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Altered Activity Patterns During Development Reduce Neural Tuning

Abstract. *Neonatal mice were reared in an acoustic environment that repetitively entrained activity in a large proportion of primary auditory afferents during the period when the frequency tuning of auditory neurons normally develops. The tuning curves obtained from these mice were significantly broader than those of normally reared mice of the same age. This suggests that the normal frequency tuning of neurons was prevented or delayed by synchronizing the pattern of activity imposed on the auditory pathway.*

Neural activity is critically involved in the normal maturation of response properties in the mammalian visual system (*1*) and a number of experiments indicate that coactivation of convergent inputs plays an important role in this process (*2*). We have investigated the role played by activity during the development of the mouse auditory pathway, using the hypothesis that coactive neural inputs are more likely to form lasting connections with a postsynaptic neuron. If this is the case, precise convergence onto postsynaptic neurons could be affected by selecting inputs with temporally correlated spike trains from an initially larger array of terminals. This idea has evolved from a theory originally put forward by Hebb (*3*) to explain the neural basis of associative learning.

Our studies were conducted on mice since their postnatal development is well defined and rapid (*4–7*). The experimental procedure was to raise young mice in an environment of repetitive clicks. Since this stimulus entrains the activity in a large proportion of auditory nerve fibers, it should have produced relatively synchronized firing patterns among these axons during development (*8*). We reasoned that in normal animals close temporal correlations in activity might provide the cues necessary to fine-tune a central auditory neuron because inputs of similar best frequency are likely to be active together. We predicted that in the absence of cues which might arise from these similar firing patterns, the ontogenetic fine-tuning of central auditory neurons would be relatively ineffective. Neurons which would have become more selective to frequency during development would consequently show abnormally broad tuning curves.

All mice were of the C57BL/6J strain. At 8 days of age litters were culled to seven animals and placed in standard laboratory cages along with their mother. The entire cage was then placed in an anechoic box within an acoustic isolation room where the mice were exposed to clicks presented at a rate of 20 per second from an overhead speaker. Each click had a peak-to-peak sound pressure level (SPL) of 88 dB (with reference to 0.0002 dyne/cm^2) and a frequency power spectrum that was essentially flat from 0 to 15 kHz (Fig. 1A). Mouse pups remained in this rearing environment until 19 to 24 days after birth, an age at which neural response properties resemble those of adults in normally reared mice (*6*). Litters to be used as normal controls were also culled to seven animals and maintained in the auditory environment provided by our animal facility.

Frequency tuning curves were taken from single units in the central nucleus of the inferior colliculus in normal and click-reared (CR) mice immediately upon removal from the click-rearing box. The animals were anesthetized with sodium pentabarbitol (Nembutal, 55 mg/kg, injected intraperitoneally), the inferior colliculus was surgically exposed, then covered with a 2 percent agar solution. Glass micropipettes filled with 2*M* NaCl and 1 percent fast green were used to record single units and compound potentials. The position of recording was marked subsequently by iontophoresing fast green. During the recording session all animals were tranquilized with chlorprothixene (Taractan, 7 mg/kg, injected intraperitoneally) (*9*), and their orally measured temperature was maintained within 1°C of one another. All auditory stimuli were presented closed field through a calibrated earphone (Stax SR-44; B & K 1/4-inch condenser microphone) fitted to the contralateral ear. The entire preparation was enclosed within an acoustic isolation chamber (IAC). Only units which responded to clicks were isolated and analyzed for frequency tuning with pulsed (1.5 per second) tone pips of 150 msec-duration (10-msec rise-fall time). Compound action potentials were recorded from the cochlear nucleus and were averaged (Tracor-Northern 1550) to detect threshold responses to pure tones (*10*).

The majority of neurons in the central nucleus of the inferior colliculus responded to a fixed range of frequencies each at characteristic intensity, and one frequency, the best frequency, generally drove the neuron when presented at lowest intensity or threshold. *Q* values are conventionally used to indicate the relative degree of tuning or stimulus selectivity of such auditory neurons (*11*). Larger bandwidths correspond to smaller *Q* values and, therefore, indicate less selectively tuned neurons.

We have obtained frequency tuning curves from 408 single units in 67 normal and CR mice. Values of *Q* were calculated for units in both groups. The mean *Q* values calculated at 20 dB above threshold (Q_{20}) for units of similar best frequency are shown in Fig. 1C along with the 95 percent confidence intervals. Units from CR animals had significantly lower Q_{20} values for characteristic frequencies in the range 10 to 15 kHz [analysis of variance, $P < 0.0005$)] and significant decreases in tuning ($P > 0.05$) were also observed in the 4 to 4.9, 8 to 8.9, and 15 to 15.9 ranges. Moreover

tuning curves were uniformly affected throughout their range so that most frequencies which drove the neurons of CR mice did so at lower than normal intensities (Fig. 1D). Again, units with best frequencies in the range from 10 to 15 kHz were most affected.

To obtain a measure of each neuron's absolute frequency range, we extrapolated the upper cutoff frequency of all tuning curves at 100 dB SPL (*12*). Units from CR animals with best frequencies in the range 10 to 14 kHz had significantly larger high-frequency cutoffs ($P < 0.01$, not shown). This result suggests that inputs that would have been functionally eliminated under normal conditions continued to be represented on central auditory neurons of CR mice.

The original hypothesis would predict a broadening of tuning curves within all regions where the click's power spectrum is flat, because units responding to all of those frequencies should be entrained. The power spectrum shows a relatively intense signal from 0 to 15 kHz and has a small region of noticeable intensity around 19 kHz (Fig. 1A). The lack of an effect in our data above 15 kHz might reasonably be explained by the sharp drop in power at that region of the click's power spectrum. Below 10 kHz the lack of an effect may be due to pup vocalizations, the normally high thresholds (Fig. 1B), or insufficient sample size. We therefore remain cautious in drawing conclusions about these seemingly unaffected populations of neurons.

One alternative explanation for our results is that the acoustic stimulation has simply damaged the cochlea. Noise-induced acoustic trauma is commonly accompanied by severely elevated thresholds (*13, 14*) and broadened tuning curves (*15*), although high-frequency cutoffs are not increased (*14, 15*). Additionally, the most dramatic traumatizing effects are commonly 1/3 to 1/2 octave above the signal's power band (*14, 16*). Except for broadening of tuning curves, these observations do not hold for our data.

To further rule out the possibility of peripheral damage, we obtained an independent measure of auditory thresholds by using the cochlear nerve compound action potential (N_1) response. We recorded the N_1 response in the cochlear nucleus to pure tones of decreasing intensity until an averaged response (up to 200 presentations) could no longer be visually detected. At the level of the cochlear nucleus, the responses of control and CR mice were virtually identical (Fig. 1B).

Although there are a small number of sharply tuned units within the affected region (10 to 15 kHz), we do not view this as being inconsistent with the hypothesis of activity-dependent tuning. Spontaneous activity may, in itself, be able to confer a proximity-dependent temporal identity on sensory afferents. In goldfish, neighboring retinal ganglion cells with similar receptive field organization often have well-correlated firing patterns in the dark (*17*). In the auditory system it is conceivable that a "spontaneous" mechanical disturbance of the basilar membrane would lead to the coactivation of afferents innervating hair cells in that region. In addition, there is evidence that the spontaneous activity of hair cells and primary afferents is not random (*18*). Although our mice were first exposed to clicks on day 8, an

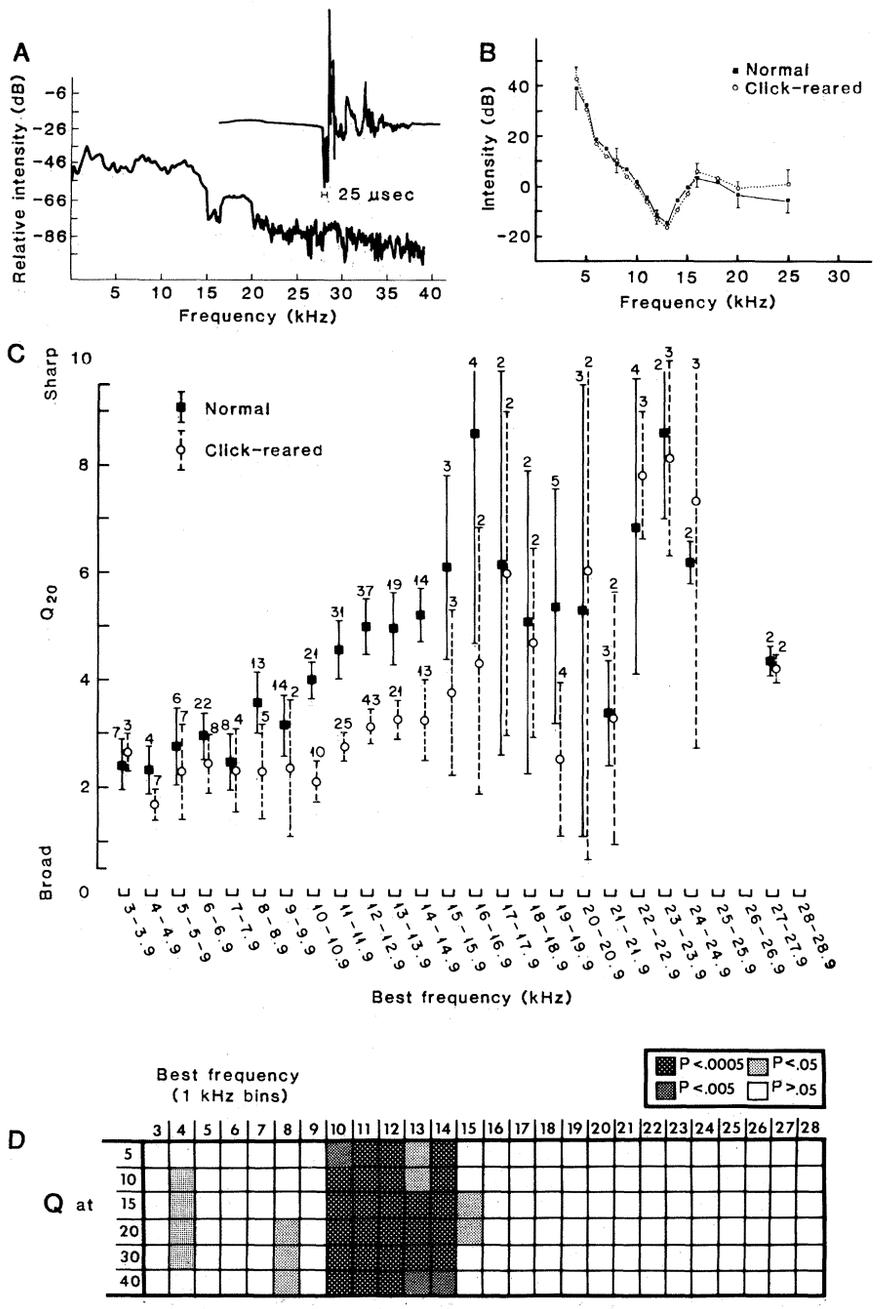

Fig. 1. (A) Fast Fourier transform of a single click (inset) used in the rearing condition. The power spectrum is relatively flat through 15 kHz. Intensity is given relative to the peak intensity of the stimulus. (B) Audiogram showing threshold of evoked compound action potential response for normal and CR mice. Error bars show 95 percent confidence intervals. (C) Summary graph showing mean Q_{20} values for units grouped in 1-kHz bins. For example, all CR units in the range 12 to 12.9 kHz were grouped together. Error bars show 95 percent confidence intervals, and small numbers indicate the number of units in each group. (D) A summary of the regions of statistical difference between units of normal and CR animals for all Q values (5 to 40 dB above threshold). A statistical difference for a particular Q value at a given kilohertz frequency bin is indicated by shading.

acoustic stimulus does not elicit a cochlear potential until day 12 (*7, 19*). However, we have recorded relatively infrequent action potentials from units in the cochlear nucleus of 10- or 11-day mouse pups, prior to ear canal opening, which could not be driven by our acoustic stimulus.

The results suggest that we have altered the normal development of synaptic connections in the auditory system despite the fact that neurons were active during ontogeny. Clopton and Winfield (*20*) have previously demonstrated that the response properties of neurons in the inferior colliculus may be biased by the rearing environment, but the rearing conditions were extremely complex, making it difficult to implicate a specific developmental mechanism. We used a very simple auditory stimulus. Repetitive click stimulation should largely mask persistent spontaneous activity, and, unlike deprivation, it avoids the potential degenerative affects of disuse (*21*). The locus of the connectivity changes we have detected remains to be determined, and we do not yet know the relative contribution of excitatory and inhibitory connections in producing the observed effects. Nevertheless, it seems likely that activity in the auditory system, as in the visual pathway, is critically involved in ontogenetic fine tuning.

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Shark Cartilage Contains Inhibitors of Tumor Angiogenesis

Abstract. *Shark cartilage contains a substance that strongly inhibits the growth of new blood vessels toward solid tumors, thereby restricting tumor growth. The abundance of this factor in shark cartilage, in contrast to cartilage from mammalian sources, may make sharks an ideal source of the inhibitor and may help to explain the rarity of neoplasms in these animals.*

Scapular cartilage in calves contains a substance that inhibits the vascularization of solid tumors (*1*). When this substance was infused into rabbits or mice, no toxic effects were observed in the animals, yet the growth of new blood vessels toward implanted tumors (V2 carcinoma and B16 melanoma) ceased and tumor growth stopped (*2, 3*). The single factor most limiting to the further study of this substance is its supply. Cartilage is present only in small quantities in mammalian species.

It occurred to us that sharks may be a potential source of this inhibitor because, unlike mammals, sharks have an endoskeleton composed entirely of cartilage. Cartilage composes about 6 percent of the shark's total body weight (*4*), compared to less than 0.6 percent in calves. In addition, some sharks are very large, about ten times heavier than calves.

Basking sharks (*Cetorhinus maximus*) 6.1 m long and weighing approximately 409 kg were obtained from Fresh Water Company, Boston. The fins and vertebrae were immediately excised, scraped with a scalpel blade to remove connective tissue, and stored at −20°C. To extract the inhibitor, a modification of the procedures used for calf cartilage (*1–3*) was employed. The shark fins were cut into 1-cm^3 pieces and extracted in a solution containing 1*M* guanidine and 0.02*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) for 41 days at room temperature. Extracts were dialyzed exhaustively against water by using membranes with a 3500-dalton cutoff and centrifuged. The supernatant was filtered through Whatman 1 filter paper and then lyophilized. Five hundred milligrams of cartilage yielded 1 mg of this extract.

The shark cartilage extract was incorporated into 1-mm^3 pellets of ethylene–vinyl acetate copolymer (40 percent vinyl acetate by weight) (*5*) at a level of 300 μg of extract and 700 μg of polymer. The polymer pellets have been shown to release over 1 μg of biologically active molecules per day for over 100 days (*3, 5*). The pellets were implanted into corneal pockets in New Zealand White rabbits. Directly behind the pellets were placed 1.5-mm^3 pieces of V2 carcinoma (*1*). These tumors induced vessels to sprout toward them from the edge of the cornea. The bioassay consisted of measuring the length of the single longest blood vessel with a slit-lamp stereomicroscope.

In experimental corneas, tumors and pellets containing shark extract were used. Control corneas were implanted with tumors and identical-sized pellets containing no extract. Earlier studies have shown that the rate of tumor neovascularization in such controls is statistically indistinguishable from the rate induced by (i) tumors and pellets containing extracts of calf cartilage with no biological activity, (ii) tumors and pellets containing proteins or polysaccharides, and (iii) tumors alone (*1, 3, 6*).

The extract of basking shark cartilage significantly inhibited tumor neovascularization. Three different tests were conducted, and inhibition was observed in every case (Fig. 1 and Fig. 2a). After 19 days all control corneas had large, three-dimensional tumors with an average maximum vessel length of 6 mm (half the diameter of the cornea) (Fig. 2b). In