Organization and Properties of GABAergic Neurons in Solitary Tract Nucleus (NTS)

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INTRODUCTION

Within central reflex circuits, the balance between excitation and inhibition determines propagation of signals through neural networks (Abbott and Regehr 2004; Sabatini and Regehr 1999; Semyanov et al. 2004). The solitary tract nucleus (NTS) is a major integrative center for a wide range of homeostatic reflex pathways (Loewy 1990; Saper 2002), and cranial visceral afferents are a primary source of excitation. GABAergic neurons are interspersed throughout the NTS (Fong et al. 2005; Izzo et al. 1992), and some play well-established roles in respiratory regulation (Abbott and Regehr 2004; Sabatini and Regehr 1999). Despite this rich physiological background, less is known about the precise patterns of organization of the relationship between ST afferents and the various potential pathways within NTS (i.e., intr-NTS) that control GABAergic neurons, especially in the absence of anesthetics that broadly facilitate GABA transmission (McDougall et al. 2008).

We hypothesized that most GABAergic NTS neurons would be connected only by indirect pathways from ST afferents. We used a transgenic mouse model that identifies a group of neurons with enhanced green fluorescent protein (EGFP). In this GIN model (GFP-expressing inhibitory neurons), fluorescent protein expression is under the control of promoters for the key synthetic enzyme, glutamic acid decarboxylase-67 (GAD67), and these EGFP-labeled neurons were designated GAD+ (Oliva et al. 2000). This approach allowed us to record from GABAergic inhibitory neurons within NTS in brain slices in which ST activation evokes excitatory and inhibitory responses (Glatzer et al. 2007). In horizontal brain stem slices, latency analysis and ST stimulus intensity recruitment relations indicated that each synaptic event, including ones in which polysynaptic indirect pathways were activated, relied on a single initiating axon with a distinct, all-or-none threshold. Most GAD+ and GAD− neurons were second-order neurons that received one direct, excitatory postsynaptic current (EPSC) from ST plus additional indirect polysynaptically driven EPSCs and inhibitory postsynaptic currents (IPSCs). This synaptic organization plus the presence of high failure rates during repeated activation of IPSCs may critically tune afferent signal propagation at the earliest stages within NTS.

METHODS

GAD-EGFP transgenic mice

An established transgenic mouse GAD-EGFP model was used that is commercially available (strain FVB-Tg(GadGFP)4570Swn/J, Jackson Laboratory) and is homozygous for inserted genes in which the GAD1 (GAD67) gene promoter controls expression of EGFP (Oliva et al. 2000). The EGFP expression in brainstems of these GIN...
mice closely resembles GAD gene expression in rat brain stem (Stornetta and Guyenet 1999), and GAD+ fluorescent neurons are widely distributed across the NTS in this model (Glatzer et al. 2007).

**NTS slices**

Hindbrains of adult male GAD-EGFP mice (6–20 wk old) were prepared as previously described (Appleyard et al. 2005; Doyle et al. 2004). Briefly, quasi-horizontal slices (250 μm thick) that contained the ST in the same plane as the NTS were cut with a sapphire knife (Delaware Diamond Knives, Wilmington, DE) in cold solutions with a vibrating microtome (Leica VT-1000S, Leica Microsystems, Bannockburn, IL). Slices were submerged in a perfusion chamber, and all recordings were performed in artificial cerebral spinal fluid composed of (mM) 125 NaCl, 3 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 10 dextrose, and 2 CaCl₂ bubbled with 95% O₂-5% CO₂ at 34–37°C and pH 7.4. Recording electrodes were filled with a solution composed of (mM) 10 NaCl, 20 KOH, 110 K-glucuronate, 11 EGTA, 1 CaCl₂, 2 MgCl₂, and 10 HEPES; pH 7.32; 295–299 mOsm. We recorded from NTS neurons within 350 μm caudal to obex and medial to the ST. The general pattern and density of EPFG-labeled neurons in and around the NTS in horizontal slices can be observed under low power (Fig. 1, A and B). The presence of epifluorescence identified individual GAD+ neurons under high magnification (Fig. 1, C and D). Patch electrodes, 2.8–4.5 MΩ, were guided to neurons using both fluorescence and differential interference contrast (DIC) optics with infrared light (Zeiss Axioskop FS2). Voltage-clamp recordings were made with a MultiClamp 700B or Axoclamp 2A and pClamp 9 data acquisition software (Axon Instruments, Union City, CA). Signals were sampled at 50-100 kHz and filtered at 5 kHz via an Axon 1325 Digitata A/D converter. Only neurons with stable holding currents <50 pA at Vₑ = −74 mV and stable input resistance ≥150 MΩ were studied further. No leak subtractions or series resistance compensations were performed. The liquid junction potential was calculated to be −13.8 mV, and all reported potentials are corrected.

**Remote activation of ST afferents**

Stimulus shocks were delivered to the ST using a concentric bipolar stimulating electrode (50-μm inner core diam, 200-μm OD, F. Haer, Bowdoinham, ME) placed at a substantial distance (~0.7–2 mm) from the recorded neurons. In some experiments, the stimulating electrode was moved medially ≤200 μm off of the visible ST to test the effectiveness of local stimulus current spread of the electrical shocks. Any cases in which seal resistance, input resistance, or holding current was altered by electrode movement were not included. Patterned bursts of five shocks (50 Hz) were generated with a duty cycle of 3–6 s (Master-8, AMPI, Jerusalem, Israel), with the intensity generally set at twice the event threshold. In some cases (10 GAD+ and 7 GAD−), neurons had no detectable synaptic response even to very high shock intensity, and such neurons were not studied further. Calculations of percentages of neurons do not include such ST-unresponsive neurons (~20% of cells). To enhance the amplitude of evoked IPSCs, some responses were recorded at depolarized holding potentials at which EPSC amplitudes were reduced and driving force for chloride was enhanced.

**Latency variability—synaptic jitter**

Latency is a key measure of the transmission process from activation of an action potential at the site of shock delivery until arrival of the observed postsynaptic response measured in the recorded neuron. Analysis of latency assesses the regularity of the onset of a synaptic event linked in time with the stimulus shock. All events were considered ST-linked if they occurred multiple times across an aggregate test series of >30 ST shock sequences (generally bursts of 5 shocks with responses designated within this sequence as EPSC1, EPSC2, etc.) and within a 5-ms window for similar waveforms. Events not meeting these ST-synchronized criteria (timing and waveform) were considered to be spontaneous events not related to ST activation, and such waveforms were excluded from analysis. Calculated jitter in the EPSC1 latency (SD of latency = synaptic jitter for >30 ST shocks) served as an index of the complexity of the conduction process along the pathway connecting ST afferents to individual NTS neurons (Doyle and Andresen 2001). Direct, monosynaptic afferent contacts were judged on the basis of the first synaptic response in the burst of five shocks and had minimal synaptic jitter, <200 μs. Synaptic event timing and therefore jitter for later events within the burst of five shocks seem to be influenced by activity-dependent processes so that jitter generally increases for later events as the burst proceeds. Because this likely involves circuitry that might include presynaptic actions on the afferent terminals, the mono- versus polysynaptic determination was judged on the basis of EPSC1. Pathways involving multiple neurons and synapses display larger jitters commensurate with timing variations along the path from ST to the recorded neuron. The length of the latency alone, however, has proven to be a poor indicator of pathway complexity in such slices (Bailey et al. 2006a). Synaptic jitter calculations included ≥30 individual latency values for each neuron. In practice, jitter values of highly synchronized ST events (ST-synchronized) were similar even with smaller samples, but high jitter responses varied greatly with smaller sample sizes. A 30-trial minimum represents a value that adequately assesses both low and high jitter responses. NTS neurons with ST-EPSC jitter <200 μs typically had low rates of synaptic failure, high amplitude, and substantial frequency-dependent depression—all hallmark characteristics of monosynaptic afferent transmission to second-order NTS neurons (Bailey et al. 2006b; Doyle and Andresen 2001).
ST stimulus intensity–recruitment relations

Action potentials are triggered and conducted in an all-or-none fashion. Thus graded increments in ST shock intensity ranged from ineffective (subthreshold) to suprathreshold values that consistently evoked synaptic responses. Once the axon threshold is exceeded, conducted action potentials are therefore intensity independent. The all-or-none character of these ST intensity–recruitment relations and the absence of additional synaptic events indicates that these responses rely on activation of single axons impinging on individual neurons (Andersen and Yang 1995; Bailey et al. 2006a,b). For testing, shock intensities were finely graded above and below threshold to establish the value for the onset of reliably evoked responses. Shock intensities ≤10 times above threshold intensity were tested generally. The appearance of new ST-synchronized events or changes in shape of synaptic responses at increased intensity indicated recruitment of additional afferent fibers, and these also exhibited discrete stimulus thresholds and latency characteristics (Bailey et al. 2006b). Events were detected and accepted for analysis as ST evoked using the above criteria. ST-evoked EPSC and IPSC amplitudes were measured as the maximum excursion from preshock baseline.

Synaptic pathway failures

Serially connected, polyneuronal pathways (>200-μs jitter) are particularly prone to synaptic failures (Bailey et al. 2006a; Doyle and Andersen 2001). Any ST shock that failed to produce an identifiable EPSC (or IPSC) within the range of response latencies for that neuron was counted as a synaptic failure. Failures rates were calculated as a percent of total ST shocks delivered at each of the positions (i.e., EPSC1, EPSC2, etc.) within ST shock bursts. For second-order NTS neurons, failure rates for EPSC1 were typically <0.1%. A minimum of 30 ST synaptic responses was examined to calculate failure rates.

Drugs

Gabazine (SR-95531), bicuculline methiodide (BMI), and 1,2,3,4-tetrahydro-6-nitro-2, 3-dioxy-benzo quinoxaline-7-sulfonamide (NBQX) were obtained from Sigma-RBI (Natick, MA).

Statistical testing

Statistical comparisons were made using paired or unpaired Student’s t-test, repeated-measures (RM) ANOVA, one- or two-way ANOVA, and the Bonferroni Dunn test for post hoc analysis, where appropriate (SigmaStat 3.5). All summary data are presented as means ± SE. P < 0.05 indicated significant differences.

RESULTS

Targeting GAD+ and GAD− neurons for whole cell recording

GAD-EGFP–labeled neurons were distributed widely along the rostral–caudal extent of the NTS including regions both medial and lateral to the ST (Fig. 1, A and B). Under high magnification and fluorescence illumination, the cell bodies of individual GAD+ neurons were easily identified for patch electrode placement (Fig. 1, C and D). Similarly, GAD− NTS neurons in such slices could be selected by excluding fluorescent neurons. Note that the stimulating electrode was placed at electrically remote distances from the recorded cells (Fig. 1, A and B), and ST shocks activated large amplitude EPSCs (Figs. 2–4). ST-activated synaptic responses were studied in 56 GAD+ and 29 GAD− NTS neurons from 54 transgenic mice.
sequential test bursts of ST shocks closely overlapped in both time and waveform (representative GAD⁺ neuron in Fig. 2A). ST activation rarely failed to evoke an EPSC (Fig. 2A). The range of absolute ST-EPSC latency values to the first shock in the burst of five was typically <1 ms within each neuron (Fig. 2B). Within ST burst responses, ST-EPSC amplitude declined steeply, a pattern consistent with marked frequency dependent depression (Fig. 2C).

On increasing shock intensity to exceed threshold, ST-EPSC activation appeared abruptly with no discernible, intermediate-amplitude responses, and further increases in shock intensity did not alter EPSC amplitudes (Fig. 2C). Note that the latency was consistent in such neurons across positions within the burst of five shocks (EPSC1–5) and was unaltered by intensity increases (n = 4, P > 0.2). Both latency and jitter, however, increased significantly for all EPSCs compared with EPSC1 in the burst (Fig. 2D; n = 4, P < 0.001). These use-dependent changes in latency and jitter for EPSC2-5 were quantitatively small, dependent on the presence of a preceding response, and unrelated to the stimulus intensity (low = medium = high intensity). Thus monosynaptic ST-EPSCs had low jitter, few failures, prominent frequency dependent depression, and all-or-none stimulus-recruitment characteristics as expected of a single axon with a single stimulus threshold. Incrementing shock intensity failed to alleviate frequency-dependent depression (n = 4, P > 0.6). Together, these afferent recruitment relations show EPSC response characteristics that consistently suggest that ST shocks recruit an individual ST axon that triggers a direct contact on the NTS neuron, i.e., a monosynaptic afferent axon. Means are presented with ±SE.

FIG. 3. Time-variant, unreliable ST-evoked EPSC characteristics identified GAD⁺ neurons with ST-indirect, polysynaptic afferent connections. All panels are from single neuron that showed only this polysynaptic response to ST activation. A: bursts of ST shocks elicited EPSCs and failures (flat lines). Right traces expand display of the 1st event (EPSC1). EPSCs were triggered at variable latency (gray oval, interval region of latency analysis) and sometimes failed (flat lines, slanted arrow). The bursts of 5 ST shocks (vertical arrows) at 50 Hz produced EPSCs with constant amplitude across the burst—i.e., little frequency-dependent depression. B: frequency histogram of latency (37 events) of neuron of A showing large latency variation spanning nearly 3 ms. Latency values centered around 6 ms with a mean synaptic jitter of 478 μs. C: ST intensity–recruitment relation displays single threshold but varied ST-EPSC amplitudes (small squares) unrelated to suprathreshold shock intensity. Failures (filled triangles) are displayed but are not part of amplitude mean calculation (large circles) from individual amplitudes (small squares). Despite polysynaptic origins, ST-indirect pathways had qualitatively similar recruitment profiles (sharp threshold and lack of recruitment above threshold) as ST-direct pathways (see Fig. 2), consistent with reliance on activation of a single afferent axon. Means are presented with ±SE.

FIG. 4. Concentric bipolar electrodes recruit synaptic responses focally with physically discrete shocks. A: photomicrograph shows horizontal brain slice and 2 positions placing the concentric bipolar electrode on the slice surface with respect to NTS and ST corresponding to synaptic results of B and C. Note that, in the ON-ST placement, the stimulating electrode spanned the width of the visible ST (ON-ST, white arrow, top left), whereas, for the OFF-ST placement (OFF-ST, top right), the electrode was directly adjacent to the visible ST (a 200-μm medial displacement from the control ON-ST site). A small white cross marks the recorded GAD⁺ neuron and recording pipette tip. The burst of 5 shocks (50 Hz) evoked similar ST-EPSC responses in all respects (original traces, bottom left and right). B: ST intensity–recruitment relation displays single threshold whether ON-ST (black circles) or just adjacent to ST (OFF-ST1, gray circles). Moving the electrode an additional 100 μm further medial to ST (OFF-ST2, open squares) failed to increment amplitude of the initial EPSC in the train (EPSC1) and for OFF-ST2 location failed to evoke the EPSC at all even though the maximum intensity was 5 times threshold. Lower shock intensities were needed to recruit EPSCs at the ON-ST position than OFF-ST 1. C: histograms of ST-EPSC latencies for ON-ST (black bars) and OFF-ST 1 (gray bars) indicate quite similar variation patterns of latency. Surprisingly, OFF-ST shocks yielded a shorter mean latency (2.26 ms, n = 20) than for ON-ST (2.13 ms, n = 23, P < 0.0002), with similar respective jitters (75 and 102 μs). Means are presented with ±SE.
aptic connection from ST (Bailey et al. 2006b; Doyle et al. 2004). Thus in such low jitter ST-EPSC NTS neurons, we found no evidence for intensity related recruitment of multiple, directly convergent ST afferent axons contacting a single GAD<sup>+</sup> neuron.

**GAD<sup>+</sup> NTS neurons receiving only indirect ST synaptic inputs**

In some GAD<sup>+</sup> neurons (18 of 56), ST shocks evoked only EPSCs with highly variable waveforms and prominent synaptic failures (Fig. 3A; representative GAD<sup>+</sup> neuron). ST-EPSCs in such neurons arrived at variable intervals so that few EPSC traces overlapped at their onset (Fig. 3A). Analysis of >30 individual ST-EPSCs showed a wide range of latencies spanning >2.5 ms, in this case resulting in high values of calculated jitter (Fig. 3B), findings consistent with a polysynaptic connection from ST to the recorded neuron. Despite the variable synaptic event timing, gradual increases in ST shock intensity showed a single discrete activation threshold (Fig. 3C). Increasing ST shock intensity failed to recruit additional synaptic components that were related in time to ST (Fig. 3C). Thus in high-jitter, ST-synchronized EPSCs, increases in shock intensity did not change the distribution of event latencies (P > 0.3). Like stimulus recruitment profiles for the monosynaptic ST-EPSCs, the discrete shock intensity threshold for polysynaptic ST-EPSCs is consistent with activation of a single ST afferent axon that initiates the response. As in the directly coupled pathways to GAD<sup>+</sup> neurons, large increases in shock intensity did not alter the synaptic waveforms or rates of synaptic failures from such high jitter, ST-dependent pathways (Fig. 3C). This performance profile for high jitter ST-EPSCs indicates ST-activated polysynaptic intra-NTS pathways that serially link to GAD<sup>+</sup> NTS neurons but with a singular threshold consistent with reliance on activation of a single ST afferent axon (Bailey et al. 2006a,b; Doyle et al. 2004).

**ST shock effectiveness is highly localized**

The large range of ST stimulus shock currents produced surprisingly discrete and reproducible shock thresholds for consistent response waveforms for the evoked synaptic currents. A concern with high stimulus intensities is the potential for current spread and activation of non-ST axons that might reach the recorded neuron. To better assess the effectiveness of current spread, the stimulating electrode was moved during some recordings to test whether changing the region of electrode contact would alter the synaptic response in its shock threshold or activate different inputs to a single recorded neuron. Initially, the stimulus-response relation for ST-EPSCs in a given neuron was established with the stimulus electrode positioned on the visible ST (on-ST; Fig. 4A, top left, and B, black circles). The stimulus electrode was moved medially just off the ST by a 200-μm displacement from the control on-ST site. This moved the electrode the equivalent of one electrode diameter (off-ST; Fig. 4B). Note that the new position was always closer to the recorded neuron and thus might be more likely to recruit non-ST axons that contacted the recorded neuron (off-ST; Fig. 4B). No changes in seal resistance, input resistance, or holding current were observed following movement of the stimulus electrode. The stimulus recruitment relationship was retested. Two patterns of synaptic response were observed for off-ST stimulation. In many cases (n = 10), while repeating the incremental stimulus-response tests in the same neuron, no synaptic responses were evoked by off-ST shocks at intensities near the on-ST threshold value. However, increasing intensity further sometimes triggered an ST-EPSC that was indistinguishable from the on-ST response in jitter and waveform but often differed slightly in latency (Fig. 4A, right). The off-ST threshold intensity was larger in these new relationships than the original (Fig. 4B, gray circles), a finding that is consistent with current spread to the original on-ST electrode location. Increasing the medial displacement of the stimulating electrode further away from the ST (more than ~300-μm medial displacement from the control on-ST site, n = 5) resulted in no ST-evoked responses regardless of intensity (Fig. 4B, open squares). Moving the electrode slightly closer resulted in restoration of ST-EPSC responses albeit at higher than on-ST intensities (Fig. 4B). Although small shifts in latency from on-ST responses (positive and negative; Fig. 4C) were observed individually, average timing, latency, and jitter values were not different (P > 0.05) between on-ST and off-ST activation of EPSCs to these neurons. These results suggest that stimulus current can spread to reach axons and, of particular interest, such shocks recruit only what seems to be the original axon activated by the on-ST electrode location. The relatively short distances from the ST (<200 μm) over which off-ST shocks can recruit responses suggests that the concentric bipolar electrodes deliver very focal stimulus currents.

**GAD<sup>-</sup> and GAD<sup>+</sup> NTS neurons are similarly ST coupled**

To test whether unlabeled NTS neurons had different ST synaptic characteristics than neurons expressing EGFP, we sampled adjacent, nonfluorescent cells and tested responses to ST activation. The latency and jitter characteristics of ST afferent-evoked synaptic responses were remarkably similar in all respects between GAD<sup>+</sup> and unlabeled, i.e., GAD<sup>-</sup>, medial NTS neurons. The distributions of latencies and jitters for neurons directly and indirectly coupled to ST ranged widely (2 to >9 ms) but overlapped with and across the groups of GAD<sup>+</sup> and GAD<sup>-</sup> neurons (Fig. 5). Note that some indirectly ST-coupled EPSCs (jitter > 200 μs) occurred at earlier latencies than many EPSCs evoked via low jitter ST connections (Fig. 5). Overall, similarly large proportions (~70%) of GAD<sup>+</sup> or GAD<sup>-</sup> neurons seemed to be monosynaptically coupled to ST axons (38 of 56 and 20 of 29, respectively). Latencies for ST-direct EPSCs were similar between GAD<sup>+</sup> and GAD<sup>-</sup> NTS neurons (4.41 ± 0.24 and 4.78 ± 0.30 ms, respectively, P = 0.46), and jitters were also similar (104.6 ± 6.78 and 116.33 ± 10.44 μs, respectively, P = 0.32). Polysynaptic, ST-EPSC latencies (6.7 ± 0.46 and 6.91 ± 0.69 ms for GAD<sup>+</sup> and GAD<sup>-</sup>, respectively) and their associated jitters (977 ± 269 and 1,332 ± 264 μs for GAD<sup>+</sup> and GAD<sup>-</sup>, respectively) were much greater than for direct connections (P < 0.001 GAD<sup>+</sup>, P < 0.001 GAD<sup>-</sup>). Thus the direct and indirect pathways that synaptically couple ST to GABAergic neurons followed patterns that were quantitatively indistinguishable between GAD<sup>+</sup> NTS neurons and adjacent GAD<sup>-</sup> NTS neurons. ST shocks of high intensity commonly evoked ST-
synchronized, polysynaptic IPSCs in addition to the ST-direct EPSCs onto second-order NTS neurons, whether neurons were GAD\(^+\) (16 of 19) or GAD\(^-\) (5 of 11). On average, indirect ST-synchronized IPSCs averaged latency (8.25 ± 0.44 ms) and jitter (951.8 ± 117.4 μs) that were similar to ST-indirect EPSCs (P > 0.05).

Frequency-dependent synaptic depression at GABAergic neurons

ST afferents have extraordinarily high and homogenous glutamate quantal release probabilities, a property that seems to contribute to strong frequency-dependent depression (Bailey et al. 2006b). In neurons with low jitter ST-EPSCs (GAD\(^+\), n = 38; GAD\(^-\), n = 20), the mean response amplitude of the first EPSC within the burst substantially exceeded those evoked in indirectly ST-coupled responses (GAD\(^+\), n = 18; GAD\(^-\), n = 9; P < 0.03; Fig. 6A). Frequency-dependent depression reduced the ST-EPSC amplitude to ~25% of control (Fig. 6A) whether neurons were GAD\(^+\) or GAD\(^-\). The rate of synaptic failures was uniformly much greater in indirectly ST-coupled responses (Fig. 6B). Failures rarely occurred at the first EPSC within ST shock bursts in neurons with direct ST connections (low ST jitter, GAD\(^+\), and GAD\(^-\); Fig. 6B), but failure rate increased in a use-dependent fashion; failures reached 10–12% for EPSCs late in stimulus trains (\(P < 0.04\), even for monosynaptically coupled responses. In contrast, failure rates were substantial even for the first ST shock and unchanged across the ST burst in indirect, polysynaptically coupled pathways (P > 0.05). Despite frequency-related augmentation of ST-EPSC failures in directly ST-coupled neurons (GAD\(^+\) or GAD\(^-\)), failure rates never approached even the lowest rates for indirectly ST-coupled neurons (\(P < 0.02\); Fig. 6B). Mean amplitudes for indirect, ST-coupled EPSCs were small and showed little frequency-dependent depression (Fig. 6C). However, indirectly ST-synchronized IPSCs failed more often than EPSCs late in ST shock bursts (P = 0.003; Fig. 6C). Overall, synaptic characteristics for polysynaptic EPSCs were similar to IPSCs and may reflect similar intra-NTS pathway organization.

FIG. 5. Summary distributions of the latency-jitter values compare ST-activated direct and indirect synaptic responses to ST stimulation in GAD\(^+\) and GAD\(^-\) NTS neurons. Each point in the scatterplot represents the latency-jitter value for a synaptic event triggered by ST shocks (e.g., EPSC1). EPSCs are represented in GAD\(^+\) neurons (filled circles, \(n = 48\)) and in GAD\(^-\) neurons (open triangles, \(n = 26\)), but inhibitory postsynaptic currents (IPSCs; stars) are combined from both types (10 GAD\(^+\) and 3 GAD\(^-\)). Synaptic responses with jitter <200 μs (below broken line) in their latencies were considered directly coupled to ST via a monosynaptic connection—ST-direct. Note that absolute latencies poorly separated the 3 data groups. The shortest latencies for synaptic events indirectly coupled to ST (jitter > 200 μs) were roughly twice the shortest latency of directly coupled EPSCs. Means are presented with ±SE.

FIG. 6. This summary of synaptic performance compares ST-activated direct and indirect synaptic responses in GAD\(^+\) and GAD\(^-\) NTS neurons. Frequency-dependent depression and the rates of synaptic failures differed between ST-evoked responses with low jitter (<200 μs, ST-direct, black bars, all EPSCs) and synaptic responses that had high jitter (EPSCs, ST-indirect, unfilled bars) in both GAD\(^-\) (left) and GAD\(^+\) (right) neurons. All bursts were 5 ST shocks at 50 Hz to evoke synaptic responses and were events numbered 1–5 (inset current trace). A: on average, amplitudes of ST-direct (black bars) EPSC1 and EPSC2 were substantially greater (\(P < 0.02\)) than ST-indirect (unfilled bars) in both GAD\(^-\) and GAD\(^+\) neurons (\(n = 38\), and 20, respectively), but this difference became nonsignificant with frequency-dependent depression of ST-direct EPSCs within the stimulus train. On average, ST-indirect EPSCs displayed no frequency-dependent depression in either GAD\(^-\) or GAD\(^+\) neurons (\(n = 18\) and 9, respectively). B: failure rates for ST-indirect EPSCs were uniformly high (i.e., failure rates were constant throughout the stimulus train), and the failure rates of ST-indirect EPSCs exceeded those for ST-direct EPSCs whether GAD\(^+\) or GAD\(^-\) neurons (\(n = 18\) and 9, respectively). C: consistent with their polysynaptic pathways from ST, IPSCs failed at high rates that increased significantly as ST shock number increased (\(P < 0.05\)). Note that IPSCs were reported as combined means from both GAD\(^-\) and GAD\(^+\) NTS neurons because of similar patterns and small samples (10 GAD\(^-\) and 3 GAD\(^+\)). Means are presented with ±SE.
Activation of any single axon is all-or-none. However, many ST axons lie in close proximity to the stimulation electrode, and different axons can have different activation thresholds. We used graded increments in ST shock intensity to recruit responses from activation of different axons that might converge on a single recorded neuron. Since most of the GAD+ NTS neurons were second order, collaterals from such neurons might contact other NTS neurons (i.e., act as interneurons), and such connections would appear as polysynaptic (high jitter), ST-synchronized IPSCs. In many neurons, ST shocks evoked a low-threshold, early-arriving EPSC with low jitter, but higher shock intensities added a late-arriving, high-jitter, ST-synchronized IPSC (Fig. 7A, gray arrow). These high-jitter IPSCs were inward currents under our experimental conditions (calculated $E_{\text{Cl}} = -60 \text{ mV}$) and often failed on successive shocks (Fig. 7A). Stepping to depolarized holding potentials ($-20$ to $-40 \text{ mV}$) inverted these late IPSCs to outward synaptic currents (Fig. 7A) as appropriate for our experimental Cl$^-$ conditions.

The latency distributions for successive, individual ST-evoked IPSCs within neurons were broad and yielded high calculated jitter values (Fig. 7B; range $>3 \text{ ms}$ with jitter $= 704 \mu\text{s}$). These jitters are consistent with mediation by polysynaptic, intra-NTS pathways. Separate recruitment analysis of the direct EPSC and the indirect IPSC showed that each event had a different ST shock intensity threshold. These distinct thresholds are consistent with origination of each synaptic event from independent ST axons and thus represent convergence of afferent-activated pathways, albeit one direct input with additional indirect inputs. The respective event shapes did not change with increasing intensity above threshold (Fig. 7C).

Thus these two events—the EPSC and the IPSC—seemed to be triggered by different ST axons. Such high-jitter, ST-synchronized IPSCs were blocked by either the GABA$_A$ antagonist, gabazine ($5 \mu$M), or independently by the non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist NBQX ($20 \mu$M). This dual glutamate/GABA antagonist for ST-evoked IPSCs (Andresen and Yang 1995) was consistent with at least a disynaptic intra-NTS pathway for translating ST activation into IPSCs (Fig. 7D). This pattern of early, low-threshold ST-EPSCs followed by late, higher-threshold, high-jitter IPSCs was found in most GAD$^+$ second-order NTS neurons (84%, 16 of 19) and nearly one half of GAD$^-$ second-order neurons (45%, 5 of 11). The timing, intensity–recruitment, and pharmacological profiles provide evidence that each high-intensity ST shock activated two different axons, each with a different threshold. In other GAD$^+$ second-order NTS neurons, late arriving EPSCs were recorded, and similar testing protocols showed high jitters, distinct, all-or-nothing stimulus intensity–recruitment profiles, and common failures, but such indirectly coupled EPSCs were blocked solely by NBQX (results not shown). Note that these results show that ST shocks activate multiple afferent fibers, but only limited pathways converge on any given single NTS neuron regardless of whether the neuron was GAD$^+$. 

**DISCUSSION**

The structural “wiring” of neural networks critically shapes information processing, and the relationship between afferent

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**ST-synchronized GABAergic inputs to GABAergic neurons**

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The structural “wiring” of neural networks critically shapes information processing, and the relationship between afferent

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**FIG. 7.** High-intensity shocks activated additional, indirect ST-coupled synaptic inputs to 2nd-order NTS neurons. A: in a representative GAD$^+$ neuron, ST-evoked synaptic responses to high-intensity ST shocks were recorded at hyperpolarized (−74 mV, left, 6 traces) and depolarized (−34 mV, right, 6 traces) potentials. At hyperpolarized potentials, each ST shock triggered both a short-latency, low-jitter inward EPSC (left bottom panel, expanded traces) that was followed by a late-arriving, inward IPSC (gray arrow). At depolarized potentials (right bottom panel, expanded traces), ST-EPSCs were attenuated, but high jitter ST-IPSCs (broken oval) became outward (E$_{\text{Cl}} = -60 \text{ mV}$). B: histogram of response latencies showed narrow, low-jitter distribution for ST-EPSCs (gray bars for analysis of gray oval in A) and broader, high-jitter distribution for ST-synchronized IPSCs (black bars for analysis of dashed oval in A). C: ST intensity–recruitment relation for this neuron shows a lower intensity threshold for direct ST-EPSC (gray, squares) than for indirectly ST-coupled IPSC (black filled circles). Average values represented by large unfilled symbols at each intensity. Failures near threshold for the IPSC were not included in calculating amplitude mean. Small symbols are individual values. D: selective antagonists identified ST-evoked responses recorded at hyperpolarized (left, mean of 6 traces) and depolarized (right, mean of 8 traces) potentials. Waveforms are multitrace means of responses to single ST shocks. In control (continuous line), the early ST-direct EPSC resisted 5 μM gabazine (−), which blocked the late IPSC. After wash recovery from gabazine, 20 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo quinoxaline-7-sulfonamido (NBQX) blocked all ST responses: both EPSCs and IPSCs ( . . . traces). GABA$_A$ receptor-mediated ST-IPSCs required non-N-methyl-D-aspartate (NMDA) receptor glutamate transmission. Similar results were recorded in a total of 18 second-order NTS neurons. Means are presented with ±SE.
Cranial visceral afferents directly activate most GABAergic NTS neurons

Synaptic timing assessed by latency jitter in relation to ST activation was a critical part of our approach. Jitter reflects temporal variation in pathway transmission over successive trials and effectively distinguished direct pathway connections from more convoluted routes from ST (Doyle and Andresen 2001). In our slices, nearly 70% of GAD+ neurons were classified as second order to ST with large-amplitude, low-jitter (<200 μs) ST-EPSCs that rarely failed and had substantial depression at 50-Hz stimulus rates (Bailey et al. 2006a; Doyle and Andresen 2001; Doyle et al. 2004). This finding is identical to a more limited recent report in this mouse model (Glatzer et al. 2007). From work in the rat, the large amplitude and reliability of such ST-direct EPSCs are attributed to chiefly two properties of ST afferents: multiple contacts on single neurons (Doyle et al. 2004; Mendelowitz et al. 1992) and a uniformly high glutamate release probability over multiple release sites that are physically close (Bailey et al. 2006a,b). Thus based on evidence from the rat, activation of a single ST axon likely produces a nearly simultaneous release of glutamate from ≤33 different release sites to produce the large, smooth EPSCs (Bailey et al. 2006b). Such large ST-EPSCs offer a high safety factor for excitation with a fidelity of afferent action potential transmission within rat NTS of 60–100% at moderate frequencies of activation (Andresen and Yang 1995; Bailey et al. 2007). ST-evoked IPSCs had latencies short enough to reflect a two-synapse intra-NTS pathway to both GAD+ and other (GAD-) NTS second-order mouse neurons as in other species (Andresen and Yang 1995; Mifflin and Felder 1988; Miles 1986; Smith et al. 1998). ST-IPSCs in these mice were always interrupted by non-NMDA receptor antagonism consistent with their initiation by ST glutamatergic synapses (Andresen and Yang 1995). Localized collaterals of GABAergic NTS neurons are described in rats and would function as local interneuron connections (Kawai and Senba 1999; Kubin et al. 2006; Smith et al. 1998).

Polysynaptic ST-driven inputs are weak and unreliable

The jitters and latencies of ST-indirect EPSCs and IPSCs received by NTS neurons (GAD+ or GAD-) were remarkably similar. A few recorded NTS GABAergic neurons had only high jitter synaptic responses. The small mean amplitudes of ST-indirect synaptic responses suggest that the synapses provided by second-order NTS neurons are quite distinct from ST afferent synapses. Indirect pathways from ST are most likely wholly contained within NTS in our thin slices and thus represent intra-NTS circuits. Although the similarities in timing and amplitude of all ST-indirect synaptic responses imply a generally similar organization, ST-indirect IPSCs failed significantly more often at the ends of brief bursts of stimuli compared with ST-indirect EPSCs. Future work might identify the mechanisms of this performance difference since it suggests that local GABAergic neurons consistently attenuate high frequencies of activation—a process that will reduce sustained inhibitory transmission.

Model and approach considerations

The results of our studies relied on several key methods of approach that importantly influenced our findings. First, our studies are based on a GAD67-EGFP reporter mouse model to select NTS neurons for recording (Glatzer et al. 2007; Williams and Smith 2006). These EGFP neurons enabled us to selectively record from populations of neurons (EGFP positive and negative). This transgenic mouse sometimes referred to as the GIN model identifies a subset of inhibitory neurons in the cortex that express somatostatin (SOM+) (Oliva et al. 2000). Co-localization with GABA is nearly complete in the spinal cord of GIN mice (Heinke et al. 2004), and single cell RT-PCR confirmed GAD67 in their vestibular neurons (Bagann et al. 2007). Different knock-in models, however, can identify different subgroups of GABAergic neurons despite targeting the same gene, GAD1 (i.e., GAD67). In recent cross-model comparisons of the neocortex, different lines of GAD-67 SOM+ interneurons fell into different groups that were distinct in their laminar location, neurochemical markers, axonal morphologies, and electrophysiological properties (Ma et al. 2006). Immunocytochemical profiling, even using the same transgenic model, however, differs across investigators (e.g., calcium binding protein calbindin staining; cf. Oliva et al. 2000 with Ma et al. 2006). Thus in our studies, the GIN-identified GABAergic neurons may represent a subset of all inhibitory interneurons present in NTS. The synaptic activation patterns—single direct ST input plus polysynaptic IPSCs and EPSCs—were quite similar between the GAD+ and all other second-order NTS neurons. This finding suggests a relative homogeneity in the predominance of second-order neurons within the organization of medial NTS,
but other subregions could differ. Thus further studies will be needed to identify whether distinct subgroups of GABAergic neurons may be present across NTS and whether particular excitatory pathways in NTS are paired with unique, companion inhibitory networks as has been suggested elsewhere (Buzsaki and Chrobak 1995).

The thin slicing of our brain sections severs axons and dendritic arbors along the plane of the cut and thus potentially interrupts ST axons courting to targets on second-order NTS neurons (Berger et al. 1984; Ezure et al. 2002). Horizontal slicing might favor one neuron class over another as it does in some dorsal horn neurons (Grudt and Perl 2002; Lu and Perl 2005). However, both transverse and horizontal NTS slices from GIN mice yielded qualitatively similar basic synaptic responses (Glatzer et al. 2007). Thus, although we cannot offer an assessment of the potential magnitude of such afferent losses, it seems quite unlikely that multiple, direct ST inputs to NTS neurons would be systematically eliminated. Since identical methods in other strains of mice recently identified dual, ST-direct EPSCs in one half of the second-order, catecholaminergic NTS neurons (Appleyard et al. 2007), the pattern of ST convergence may well be related to the specific neuron phenotype and/or to the projection target or pathway of those second order neurons.

Another key methodological consideration is our use of electrical stimulation and specifically its selectivity for activation of ST afferent axons. Clearly, electrical shocks are nonspecific and may excite multiple neurons at any excitable neuron element: cell body, axon, or dendrite. The horizontal orientation preserves more afferent axon length from rostral neuron element: cell body, axon, or dendrite. The horizontal orientation preserves more afferent axon length from rostral and caudal to cell bodies within NTS. Our concentric bipolar electrode nearly spanned the width of the visible ST and successfully recruited ESPCs $\pm 2$ mm distant from the recorded neuron. In every case, our tests measured the minimally effective constant current required to activate the synaptic response. These thresholds were sharply delineated and highly reproducible in their stimulus–response relations. Note that, as in most in vitro protocols, our supraphysiological extracellular Ca$^{2+}$ concentrations (2 mM) increase the amplitudes of our recorded synaptic responses to near their maximum (Bailey et al. 2006b). Maximal transmission improves the signal-to-noise ratios for detection of both direct and indirect pathways and thus should reduce the number of undetected synaptic inputs. The narrow thresholds and all-or-none nature of the evoked synaptic responses during fine gradations of the shock intensity are evidence that single axons are responsible for the observed synaptic responses. This evidence suggests that our stimulus and slicing protocol are highly specific for ST afferent driven pathways as intended.

Certainly, current spread from the site of electrode placement has the potential to recruit axons passing in the vicinity that are not ST-generated synaptic responses in the recorded neurons. To test this localization, we moved the electrode off of the visible ST. Despite always moving the stimulation electrode closer to the recorded cell, higher intensities only activated EPSCs closely resembling the original synaptic responses. These tests directly contradict the notion that neighboring en passant axons might be responsible for on-ST responses or that such non-ST axons commonly converge on second-order NTS neurons. These findings are similar to previous reports in thicker, larger horizontal NTS slices from rats and guinea pigs (Andresen and Yang 1990, 1995; Miles 1986). The independent recruitment of multiple ST-synchronized synaptic events within individual neurons requires that our electrode simultaneously activate multiple ST axons with each shock. In addition, dual simultaneous recordings from two second-order NTS neurons in rats (unpublished observations) likewise showed activation of independent pathways to each neuron distinguished by different thresholds from a single stimulus electrode and shock. Identical techniques identify multiple, ST-driven, convergent, polysynaptic inputs to particular subsets of NTS projection neurons (Bailey et al. 2006a). The sparse convergence profile in the ST stimulus recruitment profiles of NTS neurons contrasts with that expected in a highly convergent system such as found for optic nerve afferents to the lateral geniculate nucleus (Acuna-Goycolea et al. 2008).

**Visceral afferent convergence—slice responses resemble intact NTS recordings**

Our findings of limited convergence of ST primary afferents onto the NTS neurons are somewhat surprising. Cranial visceral afferents to NTS are diverse, and many join common peripheral nerve trunks before coursing to the CNS (e.g., the vagus). Stimulation and neuroanatomical mapping approaches suggest a loose viscerotopic organization (Kubin and Davies 1995; Loewy 1990). The physical proximity of varied afferent axons presents the potential for afferent convergence. Indeed, stimulation of multiple, whole nerve trunks in vivo supports the presence of multiple afferents converging on some individual NTS neurons (Donoghue et al. 1985; Mifflin 1996). On close examination, however, such studies commonly suggest that multiafferent convergence is relatively rare, ranging from as high as 10 to 15% of NTS neurons, for example in cat NTS (43/292 neurons) (Donoghue et al. 1985), in rabbit (7/45) (Bonham and Hasser 1993) or in other cat studies (3/28 neurons) (Ootani et al. 1995) down to as low as 1–2% in some cat studies (1/56 neurons) (Mifflin 1996). More commonly, the cross-nerve convergent inputs (e.g., carotid sinus and superior laryngeal nerves) met polysynaptic criteria in cells that received a monosynaptic input from one of the nerves (Mifflin 1996): a finding quite consistent with our results. However, the majority of certain phenotypic subsets of neurons (e.g., cat-echolaminergic) in mice received multiple monosynaptic contacts (Appleyard et al. 2007), and afferent convergence may be associated with particular pathways or types of neurons within NTS. Together, the studies suggest that second-order NTS neurons (GAD$^+$ or GAD$^-$) are organized in relatively discrete circuits, with limited synaptic inputs originating from ST afferents and thus limited different modalities (Kubin and Davies 1995).

**GABAergic second-order interneurons**

Most GAD$^+$ NTS neurons were second-order visceral afferent neurons, and ST afferent activation commonly triggered an EPSC-IPSC sequence as in most species whether intact (Mifflin et al. 1988; Miura 1975; Smith et al. 1998) or in slices (Andresen and Yang 1995; Doyle and Andresen 2001; Miles 1986). The aggregate observations in GAD$^+$ and GAD$^-$ NTS neurons suggest a consistent local pathway structure with
afferent-driven signals arriving as IPSCs indirectly along parallel pathways to second-order NTS neurons. Parallel inhibitory pathways are a common organization for feedback in afferent processing of spinal cord lamina II (Lu and Perl 2003). An interneuron function of second-order GABAergic NTS neurons does not exclude the possibility that some neurons may project outside the NTS. GABAergic NTS neurons project to paraventricular hypothalami (Kannan and Yamashita 1983; Sved et al. 1985), to rostral and caudal ventrolateral medulla (Suzuki et al. 1997), or to gastric dorsal motor neurons (Browning et al. 2002; Glazeter et al. 2007; Travagli et al. 2006). Thus GABAergic NTS neurons are critical parts of both circuits within the NTS and in NTS output.

**Homeostatic reflex integration**

Disruptions in local NTS GABAergic transmission profoundly upsets homeostatic systems, including respiratory (Wasserman et al. 2002), gastrointestinal (Travagli et al. 2006), and cardiovascular systems (Mei et al. 2003). In the case of anesthetics, targets within NTS include greatly enhanced GABAergic transmission at second-order NTS neurons (McDougall et al. 2008a), as well as descending GABAergic inputs (Jia et al. 1997; Jordan et al. 1988). The physiological drive of afferents to NTS arises from diverse, and in our studies, unknown sources that includes a major afferent phenotypic division of myelinated and unmyelinated primary afferents with systematically different discharge characteristics (Kunze and Andresen 1991; Lawson 1992; Scheuer et al. 1996; Schuld and Kunze 1997). Reflex performance, even within an afferent modality, clearly differs depending on afferent class (Fan and Andresen 1998; Fan et al. 1999), although the mechanisms remain unclear. C-type axons predominate in cranial visceral nerves (by some estimates as high as 85%; Kubin and Davies 1998; Fan and Davies 1995). This intra-NTS circuitry, however, may be particularly important in the performance of vago-vagal reflexes given their compact structure (Browning and Mendelowitz 2003; Mendelowitz 1999; Travagli et al. 2006). In summary, the overall general organization of the relationship between ST afferents and GABAergic neurons within NTS resembles a broad, common organization of afferent pathways to NTS neurons, but the limited convergence to individual GABA-synthesizing neurons leaves open the possibility for specific pathways differentiated by their specific afferent inputs (e.g., myelinated or organ-specific), cellular properties, and/or projection destination.

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