Regulation of *jun*-B Messenger RNA and AP-1 Activity by Light and a Circadian Clock

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The suprachiasmatic nuclei (SCN) of the hypothalamus comprise the primary pacemaker responsible for generation of circadian rhythms in mammals. Light stimuli that synchronize this circadian clock induce expression of the c-*fos* gene in rodent SCN, which suggests a possible role for Fos in circadian entrainment. Appropriate light stimuli also induce the expression of *jun*-B messenger RNA in the SCN of golden hamsters but only slightly elevate c-*jun* messenger RNA levels. In addition, light increases the amount of a protein complex in the SCN that binds specifically to sites on DNA known to mediate regulation by the AP-1 transcription factor. The photic regulation of both *jun*-B messenger RNA expression and AP-1 binding activity is dependent on circadian phase: only light stimuli that shift behavioral rhythms induce *jun*-B and AP-1 expression. Thus, light and the circadian pacemaker interact to regulate a specific set of immediate-early genes in the SCN that may participate in entrainment of the circadian clock.

THE CELLULAR AND MOLECULAR mechanisms underlying circadian clocks remain a mystery. However, studies of diverse organisms have shown that gene expression is important for both the generation and the control of circadian rhythms (*1–3*) and for the regulation of biochemical rhythms by the circadian system (*3, 4*). In mammals, the hypothalamic SCN are the site of a circadian pacemaking system regulating behavioral rhythms (*5*). Light entrains, or synchronizes, mammalian circadian rhythms through a specialized retinohypothalamic projection that terminates primarily in the ventrolateral portion of the SCN (*6, 7*). Fos, the product of the immediate-early gene c-*fos*, is a component of a transcriptional regulatory complex that may couple extracellular signals to changes in gene expression. Light regulates c-*fos* mRNA levels (*8, 9*) and Fos-related immunoreactivity (*8, 10*) in rodent SCN.

Two critical features of the circadian behavioral response to light (a light-induced phase shift in activity rhythms) have an observable molecular correlate in the regulation of c-*fos* gene expression. First, photic induction of both c-*fos* and circadian phase-shifting is dependent on circadian time. Light phase shifts circadian rhythms only during the subjective night (*11*) and is capable of inducing c-*fos* mRNA in hamster SCN only at these times (*9*). Second, the threshold for induction of c-*fos* mRNA is similar to the threshold for phase-shifting by light (*9*). Taken together, these results suggest that c-*fos* induction may be intimately associated with cellular processes underlying photic entrainment. Because Fos acts in concert with members of the Jun family of proteins to form the transcription factor complex AP-1 (*12*), we sought to determine whether light also regulates Jun gene expression and ultimately AP-1 activity in the SCN.

We examined the response to light of two genes that encode components of the AP-1 complex, *jun*-B (*13*) and c-*jun* (*14, 15*), in the SCN of golden hamsters. Using in situ hybridization to an RNA probe for *jun*-B mRNA, we found that 5-min light pulses at circadian time (CT) 19 (during the subjective night of the animal) cause an increase in *jun*-B mRNA levels in the SCN (Fig. 1, A through F). The cells expressing *jun*-B mRNA are located primarily in the ventrolateral region of the SCN and in an area immediately dorsal to the SCN; we did not observe any increase in *jun*-B mRNA in response to light in other brain areas. The pattern of expression in the SCN region is similar to that observed for light-induced c-*fos* mRNA expression (Fig. 1, G and H) and corresponds closely to the known pattern of retinohypothalamic tract projections in hamsters (*7*). In animals receiving no light pulse, no detectable hybridization signal was observed in the area of the SCN (Fig. 1, C and D), and a sense *jun*-B RNA probe used as a control showed no hybridization in the SCN of animals pulsed with light or maintained in the dark. In addition to inducing *jun*-B mRNA expression, a 5-min light pulse at CT19 also increased c-*jun* mRNA in the ventrolateral portion of the SCN. The magnitude of light induction of c-*jun* mRNA (approximately twofold) (*16*), however, was markedly less than that observed for *jun*-B mRNA (about 15-fold) (Figs. 2 and 3).

To determine the time course of the induction of *jun*-B mRNA, we performed in situ hybridization at various times after a 5-min light pulse (at CT19) and quantified

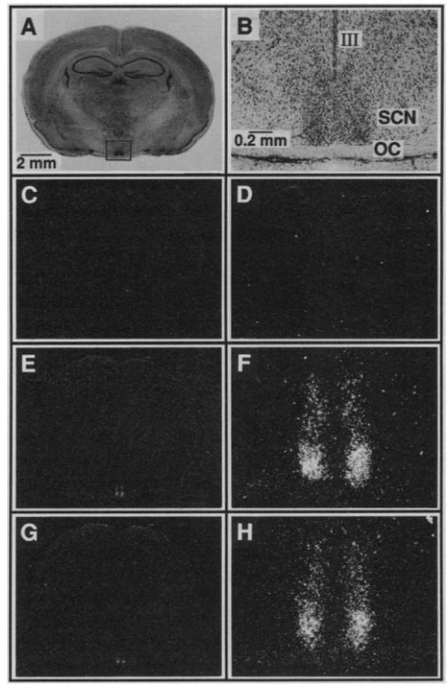

Fig. 1. Photic induction of *jun*-B and c-*fos* mRNAs in the hamster SCN detected by in situ hybridization. Hamsters were entrained to a 14-hour light–10-hour dark cycle and then placed in constant darkness for 7 days before the experiment. At CT19, hamsters received either a 5-min light stimulus (503-nm wavelength, 2.5×10^{14} photons cm^{-2} s^{-1}) or identical handling in darkness, and were then returned to darkness for 25 min. The hamsters were killed in darkness, and their brains were dissected under dim red illumination and frozen until sectioning for in situ hybridization (*25*). (**A**) A representative section of hamster brain through the SCN. Boxed area is shown in (B). (**B**) Higher magnification view indicating the location and morphology of the paired SCN. SCN, suprachiasmatic nuclei; OC, optic chiasm; III, third ventricle. Sections in (A) and (B) were stained with cresyl violet to identify neuronal cells and photographed with bright-field microscopy. (**C** and **D**) *jun*-B mRNA in the SCN of hamsters receiving no light. Sections were hybridized to an RNA probe for *jun*-B. (**E** and **F**) *jun*-B mRNA in the SCN of hamsters that received a light pulse. (**G** and **H**) c-*fos* mRNA in the SCN after a light pulse; (E) and (G) show adjacent sections of the brain, as do (F) and (H). (C), (E), and (G) are reversed-contrast photographs of x-ray film (Kodak XAR-5) autoradiographs of sections after in situ hybridization; (D), (F), and (H) show dark-field photomicrographs of hybridized brain sections after autoradiographic exposure to liquid emulsion (Kodak NTB-2) for 3 weeks.

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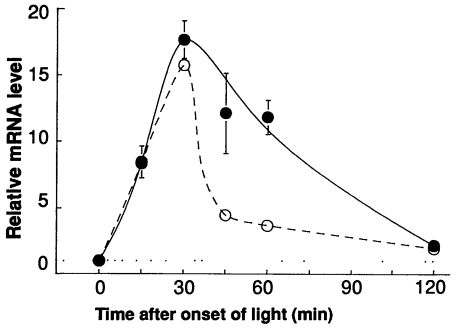

Fig. 2. Time course of *jun*-B mRNA (●) and c-*fos* mRNA (○) induction in the SCN by light. Hamsters were placed in constant darkness for 7 days before the experiment. At CT19, 5-min light pulses (503 nm, 2.5×10^{14} photons cm^{-2} s^{-1}) were delivered, followed by a return to darkness for varying durations. In situ hybridization with a *jun*-B or c-*fos* (*9*) RNA probe was performed as in Fig. 1, and the amount of hybridization in the SCN was quantified (*26*) with an Image-1/AT image processing system (Universal Imaging). The hybridization signal is expressed as a ratio of the signal to that measured in control hamsters held in the dark. Error bars show range (n = 2) of data.

the specific hybridization signal in the SCN. The elevation of *jun*-B (and c-*fos*) mRNA was rapid and transient, with a peak occurring 30 min after the onset of the light pulse (Fig. 2) (*9*). The decrease in *jun*-B mRNA was slower than for c-*fos* mRNA (Fig. 2). This temporal correlation of the expression of c-*fos* and of *jun*-B, along with the likelihood that the two genes are coexpressed in the same cells, suggests that the Fos and Jun-B proteins are available to dimerizé.

Light exposure during the subjective night, or active period, of a hamster's circadian cycle causes a phase shift (a steady-state change in phase) in the activity rhythm of the animal (*11*), resulting in entrainment of the circadian pacemaker. By contrast, light exposure during the subjective day produces no effect on the phase of a hamster's rhythm (Fig. 3A). In situ hybridization studies demonstrate that the induction of *jun*-B mRNA after a 5-min light pulse is likewise dependent on circadian phase (Fig. 3B). Light pulses at CT14, which produced a phase-delay in a hamster's activity rhythm, elevated *jun*-B mRNA levels, and light pulses at CT19 and CT21, which caused phase-advances, also induced *jun*-B mRNA. During the subjective day, at CT3 and CT9, light induced neither phase shifts nor the expression of *jun*-B mRNA. The photic induction of c-*fos* gene expression displayed this same phase dependence (Fig. 3C) (*9*). Because all animals were maintained in constant darkness before these experiments, differences between their responses to light during the subjective day and night reflect only the state of an endogenous pacemaker. This result indicates that both c-*fos* and *jun*-B mRNA are gated by the circadian pacemaker and are induced by light under the same temporal conditions.

The coinduction of *jun*-B and c-*fos* mRNAs by light makes it probable that amounts of the dimeric AP-1 complex in the SCN are increased by light stimulation at appropriate circadian times. To determine whether light indeed regulates AP-1 binding, we developed a gel mobility-shift assay method that can detect AP-1 complexes in individual SCN regions of a single animal. Whole-cell extracts were prepared from microdissected blocks of hamster brain tissue containing the SCN (*17*). These extracts were incubated with a ^{32}P-labeled oligonucleotide containing either a single or tandem AP-1 consensus sequence element (*18*) and then separated by electrophoresis on nondenaturing polyacrylamide gels. AP-1 binding activity was detectable (Fig. 4A) in animals maintained in darkness and increased 1 hour after the onset of a 5-min light pulse (delivered at CT19). The highest AP-1 binding activity occurred approximately 2 hours after light stimulation (Fig. 4A). Both of the bands of retarded mobility detected in these extracts were abolished by the addition of excess unlabeled AP-1 oligonucleotide (Fig. 4A) but not by an oligonucleotide containing an adenosine 3′,5′-monophosphate response element (CRE) consensus sequence, which demonstrates that the binding activity in these SCN extracts is specific. In tissue extracts prepared from another brain region, lateral to the SCN, amounts of AP-1 binding activity were not affected by light.

The circadian phase dependence of the photic induction of AP-1 was next determined (Fig. 4B). At CT6, during the subjective day, this protein-DNA complex was similar in extracts from hamsters receiving no light (controls) and extracts prepared 2 hours after a 5-min light pulse. After a 5-min light pulse at CT14 or at CT19 (subjective night), however, the amount of AP-1 complex in the SCN extracts was increased compared to the controls, which suggests that AP-1 activity is elevated only by light stimuli occurring during the hamster's subjective night. Addition of an excess of unlabeled AP-1 oligonucleotide abolished the observed protein-DNA complex in all of these extracts (Fig. 4B). In addition, preincubation of the SCN extracts from light-pulsed animals with a Fos antibody (to amino acids 127 through 156 of mouse Fos, in the dimerization domain) abolished the protein-DNA complex. This result indicates that Fos (or Fos-related proteins) participates in the formation of this complex. Addition of a control antibody (to neuron-specific enolase) does not affect DNA binding (Fig. 4B). Similar results were obtained with two oligonucleotides having different flanking sequences (compare Fig. 4, A and B) (*19*).

These results demonstrate that light increases the AP-1 protein complex in the SCN and imply that photic signal transduction is coupled to subsequent changes in the transcription of AP-1 target genes in the SCN. Regulation of c-*fos* and *jun*-B mRNAs by light is observed only in a restricted subset of cells within a defined region of the central nervous system known to participate in the generation of circadian rhythms. The characteristics of the induction of c-*fos* mRNA by light, together with the anatomical specificity and phase dependence of the photic stimulation of *jun*-B mRNA, suggest that light-induced gene expression may be closely linked to the process of circadian

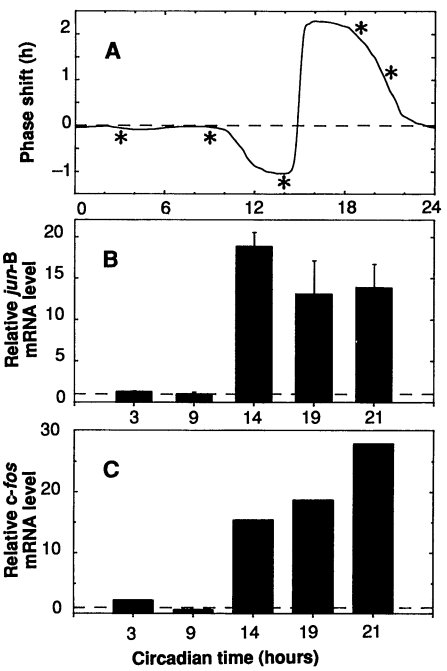

Fig. 3. Dependence of light-induced behavioral phase-shifting and *jun*-B mRNA on the circadian phase. After entrainment to a 14-hour light–10-hour dark cycle, hamsters were placed in constant darkness for 7 days before the experiment. (**A**) A phase-response curve for the golden hamster (*11*), which shows the amount of steady-state phase shift of behavioral activity rhythms produced by 60-min light pulses at different phases of the circadian cycle. Asterisks mark time of light pulses in (B) and (C). (**B**) *jun*-B mRNA in the SCN (*26*) after light pulses of 5-min duration (503 nm, 2.5×10^{14} photons cm^{-2} s^{-1}) at CT3, 9, 14, 19, or 21. Animals were returned to darkness for 25 min, and in situ hybridization was performed as in Fig. 1. Values represent the mean signal in the SCN of light-pulsed hamsters relative to the mean signal (at the same CT) in animals receiving no light (n = 2) (error bars show range of data). Hamsters receiving no light exhibited no significant *jun*-B mRNA hybridization in the SCN at all circadian times examined. (**C**) Circadian phase dependence of c-*fos* mRNA induction by light (*9*). Experiment was as described in (B). Light exposure was for 5 min, 8.0×10^{12} photons cm^{-2} s^{-1}.

entrainment (*20*). A role for transcription in the entrainment of the pacemaker is consistent with studies showing a need for macromolecular synthesis in the entrainment and expression of circadian rhythms (*1–4, 21*). As in the SCN, membrane depolarization of PC12 cells induces *jun*-B but not c-*jun* mRNA (*22*); in addition, *jun*-B can antagonize the AP-1 transcriptional activation caused by c-*jun* (*23*).

An intriguing aspect of immediate-early gene induction in this system is that the ability of light to alter expression of c-*fos* and *jun*-B in the SCN is regulated by the circadian clock. This regulation might occur through a variety of mechanisms, ranging from control by the circadian pacemaker of synaptic transmission or electrical excitability of SCN neurons to direct transcriptional regulation of c-*fos* and *jun*-B. Synthesis of the AP-1 complex is both gated by, and is in turn likely to influence, the circadian pacemaker. This proposed relation between AP-1 and the circadian clock may be analogous to the role of the *Drosophila per* gene: levels of *per* mRNA and protein cycle in a circadian manner, and expression of the wild-type Per protein is also required for normal circadian rhythms (*3*). In fact, Per is homologous to characterized transcription factors and has a nuclear localization (*24*). Transcriptional regulation may prove to be a common element of circadian timekeeping mechanisms in diverse organisms.

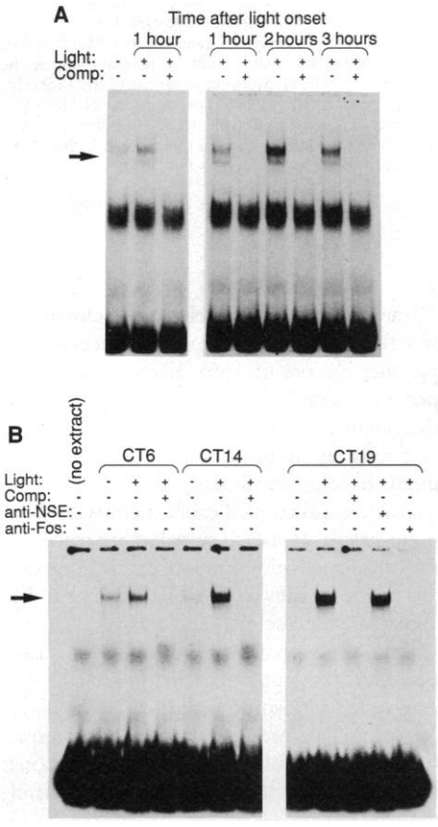

Fig. 4. Gel mobility-shift assays measuring light-induced increases in AP-1 DNA-binding activity in the SCN. Hamsters were maintained in constant darkness and activity rhythms recorded, as in Fig. 1. At the appropriate time, animals received a 5-min light pulse (503 nm, 2.5×10^{14} photons cm^{-2} s^{-1}) or the same handling in the dark and were returned to darkness until they were killed. Brains were removed in darkness, and the SCN dissected (*9, 17, 27*). (**A**) Time course of AP-1 activity after a light pulse at CT19. An AP-1 oligonucleotide with tandem AP-1 consensus binding sites (oligo 1) was used (*27*). Arrow, mobility of the two specific complexes; Comp, competition with unlabeled oligonucleotide. The nonspecific band may result from the use of total cellular instead of nuclear extract. Equivalent results were obtained in at least two independent experiments. (**B**) Circadian phase dependence of the induction of AP-1 activity by light. An oligonucleotide with a single AP-1 site (oligo 2) was used (*27*). All SCN samples were collected 2 hours after the onset of a light pulse at the CT indicated on the top of the figure or after identical handling in the dark. No extract, binding reaction performed with no SCN extract; Comp, competition with unlabeled oligonucleotide; anti-NSE, addition of an irrelevant antibody against neuron-specific enolase; anti-Fos, addition of antibody to Fos, which prevents Fos-Jun aggregation. The moderately higher amount of AP-1 in the third lane than in the second reflects sample variation. Results were verified in at least four independent experiments. Arrow, mobility of specific complex.

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16. The weak induction of c-*jun* mRNA is not due to poor hybridization of the mouse c-*jun* probe with hamster mRNA. Pentylenetetrazol-treated hamsters (70 mg per kilogram of body weight) expressed high levels of c-*jun* mRNA, detected by in situ hybridization, in the dentate gyrus and hippocampus, as in other rodents [D. W. Saffen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7795 (1988)].
17. Paired SCN from a single animal were rapidly dissected from a 1-mm-thick frontal section of fresh brain (*9*). Tissue (approximately 50 μg per paired SCN) was frozen on dry ice and stored at −80°C.
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20. There is additional evidence correlating immediate-early gene expression in the SCN and photic entrainment. (i) A noncompetitive *N*-methyl-D-aspartate antagonist, MK-801, blocks both photic phase shifts and Fos induction in the SCN [C. Colwell, M. R. Ralph, M. Menaker, *Brain Res.* **523**, 117 (1990); H. Abe, B. Rusak, H. Robertson, *Neurosci. Lett.* **127**, 9 (1991)]. (ii) A nonphotic agent, triazolam, phase shifts but does not induce Fos in the SCN (Y. Zhang, O. Van Reeth, F. W. Turek, personal communication), which suggests a specific association of Fos with photic inputs. (iii) Pentylenetetrazol induces *jun*-B and c-*jun* mRNAs in hamster brain, but not in the SCN (J. M. Kornhauser, D. E. Nelson, K. E. Mayo, J. S. Takahashi, unpublished results). (iv) A c-*fos*–*lacZ* transgene introduced into mice is regulated by light in the same manner as endogenous c-*fos* [R. J. Smeyne *et al.*, *Neuron* **8**, 13 (1992)].
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25. In situ hybridization histochemistry was performed as described (*9*). We transcribed ^{35}S-labeled riboprobes for *jun*-B from a 1.6-kb Eco RI fragment of *jun*-B cDNA (*13*) [American Type Culture Collection (ATCC) 63025] subcloned into a pGEM-7Z (Promega) vector with T7 RNA polymerase (and DNA linearized with Kpn I) to generate complementary *jun*-B probes, or SP6 polymerase (and DNA linearized with Apa I) for sense-strand probes. Riboprobes for c-*jun* mRNA were transcribed from a construct containing the entire cDNA insert (*15*) (ATCC 63026) subcloned into pGEM-7Z; SP6 polymerase (DNA linearized with Apa I) generated complementary strand probes, and T7 polymerase (DNA linearized with Kpn I) sense-strand probes. Linearized *jun*-B and c-*jun* plasmid DNAs were treated with Klenow (Promega) to remove 3′ protruding ends before riboprobe synthesis. Riboprobes for c-*fos* mRNA were prepared as described (*9*).
26. Quantification of in situ hybridization signals in the SCN was performed as described (*9*) with the

following exception: We defined the "signal" in each section by taking a measurement from a 75 by 75 μm area within the SCN and subtracting a background measurement from an area of equal size lateral to the SCN.

27. The AP-1 consensus oligonucleotides used in binding assays were oligo 1 (Oncogene Science), double-stranded 36-nucleotide, 5′-GATCCATCG**TGACTCA**GCGATCTCG**TGACTCA**GCGG-3′; oligo 2 (Promega), double-stranded 21-nucleotide, 5′-CGCTTGA**TGAGTCA**GCCGGAA-3′. (Boldface indicates consensus AP-1 binding sites.) Oligonucleotides were radiolabeled with [α-^{32}P]deoxycytidine 5′-triphosphate and terminal deoxynucleotidyl transferase and gel-purified by electrophoresis on a 15% polyacrylamide gel. Whole-cell extracts of brain tissue were prepared by sonication of each SCN sample (*17*) at 4°C in 50 μl of buffer [20 mM Hepes (pH 7.8), 125 mM NaCl, 5 mM $MgCl_2$, 12% glycerol, 0.2 mM EDTA, bovine serum albumin (BSA) (1 mg/ml), 0.1% Nonidet P-40, 5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), aprotinin (1 μg/ml), and bestatin (40 μg/ml)]. Extracts were then centrifuged at 15,600*g* for 10 min (at 4°C) and the supernatants collected. Binding assays were performed with 10 μl of SCN cell extract, 1.5 μg of poly[d(I-C)], 5 μl of buffer [50 mM Hepes (pH 7.8), 5 mM spermidine, 15 mM $MgCl_2$, 36% glycerol, BSA (3 mg/ml), 0.3% Nonidet P-40, and 15 mM DTT], and 30,000 cpm of ^{32}P-labeled oligonucleotide, with water added to a final volume of 25 μl. Reactions were incubated for 15 min on ice before addition of ^{32}P-labeled oligonucleotide, then for an additional 15 min at 22°C. When competition with unlabeled oligonucleotide was performed, a 50-fold molar excess of oligonucleotide relative to the radiolabeled probe was added to the binding assay. When antibodies were included, 1 μl was added to the reaction 15 min before the addition of the probe. The antibody to Fos is a rabbit antiserum made against the synthetic peptide corresponding to amino acids 127 to 156 of mouse Fos; rabbit antiserum against bovine neuron-specific enolase (Accurate) was used as a control antibody. Samples were subjected to electrophoresis on 4% nondenaturing polyacrylamide gels, dried, and autoradiographed.

28. We thank T. Curran, D. Linzer, and J. Mordacq for advice on AP-1 gel mobility-shift assays, and R. J. Distel and B. M. Spiegelman for use of the antibody to Fos. Supported by grants from the NIH, NSF, and McKnight Endowment Fund for Neuroscience (K.E.M.) and from the NIH, NSF, and the National Institute of Mental Health (J.S.T.).

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Technical Comments

Ultrasonic Hearing

M. L. Lenhardt *et al.* (*1*) suggest that "bone-conducted, ultrasonic stimulation may provide an alternative therapeutic approach for the rehabilitation of severe hearing loss." They argue that when speech signals are used to modulate the amplitude of an ultrasonic carrier, people detect and recognize speech sounds by physiological mechanisms other than those that normally transduce audible speech.

There is an alternative explanation for the findings of Lenhardt *et al.* that is consistent with classic auditory physiology, but does not support the possibility of using this approach to bypass a damaged cochlea. Even a slight, even-order (rectifying) nonlinearity in the transfer path from transducer to skull would result in demodulation. The speech signals, which had been modulated onto the ultrasonic carrier, could be converted back into audio frequency bone-conducted stimuli, capable of being transduced by conventional cochlear processes.

The stimuli used by Lenhardt *et al.* were intense compared with those used for bone conduction testing in the audio frequency range. Lenhardt *et al.* specify their stimuli as acceleration, in decibels with reference to 10^{-3} m/sec^2, whereas the American National Standards Institute S3.26-1981 standard (*2*) for zero hearing level for the B-71 bone vibrator averages 30.5 dB with reference to 1-μN force at 2 to 4 kHz. If this force is applied to a 5-kg head moving as a rigid body, an acceleration of 6.7×10^{-6} m/sec^2, or 43 dB below Lenhardt's reference level (*1*), would result. Response thresholds to ultrasound are reported (*1*) at +82 to +112 dB (with reference to 1×10^{-3} m/sec^2) or 125 to 155 dB above threshold accelerations at audio frequencies. If only 10% of the head mass were to effectively move in response to bone-conducted ultrasound, their thresholds would still be 105 to 135 dB more intense than audio-frequency thresholds.

A slight nonlinearity, resulting in demodulation of audible speech signals, might be difficult to observe in spectral analysis with only a 60-dB dynamic range. A better test would be to measure cochlear potentials. Foster and Wiederhold (*3*) showed that, in cats, pulsed ultrasound produced cochlear microphonics and compound action potentials that were indistinguishable from those produced by audible transient stimuli. We suspect that, were a study to be performed similar to (*3*) that used the stimuli presented by Lenhardt *et al.*, it would reveal that these stimuli were present in the cochlear microphonic and thus available in the audible range within the cochlea.

The results obtained by Lenhardt *et al.* in human subjects were not clearly better than could have been achieved by presenting the same speech stimuli in the normal audio range. Even their "deaf" subjects had *mean* thresholds [(*1*), figure 1A] that were within about 55 dB of the normal threshold at 250 Hz [we assume that the abscissa of figure 1A in (*1*) should have been labeled from 0 to 10,000 Hz for the air conduction thresholds, and that the lowest frequency tested was about 250 Hz]. Thus, some of these subjects could probably discriminate some speech stimuli when presented with adequate stimulus intensity. Two of the nine "deaf" subjects displayed accuracy scores of 20 and 30%, respectively, on a closed-set test for which random performance would produce a score of 16%. Even if these two performances had been significantly better than chance, presentation of the same speech materials as high-intensity audio stimuli might have given similar results.

One critical control experiment would be quite simple. If speech signals were reaching the cochlea as audio-range signals after demodulation, they would be maskable by audio-range noise maskers.

Until more conventional mechanisms can be excluded, it appears premature to suggest a separate ultrasonic receptor, particularly when the structure suggested to detect ultrasound, the saccule, responds to vibrations from zero to only 2 kHz in the squirrel monkey (*4*).

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Response: Dobie and Wiederhold postulate that the relatively intense ultrasonic signals used in our study (*1*) could allow demodulation of ultrasonic speech into the audio frequency range. The normal hearing ear is an excellent demodulation detector. With modulation, all listeners in our study reported hearing the sidebands and the carrier, which would have been impossible had the signals been demodulated. With deaf subjects we again found no evidence for