency was based on consideration of the responses to the first shock in the train of five (EPSC1). All events within a 5-ms window of EPSC1 were considered synchronized to the ST shock if they occurred regularly across an aggregate test series of ST shock sequences (generally bursts of five shocks) and had similar waveform. Individual events not meeting these ST-synched criteria (timing and waveform) were considered to be unrelated to ST activation and were excluded from analysis. Variability in latency (jitter) was calculated using EPSC1 as the standard deviation (SD) of latency and served as a critical index of synaptic order. In the present study, only mono-synaptic events were analyzed as judged by EPSC1 jitter of <200 μs (31). Synaptic jitter calculations included at least 30 individual latency values for each neuron. These low-jitter ST-EPSCs typically have low rates of synaptic failure, high amplitudes, and substantial frequency-dependent depression (12, 31).

ST Stimulus Intensity-Recruitment Relations

Each ST axon should be activated to fire in all-or-none fashion and thus have a distinct stimulus threshold. In the horizontal slice, increments in ST shock intensity are initially ineffectual (subthreshold) and then with increased intensity exceed threshold to consistently evoke EPSCs (6, 8, 11, 12). Once the axon threshold is exceeded, conducted action potentials are intensity independent. The all-or-none character of ST intensity-recruitment relations indicates a reliance on activation of single axons impinging on individual neurons (1, 8). For testing, shock intensities were finely graded above and below threshold to establish the threshold value for the onset of reliably evoked responses. Shock intensities up to five times above threshold intensity were tested generally. The appearance of new ST-synched events or changes in shape of synaptic responses at increased intensity indicated recruitment of additional afferent fibers. These additional synaptic events also exhibited discrete stimulus thresholds and latency characteristics but had high jitter (>200 μs, i.e., polysynaptic) events that were not analyzed (8, 12).

Synaptic Pathway Failures

ST shocks sometimes fail to evoke a synaptic response. Serially connected, polyneuronal pathways, in addition to having high-jitter latencies, are particularly prone to synaptic failures (11, 31). Any ST shock that failed to produce an identifiable EPSC within the same 5-ms window used for analysis of the response latencies was counted as a synaptic failure. Failure rates were calculated as a percentage of total number of ST shocks delivered at each of the...
positions (i.e., EPSC1, EPSC2, etc.) within ST shock bursts. A minimum of 30 ST synaptic responses was examined to calculate failure rates.

**CAP Synaptic Blockade**

Following full synaptic characterization of the recorded neurons, the transient receptor potential vanilloid type 1 agonist CAP (100 nM) was tested in a subset of cells. This CAP test took place at the end of the experiment and established the ST axon for a given EPSC was CAP sensitive or CAP resistant. Previous studies of NTS synaptic responses and nodose ganglion neurons (32) have shown that CAP sensitivity of ST afferent axons corresponds to an unmyelinated afferent, and CAP resistance depends on myelinated afferent neurons. No cases of partial blockade of ST-EPSCs were found. Only a single test of CAP was used in a given slice, and, following the CAP test, recordings ceased.

**Drugs**

All drugs were applied by bath perfusion. The blocker 1,2,3,4-tetrahydro-6-nitro-2, 3-dioxo-benzo quinoxaline-7-sulfonamide was obtained from Sigma-RBI (Natick, MA). CAP and tetrodotoxin were obtained from Tocris Cookson (Ballwin, MO) and dissolved in 100 μl DMSO before dilution with external solution to a final concentration of 100 nM. DMSO alone at the highest concentration in external solution had no effect on NTS neurons or synaptic transmission.

**Data Collection and Analysis**

**Variance-mean analysis of ST-EPSCs.** In a limited subset of neurons, variance-mean (V-M) analysis was used to assess the quantal release process for glutamate and compare this characteristic release relationships between ADN+ neurons and neurons from naive rats using methods as previously described (12). ST-EPSC amplitudes were measured as the net peak current (peak current minus the mean baseline current over 10 ms immediately preceding each stimulus shock). V was calculated as the square of SD of 30–80 successive ST-EPSC1 response amplitudes in each condition. V values were not corrected for baseline V, since the SD of the noise generally accounted for <1% of ST-EPSC amplitude in the control condition (12). For each neuron, we calculated M and V during each recording condition for each of ST-EPSC1. Although infrequent, failures were included for all V and M calculations. To modify the release probability, we constructed V-M relations for steady-state ST-EPSCs in 2, 0.5, and 0.25 mM extracellular Ca2+ with twofold Mg2+ replacement of the reduced Ca2+. Data were fit with a least squares regression method for each neuron using the equation: y = K1x + K2x^2 (Origin version 7.5, OriginLab, Northampton, MA). For comparison of V-M relations, we estimated the maximal EPSC amplitude (EPSC_{max}) for each neuron, together with the theoretical constraint at minimal release (0 V at 0 M) to generate individual V-M relations for each neuron using the equation:

\[ \text{V} = \frac{\text{EPSC}_{\text{max}}^2}{\text{M}} \]

**RESULTS**

**Neuroanatomical Identification of Aortic Baroreceptive NTS Neurons**

In rats with ADN tracer implantations, horizontal brain stem slices featured fields of fluorescent puncta medial to the ST and, under high magnification (Fig. 1A), these ADN markings formed characteristic clusters of centrally transported tracer.
dye. Clusters of dye puncta lay in close proximity to the soma membrane of individual neurons, and such dye-positive cells (ADN+) were considered to be anatomically identified second-order baroreceptive NTS neurons (Fig. 1A). Neurons with no colocalized fluorescence were designated ADN−. The relationship of ADN− neurons to ST, however, was unknown. Potentially, ADN− neurons might be other second-order neurons contacted by non-ADN ST afferents, or these cells could be higher order NTS neurons only indirectly or unrelated to ST. Without ADN labeling, such as in naive animals, second-order neurons in the medial subnucleus of NTS could be contacted by ADN or any other afferent reaching this region. These network connective distinctions cannot be resolved anatomically in this preparation and required the functional assessment of synaptic connections from ST.

**ADN+ NTS Neurons Receive Low-jitter ST-EPSCs**

To test whether ADN+ or ADN− neurons were connected to ST afferent pathways, the synaptic responses to electrical shocks to ST were assayed, and synaptic timing and amplitude to ST afferent pathways, the synaptic responses to electrical shock indicated a low synaptic jitter and narrow histogram of latencies to successive stimuli (Fig. 1C). Increasing ST shock intensity failed to alter the amplitude of the observed responses, even using shocks at multiples of the threshold intensity (Fig. 1C). The stimulus intensity recruitment relationship displayed a single distinct ST threshold intensity, and increases in intensity above threshold did not alter the response amplitude or shape (Fig. 1C). This ST stimulus activation profile is consistent with responses activated by a single-afferent input, thought to arise from a single-afferent axon (11, 12). Note that no additional ST afferent contacts were recruited by large shocks (Fig. 1B). Analysis of the latency indicated a low synaptic jitter and narrow histogram of latencies to successive stimuli (Fig. 1C). Increasing ST shock intensity failed to alter the amplitude of the observed responses, even using shocks at multiples of the threshold intensity (Fig. 1C). The stimulus intensity recruitment relationship displayed a single distinct ST threshold intensity, and increases in intensity above threshold did not alter the response amplitude or shape (Fig. 1C). This ST stimulus activation profile is consistent with responses activated by a single-afferent input, thought to arise from a single-afferent axon (11, 12). Note that no additional ST afferent contacts were recruited by large increases in ST stimulus intensity (Fig. 1C). We found no evidence that ADN+ neurons received multiple ST afferent converging on a single neuron. ADN+, tracer-identified NTS neurons had generally similar individual ST response patterns and characteristics to this example.

In their latency and amplitude characteristics, ADN dye-identified NTS neurons were generally quite similar to each other and to ADN− NTS neurons that met the criterion of having ST-EPSCs with <200-μs latency jitter (Fig. 2). Overall, in 45 NTS neurons from ADN-labeled animals, ADN+ and ADN− second-order neurons spanned a wide range of latencies but had limited jitter distributions. The highest jitter value for an ADN+ neuron (n = 32) was 192 μs, and latency-jitter paired values for individual neurons overlapped in their distributions between ADN+ and ADN− (n = 13) second-order ST contacts (Fig. 2). The range of absolute latency values (1.8–9.1 ms, Fig. 2A) in these neurons is surprisingly small, since they were physically intermixed with cell bodies within the medial portion of NTS. Neurons with different latencies were often directly adjacent and within 50 μm of one another. However, there was no systematic difference in mean latency or jitter (5.24 ± 0.27 vs. 4.72 ± 0.28 ms latency or 93.57 ± 7.66 vs. 113.01 ± 15.01 μs jitter, ADN+ and ADN−, respectively, P > 0.05). Likewise, the mean basal EPSC amplitudes and failure rates were equivalent for EPSC1 (Fig. 2B, P > 0.6). The 50-Hz bursts of five ST shocks depressed EPSC amplitudes by ~70% at EPSC5, and ST-EPSC failure rates were similar across labeled and unlabeled neurons (P > 0.2 ADN+ vs. ADN−, Fig. 2B). No anatomically identified ADN+ second-order neurons exceeded 200-μs jitter, suggesting that this electrophysiological criterion is a reliable marker for anatomically monosynaptic ST contacts.

**CAP-sensitive ST-EPSCs Are Weak and Failure Prone**

Applied to the peripheral ADN trunk, the transient receptor potential vanilloid type 1 agonist CAP selectively blocks axonal conduction of unmyelinated action potentials in the compound electroneurogram, while lightly myelinated ADN fibers continue to conduct in the Aδ range (34, 66). In slices, CAP blocks synaptic transmission along CAP-sensitive ST pathways to NTS thought to correspond to unmyelinated peripheral afferent axons (32, 43). In a subset of ADN+ NTS neurons, 100 nm CAP was used to test for blockade of ST-EPSCs (Fig. 3). CAP fully blocked evoked ST-EPSCs within 2–3 min in a subset of all neurons tested (Fig. 3A), and these neurons were classified as CAP sensitive. Note that spontaneous synaptic events remained in these neurons during CAP so that CAP actions are selective for afferent ST synaptic events (Fig. 3A). Blockade of CAP-sensitive ST-EPSCs reversed upon return to control aCSF (Fig. 3A, bottom). In the remainder of the neurons tested, the ST-EPSCs were unaffected (i.e., CAP resistant, Fig. 3B). Neurons with CAP-sensitive ST-EPSCs were considered to be second-order NTS neurons that received unmyelinated, primary visceral ST afferents, i.e., C-fiber axons. Conversely, CAP-resistant neurons were considered to receive myelinated ST afferents, i.e., A-fiber axons. In no case
did CAP partially block ST-EPSCs in sensitive neurons, i.e., produce ST-EPSCs reduced in amplitude but persistent in 100 nM CAP. Such results suggest that individual second-order NTS neurons receive either unmyelinated or myelinated ST afferents, but not both.

Within the group of ADN+ neurons, latencies of CAP-sensitive and CAP-resistant ST-EPSCs varied widely, but their distributions overlapped substantially (Fig. 4A). Although the longest latency ST-EPSC found in ADN+ neurons (Fig. 1, B and C) was CAP sensitive, average latencies and jitters (Fig. 4A) were similar (5.21 ± 0.54 vs. 4.37 ± 0.52 ms latency or 82.07 ± 9.33 vs. 109.15 ± 15.70 μs jitter, CAP sensitive n = 10 and CAP resistant n = 8, respectively, P > 0.05). However, the peak amplitudes of CAP-resistant ST-EPSCs were nearly 50% larger than in CAP-sensitive responses (P < 0.02, Fig. 4B, left). Furthermore, although the ST-EPSCs in all ADN+ neurons depressed to a similar degree (~70%) within the burst of five ST shocks, CAP-resistant ST-EPSCs remained significantly larger than CAP-insensitive responses at all burst positions (EPSC1-EPSC5, P < 0.001, Fig. 4B, left).

One of the most distinctive differences in synaptic performance between CAP-sensitive and CAP-resistant ST-EPSCs was in their failure rates. Careful inspection of the synaptic responses of some ADN+ neurons to bursts of ST shocks (Fig. 1B) showed that the first shock in the burst very rarely failed to release glutamate (i.e., EPSCs were present, Fig. 1B and Fig. 4B, right). EPSC1 failed at similar low basal rates (<1%) in all ADN+ neurons. However, in some neurons, many shocks failed to succeed in evoking an EPSC (Fig. 1B, shocks 2–5). Subsequent CAP tests revealed that CAP-sensitive, ADN+ ST-EPSCs had failure rates that increased >18-fold as the burst of five shocks progressed, while failure rates of CAP-resistant ST-EPSCs did not change within the burst (P < 0.02, Fig. 4B, right). Failures in CAP-sensitive neurons occurred at decreasing rates as the burst progressed, and in all cases the increase in failures occurred concurrently with frequency depression of EPSC amplitude (Fig. 4C). Failure amplitudes were well above detection limits, however, and CAP-resistant events of similarly low amplitudes did not fail, whereas CAP-sensitive
tive EPSCs of similar or greater amplitude failed at high rates (Fig. 4C). This frequency-dependent augmentation of synaptic failures suggests a use-dependent mechanism within the unmyelinated, presynaptic ST transmission process.

To examine whether ST transmission to ADN+ NTS neurons was unique to neurons contacted by baroreceptor afferents, similar tests were conducted in slices from naive animals, i.e., animals without dye implant surgery. In naive animals, second-order NTS neurons were identified electrophysiologically by their ST-EPSC jitter characteristics (<200 μs). Neurons were recorded within the same region as in the ADN-labeled neuron series. Latency-jitter distributions for CAP-sensitive and CAP-resistant ST-EPSCs for second-order NTS neurons (n = 47) from naive animals overlapped (Fig. 5A) and the pattern and range of values were quite similar to those in the ADN+ neurons (Fig. 4A). CAP-sensitive and CAP-resistant ST-EPSCs of unlabeled neurons overlapped in their latency-jitter distributions (Fig. 5A). With the benefit of larger sample sizes (Fig. 5A), the latencies of CAP-sensitive ST-EPSCs were significantly longer than for CAP-resistant responses (5.51 ± 0.35 ms, n = 28 vs. 4.22 ± 0.34 ms, n = 19, P = 0.014). ST-EPSC jitter values were comparable between the two naive groups (10.41 ± 6.00 vs. 8.4 ± 7.60 μs jitter, CAP sensitive and CAP resistant, respectively P > 0.05). Amplitudes of CAP-sensitive ST-EPSCs were substantially smaller than CAP-resistant responses (Fig. 5B, left, P = 0.01). Like the ADN+ neurons, ST-EPSCs failed more often in the CAP-sensitive group as the burst of five ST shocks progressed (Fig. 5B, right, P < 0.001). The general patterns were consistent with those for ADN-labeled neurons, suggesting that the differences identified by CAP were similar for all ST afferents terminating in medial NTS and not peculiar to ADN baroreceptor afferents. Thus high rates of afferent activation induce a use-dependent failure of ST afferent terminals to release glutamate only in the CAP-sensitive, unmyelinated ST inputs.

V-M Analysis Indicates High Release Probability

ST stimulation activates axons that arise from a potentially varied pool of cranial visceral afferents. It is possible that different afferents might have different glutamate release properties at their central terminations. To test this idea, we performed quantal analysis of ST-evoked EPSCs. At a given synapse, the probability of vesicle release (P0), N, and q can be estimated by determining the dependence of EPSC V on M amplitude under conditions that alter release (22, 38, 74). To compare the transmitter release characteristics at second-order NTS neurons, we conducted a V-M analysis of glutamate release for ST-EPSCs from ADN-labeled and naive animals. Within a single, representative ADN+ neuron, the mean amplitude of the ST-EPSC declined as the extracellular Ca2+ concentration was reduced, and the V increased in a characteristic fashion (Fig. 6, A and B). The V-M relationship of the ST-EPSC amplitudes at various Ca2+ conditions for this neuron (Fig. 6C) followed a parabolic model and the estimated probability of glutamate release, P0, was 0.89 in this ADN+ neuron at 2 mM Ca2+.

The V-M curves for three ADN+ neurons indicated similar basic properties for glutamate release onto these second-order neurons and resembled those constructed for second-order neurons from naive animals (Fig. 6D). Overall, the goodness of the model fit averaged 0.988 (n = 9, r2 ranging 0.912–1.000) and with the average of three ADN+ neurons as r2 = 0.997. The q averaged 30.9 ± 11.3 pA (range 17–53 pA) for ADN+ neurons and similarly 26.8 ± 2.3 pA (range 4–32 pA) for second-order neurons from naive animals (P > 0.23), while N, the number of contacts, averaged 17.4 ± 5.1 (range 12–27) sites for ADN+ neurons compared with the similar 22.4 ± 2.3 (range 9–22) sites for second-order neurons from naive animals (P > 0.6). Likewise, the P0 for glutamate was remarkably similar across neurons in the 2.0 mM Ca2+ condition at 0.919 ± 0.023 (range 0.895–0.965) for ADN+ neurons and 0.914 ± 0.003 (range 0.885–0.946) for second-order neurons from naive animals (P > 0.9). In a few cases, neurons survived long enough to be tested with CAP at the end of the V-M experiment. The ADN+ neurons tested (n = 3) were found to be CAP resistant (Fig. 6). A single naive neuron was CAP sensitive, and its V-M profile fell within the error bars of the mean values for the CAP-resistant group. This very preliminary evidence suggests that glutamate release properties may be similar across CAP-sensitive and -resistant afferents. In terms of the function of the glutamate release machinery, transmission to ADN+ neurons was indistinguishable from ST transmission to other second-order NTS neurons.

DISCUSSION

In the present studies, we exploited unique neuroanatomical features of the ADN that permitted study of aortic baroreceptor-innervated, second-order NTS neurons. The ADN DiI label arrives only through aortic baroreceptor axons, and thus the dye in our experiments is transported solely anterogradely to the brain stem (33). In most afferent-containing peripheral
nerve trunks, carbocyanine dyes label a mixture of both central afferent processes (anterograde transport), as well as central efferent processes and cell bodies of projection neurons (retrograde transport along efferent axons). In the case of the vagal nerve trunk, brain stem dye arises anterogradely in the NTS and retrogradely in DMNV neurons (33). In contrast to early anatomical studies in which the ADN was cut for horseradish peroxidase staining (21, 44), lipophilic dye implants did not require disruption of the peripheral axon and thus avoided the potential for injury-induced sprouting and terminal redistribution (37, 58).

Guided by fluorescent ADN+ terminals, we recorded from medial NTS neurons bearing these baroreceptor contacts. Our work discerned five major findings from this large cohort of aortic baroreceptive NTS neurons. First, we found that ST stimulation always evoked synaptic responses, and these ST-EPSCs conformed to monosynaptic jitter criteria (chiefly, <200 μs jitter). Thus these anatomically identified neurons confirmed that the 200-μs criterion is a reasonable electrophysiological discriminator of monosynaptic ST contacts arising from indirect polysynaptic pathways (31). Second, comparisons of synaptic transmission characteristics of ADN+ neurons with ADN− neurons meeting monosynaptic criteria for ST-EPSCs (<200 μs jitter) revealed no differences for these neurons, both within medial NTS, whether unlabeled (ADN−) or from naive animals. Thus mean latency, jitter, and frequency-dependent depression were indistinguishable between separate cohorts of second-order NTS neurons, whether ADN+ and ADN−. In addition, this lack of difference indicated by direct, quantitative synaptic comparisons (ADN+ vs. ADN− and carbocyanine vs. naive) suggests that the presence of carbocyanine tracer in ST afferents (53) does not alter afferent properties (13, 15, 73, 80). Our results from unlabeled second-order neurons were recorded from the same medial NTS subnucleus of naive animals and presumably included neurons that received ADN afferent contacts, but clearly we do not know with certainty for those individual neurons in naive animals. Third, the single threshold in the ST recruitment characteristics of EPSCs to ADN+ neurons indicated dependence on a single-afferent fiber. We found no evidence that higher intensity stimuli recruited additional, directly convergent ST afferent inputs within individual neurons. Similar, single-threshold recruitment relations were found in unlabeled second-order neurons (ADN− and neurons from naive animals). Fourth, quantal release curves described similar, high-release probabilities for glutamate-mediating transmission across ADN+, ADN−, and naive neurons. The V-M relations were consistent with broadly uniform glutamate release mechanisms across ST afferents within medial NTS. Fifth, high frequencies of ST activation generated frequent failures in CAP-sensitive but not in CAP-resistant ST-EPSCs. Such results, in conjunction with previous work in nodose neurons (43, 51), suggest that presynaptic mechanisms are responsible for this use-dependent responses differentiated between myelinated and unmyelinated (C-fiber) afferent axons. Collectively, our findings suggest that afferent-activated pathways through medial NTS may be best defined by the presynaptic afferent contacts onto individual second-order neurons, but that glutamate transmission was remarkably uniform. Afferent myelination phenotype was associated with functional distinctions that are likely to span afferent modalities arising from different peripheral sources. Such results offer a tentative outline of basic principles underlying baroreceptor integration that may apply broadly across afferent information processing within medial NTS. If these presynaptic distinctions between myelinated and unmyelinated afferents found in medial NTS are generalized, then we might speculate and anticipate that such dichotomies will be found in other subregions of NTS.
such as the commissural subnucleus, but currently evidence is lacking.

**Second-order Baroreceptive NTS Neurons Receive Reliable, Low-jitter ST Inputs**

The all-or-none stimulus intensity relations uniformly indicated that ADN+ neurons received single-afferent inputs. Our approach in using stimulus-recruitment profiles in NTS neurons is consistent with criteria widely used to distinguish single-fiber inputs in other central nervous system regions (2, 64, 65). In principle, increments in the stimulus intensity should have at least two possible recruitment effects: 1) addition of new, higher threshold axons, and/or 2) spread of stimulus currents that radiate more widely to activate axons more distant from the stimulation electrode tip. By our experimental design, we have attempted to decrease the likelihood of recruiting non-ST axons by placing our concentric bipolar stimulation electrode on the ST quite distant (1–5 mm) from the recorded cells. Stimulus spread to off-ST axons should be less likely to activate axons making contact with the recorded neurons. As our laboratory reported previously (see Fig. 4 in Ref. 9), moving the stimulation electrode off of the visible ST resulted in either no response or an elevation in threshold shock intensity for evoking the same response, a finding consistent with greater current intensity required to spread from the off-ST site to reach the same axon within the ST. When stimulation electrodes are placed quite close (~150 μm) to NTS neurons in transverse slices (20, 77), shocks commonly recruit both EPSCs and monosynaptic inhibitory postsynaptic currents (IPSCs, i.e., local nonafferent axons) (18, 77).

Single shocks to ST rarely (<1%) failed to activate an ST-EPSC, and this is quite different from excitatory transmission characteristics in many forebrain neurons in which failure rates of ~50% are common (2, 64, 65). The uniformly high reliability of ST-NTS transmission within medial NTS may relate, at least in part, to an unusually homogeneous and high probability of glutamate release. Our quantal analysis revealed that both ADN+ and other second-order NTS neurons averaged >0.91 P_R at 2 mM Ca^{2+}, similar to our laboratory’s previous studies (12). NTS release probabilities are much higher and more uniform across neurons than other central synapses under similar conditions [calyx of Held, P_R = 0.25–0.40 (54); mossy fiber-CA3 interneuron, P_R = 0.34–0.51 (47), or mossy fiber-pyramidal, P_R = 0.20–0.28 (79)]. All ST-NTS release curves had high regression coefficients for parabolic fits to V-M data, a finding consistent with uniform release site characteristics within the activated terminal fields (23). Estimates of active terminal numbers from the V-M analysis are generally consistent with ADN+ terminal staining surrounding single-cell bodies (53) (see Fig. 1). In addition to aortic baroreceptors, the medial subregion of NTS receives afferents from the heart and lungs, as well as subdiaphragmatic organs, (e.g., Refs. 29, 61), and such afferents generally display excitatory synaptic responses that similarly depress substantially to repeated activation. We do not know the staining efficiency of our ADN dye-labeling procedure. However, ADN− and second-order neurons from naive animals in our studies likely receive ST contacts from varied visceral afferents. Thus the general similarities in the present cross-sample (ADN+, ADN−, and naive) results support the conclusion that the basic glutamate transmission properties and general organization of the terminal architectures across these afferents are remarkably similar at second-order neurons within the medial NTS subnucleus.

**Afferent Pathways to Second-order Neurons in Medial NTS: Evidence for Limited Monosynaptic Convergence**

Thin horizontal slices of the brain stem offer optimal experimental access to NTS neurons for intracellular studies of afferent synaptic transmission, but clearly this procedure has the potential to damage or interrupt afferent pathways. Despite this possibility, every ADN+ neuron that we recorded responded with a low-jitter ST-EPSC to ST shocks. Such results suggest that the horizontal plane of sectioning preserves the course of ADN afferents along the ST to second-order neurons, at least over distances of up to 5 mm. If our slicing damages some ST afferent terminations, then we may be underestimating synaptic contacts to these neurons. Nonetheless, the similarity of latency and jitter distributions for ADN+ and other ADN− second-order neurons supports the view that baroreceptive afferent pathways are organized similarly to the ST pathways of the broader population of afferents in medial NTS. Since ours are functional measures, the results suggest that an afferent axon, such as one from an aortic baroreceptor, engages multiple synaptic contacts (17–22 estimated by V-M analysis) to produce EPSCs onto individual second-order neurons and rely on similar pre- and postsynaptic mechanisms. Thus our findings on ADN+ neurons are consistent with the concept that NTS is organized into “clusters, or groups, that receive their main sensory input from one type of vagal afferent,” as suggested for pulmonary afferents of medial NTS (45).

Our laboratory’s previous work in horizontal slices found that single medial NTS neurons tend to receive one direct input (monosynaptic) from a single afferent plus additional indirect, ST-activated polysynaptic inputs (7–11, 31). Here, we found no evidence for monosynaptic convergence from multiple, different afferents onto these single neurons. This in vitro result closely resembles the overall findings of studies of afferent convergence in intact animals. In intact cats and rats, extracellular recordings outlined maps of the path of single-afferent axons to their targets in NTS, and overall such work suggests that afferents commonly divide into collateral axons in a pattern that is quite similar across several afferent subtypes (26, 30). Interestingly, these collaterals of individual afferent axons span considerable rostral-caudal and medial-lateral distances before making close appositions (presumed functional synaptic contacts) with different neurons (46). Anatomically, detailed evidence concerning the distribution of terminations arising from individual afferents within NTS is quite limited. In one study, intracellular labeling of both a single-afferent pulmonary fiber, together with a single NTS neuron, showed that one collateral formed multiple synaptic terminals on a single, ventrolateral NTS neuron (3). If this particular class of pulmonary afferents is broadly typical, then together these anatomical findings plus our electrophysiological studies suggest that cranial visceral afferents may rely on multiple contacts onto single neurons and that single afferents will collateralize to repeat this contact pattern onto multiple neurons. Neither the anatomical nor the electrophysiological approach, however, answers the important questions of whether multiple,
different afferents contact individual NTS neurons or whether these branch maps indicate functional connections. From the perspective of our present findings, our afferent recruitment results in thin slices substantially agree with the anatomical and electrophysiological evidence available from intact animals.

Other approaches have been used to assess convergence from different peripheral afferent nerves onto single NTS neurons in intact animals. In general, this in vivo work has identified rather limited convergence and thus agrees well with our slice conclusions. Maximal shocks to whole peripheral nerve trunks in intact animals most commonly trigger spikes within a narrow time window in poststimulus histograms, and such surveys identified only single-afferent inputs to most single NTS neurons: 249/292 neurons (28), 38/45 neurons (17), 25/28 neurons (59), or 55/56 neurons (55). Convergence from arterial baroreceptors or cardiac mechanoreceptors, for example, was rare, <13% in NTS neurons activated by C-type pulmonary vagal afferents through right atrial injection of phenylbiguanide (60). Likewise, physiological activation of aortic or carotid sinus baroreceptors did not activate NTS neurons activated by cardiac vagal mechanoreceptors in dogs (72). Interestingly, when tested in cases of apparent convergence, responses commonly met monosynaptic criteria for only one nerve, with the remainder of inputs meeting polysynaptic criteria [e.g., carotid sinus and superior laryngeal nerves (55)].

Again, such in vivo results were consistent with our in vitro slice studies of medial NTS that have identified convergent, polysynaptic EPSCs and IPSCs driven by ST inputs, each with unique threshold intensities (8, 9, 11). However, even in thin horizontal slices, multiple (2–3) monosynaptic inputs excite particular phenotypic subsets of NTS neurons, many of which lie outside of the medial subnucleus [e.g., catecholaminergic (8)]. In horizontal NTS slices, multiple polysynaptic inputs are common in second-order medial NTS neurons and include ST-activated, polysynaptic IPSCs and EPSCs (6, 9, 11, 57), as are found in intact brain stem intracellular recordings (56, 75).

Another interesting corollary of afferent convergence is the evidence that unmyelinated and myelinated afferents do not mix at single medial NTS neurons. In our slices, CAP either completely blocked ST-EPSCs or was without effect in all ADN+ neurons, as well as other NTS neurons. This all-or-none CAP dichotomy in ADN+ neurons suggests that myelinated and unmyelinated ST afferents do not converge directly onto single NTS neurons, a finding consistent with a single axon mediating electrically activated ST-EPSCs (11, 32, 43). Again, such segregation of afferent phenotype by myelination has been observed in NTS neurons in vivo. In only 1 case out of 72, NTS neurons that were activated by unmyelinated cardiac vagal afferents also received myelinated inputs, although it is not clear whether all connections were monosynaptic (29).

**CAP-sensitive ST Afferents Fail Synaptically at High Frequencies**

Sensitivity of ST-EPSCs to CAP was all or none and subdivided NTS second-order neurons into two groups, whether ADN+ or ADN− (32). Interestingly, activation of CAP-sensitive ST-EPSCs with 50-Hz bursts of shocks revealed augmenting failure rates as each burst progressed. This form of synaptic failure progression was absent in CAP-resistant neurons, whether ADN+ or not. The mechanism for this failure difference clearly arises from the presynaptic ST afferent component of transmission. Glutamate release properties were quite uniform across afferents and in the small subset of CAP-tested neurons; the variation in individual V-M relations was not correlated with CAP sensitivity. Although further and much more extensive studies of glutamate release in CAP-sensitive and CAP-resistant neurons are necessary to resolve this issue properly, the degree of variation suggests that the basic glutamate release mechanism may be common between the two classes of afferent axons. Rather, it may be that afferent conduction and/or action potential properties of the CAP-sensitive afferents might be responsible for failure differences. Although the mechanism for these use-dependent failures is unclear, the complement of ion channels in nodose neurons differs systematically across the myelinated/unmyelinated classes (50, 70, 71, 76). Unmyelinated cranial visceral afferents have broad action potentials, lower rates of discharge, and frequency-dependent broadening within the nodose soma compared with myelinated afferents (49). CAP sensitivity of nodose and vagal ganglion neurons is found only in C-type, unmyelinated neurons, and neurons with conduction velocities in the myelinated range are uniformly CAP resistant (51). With respect to afferent frequency, the baroreflex effects of electrical activation of ADN C-fibers are nearly maximal at 5 Hz (36). Presently, little is known about the central presynaptic afferent and terminal or how closely peripheral nodose neuron properties represent central portions of these neurons.

**NTS Afferent Processing and Integration**

Our studies suggest that cranial visceral afferents and arterial baroreceptors, in particular, convey a consistently robust excitation to second-order NTS neurons within medial NTS. The mean synaptic currents (200–400 pA, on average) were quite large. Although the CAP-resistant, presumably myelinated inputs were substantially larger than the unmyelinated inputs, the synaptic responses generally generated initial spikes in the postsynaptic NTS neuron 80–90% of the time (6, 8). This synaptic conformation contrasts with the conventional view of central excitatory synaptic transmission in which integration most often occurs distally on relatively remote dendrites and single-fiber inputs may consist of single quanta (64). Thus NTS processing of afferent inputs follows a fundamentally different integration strategy in which basal communication is secured by large primary glutamate signals determined by the presynaptic, i.e., afferent properties. ST excitatory inputs appear to derive predominantly from individual afferents and thus would convey a single modality of information with a high safety factor. Instead of combining multiple small inputs converging from multiple redundant, unreliable sources, e.g., hippocampus (2), medial NTS appears to rely on limited large amplitude but highly reliable inputs. This model of medial NTS integration elevates the potential importance of modulation of the release process at presynaptic terminals, particularly by descending neural inputs, and this theme is supported by examples of powerful presynaptic regulation of cranial visceral afferent glutamate release by peptides: angiotensin, opioids, vasopressin, or cholecystokinin (7, 8, 12, 14, 39, 63).
BARORECEPTORS AND VISCERAL TRANSMISSION IN NTS

GRANTS
This work was supported by National Heart, Lung, and Blood Institute Grants HL-41119 and HL-56460.

DISCLOSURES
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

REFERENCES


