Properties of GABAergic neurons in the rostral solitary tract nucleus in mice

Min Wang¹ and Robert M. Bradley¹,²

1. Department of Biologic and Materials Sciences
   School of Dentistry
   University of Michigan
   Ann Arbor, MI 48109-1078

2. Department of Molecular and Integrative Physiology
   Medical School
   University of Michigan
   Ann Arbor, MI 48109-0622

Correspondence to:
Robert M. Bradley
Department of Biologic and Materials Sciences
School of Dentistry
University of Michigan
Ann Arbor, MI 48109-1078

Phone: (734) 763-1080
FAX: (734) 647-2110
Email rmbrad@umich.edu

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ABSTRACT

The rostral nucleus of the solitary tract (rNST) plays a pivotal role in taste processing. The rNST contains projection neurons and interneurons that differ in morphology and intrinsic membrane properties. While characteristics of the projection neurons have been detailed, similar information is lacking on the interneurons. We determined the intrinsic properties of the rNST GABAergic interneurons using a transgenic mouse model that expresses enhanced green fluorescent protein (EGFP) under the control of a GAD67 promoter. GAD67-GFP neurons were distributed throughout the rNST but were concentrated in the ventral subdivision with minimal interaction with the terminal field of the afferent input. Furthermore, the density of the GAD67-GFP neurons decreased in more rostral areas of rNST. In whole cell recordings, GAD67-GFP neurons responded with either an initial burst (73%), tonic (18%) or irregular (9%) discharge pattern of action potentials in response to membrane depolarization. These three groups also differed in passive and action potential characteristics. Initial burst neurons had small ovoid or fusiform cell bodies whereas tonic firing neurons had large multipolar or fusiform cell bodies. Irregular firing neurons had larger spherical soma. Some of the initial burst and tonic firing neurons were also spontaneously active. The GAD67-GFP neurons also could be categorized in subgroups based on co-localization with somatostatin and parvalbumin immunolabeling. Initial burst neurons would transmit the early dynamic portion of the encoded sensory stimuli while tonic firing neurons could respond to both dynamic and static components of the sensory input suggesting different roles for GAD67-GFP neurons in taste processing.
INTRODUCTION

Chemosensory information originating from taste buds in the oral cavity is relayed to the central nervous system via afferent nerve fibers of the chorda tympani (CT) and greater superficial petrosal branches (GSP) of the VIIth cranial nerve and the lingual tonsilar branch of the IXth nerve (Bradley 2006). Taste buds on the larynx and pharynx, which do not have a gustatory, function are innervated by the superior laryngeal branch of the vagus (Xth) nerve (Smith and Hanamori 1991). The central projections of these afferent neurons enter the brainstem to form the solitary tract (ST) and synapse with neurons in the rostral nucleus of the solitary tract (rNST). The rNST neurons integrate the chemosensory input and then distribute it either to more rostral relays in central taste pathways or to secretomotor, premotor or motor neurons in the brainstem (Lundy and Norgren 2004). The rNST therefore has a pivotal role in chemosensory processing, acting as a portal to the central ascending taste pathways, and is involved in the initiation of a number of important oral-facial reflex functions.

Many rNST neurons are GABAergic as defined by anatomical and immunohistochemical techniques. Early investigators identified the GABA immunoreactive neurons in hamster and rat as small ovoid cells (Davis 1993) that make up 82% of the neuronal population of the CT terminal field area of rNST (Lasiter and Kachele 1988). GABA<sub>A</sub> receptor immunoreaction was reported to be densest in the ventral rNST (King 2003). In addition, by using retrograde tracing, these small ovoid GABAergic neurons were found to not project to the parabrachial nucleus or to other brainstem regions outside of the rNST and were thus determined to be local circuit neurons. Although investigators in these earlier studies speculated that this population of
neurons played a role in inhibitory processing of taste information, technical difficulties prevented direct examination of functional characteristics of the GABAergic neurons.

More recently, GABAergic inhibitory activity in rat rNST has been studied with whole-cell patch clamp recordings in brain slices while superfusing GABA receptor agonists and antagonists over the rNST neurons. Use of GABA<sub>A</sub> and GABA<sub>B</sub> receptor agonist and antagonists confirmed that GABA<sub>A</sub> receptors were the predominant GABA receptor in rNST (Liu et al. 1993; Wang and Bradley 1993, 1995; Grabauskas and Bradley 1998a, b, 1999).

Despite the importance of inhibition in rNST sensory processing, little is known about the properties of rNST inhibitory neurons because it has not been possible to identify the GABAergic rNST neurons in electrophysiological recordings. Recently a GAD67-GFP transgenic mouse (GIN mouse - GFP expressing inhibitory neurons) has been developed that identifies a subset of inhibitory neurons in the hippocampus and neocortex that express somatostatin (Oliva et al. 2000). This mouse model has been used to characterize GABAergic neurons in a number of other locations including the spinal cord (Dougherty et al. 2005; Dougherty and Hochman 2008), neocortex (Fanselow et al. 2008) as well as the rNST (Travers et al. 2007). In these investigations electrophysiological and immunohistochemical techniques have been used to classify the GAD67-GFP neurons based on repetitive firing properties, neuron morphology and expression of neurochemical markers of GABAergic interneurons such as parvalbumin (PAV) and somatostatin (SOM) (Kubota et al. 1994; Cauli et al. 1997; Ma et al. 2006). Since the GAD67-GFP expressing neurons can readily be identified in rNST brain slices,
we have used this mouse model to characterize the properties of this particular subset of
the rNST GABAergic interneurons.

METHODS

Animals

The GIN mice used in this study are a commercially available strain (FVB-Tg(GadGFP)45704Swn/J, Jackson Laboratories, Bar Harbor, ME) in which enhanced green fluorescent protein (GFP) is expressed under the control of a GAD67 regulatory promoter (Oliva et al. 2000). All surgical procedures were carried out under National Institutes of Health and University of Michigan Animal Care and Use Committee approved protocols.

Histology

Mice (4-6 weeks old) were anesthetized with an intraperitoneal injection of a mixture of ketamine (10mg/kg) and xylazine (2mg/kg) and perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brainstem was dissected and further fixed by immersion in 4% paraformaldehyde for two hours. After fixation the brainstem was rinsed in 0.1M PBS for 30 minutes and then embedded in 4% agar.

Horizontal (n = 3) or coronal (n = 3) 50μM sections were cut using a Vibratome (Technical Products International). Selected sections were stained with Luxol fast blue and cresyl violet (Kluver and Barrera 1953) to provide an anatomical reference for the GAD67-GFP sections of rNST.

Anterograde labeling of afferent terminal fields

To explore connections between the afferent gustatory nerves and the GAD67-GFP neurons we used anterograde neural tracing in 6 mice. Mice were anesthetized as
detailed above, placed on a heating pad at 37°C and the head fixed using a custom designed head holder. The terminal field of either the CT or lingual-tonsillar branch of IX was anterogradely labeled as described by May and Hill (2006). The CT was exposed by a ventral approach and the CT nerve cut in the tympanic bulla. Crystals of Alexa Fluor 568 dextran (molecular weight, 10,000, Invitrogen, CA) were applied to the proximal cut end and sealed in place with silicone glue (Kwik-Cast, World Precision Instruments) to prevent dye spread. The IXth nerve was exposed medial to the tympanic bulla and cut near the petrosal ganglion. The proximal cut end was placed on Parafilm and crystals of Alexa Fluor 568 dextran (molecular weight, 10,000) placed on the end and sealed with silicone glue. Survival time for anterograde labeling of nerve terminal fields was 18 hours - 2 days. The skin was closed using cyanoacrylate glue and the animal was placed on a heating pad until ambulatory and observed to be drinking. The mice were then returned to their home cage.

Immunohistochemistry

Cell markers have been commonly used to describe several subpopulations of cortical GABAergic interneurons (Kubota et al. 1994; Cauli et al. 1997; Kawaguchi and Kondo 2002) and we therefore used some of the same markers to potentially differentiate similar groupings in rNST GAD67-GFP interneurons. Brainstem sections (2-3 mice per immunolabel) were floated in 0.1M PBS buffer in a 24 well cell culture dish. Sections were treated with 0.3% TritonX-100 and 5% normal goat serum in PBS for 1 hour and then incubated in primary antibody overnight at 4°C. After three 15 minute rinses with PBS, sections were incubated in either Cy³-conjugated anti-mouse antibody (1:100) or TRITC-conjugated anti-rabbit antibody (1:100) for 1 hour at room temperature. All
antibodies were diluted in 0.1M PBS containing 0.3% TritonX 100 and 5% normal goat serum. After immunoreaction the sections were rinsed three times for 15 minutes and then mounted on slides. ProLong Gold anti-fade reagent was used as a mounting medium (Invitrogen, CA). Control experiments included procedures on sections in which either the primary or secondary antibody was omitted. A Nikon C-1 confocal microscope was used to capture images of the immunolabeled sections and Neurolucida (MBF Bioscience) software used to trace the borders and subnuclei of the rNST.

Neurons were counted to quantify the extent of the colocalization of GFP cells with markers known to identify subpopulations of GABAergic interneurons. Counts of neurons were performed on three coronal sections at different levels (indicated in Fig. 1a) of the rNST (Fig. 1c, d, e). For each section a standard 100 μm X 100 μm area was selected in the medial, rostral-central and rostral-lateral subdivisions and two 100 μm X 100 μm areas in the ventral subdivision of the rNST. Single and double labeled neurons in each of these areas were counted using Neurolucida software. The total neuron counts in each subdivision of each coronal section were summed and the number of cells that were double labeled expressed as a percentage of total GFP neurons.

**Primary antibodies**

Details of primary antibodies are listed in Table 1. The antibody to NeuN has already been well characterized and reacts with most types of neurons in the CNS (Mullen et al. 1992). The GABA antibody was raised against GABA conjugated by glutaraldehyde to bovine serum albumin. The immunological properties and specificity of this antibody have been detailed with minimal cross reactivity with other amino acids (Storm-Mathisen et al. 1983). The specificity of the somatostatin-14 antibody has been
reported by the manufacturer to cross-react with somatosatin 14, somatostatin 28 and
somatostatin 25. It has no cross reactivity with a number of other peptides including
substance P, NPY, VIP, insulin and glucagon. Details of the production and specificity of
the parvalbumin monoclonal antibody have been described by Celio et al. (1988). It
reacts with parvalbumin but does not react with other members of the calcium binding
family of proteins such as calmodulin and troponin. All antibodies have been used
extensively in previous studies of the olfactory bulb, hypoglossal nucleus, neocortex and
other CNS areas (Takasu et al. 1987; Tamamaki et al. 2003; Ma et al. 2006; Naritsuka et
al. 2009).

Preparation of brainstem slices and recording

Brain slices were prepared from 22 mice using methods detailed in previous
studies (Fukami and Bradley 2005; Suwabe and Bradley 2007). Coronal or horizontal
300-μm thick slices were cut using a Vibratome (Technical Products International), and
incubated for at least 1 hour in an oxygenated artificial cerebrospinal fluid (ACSF) at
room temperature. The ACSF containing (in mM) 124 NaCl, 5 KCl, 2.5 CaCl₂, 1.3
MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 25 dextrose, was gassed with a 95% O₂, 5%
CO₂ mixture to achieve a solution pH of 7.4. Slices were transferred to a recording
chamber attached to the stage of a microscope (ECLIPSE E600-FN, Nikon) and perfused
at 2 - 2.5 ml/min with oxygenated ACSF at 33°C. GFP labeled cells were identified using
epifluorescent illumination and then observed with infrared-differential interface contrast
optics (IR-DIC). In addition, recordings were made from a small sample (n = 6) of
unlabeled neurons to potentially characterize differences with the GAD67-GFP neurons.
Recordings were made in current or voltage-clamp modes using a patch-clamp amplifier
Recording pipettes were filled with a solution that contained (in mM): 130 K-gluconate, 10 n-2-hydroxy-ethylpiperazine-\(N'\)-2 ethanesulfonic acid (HEPES), 10 ethylene glycol-bis (\(\beta\)-aminoethyl ether)-\(N,N',N',N'\)-tetraacetic acid (EGTA), 1 MgCl\(_2\), 1 CaCl\(_2\) and 2 ATP, buffered to pH 7.2 with KOH. Lucifer yellow (0.1%, Sigma) was dissolved in the pipette solution to label recorded neurons. Tip resistance of filled pipettes was 6-8 M\(\Omega\).

Electrophysiological data were analyzed using the Clampfit program (Axon Instrument). The junction potential due to potassium gluconate (10 mV) was subtracted from the membrane potential values. All passive membrane values were measured at the neurons resting membrane potential. For each neuron a standard series of hyperpolarizing and depolarizing 50 pA 1000 ms current injection steps from -250 to 200 pA were applied to measure basic membrane properties (see Fig. 4). Repetitive firing patterns were determined using current injection protocols consisting of 100 pA, 1000 ms depolarizing current steps and a –100 pA, 100 ms hyperpolarizing pulse followed by a 1000 ms depolarizing current step. Initial membrane potential was set at –60 mV by bias current injection in the current-clamp recordings. Spontaneous activity was measured at resting membrane potential by manually counting the number of action potentials in one second prior to any current injection protocols. The first action potential evoked by a 1000 ms 100 pA current injection was used to measure active membrane properties.

Voltage threshold to initiate an action potential was determined by measuring the membrane potential at the inflection point of the evoked action potential (Fedirchuk and Dai 2004). To investigate the presence of an A-type potassium current (I\(_{KA}\)) membrane potential of a subset (\(n = 6\)) of neurons was voltage-clamped at –60 mV followed by a
conditioning step to -100 mV for 500 ms and then a 1000 ms activation test step to 0 mV.

To confirm the expression of I_{KA}, a potassium channel blocker, 4-aminopyridine (4-AP), was used at 1 - 5 mM in 3 neurons.

**Neuron reconstruction**

Brainstem slices with Lucifer yellow injected GAD67-GFP neurons were fixed in 4% PFA overnight, rinsed for 30 minutes in 0.1M PBS, embedded in 4% agar and cut into 50 μM sections. Sections were mounted on slides and images were captured with a Nikon Eclipse 80i microscope equipped with a Spot RTSE camera (Diagnostic Instruments). For reconstruction, image stacks of neurons were acquired using a Nikon C-1 confocal microscope. Neurolucida software (MBF Bioscience) was used to trace and morphometrically analyze the neurons.

**Data analysis**

Statistical analysis to assess differences in passive membrane properties, action potential characteristics and morphometric measurements between GAD-GFP neuron groups was conducted using the SPSS program (SPSS Inc.). The numerical values are given as means ± SEM and statistical significance ($p < 0.05$) was assessed using a one-way ANOVA with Bonferroni post-hoc tests.

**RESULTS**

**Distribution of GAD67-GFP neurons in rNST**

In myelin and Nissl stained horizontal sections of the brainstem, the lightly stained NST is clearly distinguishable from surrounding neural tissue (Fig. 1a). The ST runs close to the lateral border of the NST (Fig. 1a). In a comparable GIN mouse horizontal section, a dense column of GAD67-GFP cells is seen to run laterally to the...
caudal ST (Fig. 1b, arrowheads). An additional band of GAD67-GFP neurons is located in the medial NST (Fig. 1b, asterisks).

In coronal sections the dorsal border of the rNST is clearly apparent (Figs. 1c-e). Based on cytoarchitecture, afferent nerve terminal field patterns and neural tracing studies, the rNST in rats and hamsters has been divided into several subdivisions (Davis and Jang 1986; Whitehead 1988; Barry et al. 1993; Halsell et al. 1996); the rostral central (RC), rostral lateral (RL), medial (M) and ventral (V) subdivisions (Fig. 1e). The mouse rNST can also be divided into the similar subdivisions (Zaidi et al 2008). The GAD67-GFP neurons are densely localized in the ventral subdivision (Figs. 1d and e). The medial and rostral lateral regions contain moderate numbers of GAD67-GFP neurons while the rostral central region has the least number of GFP cells (Fig. 1c-e). The density of GFP cells in the ventral subdivision is highest in the caudal extent of the rNST (Fig. 1e) and gradually decreases in the more rostral rNST (Fig. 1c). This apparent caudal to rostral decrease was confirmed by counting the GAD67-GFP neurons in rNST serial sections using the obex as a reference point (Fig. 1f). GAD67-GFP neurons were counted in 15-20 optical 1 μm sections centered around the midpoint of the confocal image stacks. The final count used was the average number of neurons in these optical sections multiplied by 10 to represent a count in a 10 μm section. The location of these virtual 10 μm sections was estimated relative to the obex identified from the serial sections of the brainstem.

Input from afferent gustatory nerves

As previously reported in rat and hamster (Whitehead 1988; Barry et al. 1993) the rostral central subdivision of rNST is the major termination site of the afferent CT (Fig. 2a) and IX (Fig. 2b) nerves. This pattern of input was observed in the current study and
extends for a considerable rostral-caudal distance. CT afferent fibers terminate more rostral to the IX nerve terminal field although there is considerable overlap. At higher magnifications the terminal field was not observed to extend deeply into the ventrally located GAD67-GFP neurons (Fig. 2c), although some penetration from the rostral central terminal field is apparent at the border between the rostral central and ventral subdivisions (Fig. 2c1, c2, c3). Afferent terminals were seldom seen to directly contact the GAD67-GFP neurons implying polysynaptic connections between taste afferent fibers and the GABAergic interneuron.

Co-localization of GFP with NeuN and GABA

NeuN antibody was used as a marker of mature rNST neurons (Mullen et al. 1992). All GAD67-GFP neurons co-localized with NeuN labeled cells (Fig. 3a and a1, a2, a3) but not all the NeuN neurons in the ventral subdivision were GFP positive. The number of NeuN labeled cells that were GAD67-GFP positive was determined to be 24% of the total NeuN neurons. Thus, many neurons in the ventral subdivision of the rNST are not GAD67-GFP positive GABAergic neurons.

When all GABA expressing rNST neurons were identified by immunostaining, 92% of the GAD67-GFP positive rNST neurons also immunostained with the GABA antibody (Fig. 3b and b1, b2, b3). However, despite the fact that nearly all the GAD67-GFP neurons immunoreacted for GABA, only 46% of the total GABA immunostained rNST neurons co-localized with the GAD67-GFP neurons (counts based on 4 rNST subdivisions in 4 coronal confocal sections from 2 mice). In addition, the percentage of double labeled GABA and GAD67-GFP neurons differed in the different rNST subdivisions, with the highest percentage in the ventral subdivision and the lowest
percentage in the rostral central subdivision (Table 2). Thus, like other brain areas the GAD67-GFP expressing neurons, are a subpopulation of the total GABAergic rNST neurons.

**Membrane properties and firing patterns of GFP neurons**

Recordings were made from 56 GAD67-GFP rNST neurons located primarily in the ventral and medial subnuclei of rNST. To be included in the data set, neurons had to have a stable resting membrane potential, input resistance > 100 MΩ, and overshooting action potentials. No obvious difference was noted in repetitive discharge characteristics of the GAD67-GFP neurons sampled in all rNST subdivisions. Based on response to a series of 1000 ms duration, depolarizing current injection steps, the neuron firing patterns were divided into three groups (Fig. 4). The majority of the neurons sampled (73%) responded with a short burst of action potentials at the initiation of the depolarization current step (Fig. 4a1). A second group (18%) responded with a regular or tonic firing pattern (Fig. 4b1). A third small group (9%) responded with an irregular discharge pattern (Fig. 4c1). In response to a current injection protocol consisting of membrane hyperpolarization followed by a long depolarizing step none of the neurons responded with a delayed firing or long first interspike interval that characterizes other rNST neurons (Fig. 4a2, b2, c2) (Bradley and Sweazey 1992; Tell and Bradley 1994). In response to hyperpolarizing current injection steps, some neurons in all three groups showed a “sag” in membrane potential characteristic of an I_h current (arrowhead in Fig. 4b3).
A proportion of the GAD67-GFP neuron groups were spontaneously active. 22% of the initial burst neurons had a mean spontaneous firing rate of $3 \pm 1$ Hz and 70% of the tonic firing neurons had a mean spontaneous rate of $32 \pm 5$ Hz.

In voltage clamp, rNST GAD67-GFP neurons did not respond to a hyperpolarizing-depolarizing voltage protocol with an early transient outward current (Fig. 5 a1, a2, a3). In contrast unlabeled rNST neurons respond to hyperpolarizing-depolarizing voltage protocol with a prominent transient outward current blocked by 1mM 4-AP in voltage clamp recordings (Fig. 5b1, b2, b3). Based on these observations we concluded that the GAD67-GFP neurons do not express the hyperpolarization-activated A current (Tell and Bradley 1994), but do express the hyperpolarization-activated cationic current - $I_h$ (Pape 1996).

**GAD67-GFP neuron morphology**

A total of 39 GAD67-GFP neurons were successfully filled with Lucifer yellow and grouped based on soma characteristics. Neurons had either small ovoid, large pyramidal or fusiform soma (Fig. 6). Initial burst neurons usually were characterized by small ovoid or fusiform cell bodies (Fig. 6a,d) and tonic firing neurons had large multipolar or fusiform cell bodies (Fig. 6b,e). Irregular firing neurons have larger spherical soma (Fig. 6c,f).

Initial burst GAD67-GFP neurons were recorded principally in the ventral subdivision of rNST (Fig. 6g, IB). In contrast, tonic firing GFP neurons were localized to a small area of the ventral rNST subdivision, adjacent to the underlying reticular formation (Fig. 6g, TF).
Significant differences in morphometric parameters among these groups were principally in measures of soma area (Fig. 6h), perimeter, maximum diameter and minimum diameter (data not shown). In all of these metrics, soma of initial burst neurons were significantly smaller than the soma of the other two groups. No significant differences were found among the numbers of soma form factor, primary dendrites or dendritic branches of the three groups of neurons (Fig. 6i).

Passive and active membrane properties

Analysis of the passive and active membrane properties of the initial burst, tonic and irregular repetitive discharge neuron groups revealed that they differed in several electrophysiological properties (Fig. 7). The resting membrane potential of the burst neurons was significantly less negative than tonic firing neurons but no different than the irregularly firing group (Fig. 7a). Irregularly firing neurons differed significantly from the other two groups in the values of their membrane time constants (Fig. 7c). Input resistance differed significantly only between the burst and irregularly firing groups (Fig. 7b).

Significant differences were also observed in the measures of action potential characteristics. Initial burst neurons have longer spike width than tonic firing neurons (Fig. 7f). Tonic firing neurons have spikes with significantly smaller rise and decay times and longer spike afterhyperpolarization when compared to values for neurons in the other two groups (Fig. 7g, h, i). Other significant differences include spike threshold (Fig. 7d) values between the tonic firing and the other neuron groups.

Examples of single action potentials from the three neuron groups are illustrated in Fig. 7j, k, l, to show differences in amplitude, rise and fall times, spike width and
length and amplitude of the after hyperpolarization. These data demonstrate major differences in passive membrane and action potential characteristics among groups of GAD67-GFP positive GABAergic rNST neurons.

**Neurochemical characteristics of rNST GAD67-GFP neurons**

Somatostatin staining was distributed over the entire rNST whereas parvalbumin immunolabel was concentrated in the neuropil surrounding the rNST (Fig. 8 a2, b2). Very few rNST GAD67-GFP neurons immunoreacted (~15%) with somatostatin and these were localized in the rostral lateral subdivision (Fig. 8 a3 and c1, c2 and c3). The somatostain immunoreaction in rNST was concentrated in intense fiber labeling (Fig. 8 d2). The somatostatin immunoreactive fibers surround GAD67-GFP neurons suggesting synaptic contacts (Fig. 8 d3 sampled in the rostral central subdivision).

Except for a few scattered neurons in the rostral-central subdivision there was little parvalbumin staining in the rNST (Fig. 8 b2). Some co-localization of GAD67-GFP neurons and parvalbumin was observed in the ventral border of rNST (Fig. 8b3, e3) but these neurons may be in the parvocellular reticular formation and not the rNST.

The somatostatin positive GAD67-GFP neurons were relatively small ovoid cells (Fig. 8 c3) and the parvalbumin immunolabeled GAD67-GFP neurons are larger multipolar neurons (Fig. 8 e3).
DISCUSSION

Understanding how sensory information from taste receptors in the oral cavity is processed by the rNST requires knowledge of the intrinsic properties of neurons and their temporal pattern of action potentials. Progress has been made on the properties of rNST neurons projecting to the taste relay in the parabrachial nucleus (Suwabe and Bradley 2009) but apart from a few anatomical studies little progress on biophysical and repetitive discharge properties of the GABAergic rNST interneurons has been accomplished. The results of the current study using GIN mice have revealed significant basic information on the GABAergic neurons of the rNST. Thus, by recording from these identified interneurons in mice additional information is now available on local circuit neurons important in brainstem sensory processing of taste initiated sensory information.

Based on use of NeuN and GABA immunostaining, the GAD67-GFP neurons in rNST are a subpopulation of the total GABAergic neurons interspersed with non-GABAergic neurons. Only 46% of the GABA positive cells are GAD67-GFP neurons. Even though the GAD67-GFP neurons are a subpopulation of the rNST inhibitory neurons they can be further subdivided based on significant differences in their repetitive discharge response to membrane depolarization, morphology, passive membrane characteristics and expression of somatostatin and parvalbumin. The characteristics of GABAergic interneurons in the rNST that are GAD67-GFP negative remains to be determined.

Repetitive firing patterns and membrane properties

The repetitive discharge patterns of rNST GFP neurons in response to membrane depolarization led to characterization of subgroups. The majority of neurons had an initial
burst pattern. A smaller group responded with a tonic firing pattern and a few neurons responded with an irregular train of action potentials. The passive membrane and action potential properties also differed amongst the three repetitive discharge pattern neuron groups.

In two earlier studies we used similar electrophysiological approaches to define the repetitive discharge characteristics as well as structure function correlations of neurons in rat rNST brain slices (Bradley and Sweazey 1992; King and Bradley 1994). Because these recordings were from unidentified rNST neurons (blind recordings) it was not possible to define their connections or identify them as interneurons. However, based on similarities between the results of the earlier and current studies, it is likely that some of these blind recordings originated from rNST interneurons. In these prior investigations both the initial burst and tonic firing patterns described in the current study were identified. The location of the recorded neurons in the Bradley and Sweazey (1992) study were noted at the time of the experiment on standard diagrams of the rNST coronal brain slices. The initial burst and tonic firing neurons were distributed throughout the rNST (Fig. 3 in Bradley and Sweazey 1992) and tonic firing neurons were more frequently encountered than initial burst neurons. In the King and Bradley (1994) investigation the rNST neurons were filled with biocytin during recording and therefore could be accurately localized and later morphologically classified as ovoid, elongate and multipolar neurons. In the current study initial burst neurons were all ovoid neurons but in our prior study (King and Bradley 1994) only 31% of the ovoid neurons had an initial burst response. However, none of the recordings in the early study were from neurons in the ventral subdivision (Fig. 1A, King and Bradley 1994). In addition, the distribution of
GAD67 neurons in rat rNST differ from the mouse; in rat most GAD67 immunoreactive neurons are located in the medial and central subdivisions of the rNST with relatively few located in the ventral subdivision (Fong et al. 2005).

The repetitive firing patterns, passive membrane properties and action potential characteristics reported in rNST interneurons have been demonstrated in cortical and dorsal horn interneurons (Kawaguchi 1995; Ruscheweyh and Sandkühler 2002; Ma et al. 2006). However, the frequency of occurrence of the repetitive discharge pattern groups in the dorsal horn of GIN mice differs from those in the current study (Heinke et al. 2004; Dougherty et al. 2005). For example, in spinal cord lamina I the majority of GAD67-GFP neurons responded to a depolarizing step with a single spike while neurons with tonic and burst firing patterns were encountered less frequently (Dougherty et al. 2005). In contrast, the most frequently encountered lamina II spinal cord GAD67-GFP neurons responded with an initial burst pattern (Heinke et al. 2004). Thus, while similar types of discharge pattern are described in rNST and spinal cord, their frequency of occurrence varies probably related to the different functional roles of these areas in sensory processing.

Initial burst neurons that are a characteristic of rNST were not reported in recordings in caudal NST of GIN mice; repetitive discharge characteristics were reported to be similar to other caudal NST neurons (Glatzer et al. 2007).

In other brain areas initial burst firing patterns are present in neurons with a voltage- and time dependent K⁺ current activated by depolarization designated as an M-current (Madison and Nicoll 1984; McCormick et al. 1993). It is perhaps significant that the M-current is augmented by somatostatin (Moore et al. 1988) which we have shown to be present in fibers throughout the rNST. The initial burst neurons would initiate brief
inhibitory activity and therefore potentially hyperpolarize the second order rNST neurons activating the A-current that many of these neurons express (Tell and Bradley 1994; Uteshev and Smith 2006; Suwabe and Bradley 2009). Thus, the burst neurons would play an important role in sensory processing by changing the afferent excitatory response in second order rNST neurons.

Subsets of the initial burst and tonic firing neurons are also spontaneously active. This spontaneous release of inhibitory neurotransmitter would be an important source of rNST tonic inhibition reported by investigators using both intracellular and extracellular recordings in vivo and in brain slices (Wang and Bradley 1993; Smith and Li 1998). Synaptic activation of the tonically firing neurons would augment the tonic level of inhibition.

Investigators of the rNST using extracellular recording in vivo rarely report neurons that respond to chemical stimulation of the tongue with a short burst of action potentials. In addition neurons with high levels of spontaneous activity are infrequently encountered (McCaughey, 2007). There are several possible reasons for the differences between the results of the in vivo and in vitro recordings of rNST neurons. Firstly, the goal of almost all the extracellular recordings is to determine the responses of rNST neurons to chemical stimulation of the tongue. Presumably neurons that do not respond to the chemical search stimuli are not selected for study eliminating populations of rNST neurons from analysis. Secondly, most often data reported in the in vivo studies consists of mean counts of action potential frequency and raw data records are not always provided to reveal burst properties. However, when recordings of action potentials are provided rNST neurons respond to different taste stimuli with different response patterns
including short bursts of action potentials (Doetsch and Erickson, 1970; Lemon and Margolskee, 2009). Finally, the slice preparation effectively eliminates all ascending and descending connections to rNST neurons. Investigators have shown using in vivo recording that stimulation of gustatory cortex results in the modulation of spontaneous activity and responses to chemicals of rNST neurons (Smith and Li, 2000). The elimination of the ascending and descending input may possibly alter the response properties of the in vitro recordings accounting for some of the discrepancies. However, the in vivo experiments are performed under anesthesia which could also influence rNST neuron responses. Thus, while there a number of reasons why these GAD67-GFP neuron responses have not been sampled in the in vivo studies it is important to note that these neurons as well as other GABAergic neurons are a major component of the rNST and must play an important role in sensory processing.

**Distribution of GAD67-GFP rNST neurons**

The distribution of the GAD67-GFP rNST neurons was not uniform. While the mean count of GABA\(_A\) receptor immunoreactive neurons in rNST has been reported to be 1270, half of these are located in the ventral subdivision (King 2003). Travers et al. (2007) also reported that GAD67-GFP positive neurons are densely distributed in the ventral subdivision of the rNST. The ventral subdivision is the origin of efferent projection from the rNST to the parvocellular reticular formation, an area that contains neurons targeting oromotor brainstem nuclei (Travers 1988; Beckman and Whitehead 1991). Ventral subdivision rat rNST neurons that can be retrogradely labeled by injections into the reticular formation ventral to rNST are all either elongate or multipolar cells (Halsell et al. 1996). In Golgi preparations of the hamster rNST, neurons in the
ventral subdivision have predominantly ovoid soma with only a few multipolar neurons (Whitehead 1988). Presumably the Golgi stained ovoid cells in ventral rNST correspond to the GAD67-GFP neurons characterized in the present study and the parvocellular projecting neurons are probably the population of NeuN staining neurons in the ventral subdivision that are not GFP positive.

The number of the GAD67-GFP neurons decreases in the more rostral extent of the rNST, the decrease being most marked in the ventral subdivision. The caudal-rostral change in topographical distribution of the GAD67-GFP neurons mirrors the topographical projection pattern of the anterior and posterior tongue onto the gustatory NST and may relate to the different functional roles of anterior and posterior tongue in taste processing. The anterior tongue input to rNST functions in stimulus identification while posterior tongue input plays a role in ingestive motivation (Spector and Glendinning 2009). Investigators have reported that transection of the IXth nerve in rats severely diminishes bitter taste-stimulated reflex oral gaping movements that are characteristic of aversive tasting stimuli, whereas transection of the VIIth nerve has a much less significant effect on oral-motor behavior (Travers et al. 1987; King et al. 2008). Thus, change in the ventral rNST density of GFP neurons may relate to reticular formation oromotor control circuit connections important in taste initiated acceptance and rejection behavior (Halsell et al. 1996).

Connections between afferent input and GAD67-GFP neurons

Based on terminal field labeling, except for scattered GAD67-GFP neurons in the terminal field, connections between the afferent gustatory input and the ventral subdivision GAD67-GFP neurons are not extensive. Only GFP neurons at the dorsal
border of the ventral subdivision have dendrites that extend into the afferent terminal field in the rostral-central subdivision. This suggests a polysynaptic input via second order neurons in the rostral central subdivision. GAD67-GFP neurons in the terminal field area may have monosynaptic connections as suggested in our earlier work where a high proportion of rNST neurons responded monosynaptically to ST stimulation (Wang and Bradley 1995). In the caudal visceral-sensory NST the synaptic input to GFP neurons in GIN mice has been characterized by recording neuron responses elicited by stimulation of the ST. The majority of the caudal NST GFP positive neurons have monosynaptic connections with the primary afferent input (Bailey et al. 2008). In coronal sections that include the caudal NST the ventral subdivision density of GFP cells is minimal and GFP neurons are reported to be scattered throughout the NST (see Fig. 2b, bottom panel of the serial sections) (Glatzer et al. 2007). Thus, based on location, the GFP positive neurons in the ventral rNST may have a very different synaptic connection with the primary afferent gustatory input suggesting a different functional role than caudal NST GFP neurons. Examination of the synaptic properties of the rNST GFP neurons will require further experiments.

Subpopulations of GAD67-GFP rNST neurons

In the cortex GABAergic interneurons have been divided into several subpopulations based on colocalization of calcium binding proteins and other neuropeptides (Kubota et al. 1994; Kawaguchi and Kubota 1997). In particular all the GAD67-GFP neurons in GIN mice colocalize somatostatin (Oliva et al. 2000). In the present study only a few GAD67-GFP neurons immunoreacted with somatostatin. Thus,
the GFP neurons in rNST differ from the GAD67-GFP neuron in the cortex and hippocampus.

In this study we have used GIN mice to identify and characterize GABAergic interneurons in rNST. However, only 46% of the GABA positive neurons in the rNST are GFP neurons. Thus, the GFP is not reporting other populations of GABAergic neurons. Similar results were noted for lamina I of the dorsal horn of the spinal cord and hippocampus where approximately 25% of the spinal cord and 7-22% of the hippocampus GABA immunoreactive cells are not GFP positive (Oliva et al. 2000; Dougherty et al. 2005).

In the current study very few of the GFP neuron soma were found to be parvalbumin positive while only 15% were somatostatin positive. In hippocampus virtually all GFP neurons co express somatostatin (Oliva et al. 2000). There are also marked differences in morphology of the GFP neurons in different brain areas. In rNST the GFP neurons are relatively simple bipolar neurons (ovoid neurons) while these neurons have more complex morphologies in the spinal cord (Stornetta and Guyenet 1999) and hippocampus (Oliva et al. 2000).

While somatostatin immunoreactive soma are rarely encountered in rNST, somatostatin immunoreactive fibers were observed to form a dense network surrounding both GFP and non-GFP expressing neurons. Other investigators have also reported somatostatin immunoreactive fibers throughout the NST (Kalia et al. 1984; Maley 1996) and somatostatin and GABA have been reported to coexist in other brain locations (Somogyi et al. 1984; Van den Pol 1986; Kubota et al. 1994). Somatostatin immunoreactive neurons have been demonstrated in the central nucleus of the amygdala
(CeA) (Wray and Hoffman 2007) and microinjection of retrograde tracer labels fibers that differentially distribute throughout the rostral caudal extent of the NST (Halsell 1998; Saha et al. 2000). Moreover, orthodromic activation by electrical stimulation of the CeA results in modulation of the responses of rNST neurons to taste stimuli applied to the oral cavity (Li et al. 2002) suggesting a role of the somatostatin fibers and the GAD67-GFP neurons in the centrifugal control of taste processing.

Functional considerations

Using immunohistochemical techniques previous investigators of the rNST described a single population of GABAergic interneurons (Davis 1993). However, we have now demonstrated that even a subpopulation of GABAergic interneurons, identified by expressing GFP under the control of a GAD67 promoter, can be subdivided based on repetitive firing properties and expression of neuropeptides. Thus, inhibitory processing in rNST is far more complex than suggested by the anatomical studies. The GABAergic interneurons studied here probably have polysynaptic connections with the primary afferent input and when depolarized transform the primary taste evoked sensory information. The majority of GABAergic interneurons -the initial burst neurons - would transmit the early dynamic portion of the encoded sensory stimuli. In contrast the smaller population of tonic firing neurons would respond to both the dynamic and static components of the sensory input. Moreover, because of their concentration in a subdivision of the rNST known to project to brainstem circuits involved in tongue and facial muscle movements, these rNST GABAergic interneurons have an important role in taste guided muscle movements. The rostral-caudal differences in GFP positive neuron
distribution suggest different functional roles of the GABAergic interneurons related to
the source of the receptive field connections.
Supported by National Institute on Deafness and Other Communication Disorders Grant number DC-000288 to Robert M. Bradley.
REFERENCES


Halsell CB, Travers SP, Travers JB. Ascending and descending projections from the rostral nucleus of the solitary tract originate from separate neuronal populations. *Neuroscience* 72: 185-197, 1996.


Somogyi P, Hodgson AJ, Smith AD, Nunzi MG, Gorio A, Wu JY. Different populations of GABAergic neurons in the visual cortex and hippocampus of cat
contain somatostatin- or cholecystokinin- immunoreactive material. *J Neurosci* 4: 2590-2603, 1984.


Figure Legends

FIG 1. Distribution of GAD-GFP neurons in the rostral nucleus of solitary tract (NST) in mouse brainstem.  

a. Horizontal section of the mouse brainstem stained with luxol fast blue and cresyl violet to show the NST. The blue stained solitary tracts (ST) are located adjacent to the lateral border of the nucleus. R = rostral, L = lateral. 

b. A similar horizontal section from a GIN mouse showing the fluorescently labeled GFP neurons. A dense band of GFP neurons are located lateral to the ST in the caudal NST (arrowheads). A further concentration of GAD67-GFP neurons is localized in the more rostral medial NST (asterisks). 

Bar = 200 μm. 

c, d, e. Selected coronal sections of rNST at the levels indicated in a. The standard subdivisions of the rNST are outlined in e. Medial subdivision = M, rostral central subdivision = RC, rostral lateral subdivision = RL, and ventral subdivision = V. GFP neurons are densely concentrated in the V subdivision. The other subdivisions also contained some GFP positive neurons. The RC subdivision had the least amount of GFP neurons. ST = solitary tract. Inset in e is a low magnification photomicrograph of the right dorsal quadrant of a coronal section of the brain stem indicating the location of the higher magnification images of c, d, and e (white box outlines location of higher magnification images). Bar in both high and low magnification images = 100μm. 

c, d, e, series of selected coronal sections are arranged from from rostral (c) to caudal (e) rNST. There is 150μm distance between c and d and 100μm between d and e. GFP neurons gradually decreased in density from caudal to rostral NST. Bar = 100 μm. f. Total counts of GAD67-GFP neurons in all subdivisions of rNST coronal sections at different caudal to rostral distances from the
obex. The number of cases used to calculate each point is indicated above each data point.

FIG. 2. Terminal field pattern of CT and IX afferent nerves in GIN mice. **a.** Fluorescently labeled (red) chorda tympani (CT) nerve terminal field in a rostral-caudal serial sections. The top panel shows the entrance of the CT nerve followed by panels of serial sections of NST from rostral to caudal. The numbers on the right of the sections are the distances between the sections and the obex. Bar = 100 μm. **b.** Fluorescently labeled (red) glossopharyngeal (IX) nerve terminal field in a rostral-caudal serial sections. The top panel shows the entrance of the IX nerve followed by panels of serial sections of NST from rostral to caudal. The numbers on the right of the sections are the distances between the sections relative to the obex. D = dorsal and M = medial. Bar = 100 μm. **c.** Higher magnification image of CT terminal field showing that the afferent fibers are mainly restricted to the rostral central subdivision with minimal penetration into the ventral subdivision where GFP neurons are located. Bar =100 μm. **c1, c2, c3.** Higher magnification images of c at the three indicated locations illustrating minimal penetration of the terminal field fibers into the ventral subdivision. Bar = 10 μm.

FIG. 3. Co-localization of GFP neurons with NeuN and GABA. **a.** Co-localization of NeuN (red) positive cells with a subpopulation of GFP (green) cells in rNST. **a1, a2, a3.** Higher magnification of the area outlined in a showing co-localized GFP and NeuN positive cells (arrows) in the merged image. **b.** Co-localization of GABA (red) positive and GFP neurons. Bar = 100 μm. **b1, b2, b3.** Higher magnification of the area outlined in b showing co-localization of GABA positive and GFP neurons (arrows) in the merged images. Bar = 10 μm.
FIG. 4. Repetitive firing patterns of the rNST GFP neuron responses to a 1000 ms depolarizing current pulse. a1 Initial burst firing, b1 tonic firing and c1 irregular firing. a2, b2, c2 recorded at membrane potential set at -60 mV by bias current injection.

Repetitive firing patterns of the same GFP neurons to a 100 ms hyperpolarizing current pulse followed by a 1000 ms depolarizing current pulse at resting membrane potential. Membrane hyperpolarization had no effect on the repetitive discharge pattern evoked by membrane depolarization. All neurons were maintained at – 60 mV by current injection.

a3, b3, c3. Responses of the same neurons to a series of hyperpolarizing and depolarizing current injections at the neuron resting membrane potential. Note the membrane sag in b3 (arrow).

FIG. 5. Voltage clamp recordings from rNST GFP and non-GFP labeled neurons. GFP neuron responds to hyperpolarization followed by depolarization without an initial outward current (a1, a2, a3). In contrast the non-GFP neuron responded to the same voltage protocol with an initial transient outward current (b1) that is blocked by application of 4-AP (b2). b3 is an arithmetical subtraction of the membrane voltage records in b1 and b2.

FIG. 6. rNST GAD67-GFP neuron morphology. Examples of Lucifer yellow injected neurons of each of the three firing patterns are illustrated in a, b and c with tracings of the same neurons in d, e and f. Bar = 10 μm. g. Location of filled neurons mapped onto a diagram of a coronal section of the left rNST. All neurons are of the initial burst pattern except those marked TF (tonic firing), and IF (irregularly firing). Bar = 250 μm. h, i. comparison of the morphometric characteristics of the soma and dendrites of neurons
with burst, tonic and irregular firing patterns. Data are presented as means ± SE. *
indicates significant differences between the means at the p < 0.05 level.

FIG. 7. Passive and action potential characteristics of initial burst, tonic and irregular firing GFP neuron groups. **Passive membrane properties.** **D, E, F, G, H, I.** action potential characteristics. The number of neurons in each group are indicated in **A, and** data is presented as the mean ± SE. * indicates significant differences between means at the p<0.05 level. **J, K, L.** Examples of the action potentials of initial burst, tonic and irregular firing neuron groups.

FIG. 8. Distribution of somatostatin and parvalbumin immunoreaction in GIN mouse rNST. **A.** Somatostatin positive fibers are distributed throughout the rNST. **A1** GFP neurons (green); **A2** somatostatin positive fibers (red); **A3** merged image showing GFP and somatostatin immunoreaction. **B.** Parvalbumin is largely absent from the rNST. Bar = 100μm. **B1** GFP neurons (green); **B2** parvalbumin positive immunoreaction (red); **B3** merged images of GFP and parvalbumin immunoreaction. **C1, C2 and C3.** Higher magnification images of co-localized somatostatin and GFP neurons indicated in the white box in **A3.** Bar = 10 μm. **D1, D2 and D3.** Higher magnification images of the somatostatin immunoreacted fibers and GFP neurons in the rostral central subdivision. In the merged image (**D3**) the GFP neurons are observed to be surrounded by somatostatin positive fibers. Bar = 10 μm. **E.** Higher magnification images of co-localized parvalbumin and GFP neurons. **E.** Few GFP neurons in rNST co-localize with parvalbumin except at the ventral border (box in **B3**).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Source</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuN</td>
<td>Purified cell nuclei from mouse brain</td>
<td>Millipore (Chemicon, Temecula, CA), mouse monoclonal, MAB 377</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid conjugated to BSA</td>
<td>Sigma-Aldrich, rabbit polyclonal, A-2052</td>
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<tr>
<td>Somatostatin - 14</td>
<td>Synthetic peptide AGCLNFFWKFTFTSC of somatostatin - 14</td>
<td>Bachem (Torrence, CA), rabbit polyclonal, T-4103</td>
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<tr>
<td>Parvalbumin</td>
<td>Purified frog muscle parvalbumin</td>
<td>Sigma-Aldrich, mouse monoclonal P-3088</td>
<td>1:1000</td>
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Table 2. Percent of co-localization of GAD67-GFP and GABA immunoreactive neurons in rNST subdivisions*

<table>
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<tr>
<th>rNST sub-division</th>
<th>Percentage of neurons double labeled with GABA and GAD67-GFP</th>
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<tbody>
<tr>
<td>Rostral medial</td>
<td>28%</td>
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<tr>
<td>Rostral central</td>
<td>27%</td>
</tr>
<tr>
<td>Rostral lateral</td>
<td>33%</td>
</tr>
<tr>
<td>Ventral</td>
<td>64%</td>
</tr>
</tbody>
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* Based on 4 coronal confocal sections in 2 mice