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The Sharpening of Frequency Tuning Curves Requires Patterned Activity during Development in the Mouse, *Mus musculus*[1]

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Abstract

Neural activity has been implicated as having both a trophic function and a role in synaptic specificity. Sensory deprivation studies in a large number of developing systems have resulted in the pathological morphology of neurons and abnormal response properties. If the relative timing of discharge among afferent terminals is a cue employed by the developing system to refine the array of synaptic connections, then altering the discharge patterns should hinder this process. In the present experiments, we investigate the role played by the temporal pattern of neural activity during the ontogeny of frequency tuning in the mouse central auditory system. Postnatal animals were exposed to acoustic stimuli, repetitive clicks, that continuously entrained a large proportion of primary afferents from the onset of hearing until an age at which tuning curves should have been adult-like. The amount of fatigue to repetitive clicks was characterized at the level of the eighth nerve and inferior colliculus in normal animals.

Frequency tuning curves obtained from the inferior colliculus were used as an assay for the specificity of neural connections. Click-reared animals had significantly broader tuning curves than did normally reared mice, particularly for units with best frequencies in the 10- to 15-kHz range. Furthermore, it was found that this change could not be attributed to the selective loss of the sustained component of the response. The affected range is interpreted in terms of the frequency spectrum of the click and the fact that lower frequency regions of the inferior colliculus were found to habituate rapidly. The click-rearing environment did not appear to affect unit spontaneous activity or response latency, nor did it alter the tonotopic map in the inferior colliculus. We argue against the possibility of cochlear damage based on threshold and high frequency cutoff measurements.

Mice were reared in a second acoustic environment, repetitive pulses of two added frequencies, as a control for the effects of the click stimulus. This rearing paradigm did not lead to a broadening of tuning curves. It did, however, alter the properties of bimodal tuning curves. For units with bimodal tuning curves having best frequencies in the range of the rearing frequencies, it was found that the second excitatory area had a lower than normal threshold. In addition, the frequency range separating the peaks of the two excitatory regions was statistically smaller. These results are discussed with reference to the specific frequencies used in the rearing paradigm. We conclude from these results that neural response properties and, therefore, synaptic connections reach a mature state at least in part due to the relative timing of action potentials in individual neurons.

Many neuronal systems exhibit an overproliferation of excitatory synaptic inputs during early development (Redfern, 1970; Lichtman, 1977; Mariani and Changeaux, 1981; Jackson and Parks, 1982). It has been suggested that selective elimination from among this polyneuronal innervation leads to a mature connectivity pattern that is optimal for the function of sensory and motor systems. In addition, a degree of functional maturity is probably achieved through the establishment of inhibitory synapses (Granit and Phillips, 1956; Hartline et al., 1956; Erulkar, 1959; Sillito, 1975). Such a dynamic situation presents an opportunity for electrical properties of the nervous system, evoked and maintained activity, to contribute to the establishment of specific connections.

It is generally recognized that active inputs retain an advantage over less active ones both in their ability to secure space in the target zone and in their ability to drive postsynaptic neurons (Wiesel and Hubel, 1965; Shatz and Stryker, 1978; Silverman and Clopton, 1977). This concept has since been refined to account for the retention of only a subset of inputs from an initially larger population: synapses that are coactive are stabilized at the expense of those which fire asynchronously. The salient feature of a presynaptic fiber's activity would not simply be the amount, but the timing of its action potentials relative to the firing of other inputs (Hebb, 1949; Marr, 1960; Stent, 1973). In the present experiments we test the hypothesis that the pattern of afferent activity is a parameter critical to the normal development of frequency tuning in the mouse central auditory system.

Reports demonstrating changes in neural response properties induced by the sensory environment are often difficult to interpret because of the failure to define the full effect of the manipulation on neuronal activity and survival (Rubel, 1978). Experiments of this sort have been approximated by depriving the developing sensory system of environmental stimuli. One would predict that if an input's temporal firing pattern served as a cue during synaptic sorting, then a decrease in activity would effectively limit this cue and lead to mistakes in connectivity. In particular, the receptive field sizes of postsynaptic cells should be larger if the inappropriate inputs they receive during development are not eliminated. This result has been obtained for units in the cortex of visually deprived kittens (Singer

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and Tretter, 1976; Leventhal and Hirsch, 1980). However, the interpretation of these results is limited by the fact that sensory deprivation has been shown to severely retard morphological maturation or lead to degenerative damage (Wiesel and Hubel, 1963; Coleman and O'Connor, 1979; Webster and Webster, 1979; Deitch and Rubel, 1982; Kalil et al., 1983; Kupperman and Kasamatsu, 1983).

Previously, we have reported a disruption of frequency tuning by central auditory neurons when neonatal mice were reared in an acoustic environment (click stimuli) which repetitively entrained a large proportion of the afferent population (Sanes and Constantine-Paton, 1983). The mice were exposed to this environment during the time when frequency selectivity normally matures (Shnerson and Willott, 1979; Saunders et al., 1980; Shnerson and Pujol, 1981). These results indicated that, in the presense of synchronous afferent activity patterns (e.g., when afferents no longer fire at diverse times), the sharpening of frequency tuning did not occur.

We now consider in more detail the neurophysiological properties of neurons in the inferior colliculus (IC) of these click-reared mice. Tuning curves obtained as a function of either the onset component or the sustained component of the poststimulus response (Geisler et al., 1969; Moore and Irvine, 1981) are used to test for differential affects or the loss of a response type. These results are discussed in light of data on the development of the response to repetitive clicks in the cochlear nerve and IC. The results of a second experiment, involving mice reared with repetitive pulses of paired tones, are presented as a control for the effects of click rearing. The effects of this pulsed two-tone environment on bimodal tuning curves are also discussed in terms of the aforementioned temporal activity hypothesis.

Materials and Methods

Rearing conditions. Single litters of 8-day mice (C57BL/6J) were culled to seven pups and placed along with their mother in a wooden box (3 × 3 × 3 feet) lined with anechoic foam. The animals were contained within a smaller plastic cage, the top of which was relatively open to sound. Both food and water were supplied and an 8-hr light/16-hr dark cycle was maintained. Bedding in the cage was changed once during the rearing period. A speaker (Realistic model 40-1286) with an effective frequency range of 57 Hz to 16 kHz was mounted on the wooden box facing the top of the plastic cage. The entire rearing apparatus was housed in a sound-attenuated room.

Repetitive clicks of 20/sec were continuously presented through the speaker from postnatal day 8 until the day of recording, which varied from days 19 to 24. The clicks were produced by feeding a 1-V rectangular wave of 20 μs (Grass S-44 stimulator) into a 30-W audio amplifier (Hitachi HA-330) and applying this signal to the speaker. The volume was adjusted such that the sound level of a click was 88.5 dB SPL measured with a condenser microphone (Bruel & Kjar ¼ inch) positioned approximately 20 cm above the mouse pups. The peak-to-peak amplitude of the click, as displayed on an oscilloscope, was converted to a root mean square value. It was then referenced to the voltage value of the condenser microphone that was associated with a sound pressure level of 0.0002 dyne/cm^2. The spectral components of this click were determined by storing the signal on a TEAC A-2340 tape recorder and analyzing it with a fast Fourier transform real-time spectrum analyzer (Princeton Applied Research, model 4512; see Fig. 1). The signal was highpass filtered at 100 Hz before being processed.

A second acoustic rearing environment consisted of two frequencies that were first added electronically and then pulsed at a rate of 20/sec. The two frequencies, 11 and 14 kHz, were supplied by sine wave generators (General Radio model 1309A and Hewlett-Packard model 200CD) and shaped with an externally controlled switch (Grason-Stadler 829C). The resultant sound envelope had an 8-msec duration and a 2.5-msec rise/fall time. The peak amplitude of this signal was 61 dB SPL, again measured approximately 20 cm above the mouse pups. The acoustic signal from the condenser microphone was amplified and fed into a spectrum analyzer (R. C. Electronics) to obtain the true frequency components present. All other conditions were similar to those used for click rearing.

Surgical preparation. Animals were weighed and anesthetized with sodium pentobarbitol (Nembutal, 55 mg/kg, i.p.). They were then placed on a stage equipped with a headholder and heating pad, and a craniotomy was performed over the right IC using a dental drill and fine forceps. The dura mater was folded back and bleeding was controlled by covering these areas with small pieces of fascia.

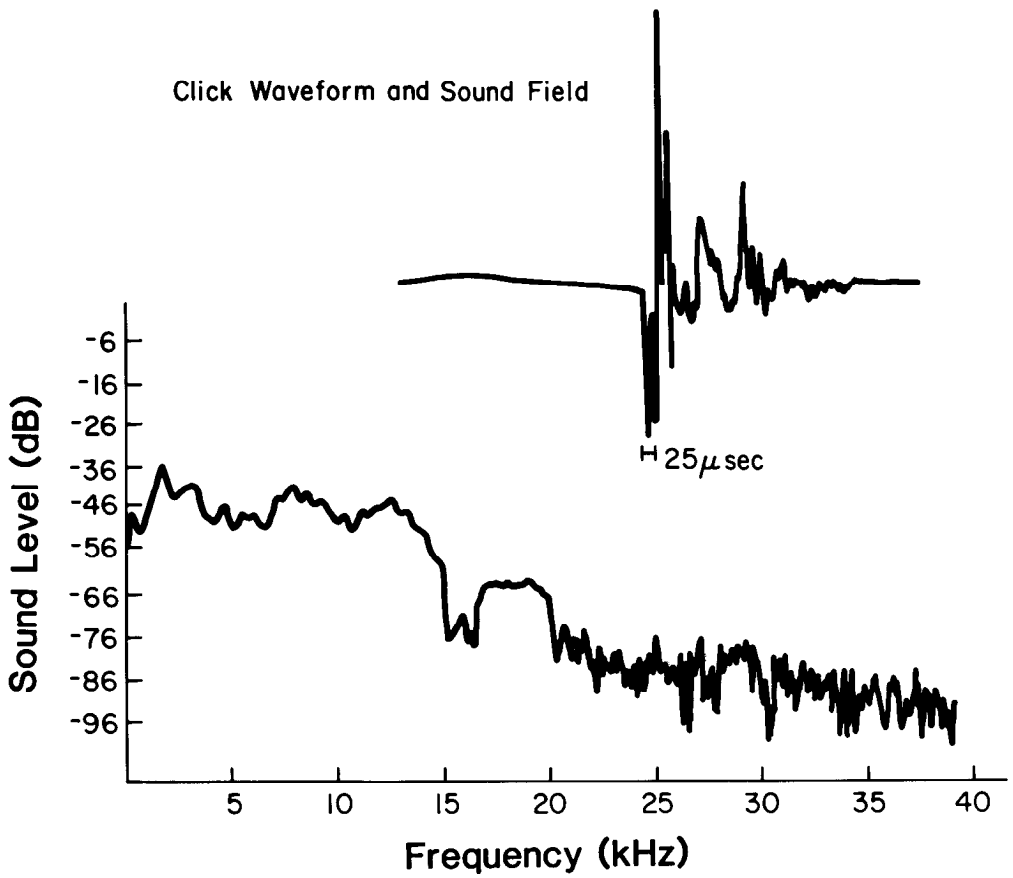


Figure 1. Acoustic waveform and frequency spectrum of the click stimulus used in the rearing paradigm. The waveform was obtained as the output voltage of a sensitive pressure transducing condenser microphone. The frequency spectrum was computed electronically from the waveform and shows that each click has an appreciable amount of energy from 0 to 15 kHz. After this point the pressure wave possesses relatively little energy.

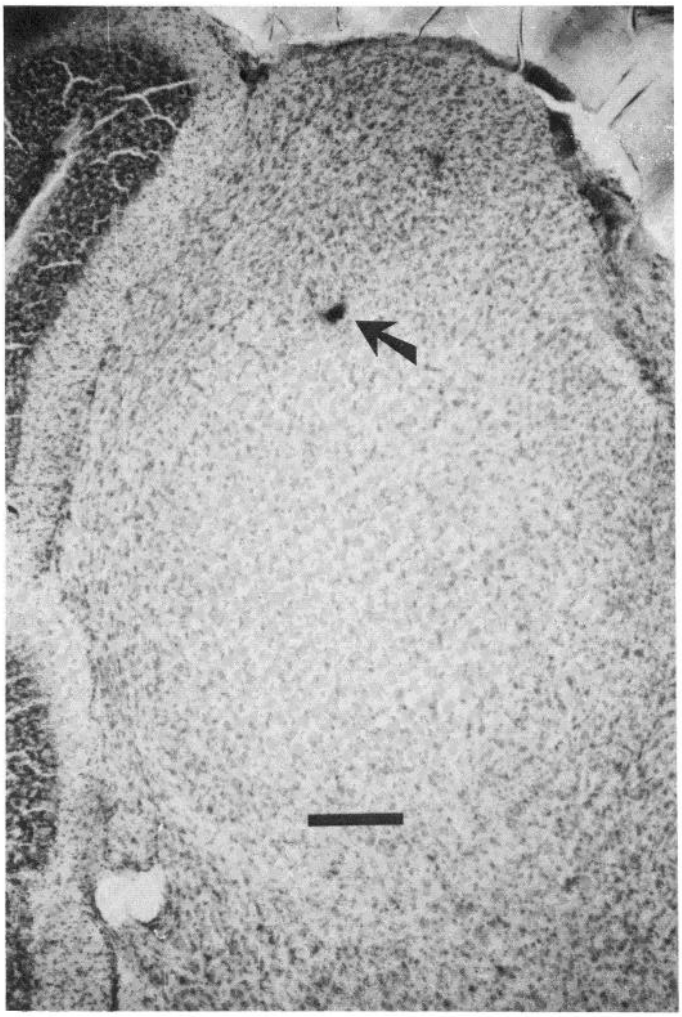


Figure 2. Fast green electrode mark localized in a sagittal section through the IC of an animal that had been reared in repetitive clicks. The dark spot (*arrow*) at the end of the electrode tract indicates the position of an electrode tip which recorded from a unit with a best frequency of 12.4 kHz. The depth of the electrode tip was 678 μm as taken from the hydraulic microdrive and 612 μm as measured from the tissue section. Cresyl violet stain. *Calibration bar*, 200 μm.

When all bleeding had ceased, a warm 2% agar solution was superfused on the exposed area providing a clear view of the recording site and further stabilizing the nervous system. The mouse was then allowed to recover from the surgical level of anesthesia. Subsequently, a small dose of chlorprothixene (Taractan, 7 mg/kg, i.p.) was injected to maintain the animal during the recording session (Drager, 1975). Temperature was monitored in the oral cavity with a self-manufactured thermistor thermometer. Animals were stabilized within 1°C of one another from one experiment to another, the absolute temperature being 34°C.

Single-unit recordings. Standard electrophysiological techniques were used to record from the central nucleus of IC. Glass micropipettes filled with a 2 M NaCl solution containing concentrated fast green (approximately 1%), and having a resistance of 2 to 8 megohms, served as recording electrodes. These were lowered through the agar under visual inspection until a click from the audio monitor signified that they first touched the dorsal IC. Electrode positions were subsequently controlled with a hydraulic microdrive (Narashige) from outside the sound-proof booth (Industrial Acoustics Company) that housed the entire preparation.

Stimulus-evoked discharges were processed with a window discriminator (W-P Instruments model 120) and counted on line. Units were isolated using repetitive clicks of 8/sec as a search stimulus, and only those units that responded reliably were analyzed. Although the vast majority of units from animals reared in the pulsed two-tone environment responded to clicks, this was not used as a selection criterion. When a unit was isolated, its frequency threshold curve was determined. A positive response was defined as a 50% occurrence of one of the following criteria: (*1*) a latency-locked spike, or (*2*) a firing rate double that expected from spontaneous activity alone. Originally, a unit's frequency domain was characterized with either or both response criteria, whichever gave the lowest threshold for a particular stimulus frequency. The analysis was subsequently repeated using exclusively one response criterion or the other. Both sets of data are considered below.

The frequency at which minimum amplitude is able to elicit a response is defined as the best or characteristic frequency of the unit. The plot of frequency versus stimulus amplitude, a tuning curve, may be described as either sharp (i.e., responding to few frequencies) or broad (i.e., responding to many frequencies). The sharpness of a tuning curve has been mathematically defined as the characteristic frequency divided by the frequency bandwidth at a given number of decibels above threshold, a *Q* value (Kiang et al., 1965).

To mark the position of the electrode tip, approximately 12 μA of current were passed through the micropipette for 20 min. The small dye spot was later localized in 60-μm frozen sections (Fig. 2). Units falling outside the central nucleus of the IC were discarded. After performing this manipulation more than 100 times, we developed an impression of the electrode tip location from external landmarks and the response characteristics of the central nucleus (Merzenich and Reid, 1974; Aitkin et al., 1975; Willott and Urban, 1978). At this point we discontinued the marking procedure for each electrode track.

Acoustic stimulus presentation. Pure tones generated by an adjustable oscillator (General Radio model 1310-A) were fed into a custom made switch which had an externally controlled duty cycle. The switch was adjusted to produce 150-msec tone pips with logarithmic rise and decay times of 10 msec. The tone pulses were always presented at a rate of 1.5/sec. Click stimuli were produced with a 1-V rectangular wave of 100 μsec. Shorter-duration rectangular waves did not produce a click of sufficient amplitude. The shaped wave and click stimuli were amplified (Hitachi HA-330 amplifier) and passed through an attenuator (Hewlett-Packard model 350B) before being applied to an earspeaker (Stax SR-44). The earspeaker was coupled down to the size of the aural opening and all auditory stimuli were presented closed field.

The earspeakers were calibrated by coupling the output to a condenser microphone and obtaining the maximum sound level for each of the probe frequencies to be used. The procedure was repeated for several attenuator settings. In addition, we coupled the earspeaker probe to the condenser microphone with the middle ear cavity interposed (two animals) and found that there were no major resonant frequencies, compared to the calibration system. The system was calibrated often during the course of the study and was found not to vary by more than 4 dB from one calibration to another.

Compound action potential analysis. Compound action potentials were recorded in both the cochlear nucleus and the IC. The former was exposed by aspirating the overlying cerebellum ipsilateral to the stimulated ear. In one set of experiments, we recorded the N_1 (cochlear nerve) potential to pure tones of decreasing intensity until an averaged (Tracor-Northern 1550 Signal Averager) response could no longer be visually detected. In this way, threshold audiograms were obtained for both normal and click-reared mice.

A second experiment, which made use of compound potentials, was directed toward determining how well a population of neurons followed repetitive clicks at two ages, 14 and 18 days postnatal. Compound potentials in the IC were first characterized by the range of frequencies to which they responded. The averaged (R. C. Electronics Computerscope) amplitudes of the compound potentials (e.g., 20 responses) were then obtained for several click repetition rates at the beginning and 5 min after the stimulus regimen had begun. The amplitudes of the averaged responses are all presented relative to the amplitude of the response obtained initially for the 0.5/sec repetition rate. An identical protocol was employed for the N_1 potential recorded in the cochlear nucleus, except that it was more difficult to characterize the frequency range of fibers contributing to this compound response. A full report of the development of the click following response is in preparation (D. H. Sanes and M. Constantine-Paton, manuscript submitted for publication).

Results

Repetitive clicks (see Fig. 1) were used to stimulate the developing auditory system such that primary afferents discharged synchronously to each pulse. If normally occurring differences in the temporal discharge pattern of afferent fibers are important to frequency tuning, then, in their absence, elimination of inappropriate connections should not occur during development. The working hypothesis is diagrammed in Figure 3. It predicts that afferents representing an

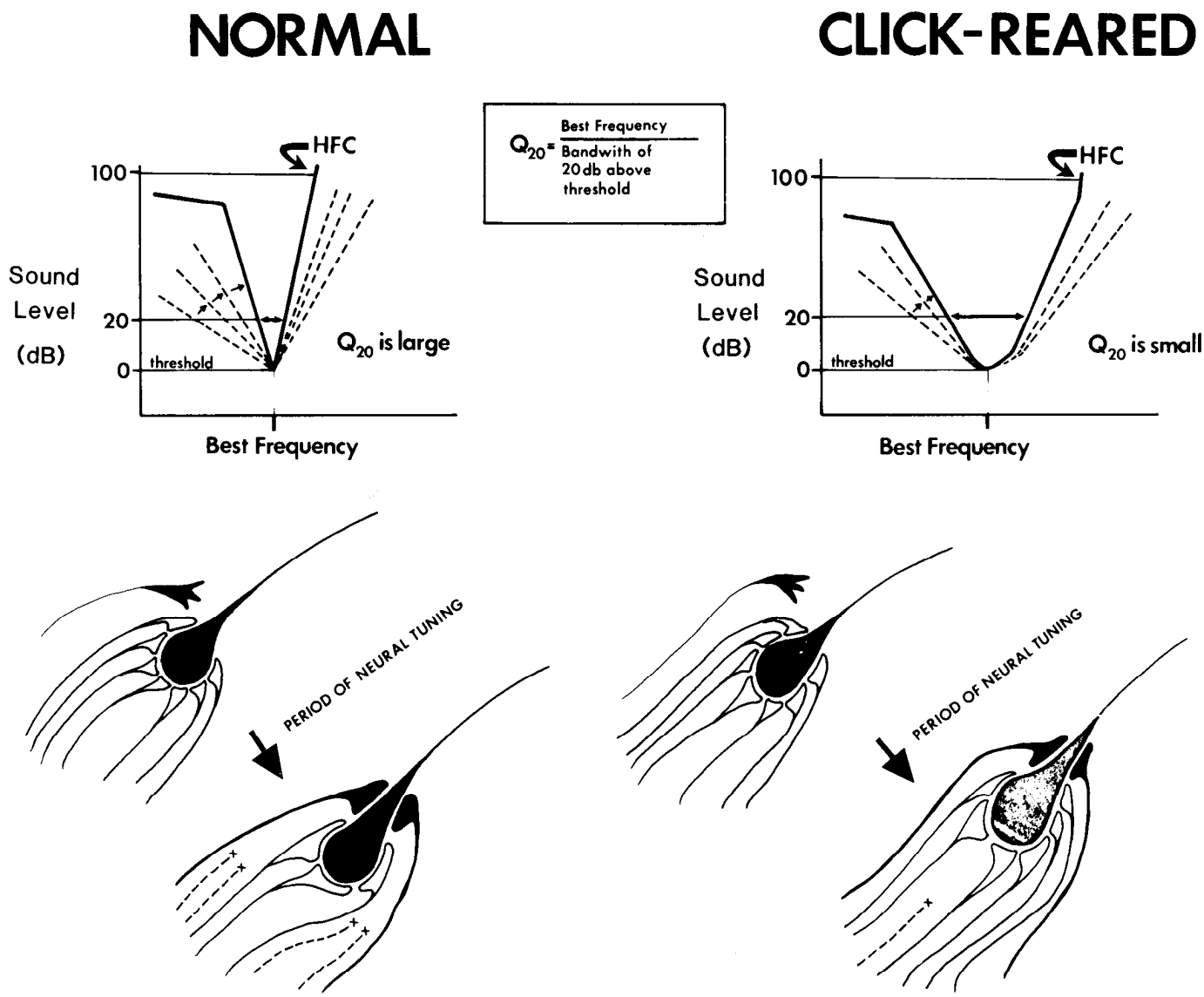


Figure 3. A schematic representation of the development of frequency tuning curves after normal and click rearing with our model of the synaptic changes underlying it. In the normal situation (*left*), the tuning curve is originally broad (*dashed lines*) and gradually narrows during development. Below the tuning curve plot we represent this narrowing of frequency response as being due to both the elimination of inappropriate excitatory inputs (*open terminals*) and the addition of inhibitory inputs (*solid terminals*). In the click-reared situation (*right*), the tuning curve does not attain the normal sharp degree of tuning. We represent this as being due to the retention of inappropriate excitatory inputs (see the introduction for a full statement of the hypothesis). We have ignored the ontogenetic decrease in tuning curve thresholds in this simplified model. This figure also presents two parameters of tuning curves which were compared in the present analysis. The first, Q_{20}, is equal to the best frequency divided by the frequency bandwidth at 20 dB above threshold. A comparison of the normal and click-reared plots shows that the Q_{20} is large for sharply tuned units and small for broadly tuned units. A second measure of tuning, the high frequency cutoff (*HFC*), is the frequency at which the tuning curve crosses the 100 dB SPL level.

abnormally wide range of frequencies fail to be eliminated, resulting in central auditory neurons with broad tuning curves.

The development of the click-following response. The degree to which repetitive clicks used in these experiments evoked a response from the primary afferent population was assessed by observing the decrement in the click-evoked compound action potential (CAP). Recordings made at the level of the cochlear nucleus showed that, at 16 days postnatal, the click-evoked CAP did not show a significant reduction in amplitude over the course of a 6-hr stimulation regimen (Fig. 4*A*). The ability to follow click stimuli was more fully characterized at the level of the eighth nerve and IC. The amplitudes of CAPs evoked by clicks presented at increasing repetition rates were compared to a base line value, defined as the amplitude obtained for a click repetition rate of 0.5/sec at the beginning of a stimulus regimen. A decrease in the amplitude of the compound response was taken as an indication that fewer units were responding. Mice of two ages, 14 and 18 days postnatal, were used in these studies. Four animals in both age groups were examined for the IC recordings and two animals in each age group were examined for the eighth nerve recordings.

Figure 4*B* plots the relative amplitude of the CAP response against click repetition rate in 14-day mouse pups after 5 min of stimulation. There was a clear indication that the collicular response both in low (3 to 9 kHz) and in higher (9 to 17 kHz) frequency regions of the IC had habituated relative to the N_1 response. Nevertheless, for the click repetition rate used in the rearing study (20 pulses/sec), the primary afferents continued to respond at approximately 70% of their original level. By day 18 (Fig. 4C), the compound response from the colliculus was approximately 70% of its original level, whereas the N_1 response barely showed a decline. These data indicated that click stimuli did entrain a large number of primary afferents, even in the first few days after the external auditory meatus opens. At the level of the IC, however, the response to clicks was

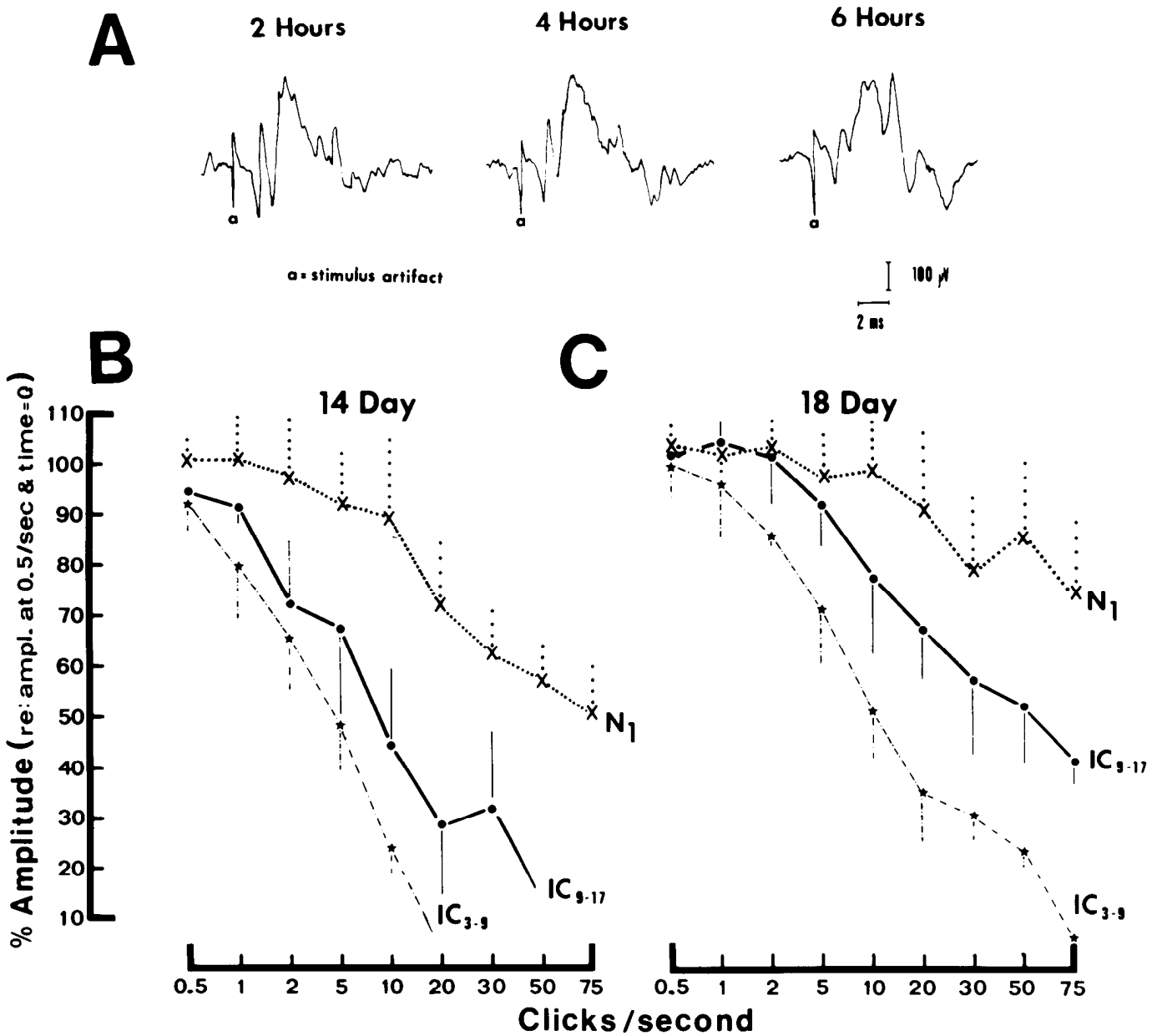


Figure 4. The development of the click-following response at the level of the cochlear nerve and IC. *A*, Single traces of a click-evoked response recorded in the cochlear nucleus of a 16-day animal. The amplitude does not wane significantly over the course of 6 hr. *B*, The relative amplitude of 20 averaged click-evoked CAPs after 5 min of stimulation. The compound potential amplitudes were referenced to the value obtained at the 0.5/sec rate during the first 20 presentations. The three *lines* represent the cochlear nerve (N_1), the higher frequency region of the IC (IC_{9-17}), and the lower frequency region of the IC (IC_{3-9}). At 14 days the N_1 response has decreased to 70% of its original amplitude, whereas the response from IC_{9-17} has decreased to 30% of its original value. *C*, A plot of data similar to that shown in *B*, except that animals were 18 days postnatal. By 18 days the N_1 response hardly decreases and the IC_{9-17} only decreases by 30%. Mean and 95% confidence intervals are shown.

only 30% of its original amplitude after 5 min of stimulation during the first few days of hearing.

A finding of some relevance to this study was that IC CAPs characterized as responding to a lower range of frequencies (3 to 9 kHz) seemed to show a more severe decrease in amplitude as click repetition rate and time increased (Fig. 4, *B* and *C*). This difference in neural populations responding to low versus higher frequency ranges will be discussed below with reference to the effects of click rearing.

The effect of click rearing on frequency tuning. A total of 591 tuning curves was obtained from 47 normal (NM) and 56 click reared (CM) animals as described under "Materials and Methods." With the addition of 183 single units to the previously reported data base (Sanes and Constantine-Paton, 1983), we found that the *Q* values of units recorded in CM mice were significantly smaller, indicating that auditory neurons were more broadly tuned. The graph in Figure 5*C* shows the Q_{20} values for units grouped in 1-kHz best frequency bins (e.g., units with best frequencies from 10 to 10.9 kHz are grouped together). For these units, a threshold response to a particular frequency was taken to be either an onset or a sustained discharge, depending upon which response criterion gave the lower threshold value.

The addition of units to the data base indicated a more substantial effect below 10 kHz than was originally evident (Sanes and Constantine-Paton, 1983). Tuning curves were statistically broader at 20 dB above threshold for units having best frequencies in the ranges 4 to 4.9, 6 to 6.9, 8 to 8.9, 10 to 15.9, and 19 to 19.9 kHz (*t* test, $p < 0.0005$). Bandwidths were measured and *Q* values were calcu-

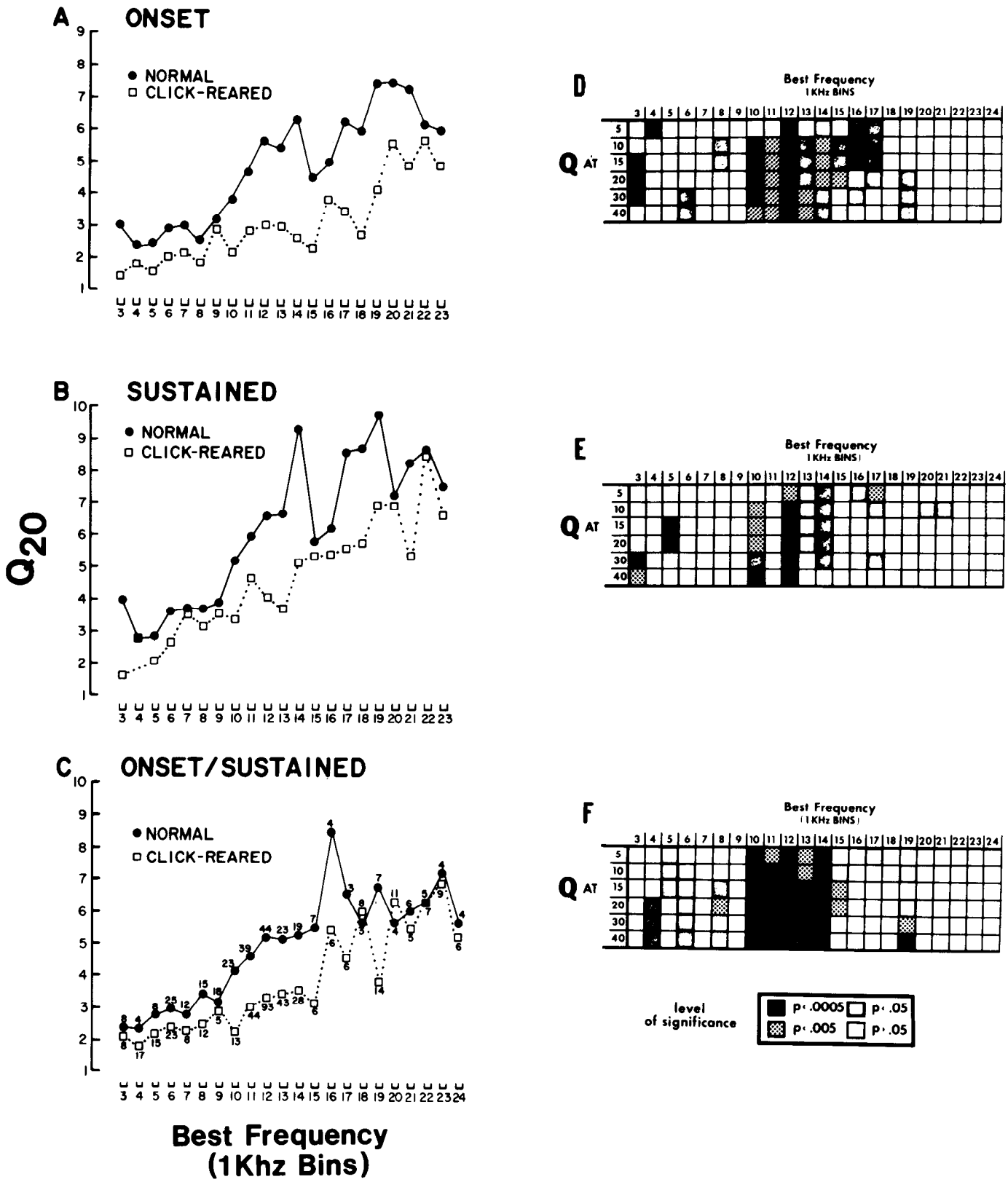


Figure 5. The differences in tuning between units from normal and click-reared animals. *A* to *C*, Q_{20} values of units from normal versus click-reared animals. Units were grouped in the 1-kHz best frequency bins, and the mean Q_{20} value was calculated. The three *graphs* are from tuning curves obtained with different response criteria (see "Materials and Methods"). The *numbers* next to the mean Q_{20} values in C refer to the number of units from which each mean was calculated. In *D* to *F*, summaries of the statistical differences between *Q* values obtained at 5, 10, 15, 20, 30, and 40 dB above threshold are presented. Each *table* corresponds to the graph that lies to its left. Reading across the "*Q* at 20" line on a given summary table would detail where statistical differences exist for the plot of Q_{20} versus best frequency. All *graphs* and *summary tables* indicate that units with best frequency in the 10- to 15-kHz range are statistically broader in click-reared animals.

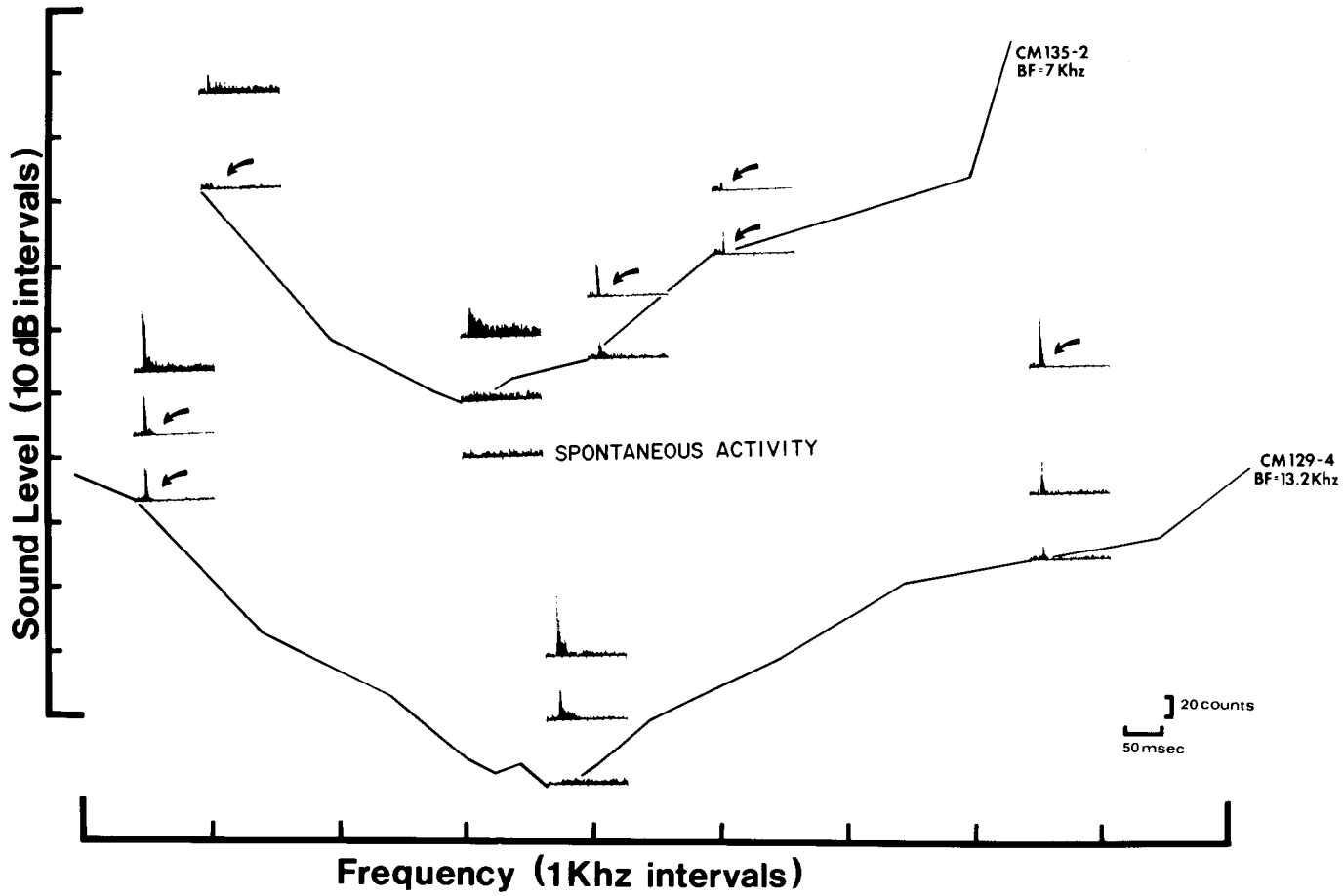


Figure 6. Frequency tuning curves obtained in click-reared animals along with representative poststimulus time histograms. The frequency and amplitude axes are marked at regular intervals and the unit best frequency (*BF*) is given to the *right* of each *curve*. The poststimulus time histograms were obtained by presenting the relevant frequencies, approximately 50 times, at or above threshold. It was commonly found that as one stimulated with a more peripheral frequency or at a greater amplitude, the poststimulus response changed from having a sustained component to having only an onset component (*arrows*).

lated at five other levels above threshold: 5, 10, 15, 30, and 40 dB. A summary of the statistical differences between NM and CM groups at each of these levels is shown in Figure 5*F*. It is clear from this figure that, within the range of best frequencies extending from 10 to 14.9 kHz, auditory neurons were less selective to frequency at all intensities examined.

Frequency tuning as a function of response type. In our previous analysis (Sanes and Constantine-Paton, 1983), the frequency sensitivity (tuning) of each neuron was predominantly determined using the onset criterion, because it gave the lowest threshold. However, the mouse IC is known to contain both phasic and tonic neural response types (Willott and Urban, 1978). It was therefore important to address the question of whether a particular portion of a neuron's response area, the sustained response, was being lost or altered during development. Tuning curves were obtained in two ways from a second set of NM and CM animals. For the vast majority of isolated units, the threshold response was plotted two times, once on the basis of an onset discharge and once using the sustained discharge as the criterion.

One hundred eighteen units from 21 NM and 130 units from 27 CM mice were characterized in this study. The broadest tuning curves consisted almost exclusively of thesholds obtained with the onset criterion. Bandwidth differences were determined for both onset and sustained criteria, and *Q* values were calculated. The Q_{20} values are shown in Figure 5, *A* and *B*, and the statistical differences are summarized in Figure 5, *D* and *E*. The graphs show that, although the effect of click rearing was seen with either method of threshold determination, the onset criterion was more likely to highlight the difference between units from CM and NM groups. When the onset criterion was used, there were more best frequency bins which showed statistically broader tuning than when the sustained criterion was used, especially for units with best frequencies above 14.9 kHz (Fig. 5, *D* and *E*).

It was commonly found that the poststimulus discharge pattern of a unit differed in distinct regions of its tuning curve. The lowest intensity stimuli evoked sustained responses near the unit's best frequency, whereas an onset response provided the lowest excitatory thresholds as one stimulated with successively peripheral frequencies. This sort of response heterogeneity is common in the auditory system and appears to reflect the complex interactions between excitatory and inhibitory systems (Greenwood and Maruyama, 1965; Pfeiffer, 1966). Figure 6 shows frequency tuning curves obtained from CM animals along with representative poststimulus time histograms. Most units gave a primary-like response to tonal stimuli at or near the best frequency threshold. As one increased intensity or stimulated with peripheral frequencies, pauses in the excitatory response occurred or solely onset spikes remained as the response. The processes leading to this change seemed to involve active inhibition since the rate of discharge frequently dropped below spontaneous levels.

Several frequency tuning curves obtained from CM animals were particularly revealing. These are shown in Figure 7. The difference in tuning curves obtained with the onset versus the sustained criterion is evident. There are two additional points worth noting: (*1*) it was not uncommon to find units with bimodal frequency tuning if they were characterized with the sustained criterion, yet these same

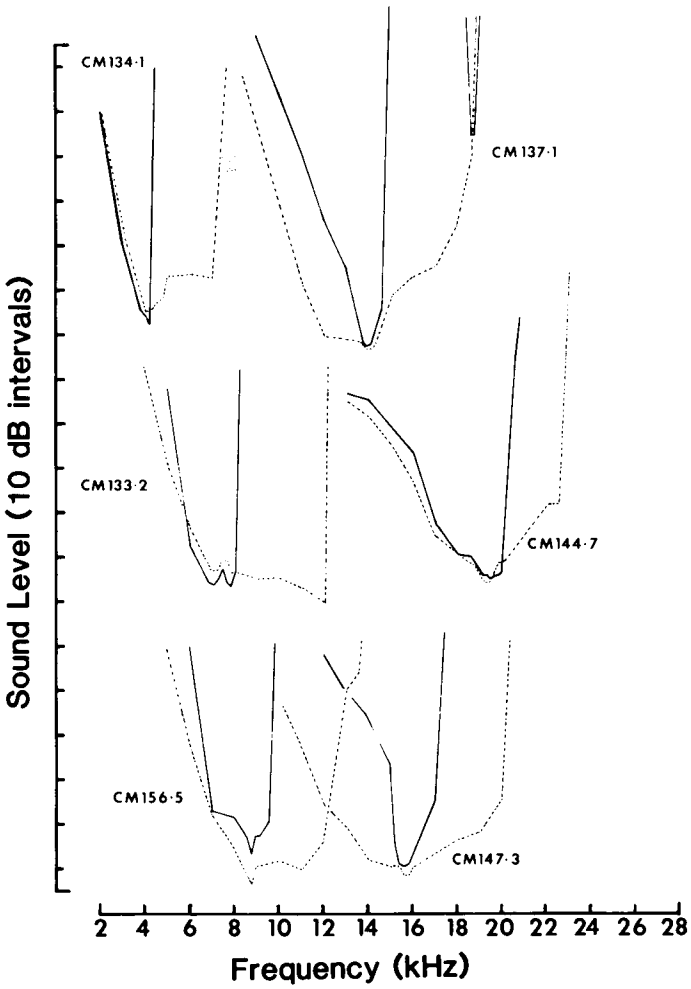


Figure 7. Tuning curves from click-reared animals obtained with two different response criteria. The *solid lines* delineate the excitatory frequency range when units were characterized with sustained criteria, and the *dashed lines* delineate the range seen when the same units were characterized with onset criteria (see "Materials and Methods."). The amplitude axis is drawn such that the absolute threshold of each unit is not evident. The onset criterion typically evokes a response from a broader range of frequencies.

units would be broadly tuned if classified with the onset criterion; (*2*) the greatest difference in tuning revealed by the onset criterion occurred in the high frequency region of a neuron's receptive field. In one severe case (CM133-2), this high frequency extension apparently "alters" the neuron's best frequency.

The effect of click rearing on high frequency cutoff. The upper frequency range of each unit was extrapolated from the intersection of its high frequency slope with 100 dB SPL (see Fig. 3). Results from this analysis are shown in Figure 8. As for the analysis of bandwidth presented above, the greatest increase in high frequency cutoff was seen for units with best frequencies in the range 10 to 15.9 kHz. A comparison of units that were independently characterized using the onset or sustained response criteria (Fig. 8, *top* and *middle*) reveals that the onset criterion is, once again, a more accurate indicator of the range of frequencies capable of driving the cell. This is particularly evident for units with best frequencies below 9 kHz. The high frequency cutoff data, taken together with increases in the bandwidth, suggest that completely novel inputs are functionally represented on the postsynaptic neurons.

Additional physiological parameters. There are several potential explanations for the data presented above. The changes observed may be variously attributed to cochlear damage, to anesthetic state, to nutritional differences, to an age bias, or to recording site differences. In this section, data on latency, spontaneous activity, electrode position, and thresholds of the units studied are presented to address these alternative explanations.

An examination of both the latency (relative to the electrical artifact produced at the electrode by the current flowing through the earspeaker) to respond to clicks (at 8/sec) and spontaneous activity levels (Fig. 9, *A* and *C*) did not point out any consistent statistical differences between NM and CM animals. There is a trend, however, for CM units to have slightly elevated response latencies. The latency measurements were obtained after a train of stimuli had begun and, therefore, did not necessarily reflect the shortest possible latency to a single click. The trend is neither consistent with nor is it related to the degree of tuning evidenced by the particular units examined. The units with best frequencies in the range 11.9 to 14 kHz have nearly identical mean latencies, yet this is the same frequency range in which tuning is most broadened after click rearing (Fig. 5*A*). Only the data for units characterized with the onset criterion are shown in Figure 9; however, the latency analysis for sustained response-type units is essentially identical.

Fast green marking of electrode position was originally used to ensure that the isolated units were found in the central nucleus of the IC and to determine the appropriate surface landmarks and depths for reliable sampling from the central nucleus of the IC. In later experiments, recording site depth was read from the calibrated hydraulic microdrive, making it possible to construct a tonotopic map for both NM and CM mice. These data are shown in Figure 9*B*. There were no statistical differences for unit depth between NM and CM animals, and the tonotopic map was similar to that obtained by Harnischfeger (1978). This would be expected if normal tonotopic alignment results from a variety of cell surface interactions rather than from activity-dependent processes (Sperry, 1963; Harris, 1980). Moreover, the similar distribution indicated that a position- or frequency-dependent sampling bias cannot account for the tuning differences observed in CM mice.

The possibility that age or weight differences between CM and NM mice might have biased our results also seems unlikely. For a given age group, the average weight of the animals recorded from did not differ statistically (data not shown). In addition, there was no significant difference in age for any of the 1-kHz best frequency bins, with the exception of the 4- to 4.9- and 12- to 12.9-kHz bins in which the CM group was approximately 1 day older (*t* test, $p < 0.05$).

The most reasonable alternative explanation for a broadening of tuning when mice are reared in an acoustically altered environment is that the cochlea has been damaged. Broadening of tuning curves and a concomitant increase in the best frequency threshold have been repeatedly linked to such cochlear damage (Wever and Smith, 1944; Cody and Johnstone, 1980; Van Heusden and Smoorenburg, 1981). We analyzed thresholds at the level of the cochlea, by determining frequency thresholds for the N_1 CAP. As Figure 10*A* demonstrates, there were no differences in threshold at the level of the cochlea. The thresholds from single units recorded in the IC of NM and CM mice were also compared (Fig. 10*B*), and the difference was very slight (e.g., <5 dB). A statistical difference (*t* test, $p < 0.05$) did exist for the 10- to 10.9- and 15- to 15.9-kHz best frequency bins, but the differences in mean thresholds were far less than that commonly seen in acoustically traumatized animals (Cody and Johnstone, 1980; Van Heusden and Smoorenburg, 1981). The thresholds in Figure 10*B* are from the tuning curves taken with either onset or sustained responses, depending on which response was observed at the lowest stimulus intensity. When thresholds from units characterized with the onset criterion were compared, there were no statistical differences between NM and CM animals, nor did the means diverge by more than 2 dB.

Specificity of the click-rearing environment. The click stimulus was chosen for the rearing paradigm because it encompassed the greatest range of frequencies and, therefore, should have entrained the largest number of afferents. To test whether or not the broad-

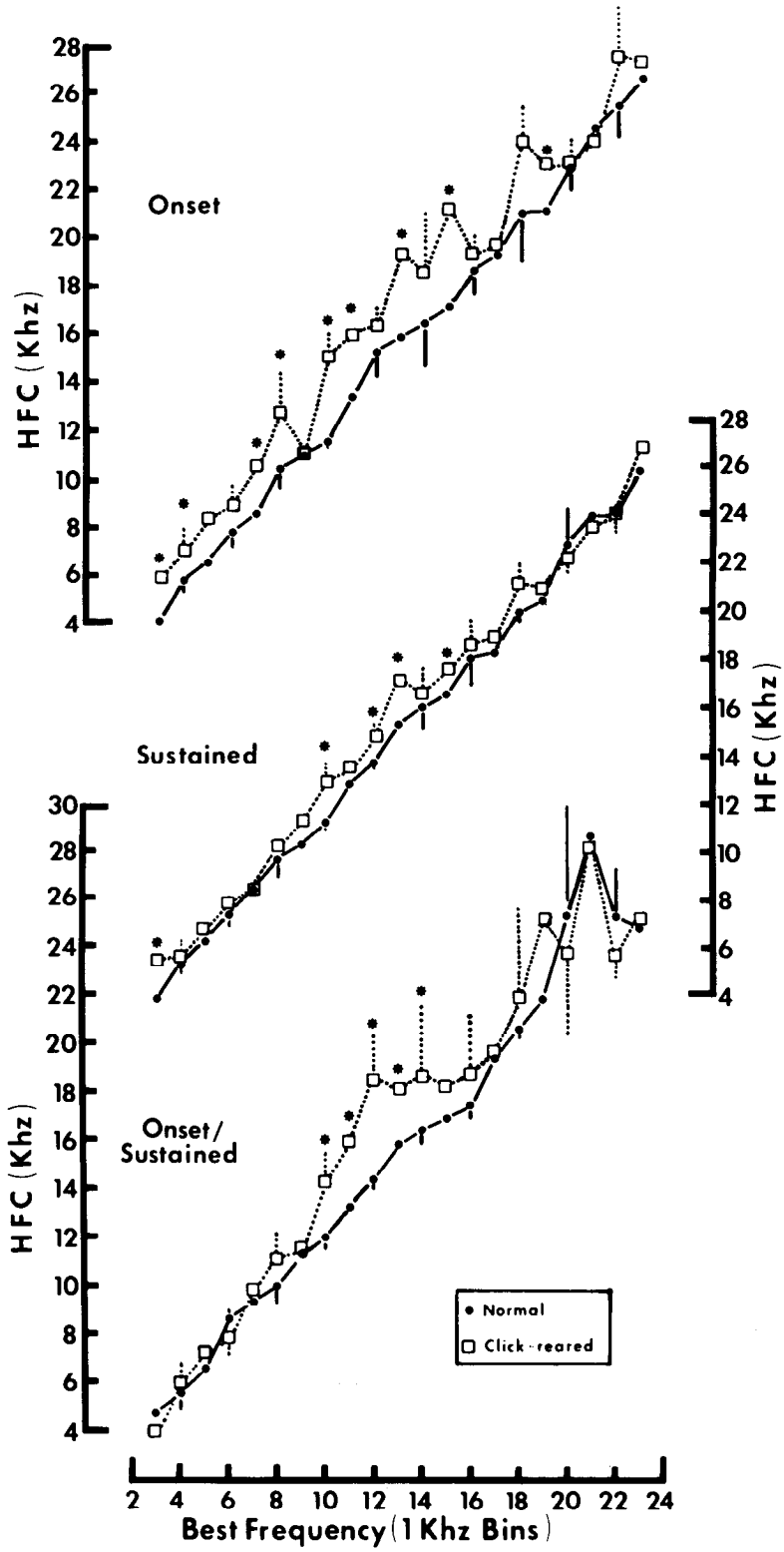


Figure 8. Plots of high frequency cutoff (*HFC;* see Fig. 3) versus best frequency for units characterized in three different ways (see "Materials and Methods"). The mean and 95% confidence intervals were computed for units within 1 kHz best frequency bins, as for Figure 5, *A* to *C*. The larger high frequency cutoffs indicate that units responded to a greater range of frequencies. The effect of click rearing was predominantly on units in the 10- to 15-kHz best frequency range. *Asterisks* connote a statistical difference between units from normal and click-reared animals (*t* test, $p < 0.05$; smaller for some bins).

ening of tuning curves was a response to the specific spectral characteristics of the click stimuli, we raised mouse pups in a second sound environment. In this case, two frequencies, 11 and 14 kHz, were electronically added and pulsed at 20/sec (see "Materials and Methods"). The signals' power spectrum confirmed that the acoustic waveform did consist of two discrete frequencies (Fig. 11). We expected that two small sets of afferents would be repetitively entrained in this environment, and that tuning would consequently remain unaffected. Tuning curves for 77 units were obtained from 16 animals reared in this environment. An analysis of the frequency tuning in units from these animals was performed to determine whether they were more broadly tuned. The Q_{20} values of these units were no different from those found in units from NM animals in the region most dramatically affected by the click-rearing (10 to 13.9 kHz) paradigm (Fig. 12).

The effects of two-tone stimulation on bimodal tuning curves. Several investigators have described auditory neurons with two discrete excitatory areas in the central nuclei of normal animals (Moushegian et al., 1962; Gerstein et al., 1968; Moller, 1969; Goldberg and Brownell, 1973; Willott and Urban, 1978). It was of interest,

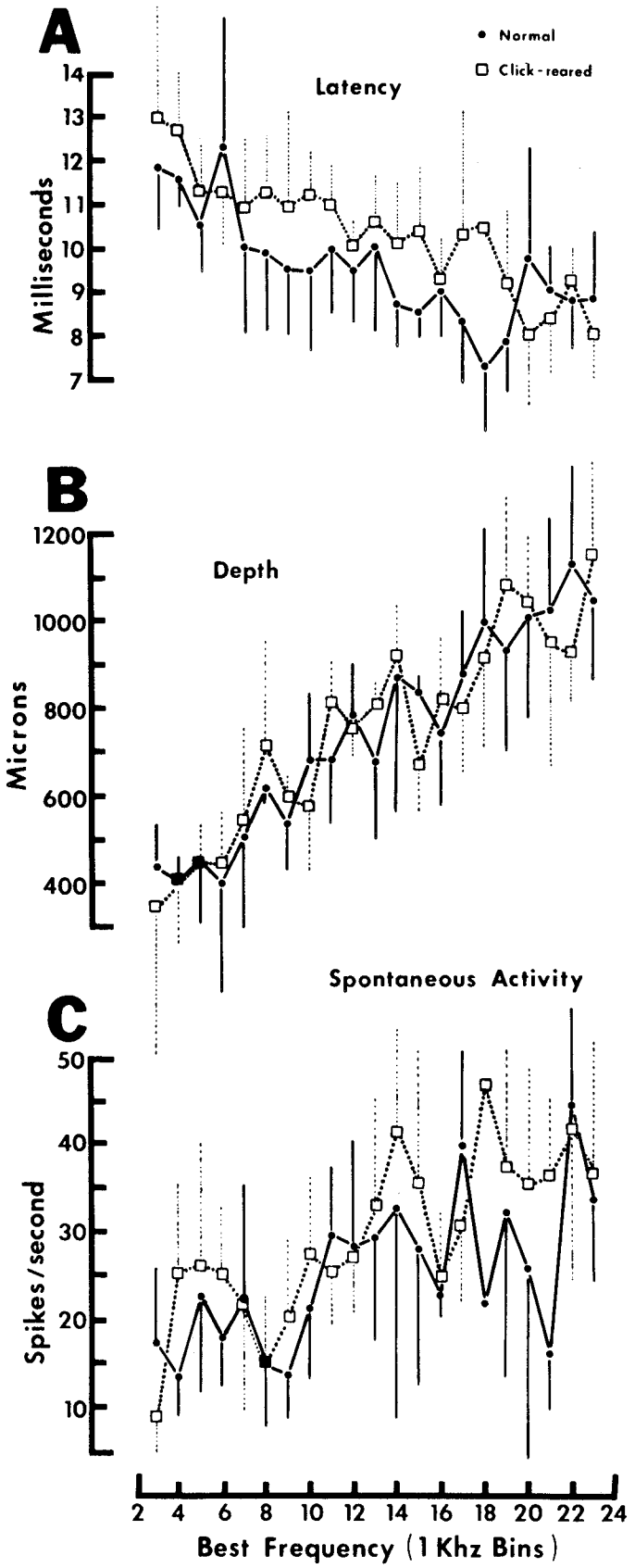


Figure 9. Measurements of response latency, recording site depth, and spontaneous activity for IC units from normal and click-reared animals. All data were taken from units characterized with the onset criterion. Units were again grouped in 1-kHz best frequency bins for these analyses. *A*, The latency graph plots the fastest time to respond to an ongoing presentation of clicks (see "Materials and Methods") against the best frequency bin. There is a general trend toward decreasing latency with increasing best frequency. Small differences in mean values do exist between units from normal and click-reared animals, but these are not statistically significant. *B*, The depth graph plots the recording site depth in micrometers against the best frequency bin. There are no differences between units from normal and click-reared animals. *C*, The spontaneous activity graph plots the number of discharges per second against the best frequency bin. The determination was made by counting the number of spikes for 1 to 2 min. There are no apparent trends or statistical differences between units from normal and click-reared animals. Mean and 95% confidence intervals are drawn in all plots.

therefore, to determine whether animals reared in the two-tone environment showed an increased percentage of such units. The specific prediction was that there should be a large number of bimodal units with response areas corresponding to the two frequencies used in the rearing paradigm, 11 and 14 kHz. This prediction did not appear to be borne out by the data, although we sampled several units with a best frequency near one of the rearing frequencies (Fig. 12).

However, two differences in the bimodal tuning of units from animals reared in the two-tone environment were apparent. Representative bimodal tuning curves from NM and experimental (BiM) animals are shown in Figure 13. It can be seen that both groups of animals show their predominant excitatory area for the lower frequency region. Since each unit had two excitatory areas, there were also two best frequencies associated with them. The following analyses deal only with units having a best frequency in the range 10 to 15 kHz. It must also be noted that bimodal units from CM animals were usually broad if characterized using onset criteria (Fig. 7). Therefore, the bimodal curves from CM animals that were generated using sustained response criteria were used for the comparisons.

We first looked at the range of frequencies separating the two best frequency peaks (i.e., interpeak interval). The units from BiM animals had a statistically smaller (t test, $p < 0.001$) interpeak interval than did units from NM and CM animals (Fig. 14). The normal interpeak interval is close to 4 kHz, whereas that of BiM animals was closer to the 3-kHz interval that separated the rearing tones. A second parameter of bimodal tuning curves is the difference between the two best frequency thresholds (i.e., interthreshold interval). It was found that the interthreshold interval of units from BiM animals was smaller (t test, $p < 0.001$) than those for NM and CM animals (Fig. 14). The interthreshold interval for CM units was also found to be statistically smaller than that of NM units. Since the thresholds of the first peak in BiM units were normal, this implies that the second excitatory peaks generally had lower than normal thresholds.

Two additional parameters were examined and found not to differ statistically between populations. The Q_{20} values of the higher excitatory frequency regions showed a trend toward being smaller in BiM and CM animals. A final measure obtained from bimodal curves was the smallest intensity difference between a best frequency peak and the intervening region of increased threshold (Fig. 14). The relatively large numbers obtained, greater than 20 dB, indicated that we were not simply looking at small threshold fluctuations in tuning curves and labeling them as bimodal. Again, there were no statistical differences between units from any of the populations examined (Fig. 14).

Discussion

The experiments presented in this report were designed to test the hypothesis that temporal patterns of neural activity provide structuring information to the developing nervous system. The most desirable test of this hypothesis would involve imposing a known pattern of activity on the afferent population and predicting the resultant response properties, reflecting the pattern of connectivity. Recently, the role of patterned activity during the development of ocular dominance columns was directly tested (Stryker and Strick-

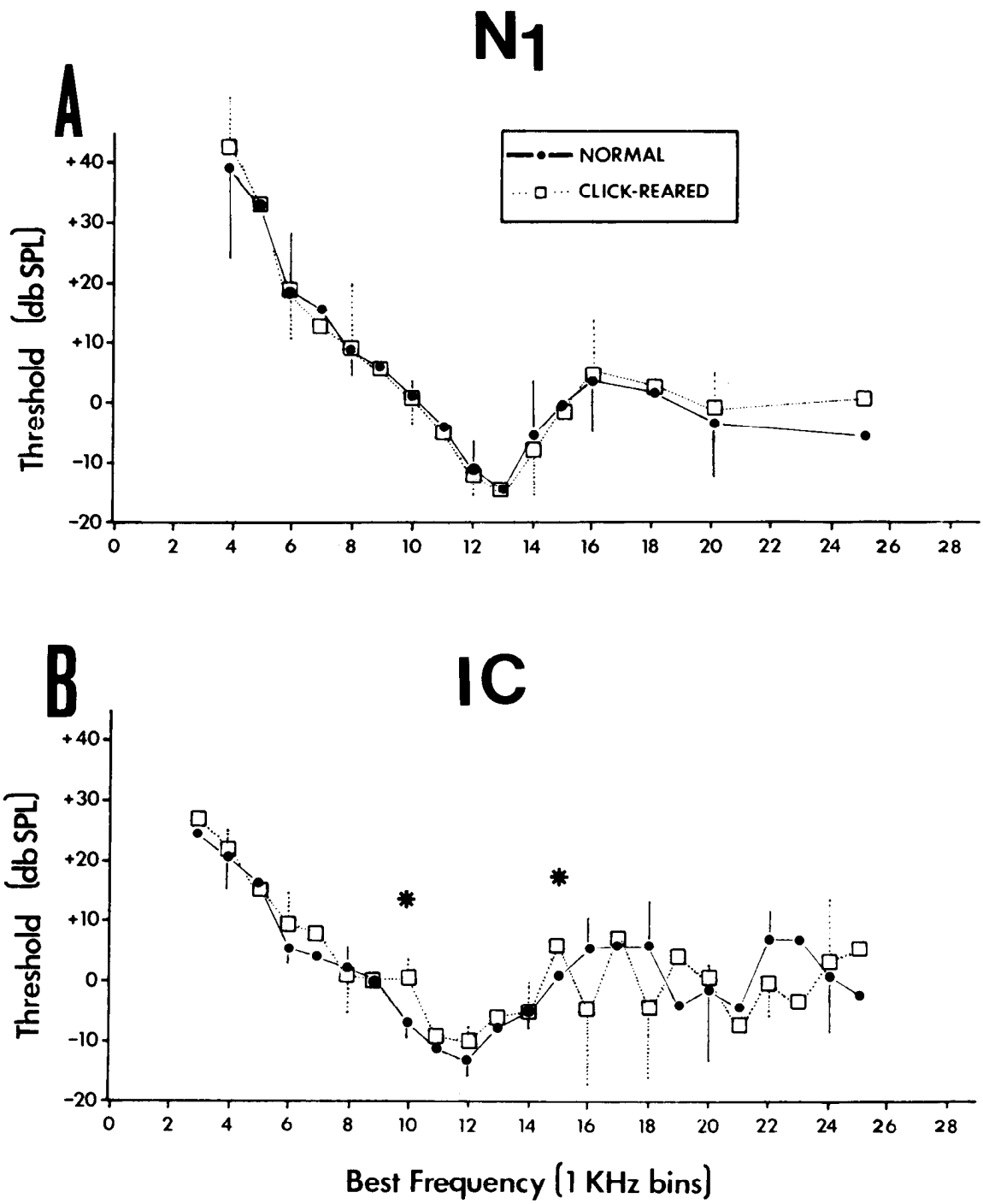


Figure 10. Threshold audiograms at the level of the cochlear nerve and IC. *A*, A graph of the threshold for obtaining an average N_1 response plotted against the stimulating frequency used to evoke the compound potential. There are no statistical differences between normal and click-reared animals at the level of the cochlear nerve. *B*, A plot of mean threshold values obtained from single units recorded in the IC versus the best frequency bin. The data are taken from units characterized with the onset/sustained response criterion (see "Materials and Methods"). *Asterisks* indicate a statistical difference (t test, $p < 0.05$) between units from normal and click-reared animals. The 95% confidence intervals are given at alternate mean values in both graphs.

land, 1984). Layer IV of the cat visual cortex has alternating functional areas such that postsynaptic neurons are driven by first one eye or the other (Hubel and Wiesel, 1962). Previously it has been shown that intraocular injections of tetrodotoxin, a sodium channel blocker which prevents action potentials, prevents the afferent segregation necessary for ocular dominance column formation during development (Stryker, 1981). If the relative pattern of activity emanating from each of the two eyes is important in this segregation process, then synchronously driving both optic nerves should also prevent segregation. Conversely, stimulating the two nerves asynchronously should lead to the normal columnar organization. Both of these predictions were met when the optic nerves were electrically driven in the appropriate pattern (Stryker and Strickland, 1984).

The development of the click-following response. The click stimuli were chosen to repetitively entrain afferents, and it was important to evaluate the likelihood that this occurred. From compound potential recordings made in the cochlear nucleus and IC (Fig. 4) we were able to say that a large number of primary afferents remained entrained at day 14. By day 18 the adaptation of primary afferents appeared to be negligible. These measurements were taken 5 min

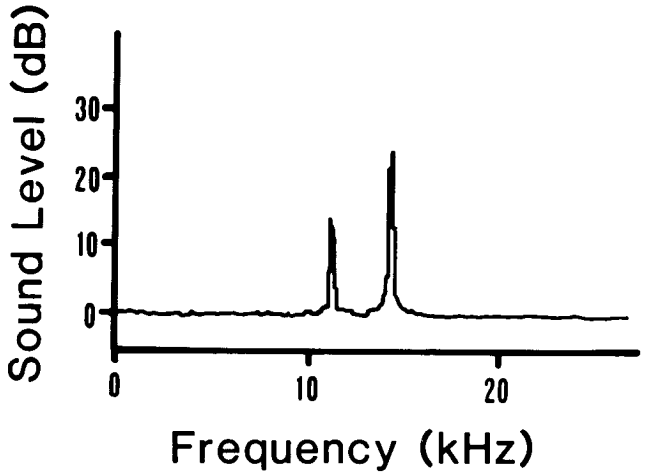


Figure 11. The frequency spectrum of the paired tone stimulus used in the rearing paradigm. The spectrum was computed electronically from the waveform as obtained with a condenser microphone. The frequencies that were added, 11 and 14 kHz, appear to be transduced by the speaker with very little spectral spreading between the peaks.

after the stimulus regimen began, a time after which adaptation appears to be minimal (Fig. 4*A*; see Peake et al., 1962). The N_1 potential that is evoked by click stimuli cannot easily be characterized in the frequency domain, making it difficult to ascertain whether neurons in the affected region (i.e., 10 to 15.9 kHz) are among the fibers being entrained. The N_2 potential, which most likely reflects both eighth nerve and cochlear nucleus contributions (Tasaki, 1954; Kiang et al., 1965; Ozdamar and Dallos, 1978), seemed to be sensitive to a discrete range of frequencies. An analysis of the click-evoked N_2 potentials which have a frequency range of 9 to 17 kHz yields results very similar to those obtained for N_1 (D. H. Sanes and M. Constantine-Paton, manuscript submitted for publication).

The efficacy of click stimuli at the level of the auditory midbrain, where tuning curves were subsequently examined, was not as clearcut. At 14 days there appeared to be a rather large habituation to click stimuli (Fig. 4*B*) at a presentation rate of 20/sec. The possibility exists that units are dropping out completely. However, based on our results (D. H. Sanes and M. Constantine-Paton, manuscript submitted for publication) in developing animals and other published results (Kiang et al., 1965), we believe that a large number of units may continue to respond at a lower rate. If neurons were firing to 30% of the stimuli on day 14 (e.g., as opposed to 30% of the neurons responding to each click), then one would expect a firing rate of approximately 6 spikes/sec. A firing rate of this magnitude would make it unlikely that collicular neurons were experiencing a deprivation environment, rather than the one we had intended. Shnerson and Willott (1979) have recorded the spontaneous activity rates in animals aged 12 to 14 days, and they typically fire at a rate of 1/sec.

The effects of click rearing. The original hypothesis would predict broader tuning curves within all regions of the clicks power spectrum, the frequencies which entrain the primary afferents. However, immature tuning properties occurred for only a subset of the units we recorded from, those with best frequencies between 10 and 15.9 kHz. Whereas the click power spectrum showed a relatively intense signal through 15 kHz, the lack of a consistent effect above 15.9 kHz might reasonably be explained by the sharp decrease of power in that region of the click's spectrum (Fig. 1). The lack of a consistent effect below 10 kHz is difficult to dismiss since the clicks provide relatively equal power throughout this frequency range. However, it is significant to note that, in the 3- to 9-kHz range, the click-evoked response from the IC decreased considerably with repeated stimulation (Fig. 4). This suggests that a relative failure to entrain neurons during the rearing period may be partly responsible for this uneffected frequency range.

A second consideration is the variable response latency that is introduced by the cochlea. This could effectively limit the ability to synchronize auditory fibers with click stimuli because the travel time along the basilar membrane has its greatest range for lower frequencies (Kiang et al., 1965; Evans, 1972). Finally, it is possible that the normally high auditory thresholds in the 3- to 9-kHz frequency range (Fig. 10) could decrease the efficacy of an entraining auditory stimulus.

There were no obvious physiological signs of deprivation at the level of the IC. Cells were not sluggish as is often the case for units

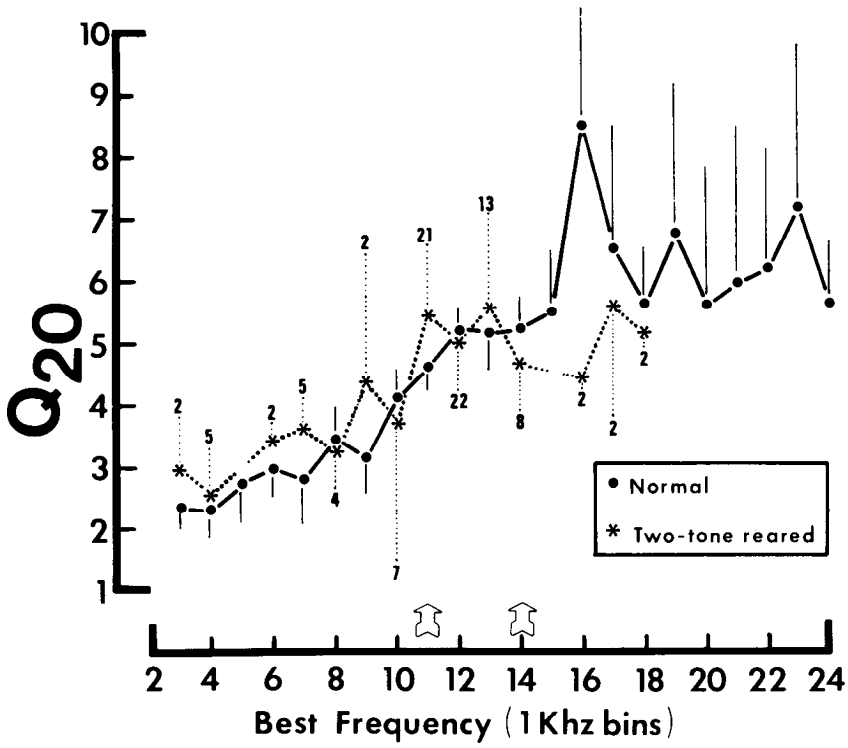


Figure 12. The frequency tuning of IC units from animals reared in the repetitive two-tone environment. The Q_{20} values from these units are plotted against the best frequency bin. Also plotted are the Q_{20} values from Figure 5C, which were obtained with the sustained/onset response criterion. There are no statistical differences between units from normal and two tone-reared animals. The 95% confidence interval and the number of units in the 1-kHz best frequency bin are given along with each mean. The *arrows* on the *abscissa* represent the two frequencies used in the rearing environment. The *numbers* above each mean represent the number of units sampled.

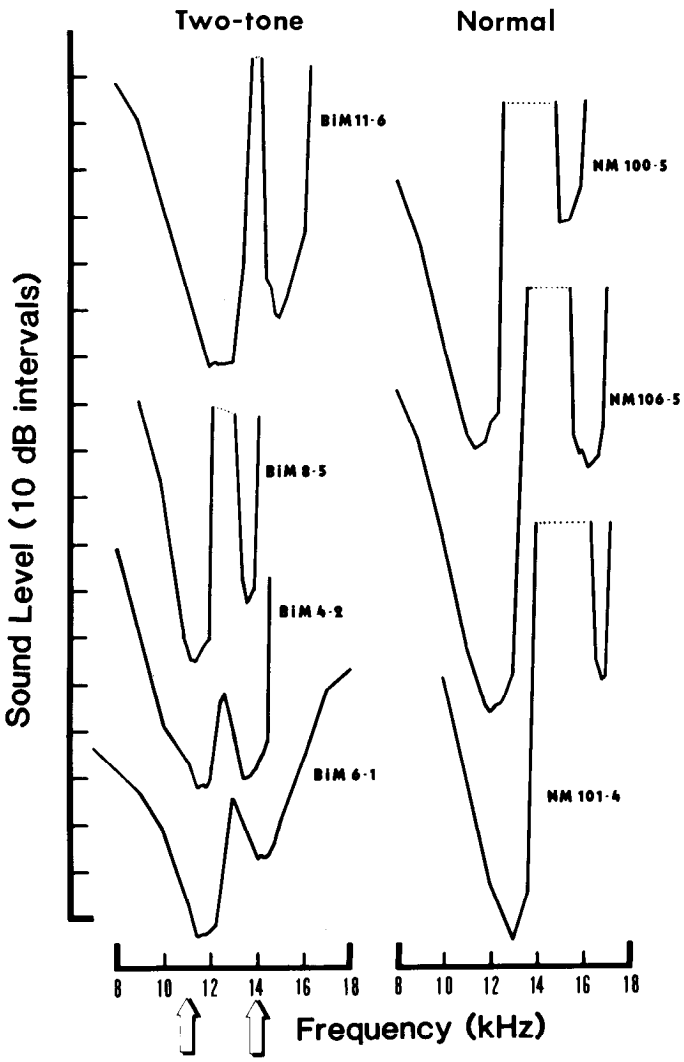


Figure 13. Bimodal frequency tuning curves obtained from animals reared normally or in the repetitive two-tone environment. The *arrows* under the *two-tone abscissa* represent the two frequencies used in the rearing environment. Absolute thresholds are not shown in these plots. These examples indicate that the second (i.e., higher frequency) of the two excitatory areas has a lower threshold in BiM units. Frequencies between the two excitatory regions (i.e., *dotted lines*) either did not elicit a response or decreased the unit's spontaneous activity.

in visually deprived kittens (Wiesel and Hubel, 1963; Leventhal and Hirsch, 1980), nor did they exhibit any consistent decrease in spontaneous activity (Fig. 9*B*) as did units with best frequencies above 10 kHz in acoustically deprived cats (Liberman, 1978). Although there was a trend for slightly longer latencies in CM animals (Fig. 9*A*), there were no statistical differences with NM animals, and some of the most affected units (e.g., those with best frequencies in the range 11.9 to 13.9 kHz) had identical mean latencies. An effect of latency has previously been documented in collicular units of binaurally deprived rats (Clopton and Silverman, 1978). In this case, units with a best frequency above 10 kHz fell into two populations having either normal latencies or ones that were 2 to 4 msec longer. We did not see two separate populations of latencies, nor did we observe mean latencies of CM units much more than 1 msec above the mean latencies of NM units.

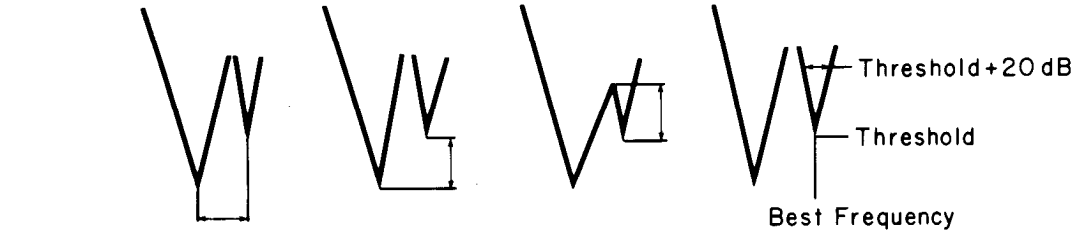
An important aspect of the present study was to test whether a response type changed or was lost due to the rearing environment, leading to the result of statistically broadened tuning. Originally, unit thresholds were determined by the presence of either an onset or a sustained response, whichever was elicited at lower stimulus intensity. In the present study we found a reduction in tuning when only the sustained response criterion was employed, but the onset criterion appeared to be a more sensitive indicator of the range of excitatory frequencies in both NM and CM animals. Nevertheless, it was clear that the sustained response type had not been lost during the click-rearing period, leading to an abundance of broadly tuned onset-type units. We tentatively offer the following interpretation of the onset response and its relationship to broadened tuning curves.

Intracellular records from central auditory neurons commonly reveal that the initial EPSPs, which may result in spiking, have shorter latencies than IPSPs, which appear to suppress discharge (Nelson and Erulkar, 1963; Starr and Britt, 1970; Oertel, 1983, Rhode et al., 1983). In addition, the IPSPs are often evoked by frequencies adjacent to the excitatory area (Starr and Britt, 1970). These inhibitory inputs may serve to alter the temporal pattern of discharge from a sustained to an onset response type (Greenwood and Maruyama, 1965). Our working hypothesis was that the manipulation would result in the maintenance of anomalous excitatory connections. This means that stimulus frequencies which typically inhibited the postsynaptic cell would, instead, be expected to elicit an onset spike by virtue of a fast latency EPSP, followed by the normally occurring IPSP. Therefore, the differences in tuning obtained with onset or sustained criteria are not completely unexpected.

An alternative explanation for broadened tuning curves is that the cochlea was damaged by the pulsed clicks during development. We argue against this explanation for the following reasons. First, acoustic trauma is always accompanied by elevated thresholds (Wever and Smith, 1944; Cody and Johnstone, 1980), whereas the thresholds at both the level of the eighth nerve and at the IC were normal in CM mice (Fig. 10). Second, the high frequency cutoff of units in acoustically traumatized animals is commonly unchanged (Cody and Johnstone, 1980; Van Heusden and Smoorenburg, 1981), but those of units in CM animals are significantly increased (Fig. 8). Finally, when mouse pups were reared in an acoustic environment consisting of pulsed tones rather than clicks, we did not find a change in frequency tuning (Fig. 12). This suggests that the acoustic environment was not powerful enough to injure the cochlea, since the clicks were present for an even shorter time than the tone pips. Nevertheless, we cannot rule out the possibility that the frequency tuning of the basilar membrane was affected independent of a threshold shift.

The effects of a pulsed two-tone environment. Several mice were reared in an acoustic environment consisting of two added tones (i.e., 11 and 14 kHz) which were pulsed at 20/sec. Bimodal tuning curves (Fig. 13) in the frequency range of the rearing environment (i.e., 10 to 15 kHz) were compared to those obtained from NM and CM animals. The most surprising finding was that the second excitatory area had a lower threshold in units from both the two tone-reared and CM animals, compared to bimodal curves from NM animals (Fig. 14). This result is consistent with the hypothesis that coactive inputs are preferentially stabilized, if we assume that two discrete populations of afferents were repetitively co-entrained by the pair of tones. It is interesting to note that many of the bimodal tuning curves from CM animals would be classified as broadly tuned units when characterized with the onset criterion (Fig. 7). The frequencies between the two excitatory regions appeared to actively inhibit these units (Gerstein et al., 1968; Goldberg and Brownell, 1973; Willott and Urban, 1978; D. H. Sanes and M. Constantine-Paton, personal observations) in CM animals, except for an onset spike. These observations re-emphasize the importance of excitatory and inhibitory interactions when considering the physiological refinement of response properties.

A second suggestive observation concerns the interval between the two best frequency peaks in bimodally tuned units. The intervals were found to be statistically smaller than those from NM or CM animals (Fig. 14). The actual mean interval for units from two tone-reared animals, 2.6 kHz, is very close to the frequency interval of the rearing tones, 3 kHz. The normal interpeak interval is closer to 4



	Inter-Peak Interval (Khz)	Inter-Threshold Interval (dB)	Smallest Drop in Threshold (dB)	Q_{20}
Normal N=12	3.8±0.2	48.8±5.1	32.8±3.3	19.6±4
Two-tone Reared N=21	2.6±0.3	12.5±3.8	32.9±11.8	16±4.9
Click Reared N=15	3.8±0.8	28.2±7.8	27.3±9.8	16±8.6

Figure 14. A summary of the parameters of bimodal tuning curves which were measured and compared between normal, click-reared, and two tone-reared groups. The representative bimodal tuning curve *above* each *column* indicates the parameter that is being measured with *arrowheaded lines*. The number of units in each group is given to the *left* of each *row*. *Asterisks* indicate a statistical difference (see "Results") between the two groups spanned by the respective line.

kHz. One must be cautious when attributing these changes to the predominant rearing stimuli because we have not verified that the expected populations of fibers, those with best frequencies of approximately 11 and 14 kHz, are co-entrained during development. In light of these results, it will be interesting to follow the development of bimodal tuning curves, particularly the threshold and breadth of the high frequency region.

The experiments described here indicate that the relative timing of neural activity is an important cue used by the developing auditory system. When this cue is effectively altered by imposing the same temporal discharge pattern on most auditory affferents, the frequency selectivity of individual neurons appears to remain in the immature broadly tuned state. We have operated under the assumption that inappropriate excitatory connections are retained in these broadly tuned units, but this need not be the case. Our results are equally compatible with a mechanism that reduces the amount of inhibition evoked by tonal stimuli.

An extraordinary range of response properties must be generated in the central auditory system during its development. Behavioral observations indicate that immature animals must be exposed to a normal acoustic environment in order for them to make the fine sensory discriminations which adults commonly perform. Monaural and binaural attenuation experiments indicate that there is a resultant decrease in frequency discrimination (Kerr et al., 1979) and the ability to localize sound stimuli (Clements and Kelly, 1978; Knudsen et al., 1982). We suggest that the mechanism by which complex response properties develop makes use of the unique temporal patterns of activity found in an afferent population. It will be critical to compare the neurophysiological and behavioral deficits resulting from well defined rearing environments to fully evaluate this thesis.

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