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Research Report

Ultrasonic evoked responses in rat cochlear nucleus

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ABSTRACT

Numerous studies have reported auditory brainstem responses evoked by stimuli within the "normal" hearing range of rats, with maximum sensitivity peaking around 16 kHz. Yet rats also emit and respond to sounds in the ultrasonic (US) frequency range (30–100 kHz). However, very few electrophysiological studies have recorded auditory brainstem responses using US stimuli, and none have exceeded 70 kHz. We report here short-latency (1–3 ms) evoked potentials recorded in rat cochlear nucleus (CN) to US stimuli ranging from 40 to 90 kHz. Robust responses were recorded in 33 of 36 CN recording sites to stimuli ranging from 40 to 60 kHz; and twenty-eight of these sites continued to yield well-defined responses out to 90 kHz. Latencies systematically increased and overall amplitudes decreased with increasing US frequency. Amplitudes differed significantly in the three CN subnuclei, being largest in posterior-ventral (PVCN) and smallest in anterior-ventral (AVCN). The fact that well-defined responses can be recorded to stimuli as high as 90 kHz significantly extends the recorded upper frequency range of neural activity in the brainstem auditory pathway of the rat. These evoked potential results agree with the well-documented behavioral repertoire of rats in the US frequency range.

1. Introduction

It has been known for more than 50 years that rodents emit ultrasonic vocalizations (USVs) at frequencies higher than 30 kHz (Anderson, 1954). Mouse pups emit ultrasonic "isolation calls" when removed from the nest (Haack et al., 1983), and adult males emit USVs when exposed to female pheromones (Gourbal et al., 2004). The frequency distribution of mouse pup calls clusters around 67 kHz, while adult USVs cluster around 80 kHz (Liu et al., 2003). Holy and Guo (2005) have shown that adult mouse vocalizations have the characteristics of songs, with temporal structures that may contain components as brief as 1 ms.

Rats communicate extensively through USVs in two frequency bands: 20–30 and 40–70 kHz (Sewell, 1970; Sales and Pye, 1975). Infant rats emit 40-kHz isolation calls (Blumberg and Alberts, 1991). Adolescent and adult rats emit 22-kHz USVs during unconditioned and conditioned fear responses (Choi and Brown, 2003) and 50-kHz USVs during play behavior, mating, and anticipation of rewarding events (Burgdorf et al.,

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2000, 2005; Panksepp and Burgdorf, 2003; McGinnis and Vakulenko, 2003). Studies of the motor control of USVs have shown in infant rats that aspiration of neocortex and hippocampus and precollicular decerebration do not inhibit USVs in response to cooling; thus brainstem neural circuitry appears sufficient to support the production of USVs (Middlemis-Brown et al., 2005). The involvement of the brainstem in USV motor output suggests that input processing may also be crucial at this level.

Although high-frequency rat vocalizations are customarily termed 50-κ $_{a}$, the actual frequency range can extend up to and beyond 80 kHz (Sales, 1972; White et al., 1990). A common variant of 50-kHz USVs involves a frequency modulated (FM) trill component with high frequencies extending beyond 70 kHz (Burgdorf and Panksepp, 2006; Burgdorf et al., 2007). And carbachol injection into nucleus accumbens of the rat can induce calls out to 96 kHz (Fendt et al., 2006). These results demonstrate quite high USV frequencies in the rat, presumably having behavioral and communicative significance.

In spite of an extensive literature dealing with vocalization frequencies and the social and behavioral significance of rodent USVs, little research has investigated electrophysiological responses to ultrasonic (US) stimuli. Hofstetter and Ehret (1992) identified an ultrasonic field in the mouse auditory cortex. Interestingly, female mice are more attracted to infant ultrasounds when presented to the right ear, suggesting a left-hemisphere capacity that mimics human speech perception (Ehret, 1987). Stiebler et al. (1997) 3692aeq

deviations below the mean of all amplitudes evoked by the 40-kHz stimulus (also confirmed by abnormal or missing peaks upon visual inspection).

Using a computer cursor program the peak latencies and peak-to-trough amplitudes were measured for six US stimuli (40, 50, 60, 70, 80, and 90 kHz) in the 33 electrode sites yielding measurable responses. All 33 sites showed well-defined responses to stimuli ranging from 40 to 60 kHz, but five sites (DCN=1, AVCN=2, PVCN=2) did not respond above 60 kHz. However the 28 remaining sites continued to show responses out to 90 kHz.

Fig. 1 presents typical evoked response waveforms from the three CN subnuclei (one animal for each subnucleus; superimposed waveforms from bilateral recording sites during ipsilateral ear stimulation). The results show robust responses out to 90 kHz consisting of a dual-wave (P1–N1–P2–N2) pattern with a high degree of similarity in the superimposed (bilateral) waveforms. The results also show a consistent pattern in which all evoked potential components in the 40- to 60-kHz stimulus range undergo a gradual reduction in amplitude and a slight increase in latency with increasing US frequency. In the 70- to 90-kHz range, however, the latency shift is more pronounced resulting in an overall graphic pattern in which responses at higher US frequencies are time-shifted versions of responses evoked by lower frequency stimuli. It should be noted, however, that the maximum latency shift in going from 40- to 90-kHz stimulation, although quite reliable, is less than 1 ms. It should also be noted that the total duration of each dual-wave pattern is

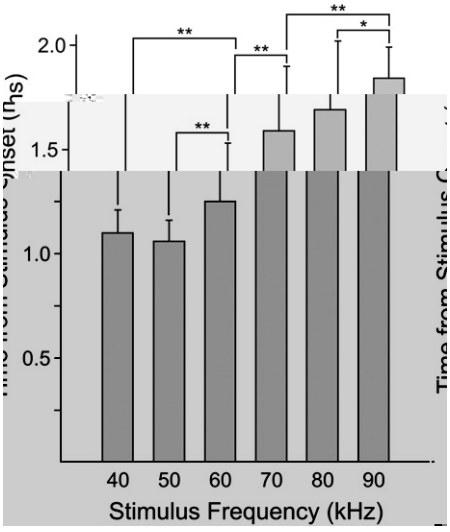

Fig. 2 – Graph of highly significant [$F(5,150)=119.5$, $p<0.0001$] effect of ultrasonic stimulus frequency on evoked response latency (initial peak of the biphasic response seen in Fig. 1); data plotted are mean latencies and standard deviation error bars. The overall results (subnuclei combined) show virtually identical latencies for 40 and 50 kHz, but consistently increasing latencies for all higher stimulus frequencies. Post hoc t-tests of significant mean differences are indicated by connecting brackets and asterisks ($^{*}p<0.05$; $^{**}p<0.01$). Only the mean latencies for adjacent or nearby stimulus frequencies are tested since wider frequency separations would obviously yield highly significant differences.

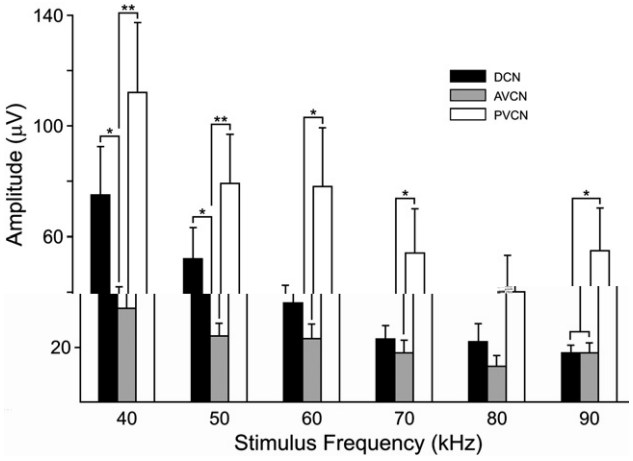

Fig. 3 – Graph showing the significant [$F(2,30)=4.66$, $p=0.0172$] overall amplitude main effect for CN subnuclei. This effect is due to a consistent pattern of larger PVCN amplitudes and smaller AVCN amplitudes across the entire stimulus frequency range. This graph also indicates a significant [$F(10,150)=2.15$, $p=0.0240$] Frequency × Subnucleus interaction. The pattern of this interaction shows DCN amplitudes midway between AVCN and PVCN at 40 and 50 kHz, but approaching AVCN at higher frequencies (becoming identical at 90 kHz). The interaction also shows large but declining PVCN amplitudes with increasing frequency, except at 90 kHz, where the amplitude increases somewhat. Post hoc t-tests of significant mean differences are indicated by connecting brackets and asterisks ($^{*}p<0.05$; $^{**}p<0.01$). Mean amplitude values are in absolute microvolts (μV), and error bars are standard error of the mean (not standard deviations as in Fig. 2).

stable at approximately 1.5 ms, with a complete absence of waves beyond approximately 3 ms.

2.3. *Analysis of latencies*

Analysis of variance (ANOVA) showed that there were no significant latency differences between the three CN subnuclei. However, there was a highly significant [(5,150)=119.5, <0.0001] overall main effect for frequency (Fig. 2). The results show virtually identical latencies for 40–50 kHz, but consistently increasing latencies thereafter for all higher US frequencies. Post hoc -tests confirmed significantly increasing latencies as a function of stimulus frequency beyond 50 kHz, except for the non-significant difference between 70 and 80 kHz.

Fig. 2 graphs the post hoc results only for adjacent or nearby frequencies. Obviously wider comparisons would yield highly significant results. Indeed the -values for 40–90- and 50–90-kHz comparisons are 22.74 and 24.08, respectively (<0.0001). However while the latency differences are statistically significant, the absolute latency differences are quite small. Thus the smallest significant mean difference (80–90 kHz, <0.05) equals 0.15 ms, and the largest (50–90 kHz, <0.0001) is 0.78 ms. The ability to reliably measure such small latency differences was afforded by a fast data sample rate (100 k/s).

2.4. *Differential amplitude patterns in CN subnuclei*

Repeated measures ANOVA showed a significant [(2,30)= 4.66, =0.0172] overall amplitude main effect for CN subnuclei. This was due to a consistent pattern of larger amplitudes in PVCN and smaller amplitudes in AVCN across the entire frequency range (Fig. 3; due to wide amplitude variability the graphed error bars are standard errors of the mean, not standard deviations). There was also a significant [(10,150)=2.15, =0.0240] Frequency × Subnucleus interaction. The pattern of this interaction showed DCN amplitudes midway between AVCN and PVCN at 40 and 50 kHz, but approaching the lower AVCN amplitudes at higher frequencies (becoming identical at 90 kHz). The interaction also shows declining PVCN amplitudes with increasing frequency, except at 90 kHz, where the amplitude increases relative to 80 kHz.

2.5. *Missing response at 80 kHz*

An interesting pattern was seen in several animals in our unpublished pilot study in which the evoked response was absent at 80 kHz, but well defined at all other stimulus frequencies, including 90 kHz. In the present study, this pattern was again observed (Fig. 4) in four animals ($_{DCN}$=2, $_{AVCN}$=1, $_{PVCN}$=1). Although the number of animals showing this pattern is small, it is of interest that in each case the missing 80-kHz response occurred only in recordings from right CN, that is, recordings from left CN were robust at 80 kHz (left CN recordings not shown in Fig. 4).

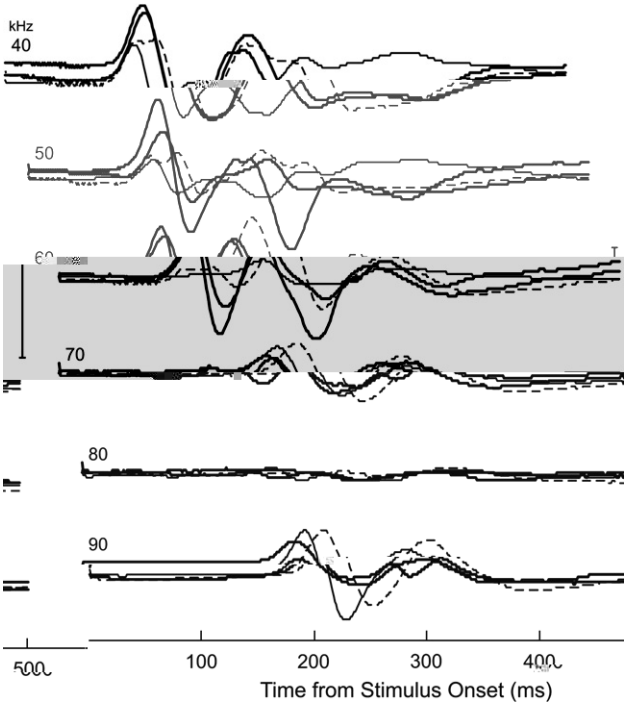

Fig. 4 – Superimposed response waveforms evoked by ultrasonic stimuli (40–90 kHz) recorded from four different animals (DCN, thick lines, *n*=2; AVCN, thin dashed line, *n*=1; PVCN, thin solid line, *n*=1). The data illustrate a pattern in which the response is absent to the 80-kHz stimulus, but well defined at all other frequencies, including 90 kHz. Vertical calibration equals 75 μV (PVCN and DCN) and 25 μV (AVCN).

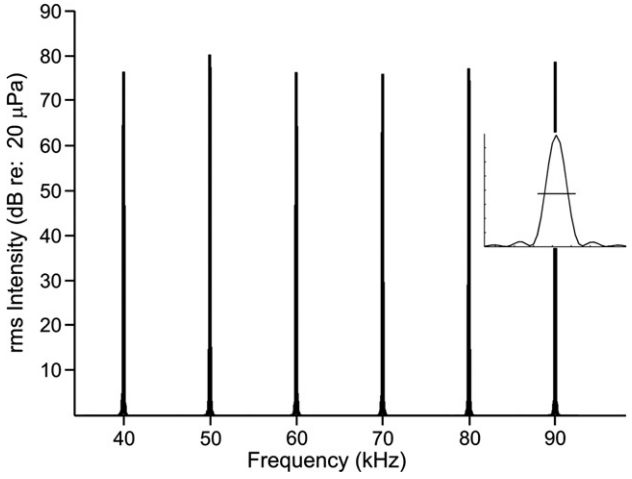

Fig. 5 – Acoustical intensities of ultrasonic stimuli ranging in frequency from 40 to 90 kHz. Main figure shows stable rms (root–mean–square) intensities varying between 75 and 79 dB. Insert: 90-kHz stimulus plotted with expanded *x*-axis frequency scale showing greater spectral detail (horizontal bar represents 156-Hz bandwidth).

3. Discussion

The present results demonstrate robust auditory evoked potentials recorded from rat CN in response to US stimuli with a frequency range of 40–90 kHz. It is reasonable to expect electrophysiological responses to stimuli at such high frequencies since behavioral auditory sensitivity in the rat has been documented up to 80 kHz (Fay, 1988, 1992; Kelly and Maserton, 1977; Warfield, 1973; White et al., 1990). Moreover, rats communicate extensively in a 40- to 70-kHz band (Burgdorf and Panksepp, 2006; Sewell, 1970; Sales, 1972; Sales and Pye, 1975). But even higher frequencies are implicated since "50-kHz" calls can extend beyond 80 kHz (Sales, 1972; White et al., 1990), and the frequency modulated trill component extends beyond 70 kHz (Burgdorf and Panksepp, 2006; Burgdorf et al., 2007). Indeed rat USVs have been recorded as high as 96 kHz, albeit under conditions of carbachol injection into nucleus accumbens (Fendt et al., 2006).

Taberner and Liberman (2005) recorded characteristic frequencies of auditory nerve fibers in the mouse out to 69.8 kHz, and Zheng et al. (1992) recorded rat auditory nerve responses out to 67 kHz. Stiebler et al. (1997) described a non-tonotopically organized ultrasonic field in the mouse auditory cortex that responded to about 70 kHz, and Inoue et al. (1988) recorded cortical evoked potentials in the rat to stimuli as high as 70 kHz. These studies all share an upper response limit of approximately 70 kHz. Taberner and Liberman acknowledge that their high-frequency limit of 70 kHz was artificially imposed by the emitter microphone. Thus relatively few studies have employed US stimuli above 70 kHz, and we not aware of any evoked potential studies to do so. This may be due in part to a lack of interest or awareness concerning the role of USVs

in higher frequency ranges, or more likely due to limitations in generally available acoustical instrumentation. This is especially true of studies producing, as opposed to recording, US frequencies (yet even Fendt et al., 2006 acknowledge that their USV upper limit of 96 kHz, while extremely high, was nevertheless imposed by sound recording limitations). Fig. 5 shows that stimulus intensities in the present experiment were stable from 40 to 90 kHz. The results of the present study, showing reliable CN evoked potentials to frequencies as high as 90 kHz, no doubt benefit from the inherent fidelity of our US stimuli.

The frequency output of the emitter microphone used in the present study drops off above its resonant frequency of approximately 140 kHz (Frederiksen, 1977). This is well beyond our highest frequency of interest (90 kHz). Yet the small geometries of the rat's ear canal can introduce variable and unknown high-frequency impedance to the transmitter, resulting in variable resonances. Individual differences in ear canal geometries or slight differences in tube placement within the ear canal might account for such results as the absence of a response only at 80 kHz (Fig. 4). An acoustical rather than a neural interpretation of the missing 80-kHz response seems most parsimonious, since in every case (four out of four) the neural response on the opposite (left) side was well defined at 80 kHz. Only further research can verify whether this asymmetric frequency-specific response pattern is a unique form of US auditory lateralization.

Using c-fos immunocytochemistry, Friauf (1992) showed tonotopic organization to stimuli as high as 50 kHz in all three CN subdivisions of adult and developing rats, with high frequencies represented dorsomedially. Using microelectrode recordings from single neurons, Yajima and Hayashi (1989) showed tonotopic organization in rat DCN with higher US frequencies (50–53 kHz) represented medially. Although use of macro-electrodes in the present study conferred the advantage of recording summated field potentials from populations of neurons, thereby increasing overall frequency and spatial sensitivity, the present results are limited by fixed electrode placements that cannot yield US tonotopic maps. Tonotopic experiments using US frequencies above 50 kHz are necessary and might reveal restricted regions of CN responsive to US frequencies as high as 90 kHz (or higher). Thus in spite of the broader spatial sensitivity conferred by using macro-electrodes, slight variations in electrode placements within the anatomical fine structure of each CN subnucleus might account for some of the individual differences in the present results.

It should be noted, however, that there was nevertheless an obvious and confirmatory limit to the spatial sensitivity of our CN recordings. Thus because there were no "far-field" evoked response components beyond approximately 2.5–3.0 ms, it is certain that all recordings were limited to the CN and did not engage more rostral auditory nuclei such as superior olive, lateral lemniscus, or inferior colliculus, which would be activated only after further synaptic and transmission delays of several additional milliseconds.

The systematic shift to longer latencies as a function of increasing US frequency was highly significant ((5,150)= 119.5, <0.0001; Fig. 2). It should be noted, however, that absolute mean latencies ranged only from approximately 1 ms at 40 and 50 kHz to less than 2 ms for the longer 90-kHz response. These response latencies are within the distribution of initial short-latency potentials evoked by a 16-kHz stimulus also localized to the CN (Ping et al., 2007). Moreover, the reliable dual biphasic-wave complex (Figs. 1 and 4), occurring within an elapsed time interval of no more than 1.5 ms, indicates rapid neural processing. The overall latency shift, coupled with decreasing response amplitudes, suggests relatively reduced but apparently supra-threshold hearing levels at higher US frequencies.

The significant [(2,30)=4.66, =0.0172] overall main effect for CN amplitude was due mainly to largest amplitudes in PVCN and smallest in AVCN (Fig. 3). These two subnuclei receive distinctive patterns of innervation from the eighth nerve as it bifurcates into ascending (AVCN) and descending (PVCN and DCN) branches. Most AVCN neurons have extremely high levels of ongoing "spontaneous" activity, even in silence (Rubel et al., 2004). The PVCN, on the other hand, is populated predominantly by "onset" neurons with broad tuning curves implying that auditory nerve fibers with substantially different characteristic frequencies (CFs) provide converging inputs (Rhode, 1991). Moreover, octopus cells in PVCN provide onset responses with some of the highest temporal precision of any neurons in the brain and thus would be ideally suited to respond in the ultrasonic range. Neuron properties such as these could provide the basis for the observed evoked response differences between AVCN and PVCN, especially the large amplitude responses seen in PVCN across all tested US frequencies.

There was also a significant [(10,150)=2.15, =0.0240] Frequency × Subnucleus interaction. The pattern of this interaction (also Fig. 3) showed DCN amplitudes approximately midway between AVCN and PVCN at 40 and 50 kHz but decreasing and approaching the lower AVCN amplitudes at higher US frequencies (becoming identical at 90 kHz). The DCN is described as the most complex of the three CN subnuclei due mainly to extensive GABAergic inhibitory interneurons that can completely suppress onset unit activity ("onset-inhibitory" neurons) (Cant, 1992; Rhode, 1991). Moreover, there is evidence to suggest that DCN and AVCN might be more tightly coupled in overall response patterns and distinct from PVCN, since connections from the DCN to the AVCN are inhibitory and organized tonotopically (Paolini et al., 1998a,b).

It is difficult to extrapolate from past studies of the physiological–morphological properties of CN neurons, because none have utilized stimulus frequencies above approximately 50–60 kHz. Moreover, the brief (1 ms) stimulus used here bypasses so-called "buildup" (firing rates gradually increase over 10 to 100 ms), "pauser" (discharges cease from 3 to 25 ms following the initial spike), or "chopper" (temporally regular responses acting as free-running, driven oscillators) neurons (Rhode, 1991). It thus remains for future research to clarify our understanding of the physiological–morphological properties of different CN neuron types when stimulus frequencies are extended into an ultrasonic range at least as high as 90 kHz.

3.1. *Conclusions*

The present results demonstrate robust auditory evoked potentials recorded from the rat CN in response to ultrasonic frequencies. Responses were well defined in all three subnuclei

(DCN, AVCN, PVCN), although there were significant differences in amplitude. A majority of recording sites (28 out of 33) showed responses to frequencies as high as 90 kHz. This significantly extends the frequency range of recorded electrophysiological responses in the initial brainstem auditory nucleus of the rat. The presence of evoked potentials in this higher range of US frequencies is in agreement with the well documented behavioral repertoire of rats that emit and respond to auditory stimuli in this same frequency range.

4. Experimental procedures

4.1. *Animal preparation*

Eighteen male Sprague–Dawley rats (age 10–12 weeks, weight 290–350 g) obtained from Beijing Vital River Experimental Animals Technology Ltd. (Beijing, China) were anesthetized with chloral hydrate (400 mg kg^{-1}, intraperitoneally) and placed in a Kopf small animal stereotaxic instrument. The scalp was incised, the skull exposed, and 0.75-mm diameter holes drilled at appropriate anterior–posterior/medial–lateral stereotaxic coordinates. Metal electrodes insulated except at the 0.25-mm diameter tip were then lowered to the target depth and the assembly fastened to the skull with dental acrylic. Stereotaxic coordinates (in mm) were dorsal cochlear nucleus (DCN) AP=−11.3, ML=±3.8, DV=−7.3 to −7.5; posterior-ventral cochlear nucleus (PVCN): AP=−11.0, ML=±3.8, DV=−8.4; anterior-ventral cochlear nucleus (AVCN: AP=−10.0, ML=±4.3, DV=−8.6 to −8.7 (Paxinos and Watson, 1997). All animals were implanted bilaterally, with six animals each assigned to DCN, AVCN, and PVCN. Electrode impedance measured immediately after implantation showed acceptable low values (mean=12.8 kΩ, SD=2.7, highest impedance=18 kΩ).

The anesthetic and experimental protocol met all requirements regarding the care and use of small animal subjects in accordance with guidelines of the Beijing Laboratory Animal Center, guidelines of the Canadian Council of Animal Care, and Policies on the Use of Animals and Humans in Neuroscience Research (Society for Neuroscience, 2006).

4.2. *Auditory stimulation*

The acoustic stimuli consisted of six ultrasonic (40, 50, 60, 70, 80 and 90 kHz) tone pips, 1-ms duration, 0.3-ms linear rise/fall and 0.4-ms plateau. Stimulus repetition rate was 11 s^{-1}. Digital stimulus waveforms were delivered by a Berkeley Nucleonics Inc. Model 630 arbitrary function generator running at an internal clock rate of 1.25 MHz (i.e., 1250 data points produced a 1-ms stimulus waveform). Output from the function generator was delivered to a Stewart PA-100B power amplifier (linear to 100 kHz) which in turn drove a Brüel and Kjær (B and K) 1/8-in. pressure-field condenser microphone used as an emitter source (Frederiksen, 1977). US stimuli were delivered monaurally into each ear via a rubber tube (3/16-in. O.D., extending 1 cm beyond the microphone tip) to prevent the microphone from directly touching the animal and to provide flexible entry into the ear canal (inserted as far as possible without distorting or blocking the tube end). The tube was positioned and held firmly in the ear canal by adjusting a multi-position clamp attached to the B and K microphone. The ear canal was not otherwise closed. Initial position adjustments of the microphone were made to verify optimal evoked response amplitudes.

Stimulus sound pressure levels were measured by a second 1/8-in. B and K microphone coupled to a B and K Type 3560 Acoustic Analyzer capable of computing fast Fourier transform (FFT) results out to 200 kHz. The analyzing microphone was placed exactly at the exit end of the rubber tube attached to the emitting microphone so as to measure sound levels as they would exist upon entering the rat's ear canal. Root–mean–square stimulus intensities were between 75 and 79 dB SPL across the frequency range (Fig. 5).

4.3. *EEG recording and data acquisition*

Electroencephalographic (EEG) activity ipsilateral to the stimulated ear was recorded from CN by a Grass P511 amplifier (gain 20 k, band pass 300 Hz–30 kHz, −6 dB down). The implanted CN electrode was referenced to a needle electrode in the footpad. EEG recording quality was monitored on an oscilloscope and displayed on the computer screen in real-time. Each sample epoch was initiated by triggering the arbitrary function generator which delivered the stimulus waveform independent of time-locked computer acquisition of EEG data sampled at 100 ks^{-1} by an interrupt-driven Scientific Solutions Lab Master analog-to-digital converter.

Custom data acquisition software developed for our rodent ABR studies (Galbraith et al., 2006) implemented effective on-line artifact rejection. All trials containing extreme amplitudes (e.g., electrocardiogram artifact) were rejected and the stimulus repeated.

4.4. *Computer averaging of ABRs*

Averaged evoked responses were based on 500 artifact-free trials. Final averaged ABR waveforms were displayed and saved for later off-line analysis.

4.5. *Off-line data analysis*

ABR peaks were measured off-line by means of a computer cursor program that displayed latencies and amplitudes. When correctly detected, or manually corrected if necessary, the results were saved for further analysis. The cursor program was used to measure the absolute peak latencies and peak and trough amplitudes of the dual-biphasic wave complex (P1–N1–P2–N2) that characterized virtually all evoked response recordings. The results were plotted and entered into a spreadsheet for subsequent statistical analyses.

4.6. *Histology*

At the end of testing each animal was euthanized with an overdose of chloral hydrate. Lesions were made at the recording electrode tip by an anodal DC current (500 μA for 10 s). The brains were removed, stored in 10% formalin with 30% sucrose until they sank, and then sectioned at 50-μm intervals in the frontal plane in a cryostat (−20 °C) and the sections examined to assess electrode placements in CN subnuclei.

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