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Substrates of Auditory Frequency Integration in a Nucleus of the Lateral Lemniscus

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Abstract

In the intermediate nucleus of the lateral lemniscus (INLL), some neurons display a form of spectral integration in which excitatory responses to sounds at their best frequency are inhibited by sounds within a frequency band at least one octave lower. Previous work showed that this response property depends on low-frequency-tuned glycinergic input. To identify all sources of inputs to these INLL neurons, and in particular the low-frequency glycinergic input, we combined retrograde tracing with immunohistochemistry for the neurotransmitter glycine. We deposited a retrograde tracer at recording sites displaying either high best frequencies (>75 kHz) in conjunction with combination-sensitive inhibition, or at sites displaying low best frequencies (23–30 kHz). Most retrogradely labeled cells were located in the ipsilateral medial nucleus of the trapezoid body (MNTB) and contralateral anteroventral cochlear nucleus. Consistent labeling, but in fewer numbers, was observed in the ipsilateral lateral nucleus of the trapezoid body (LNTB), contralateral posteroventral cochlear nucleus, and a few other brainstem nuclei. When tracer deposits were combined with glycine immunohistochemistry, most double-labeled cells were observed in the ipsilateral MNTB (84%), with fewer in LNTB (13%). After tracer deposits at combination-sensitive recording sites, a striking result was that MNTB labeling occurred in both medial and lateral regions. This labeling appeared to overlap the MNTB labeling that resulted from tracer deposits in low-frequency recording sites of INLL. These findings suggest that MNTB is the most likely source of low-frequency glycinergic input to INLL neurons with high best frequencies and combination-sensitive inhibition. This work establishes an anatomical basis for frequency integration in the auditory brainstem.

Keywords

combination sensitivity; spectral integration; glycine; mustached bat; INLL; *Pteronotus parnellii*

Although the initial stages of the auditory system create and maintain a spectral decomposition of sounds, acoustically guided behavior and perception are based on integration of information across spectral elements. Whether the task is localization of sounds (Hebrank and Wright, 1974, Knudsen and Konishi 1979; Middlebrooks, 1992; Populin and Yin, 1998), the analysis of sonar echoes (Simmons et al., 2004; Genzel and

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Wiegrebe, 2008), or responses to conspecific vocalizations in social interactions (Park and Dooling, 1985; Boothroyd et al., 1996; Shannon et al., 2004; Moore, 2008), the ability to integrate information across the audible sound spectrum is fundamental to an appropriate behavioral response.

How is integration across the sound spectrum performed in the brain? In one sense, single neurons of the auditory nerve already perform some forms of spectral integration, since they display two-tone suppression and their tuning curves often include low frequency tails (Sachs and Kiang, 1968; Arthur et al., 1971; Kiang and Moxon, 1974). Further, there is abundant evidence that responses of central auditory neurons are affected by frequencies outside their classical tuning curves (Young and Brownell, 1976; Suga et al., 1978; Shamma and Symmes, 1985; Margoliash and Fortune, 1992; Mittmann and Wenstrup, 1995; Rauschecker et al., 1995; Ohlemiller et al., 1996; Imig et al., 1997; Sutter et al., 1999), suggesting that these responses depend on interactions within the central nervous system. The circuitry underlying these interactions most likely depends on projections outside of the normal frequency-by-frequency pattern of connections within the auditory system, but such circuitry is generally not understood.

The current study examines circuitry underlying spectral integration in the brainstem of the mustached bat, a mammalian species that depends on the analysis of spectrally complex biosonar and social vocalizations. Many neurons in the auditory midbrain, thalamus, and forebrain of this bat display a form of temporally sensitive spectral integration called combination sensitivity, in which the neuron's excitatory response to one frequency band is inhibited or facilitated by another frequency element that is one to three octaves lower (Suga et al., 1978; O'Neill and Suga, 1979; O'Neill, 1985; Olsen and Suga, 1991; Mittmann and Wenstrup, 1995; Wenstrup, 1999). While the facilitatory interaction originates within the midbrain's inferior colliculus (IC) (Portfors and Wenstrup, 2001; Wenstrup and Leroy, 2001; Nataraj and Wenstrup, 2005; Marsh et al., 2006), the inhibitory spectral interaction originates within the intermediate and ventral nuclei of the lateral lemniscus and depends on low frequency-tuned glycinergic inhibition of high-frequency-tuned excitatory responses (Peterson et al., 2009). The present study examines the source of this low-frequency, glycinergic input to provide evidence of a spectrally integrative circuit within the auditory brainstem.

EXPERIMENTAL PROCEDURES

Seventeen adult mustached bats (*Pteronotus parnellii*), captured in Trinidad and Tobago, were used to examine sources of inputs to combinatorial neurons of the intermediate nucleus of the lateral lemniscus (INLL). Our procedures were approved by the Institutional Animal Care and Use Committee at the Northeastern Ohio Universities Colleges of Medicine and Pharmacy. These procedures follow guidelines set by the National Institutes of Health for the care and use of laboratory animals.

Surgical procedures

Surgery was performed to fix a post on the animal's skull for stereotaxic positioning of electrodes in nuclei of the lateral lemniscus (NLL). Before surgery, each animal received an intraperitoneal injection of butorphanol (5 mg/kg, Torbugesic, Fort Dodge Animal Health, Fort Dodge, IA) and was anesthetized with Isoflurane (1.5–2.0% in oxygen; Abbott Laboratories, North Chicago, IL). After the anesthetic abolished nociceptive reflexes, the hair on the bat's head was removed with depilatory lotion. A midline incision was made in the skin and the underlying muscles were reflected laterally to expose the skull. A metal pin was cemented onto the skull to secure the head to a stereotaxic apparatus used during the experiments and a tungsten wire was cemented into a small hole in the skull overlying the

cerebral cortex to serve as a reference electrode. For access to NLL, a small hole was made on the dorsal surface of the skull overlying the caudal IC. A local anesthetic (lidocaine) was applied to the surgical area. The bat recovered from surgery for at least two days prior to physiological recordings.

Acoustic stimulation

Search stimuli (noise bursts: 61 ms duration, 0.5-ms rise-fall time, 4 per second) and test stimuli (tone bursts or combinations of tone bursts: 4 to 61 ms, 0.5-ms rise-fall time, 4 per second) were digitally synthesized and downloaded onto a digital signal processor (AP2 Multi-Processor DSP card; Tucker-Davis Technologies, Alachua, FL). They were converted to analog signals at a sampling rate of 500 kHz (model DA3-2; Tucker-Davis Technologies; Alachua, FL), filtered (model FT6-2; Tucker-Davis Technologies, Alachua, FL), attenuated (model PA4; Tucker-Davis technologies, Alachua, FL), summed (model SM3; Tucker-Davis technologies, Alachua, FL), amplified (model HCA-800II; Parasound, San Francisco, CA), and then sent to the loudspeaker (Infinity EMIT-B; Harmon International Industries, Woodbury, NY). The speaker was placed 10 cm away from the animal and 25° into the sound field contralateral to the NLL under study. The output of the acoustic system was tested with a calibrated microphone (model 4135; Brüel and Kjaer, Naerum, Denmark). The system response had a gradual roll-off of approximately 3 dB per 10 kHz. Harmonic distortion was not detectable 60 dB below the signal level using a fast Fourier analysis of the digitized microphone signal (Tucker-Davis Technologies, model AD2).

Neurophysiological recording

Electrophysiological recordings were similar to those described in a previous study of the lateral lemniscal nuclei (Peterson et al., 2009). The bat was placed within a custom-made stereotaxic apparatus housed in a single-walled Industrial Acoustic chamber (New York, NY), lined with polyurethane foam to reduce echoes. If the bat showed signs of discomfort, it was lightly sedated with butorphanol (0.05 to 0.1 mg/kg, subcutaneous). Recording sessions typically lasted 4–6 hours and were limited to one session per day.

The evoked activity of clusters of a few neurons (multiunit responses) was recorded using micropipette electrodes filled with 1 M NaCl (tip diameter: 2–5 μm) or with a solution containing a neural tracer for axonal transport studies (tip diameter: 8–12 μm). Electrodes were advanced by a hydraulic micropositioner (David Kopf Instruments, model 650) from the dorsal surface of the caudal IC to below the ventral nucleus of the lateral lemniscus (VNLL). Extracellular action potentials from the recording electrodes were amplified, bandpass filtered (600–6000 Hz), and sent through a spike signal enhancer (model 40–46-1; Fredrick Haer Company, Bowdoinham, ME) before being digitized at a 40 kHz sampling rate (model AD2; Tucker-Davis Technologies). A second AP2 digital signal processor (Tucker-Davis Technologies, Alachua, FL) uploaded the digital signal to the computer. The custom-made software calculated the time of occurrence of the spikes and displayed post stimulus time histograms, rasters, and basic statistics on the neural responses in real time. During the first few days of recording, electrodes without tracer were placed to map neural activity within the IC and NLL. Responses to tone bursts were examined at 50–100 μm intervals to identify best frequency (BF: the frequency requiring the lowest intensity to elicit stimulus-locked spikes) and minimum threshold at best frequency (the lowest sound level to elicit stimulus-locked spikes). These penetrations established the borders and frequency organization in NLL for subsequent tracer deposits.

Tracer deposits were made at high-BF sites ($\text{BF} \geq 75 \text{ kHz}$) showing combination-sensitive inhibition or at low-BF sites tuned to frequencies of 23–30 kHz. In penetrations using electrodes filled with tracer, NLL multiunit responses were examined in detail. First, BF and

minimum threshold were obtained audiovisually. Subsequent quantitative tests examined responsiveness across a broad frequency range (10–110 kHz) at one or more attenuation values and across a range of sound levels at BF. We used a two-tone stimulus paradigm to evaluate the presence of low frequency inhibition of high-BF excitatory responses. One tone was set to the multiunit BF and presented 10 dB above its threshold. The second tone (a lower frequency signal) was set to frequencies within the first harmonic of the biosonar call (23–30 kHz) and varied over a range of intensities and timing (delays) relative to the BF signal. Delay sensitivity was evaluated in 2-ms steps. Typically, short duration stimuli (4 ms) were used to reveal maximum temporal sensitivity. Neurons were considered to show low frequency inhibition of high-BF signals if the low frequency tone suppressed the excitatory response to the BF tone by at least 20%. These criteria are consistent with features of combination sensitivity in INLL that depend on glycinergic inhibition (Peterson et al., 2009). In experiments featuring tracer deposits tuned to BFs in the 23–30 kHz range, only frequency response and rate-level functions were obtained. After completion of all tests, a retrograde tracer was deposited.

To achieve restricted tracer deposits, most axonal transport experiments used FluoroRuby (FR; tetramethylrhodamine dextran, MW 10,000, 10% in filtered 0.9% saline, Molecular Probes, Eugene, OR). FR was ejected using pulsed current (+ 5 μ A; 7 s on/off) for 10–15 minutes. Some experiments used FluoroGold (2% in filtered 0.9% saline; FluoroChrome, Inc., Englewood, CO). FluoroGold was ejected using pulsed current (+ 2 – +5 μ A) for 3–8 minutes. The electrode was kept in the brain at least for five minutes to minimize accidental ejection of residual tracer during the retraction of the electrode.

Histological methods

Animals used in transport studies survived 5–7 days before they were euthanized. Each animal was killed with an overdose of Fatal Plus (>100 mg/kg, i.p., Vortech, Dearborn, MI). Following loss of corneal and withdrawal reflexes, the animal was perfused through the left ventricle with phosphate buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The skull was removed and the brain was blocked in a transverse plane angled 15° dorso-caudal to ventro-rostral. After removal, the brain was stored overnight at 4°C in a 30% sucrose-phosphate buffer solution, and then sectioned on a freezing microtome at a thickness of 35–40 μ m. Sections were collected from the cochlear nucleus through the auditory cortex in three alternating series. Typically, one series was mounted on gelatin-coated slides and stained with cresyl violet to identify cytoarchitectural boundaries. One or two series (to be used for fluorescence) were mounted on gelatin-coated slides and coverslipped with DPX.

In several experiments, the third series was reserved for glycine immunocytochemistry using anti-sera compatible with paraformaldehyde fixation. Sections were first treated in 0.3% hydrogen peroxide in PBS for 30 minutes, in order to suppress endogenous peroxidase activity. After three 5-minute rinses in PBS, the sections were placed in a blocking solution of 0.5% Triton X, 10% normal goat serum, and 0.2% bovine serum albumin overnight at 4°C. Following two 5-minute washes in PBS, the tissue was then incubated with a polyclonal rabbit anti-glycine (1/ 1000, Immunosolution PTY Ltd, Everton Park, Australia, # IG1001) in PBS containing 1% Triton-X, 10% normal goat serum and 0.2% bovine serum albumin for 48 hours at 4°C. After three 5-minute rinses, the tissue was incubated for 1.5 hours with secondary antibody (1/ 250; goat anti-rabbit; Alexa 488, Molecular Probes, #A11008) with 10% normal goat serum and 0.2% bovine serum albumin in PBS. After two 5-minute washes with PBS and then phosphate buffer, sections were mounted, cleared, and then coverslipped with DPX. All steps were performed at room temperature unless noted. The optimum amount of labeling was determined by testing antibody dilutions of 1:750, 1:1000, and 1:1500. In some sections, immunocytochemistry was performed with omission

of primary (Immunosolution, # IG1001) or secondary antibodies (Molecular Probes, #A11008). These controls did not result in cell-specific labeling in histological sections.

Data analysis

Retrogradely labeled, immunolabeled and double-labeled cells were plotted using a Zeiss Axioplan II microscope and Neurolucida reconstruction system (MBF Bioscience, Williston, VT, USA). Outlines of brainstem nuclei and subdivisions were drawn in Neurolucida from the unstained DPX-mounted sections, with adjacent Nissl-stained sections serving as references. Cytoarchitectonic boundaries are based on previous work in mustached bats (Zook and Casseday, 1982; Kossel and Vater, 1990; Vater, 1995). Tracer deposit sites and labeled brainstem neurons were photographed with a Zeiss AxioCam HR3 and AxioVision software (version 4.6; Zeiss) mounted on a Zeiss Imager Z1m fluorescence microscope. Adobe Photoshop CS3 was used to overlay injection images, to adjust brightness and contrast, and to add labels.

RESULTS

Inhibitory combination-sensitive responses in INLL require excitatory inputs tuned to the neuron's high BF and a glycinergic input tuned at least an octave lower in frequency (Peterson et al., 2009). The results describe circuitry that could underlie this complex response. To identify brainstem inputs to INLL neurons showing inhibitory combination sensitivity, we placed physiologically defined tracer deposits in INLL. To identify low-frequency-tuned inputs to high-BF combination-sensitive recording sites, we compared the projection patterns obtained after deposits at these high-BF recording sites with those obtained after deposits at INLL sites tuned to low frequencies. To identify the sources of glycinergic input to INLL neurons, especially low-frequency-tuned inputs, we combined the tracer deposits with glycine immunocytochemistry.

Deposit sites

Deposits of FR were highly restricted within INLL (Fig. 1). In five cases with successful transport, deposits were placed in the ventrolateral INLL at sites that displayed a high best excitatory frequency (75–84 kHz) and inhibition tuned to lower frequencies in the range 29–30 kHz. We chose this high BF band because it was sufficiently distant from representations of 23–30 kHz both in INLL and in the adjacent multipolar subdivision of the ventral nucleus of the lateral lemniscus (VNLLm) (Fig. 2). The deposit illustrated in Figure 1A was placed along an electrode penetration that recorded increasing best frequencies in INLL (Fig. 2A) in accord with the anatomically demonstrated tonotopic organization (Zook and Casseday, 1985; Frisina et al., 1989; Wenstrup et al., 1994). At the deposit site, multiunit responses were maximal near 75 kHz (Fig. 2B) and displayed a monotonically increasing response (Fig. 2C). Although there was some response to low frequency signals (Fig. 2B), there was little at the neuron's best inhibitory frequency of 29 kHz (Fig. 2C). When the best excitatory and inhibitory frequencies were combined, the low frequency tone suppressed the high frequency excitatory response at delays near 0 ms, (simultaneous presentation, Fig. 2D). At all combination-sensitive tracer deposit sites, inhibition was best at simultaneous presentation. These responses are consistent with features of combination-sensitive inhibition in INLL that are mediated by low frequency glycinergic input (Peterson et al., 2009).

In two cases, restricted FR deposits were placed in the dorsomedial INLL at sites tuned to lower frequencies, 24 kHz or 26 kHz (Fig. 1B). The deposit in Fig 1B occurred within an electrode penetration that revealed the expected tonotopic increase in frequencies with

greater depth in INLL (Fig. 2E). Even at high sound levels, there was no response to frequencies above 55 kHz at the deposit site (Fig. 2F).

We did not observe any label suggesting that axons passed through the high-BF, ventrolateral INLL en route to the low-BF, dorsomedial INLL. Thus, tracer deposits in the ventrolateral part did not label terminals in the dorsomedial INLL, nor did deposits in the dorsomedial part result in filled axons passing through the ventrolateral part.

Retrograde labeling after INLL deposits

FR deposits in INLL resulted in relatively small numbers of well-labeled somata throughout the brainstem (Fig. 3). Of seven successful transport cases, all but one had between 48 and 76 labeled brainstem cells (as plotted in a 1-of-3 series of sections). The general features of retrograde labeling were similar for deposits at both high-BF combination-sensitive sites and low-BF recording sites (Fig 4–Fig 6). Thus, the greatest number of labeled neurons was consistently in the ipsilateral medial nucleus of the trapezoid body (MNTB), contralateral ventral cochlear nucleus (VCN), and ipsilateral lateral nucleus of the trapezoid body (LNTB). Fewer or less consistent numbers of labeled cells were observed in other ipsilateral structures, including the ventral periolivary nucleus (VPO), VCN, and multipolar subdivision of the ventral nucleus of the lateral lemniscus (VNLLm). Very little or no labeling was observed in the columnar subdivision of VNLL (VNLLc), the dorsal cochlear nucleus bilaterally, the entire contralateral superior olivary complex, and the medial and lateral superior olives on the ipsilateral side. A FluoroGold case, with a deposit limited to the INLL, displayed a nearly identical pattern (Supplemental Figure). These results closely match those reported in a study of INLL inputs in the big brown bat (Huffman and Covey, 1995).

The main difference in retrograde labeling after deposits in high-BF, combination-sensitive sites versus low-BF sites related to the locations of cells *within* auditory brainstem nuclei. After FR deposits at combination-sensitive recording sites, labeling in the anteroventral cochlear nucleus (AVCN) was located more caudally in both the anterior and posterior parts (Fig. 4A), compared to that observed after low frequency deposits (Fig. 4B). In the posteroventral cochlear nucleus (PVCN), labeling after high-BF deposits was in the lateral part (Fig. 4A) whereas labeling after low-BF deposits was in the medial part (Fig. 4B). In both AVCN and PVCN, the labeling patterns corresponded to the tonotopic organization established in previous studies (Ross et al., 1988; Kössl and Vater, 2000; Marsh et al., 2006).

The labeling in the ipsilateral MNTB showed a different pattern. High-BF combination-sensitive deposits labeled cells in both the medial and lateral parts of MNTB, whereas low-BF deposits labeled cells only in the lateral MNTB. Previous work suggests that best frequencies are arranged from low (lateral) to high (medial) in the MNTB of mustached bats (Zook and Casseday, 1985; Zook and Leake, 1989; Kuwabara and Zook, 1992). In each case with deposits at combination-sensitive recording sites, labeled cells were located in both the lateral and medial MNTB (Fig. 4A, 7A). In contrast, the two low frequency deposits in INLL labeled only the lateral part of MNTB (Fig. 4B, 7B). These observations suggest that INLL sites characterized by high BFs and low frequency inhibition receive MNTB input from two frequency bands. Low frequency INLL sites receive input only from the lateral, low frequency part of MNTB.

There was no clear frequency-specific difference in the distribution of retrograde labeling in either the ipsilateral LNTB or VPO.

Glycinergic inputs to combination-sensitive INLL sites

We examined glycine immunohistochemistry in eight mustached bats, four of which received FR deposits at high-BF combination-sensitive sites in INLL. Among auditory nuclei, anti-glycine immunolabeling was particularly robust in cells of the MNTB (Fig. 8A–D, *glycine*) and the columnar subdivision of VNLL (VNLLc, not shown). However, nearly every major auditory brainstem structure contained well-labeled neurons (e.g., Fig. 8E–F, *glycine*).

Plots of glycine labeling throughout the auditory brainstem (Fig. 9) reveal that MNTB and VNLLc contain large numbers of immunolabeled cells, while LNTB and VCN contained a substantial number of such cells. Numbers of immunolabeled cells were fewer in the medial and lateral superior olive, VNLLm, and INLL. The inferior colliculus and the dorsal nucleus of the lateral lemniscus (DNLL) contained no labeled somata. The overall pattern of somatic labeling in the brainstem corresponded closely to previous reports in mustached bats (Vater, 1995; Winer et al., 1995).

We observed double-labeled cells in each case combining glycine immunolabeling with FR deposits at combination-sensitive recording sites (Fig. 8, Fig. 9). In the four cases, 19–38% of FR labeled cells (mean, 26%) were also glycine positive. The vast majority was in the MNTB (84% all double-labeled cells, range, 73–95%, Fig. 5). Across the four cases, 44–75% (mean, 56%) of FR labeled cells in MNTB were also glycine positive. Importantly, double-labeled cells were present in both the medial and lateral parts of MNTB (Fig. 8A–D, Fig. 9). These results show that both the lateral, low-BF part of MNTB and the medial, high-BF part of MNTB each contribute glycinergic input to neurons within the uptake zone associated with high-BF combination-sensitive deposit sites in INLL.

The only other structure with a significant number of double-labeled cells was LNTB, with 13% of all double labeling (range, 5–27%, Fig. 5, Fig. 8, Fig. 9). Across cases, 23–50% (mean, 40%) of the FR-labeled cells in LNTB were also glycine immunopositive. Both lateral and medial cells were double-labeled in LNTB. However, there was no clear relationship between the locations of labeled cells in LNTB and the BF of the deposit site.

Although the contralateral VCN provided the second largest input to inhibitory combination-sensitive neurons of INLL, there were no double-labeled cells in any division of VCN in any case (Fig. 5, Fig. 8G, Fig. 9).

DISCUSSION

This study examined circuitry underlying a form of auditory spectral integration characterized by excitation evoked by sounds within one frequency band and inhibition evoked by sounds within a distant frequency band. In the mustached bat, such integration arises in nuclei of the lateral lemniscus and depends on low frequency-tuned glycinergic inhibition (Peterson et al., 2009). The major result of the present study is that high-BF, combination-sensitive INLL neurons receive glycinergic input from the lateral, low frequency parts of MNTB to create combination-sensitive inhibition. This demonstrates new roles for MNTB and INLL neurons in the processing of complex sounds, and indicates that circuitry underlying integration of information from distant spectral elements occurs in the auditory brainstem.

Excitatory and Inhibitory Inputs to INLL Neurons

Afferents to INLL neurons in the mustached bat originate primarily from the ipsilateral MNTB and contralateral VCN, with weaker but consistent input from ipsilateral LNTB, VPO and VCN (Zook and Casseday, 1987; this study). This pattern corresponds closely to

that in big brown bats (Huffman and Covey, 1995). In rats and cats, INLL receives major VCN and MNTB inputs but in addition receives projections from the ipsilateral VNLL, medial and lateral superior olives and superior paraolivary nucleus (in rat) (Glendenning et al., 1981; Kelly et al., 2009). In the dorsal part of the rat VNLL, labeling similar to the bat species has been reported (Kelly et al., 2009). These patterns of INLL input also agree with other studies using anterograde transport methods (Spangler et al., 1985; Zook and Casseday, 1985; Vater and Feng, 1990; Benson and Cant, 2008).

Of nuclei described above, the main source of excitatory input to INLL neurons in bats and a major excitatory input in other species is VCN. We found no evidence that glycinergic VCN neurons project to INLL. In guinea pigs, VCN input to INLL appears to involve excitatory amino acids (Suneja et al., 1995).

Although both glycinergic and GABAergic inputs supply inhibition to INLL (Vater et al., 1992; Winer et al., 1995; Kutscher and Covey, 2009; Peterson et al., 2009), MNTB likely provides the main source of inhibition. Across species, most or all MNTB neurons are glycinergic, (Helfert et al., 1989; Adams and Mugnaini, 1990; Vater, 1995; Winer et al., 1995). In studies of the mustached bat MNTB using 1–2 μm -thick sections, all neurons are reported to be glycine immunopositive except a few non-principal cells located along the margins of the nucleus (Vater, 1995; Winer et al., 1995). In our material, somewhat over 50% of MNTB cells were glycine immunopositive. Our lower percentage of glycinergic MNTB neurons is most likely the result of limited penetration of the antibody through 35–40 μm sections. In fact, it was clear that immunostaining did not always extend through the entire thickness of the sections. Our interpretation, consistent with previous results (Vater, 1995; Winer et al., 1995), is that *all* FR-labeled MNTB cells in our experiments are glycinergic.

Approximately 84% of double-labeled (GLY+, FR+) neurons in our study were located in MNTB. A much smaller number of glycinergic neurons project to INLL from LNTB. Although we did not examine sources of GABAergic input to INLL, previous immunohistochemical studies show that nuclei projecting to our INLL tracer deposit sites do not include many GABAergic neurons (Vater et al., 1992; Vater, 1995; Winer et al., 1995). Overall, our data and previous studies suggest that INLL neurons receive predominant excitatory input from VCN and predominant inhibitory input from glycinergic neurons of the MNTB.

Circuitry Underlying Spectral Integration in INLL

Neurons in INLL display a form a spectral integration termed combination-sensitive inhibition, in which excitatory responses to high-BF signals are inhibited by simultaneous signals in the 23–30 kHz range (Portfors and Wenstrup, 2001; Peterson et al., 2009). Essential circuitry associated with these functional properties is illustrated in Figure 10. For high frequency excitation, the source is most likely the contralateral VCN. For low frequency inhibition, which is fast and exclusively glycinergic (Peterson et al., 2009), the ipsilateral MNTB seems to be the most likely candidate.

What is the evidence that MNTB provides a non-tonotopic, low frequency-tuned input to combination-sensitive INLL neurons? The results here show that both the lateral and medial parts of MNTB project to the ventrolateral, high-frequency region of the INLL that contains combination-sensitive responses. It is the lateral part, however, that is closely associated with low frequency responsiveness, due to its connection to low frequency parts of INLL (this study), VCN (Zook and Leake, 1989), lateral superior olive (LSO), and the medial superior olive (MSO) (Zook and Casseday, 1985; Covey et al., 1991; Kuwabara and Zook, 1991, 1992). These same studies show that the medial part of MNTB is associated with

known high frequency regions of VCN, MSO, and LSO. This topographic pattern of frequency representation is consistent with other mammals (Guinan et al., 1972; Spangler et al., 1985; Vater and Feng, 1990; Kuwabara and Zook, 1991, 1992; Sommer et al., 1993; Kim and Kandler, 2003). In our results, the unexpected retrograde labeling in the lateral MNTB following deposits at combination-sensitive INLL sites does not appear to result from interruption of fibers in transit to the low frequency part of INLL. These considerations strengthen the interpretation that the lateral MNTB provides low-frequency-tuned glycinergic inputs to high-BF neurons in INLL. Another nucleus, LNTB, may contribute in some minor way to this circuit, but its role is questionable because its glycinergic input to INLL is a small fraction of the MNTB input (this study) and because it provides inhibition by *ipsilateral* sounds to the INLL under study (Zook and Casseday, 1985; Kuwabara and Zook, 1992). We therefore conclude that MNTB provides the low-frequency-tuned glycinergic inhibition that is an essential feature of combination-sensitive responses of INLL neurons (Fig. 10).

The participation of MNTB in cross-frequency integrative responses is surprising given its well-known role in binaural circuits for which frequency matching has been emphasized (Boudreau and Tsuchitani, 1968; Guinan et al., 1972; Thompson and Schofield, 2000; Sanes and Friauf, 2000; Kim and Kandler, 2003). This major nucleus of the auditory brainstem had been viewed primarily as a participant in the lateral superior olivary circuit analyzing interaural intensity cues, but several studies have brought attention to its input to the medial superior olive in connection with analyses of interaural timing (Kuwabara and Zook, 1992; Brand et al., 2002; Pecka et al., 2008) and its role in offset responses of the rodent SPN (Kadner et al., 2006; Kulesza et al., 2007). The present study, together with previous work (Peterson et al., 2009), shows that glycinergic inhibition originating in MNTB contributes to spectrally integrative responses, a significant feature of auditory processing and a novel function associated with this nucleus.

There are several examples of central auditory interactions that involve spectral integration across widely separated frequency bands. However, connections that underlie these interactions are generally not well understood. In DCN, the best-known example, neurons with type IV responses display broadly tuned inhibition (Young and Brownell, 1976; Spirou and Young, 1991; Nelken and Young, 1994) that is glycinergic (Doucet et al., 1999; Davis and Young, 2000) and appears to originate in stellate cells of the VCN (Doucet and Ryugo, 1997; Arnott et al., 2004). Spectral integration is performed either by the stellate cells in VCN (Nelken and Young, 1994; Winter et al., 1995) or by integration of stellate cell input by DCN neurons with type IV responses (Lomakin and Davis, 2008). In the mustached bat IC, spectral integration is more specific; facilitated responses to combinations of low and high frequency signals are created by brainstem glycinergic inputs tuned both to the neuron's BF and to a frequency 1–3 octaves below (Wenstrup et al., 1999; Wenstrup and Leroy, 2001; Nataraj and Wenstrup, 2005; Sanchez et al., 2008). In these facilitated neurons, frequency integration may result from a non-tonotopic projection from either INLL or VNLL to high frequency parts of IC (Wenstrup et al., 1999). The present study thus provides one of the few examples in the auditory system where connections underlying integration across distinct frequency bands have been documented.

Contribution of INLL Processing to Auditory Spectral Integration

Although INLL shares common inputs across a range of mammals, and in some species forms one of the largest inputs to the IC (Zook and Casseday, 1982; Covey and Casseday, 1986; Wenstrup et al., 1999), little is known of its contribution to response properties of IC neurons. In the mustached bat, however, the combination-sensitive inhibition displayed by INLL neurons is closely linked to similar responses in IC neurons (Portfors and Wenstrup, 1999; Nataraj and Wenstrup, 2005, 2006; Gans et al., 2009; Peterson et al., 2009). There is

strong evidence that the inhibitory interaction does not originate in the IC (Nataraj and Wenstrup, 2005, 2006; Peterson et al., 2008). Further, such neurons in IC receive their largest inputs from INLL regions that display combination sensitivity (Wenstrup et al., 1999). Finally, it is clear that many INLL neurons are excitatory (Winer et al., 1995; Saint Marie et al., 1997; Riquelme et al., 2001), despite the widespread view that the INLL-IC projection is inhibitory. Our conclusion, therefore, is that INLL imposes its inhibitory combination-sensitive response property onto many IC neurons via excitatory connections (Fig. 10).

As a result of this INLL input, the IC neuron's excitatory response to frequencies near BF occurs only if signal energy at frequencies in the 23–30 kHz range is low. During echolocation behavior, these neurons would respond poorly to the bat's sonar call emission, because the 23–30 kHz fundamental is sufficiently strong to activate inhibition. However, the neurons should respond well to sonar echoes in which the level of the sonar fundamental (at 23–30 kHz) has been sufficiently attenuated. Responses to social vocalizations similarly depend on the presence or absence of high energy in the 23–30 kHz range.

In IC, another target of INLL combination-sensitive neurons takes this processing a step further, combining the inherited inhibitory combination-sensitivity with facilitatory interactions that evoke the strongest response only when low and high frequency signals occur at a certain interval, e.g., at a certain pulse-echo delay (Portfors and Wenstrup, 1999; Nataraj and Wenstrup, 2005; Sanchez et al., 2008). The result is a neuron that responds to a broad array of acoustic stimuli, but which in particular situations (e.g., echolocation) shows remarkable selectivity. The inhibitory combination-sensitive input from INLL is a key component, constraining the responsiveness of the neuron to spectrally complex sounds.

There is widespread evidence that neurons in the auditory midbrain and forebrain display a similar, frequency-specific inhibition of distant excitatory frequencies (Shamma and Symmes, 1985; Imig et al. 1997; Portfors and Wenstrup, 1999; Sutter et al., 1999; Kadia and Wang 2003; Portfors and Felix, 2005). The origin and mechanisms of these are unknown, but may well involve spectral integration in the INLL. This study shows that complex interactions like spectral integration may depend on circuitry within the early stages of the ascending auditory pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

LIST OF TEXT ABBREVIATIONS

AVCN	anteroventral cochlear nucleus
BF	best frequency
DCN	dorsal cochlear nucleus
DNLL	dorsal nucleus of lateral lemniscus
FR	FluoroRuby
IC	inferior colliculus
INLL	intermediate nucleus of lateral lemniscus
LNTB	lateral nucleus of trapezoid body
LSO	lateral superior olive

MNTB	medial nucleus of trapezoid body
MSO	medial superior olive
NLL	nuclei of lateral lemniscus
PBS	phosphate buffered saline
PVCN	posteroventral cochlear nucleus
VCN	ventral cochlear nucleus
VNLLc	columnar division of ventral nucleus of lateral lemniscus
VNLLm	multipolar division of ventral nucleus of lateral lemniscus
VPO	ventral periolivary nucleus

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Figure 1.

FluoroRuby (FR) deposits in INLL were restricted in size. **A.** FR deposit at a multiunit recording site in Bat 708 with BF near 75 kHz and inhibitory combination sensitivity. Physiology within penetration and at deposit site shown in Fig. 2A–D. **B.** FR deposit at multiunit recording site in Bat 704 with BF near 26 kHz. Physiology within penetration and at deposit site shown in Fig. 2E,F. Each panel is an overlay of two images, one taken with rhodamine filters to visualize FR and a second taken with fluorescein filters to reveal cytoarchitecture. Dorsal is up and lateral is to the left. Scale bar: 200 μ m for low magnification, 100 μ m for high magnification inset. *Abbreviations in this figure:* DNLL, dorsal nucleus of lateral lemniscus; INLL, intermediate nucleus of lateral lemniscus; VNLLm, multipolar division of ventral nucleus of lateral lemniscus; VNLLc, columnar division of ventral nucleus of lateral lemniscus. *All other anatomical abbreviations:* AVCN, anteroventral cochlear nucleus; AVa, anterior division of anteroventral cochlear nucleus; AVm, marginal division of anteroventral cochlear nucleus; AVp, posterior division of anteroventral cochlear nucleus; D, dorsal; L, lateral; ICC, central nucleus of inferior colliculus; MNTB, medial nucleus of trapezoid body; LNTB, lateral nucleus of trapezoid body; LSO, lateral superior olivary nucleus; MSO, medial superior olivary nucleus; PVCN, posteroventral cochlear nucleus; PVI, lateral division of posteroventral cochlear nucleus; PVm, medial division of posteroventral cochlear nucleus; VIII, root of eighth cranial nerve; VNTB, ventral nucleus of trapezoid body; VPO, ventral periolivary nucleus.

Figure 2.

Features of auditory responses associated with FR deposits at a high-BF, combination-sensitive recording site (A–D) and at a low-BF recording site (E,F). **A.** Schematic illustration of penetration and deposit through NLL. Progression of BFs follows established tonotopic organization of INLL (see text). **B.** Frequency response at deposit site in **A**, obtained with 31 ms tone bursts at 30 decibels (dB) below maximum speaker output. Some response to low frequencies is typical among high-BF neurons in NLL (Peterson et al., 2009). **C.** Rate-level functions for BF and best inhibitory frequency (LF: low frequency) in response to 4 ms tone bursts. **D.** Delay-sensitive inhibition is revealed by two-tone delay tests. The response to the BF signal (75 kHz) was strongly inhibited when the best inhibiting

signal (29 kHz) was presented simultaneously. Both signals were 4 ms duration, BF signal presented at 51 dB SPL, 29 kHz signal presented at 59 dB SPL. **E.** Schematic illustration of penetration leading to FR deposit at low-BF recording site. **F.** Frequency response at deposit site in **E**, obtained with 31 ms tone bursts attenuated 30 dB below maximum speaker output. For abbreviations, see Figure 1 legend.

Figure 3.

FR deposits produced well-labeled neurons in the auditory brainstem. **A.** contralateral AVa, Bat 666. **B.** ipsilateral AVa, Bat 666. **C** and **D.** contralateral AVp, Bats 687 and 704, respectively. **E.** contralateral PVI, Bat 687. **F.** contralateral PVm, Bat 704. **G.** ipsilateral MNTB (lateral), Bat 666. **H** and **I.** ipsilateral MNTB (medial), Bat 666. **J.** ipsilateral VPO, Bat 687. **K** and **L.** ipsilateral LNTB, Bats 687 and 666. Scale bar for all photomicrographs: 20 μ m. For abbreviations, see Figure 1 legend.

Figure 4.

FR deposits at combination-sensitive recording site (Bat 708) or low-BF recording site (Bat 704) labeled cells (black circles) in the same auditory nuclei: contralateral AVCN and PVCN, ipsilateral MNTB, VPO, LNTB, and VNLL. The main differences were in the distribution within nuclei. In AVa, labeling was observed after combination-sensitive deposits (**A**, sections 38 and 46) but not low-BF deposits (**B**, sections 23, 38, and 41). In PVCN, labeling was in PVI after combination-sensitive deposits (**A**, section 29), but labeling after low-BF deposits was in PVm (**B**, section 14). In MNTB, labeling after combination-sensitive deposits was located in both medial and lateral parts (**A**), but labeling after low-BF deposits was restricted to the lateral part (**B**). These cases are also illustrated in Figure 1 and

Figure 2. Transverse sections are arranged from rostral (*top*) to caudal (*bottom*). Tracer deposit sites are depicted here by a *star*. For abbreviations, see Figure 1 legend.

Figure 5.

Retrograde labeling after FR deposits at combination-sensitive recording sites resulted in consistent labeling patterns across cases. Bar chart indicates, for each successful case, the percentage of brainstem labeled neurons found in each auditory structure. The *total length* of a bar (*filled + unfilled*) indicates the total percentage. The *unfilled* part of the bar indicates the percentage of FR-labeled cells that are double-labeled for FR and glycine. No glycine immunolabeling was performed for case 688. The MNTB and AVCN always contained the largest proportion of FR-labeled cells. The MNTB always contained the largest proportion of double-labeled cells. For each case, cells were counted in a one-of-three series of sections. For abbreviations, see Figure 1 legend.

Figure 6.

Retrograde labeling after FR deposits at low-BF recording sites resulted in consistent labeling patterns across cases. Bar chart as in Figure 5. No glycine immunolabeling was performed in these experiments. As in the cases featuring combination-sensitive FR deposits, the MNTB and AVCN contained the largest proportion of FR-labeled cells. For each case, cells were counted in a one-of-three series of sections. For abbreviations, see Figure 1 legend.

Figure 7. Combination-sensitive FR deposits in INLL retrogradely labeled both lateral and medial MNTB. **A.** Retrograde labeling after FR deposits at combination-sensitive recording sites in INLL. In all such cases, there were discontinuous foci of labeled cells in MNTB. One focus was located in the dorsolateral MNTB while the other was located more medially. **B.** Retrograde labeling after FR deposit at low-BF recording site in INLL. In both such cases, there was a single focus of labeled cells in the dorsolateral MNTB. These results indicate that combination-sensitive recording sites in INLL receive input from both low-BF and high-BF neurons in MNTB. Each column features the sequence of FR-labeled MNTB

sections from a one-in-three series in the indicated experimental case. INLL deposit sites are shown at top of each column.

Figure 8.

Combination-sensitive recording sites in INLL receive glycinergic input from MNTB and LNTB. **A** and **B**. Each column depicts FR labeling, glycine immunolabeling, and merged images for sites in the medial (high-BF) region of MNTB, Bat 708. **C** and **D**. Each column depicts FR labeling, glycine immunolabeling, and merged images for sites in the lateral (low-BF) region of MNTB, Bats, 666 and 708, respectively. **E** and **F**. Columns depict FR labeling, glycine immunolabeling, and merged images in LNTB. Bats 666 and 708, respectively. **G**. Columns depict FR labeling, glycine immunolabeling, and merged images in AVCN. Bat 666. No cells were double-labeled in the cochlear nucleus. *Arrows* indicate

double-labeled cells; *flat arrowheads* indicate FR-only; *indented arrowheads (carets)* indicate glycine only. Scale bar: 10 μm for **A–E**, 20 μm for **F, G**.

Figure 9.

Glycine immunolabeled cells (*gray symbols*) and double-labeled cells (*black circles*) after FR deposits at high-BF, combination-sensitive recording site. MNTB and VNLLc contained the highest density of glycine-positive cells. Double-labeled cells in MNTB occurred in both medial and lateral MNTB as well as in LNTB. No double labeling was observed in other auditory nuclei in this case. *Insets at left* provide higher power views of MNTB and LNTB labeling. FR deposit site depicted by a *star*. Glycine immunolabeling is plotted only for auditory nuclei. For abbreviations, see Figure 1 legend.

Figure 10.

Summary of the essential circuitry and response properties underlying inhibitory combination-sensitive responses in INLL and IC. While the results demonstrated additional inputs to INLL, those other inputs are not essential to combination-sensitive inhibition and are therefore not included here. *Solid projection line* indicates excitatory high-frequency input; *dashed projection line* indicates glycinergic low-frequency input. *Unfilled tuning curves* are excitatory, *filled tuning curves* are inhibitory.