Friend erythroleukemia cells. Adherence and differentiation of HL-60 cells were not induced by promoters, such as anthralin, that are unrelated in chemical structure to the plant diterpenes and are much less active in vivo. A similar difference between the effects of phorbol diester tumor promoters and other types of tumor promoters on cells in culture has been observed by others. Driedger and Blumberg (*19*) found that TPA and nonphorbol tumor promoters had different effects on the large-external-transformation-sensitive (LETS) glycoprotein and on deoxyglucose transport in chick embryo fibroblasts. Wigler *et al.* (*20*) found in the same cells that only diterpene esters, and not other types of tumor promoters, induced plasminogen activator. It is possible, therefore, that many of the effects of tumor promoters on cells in vitro are unrelated to the mechanism of promotion or that different chemical classes of promoters have completely different mechanisms of promoting action. Driedger and Blumberg (*19*) reached a similar conclusion. It is also of interest in this connection that a compound such as fluocinolone acetonide, which can inhibit TPA-induced tumor promotion in vivo (*16*) and TPA-induced DNA synthesis in mouse epidermal cells in vitro (*21*), had no effect on the induction of differentiation by TPA in HL-60 cells.

It is also interesting that mezerein, one of the plant esters that was an effective inducer of differentiation in the HL-60 cell line, has been shown to exert antileukemic activity against the P388 murine lymphocytic leukemia (*22*). Its ability to force proliferating human promyelocytic leukemia cells to differentiate into nonproliferating macrophage-like cells suggests that it and similar compounds may merit further investigation as antitumor drugs with this particular mechanism of action. It should be stressed that the loss of proliferative capacity induced in HL-60 cells by mezerein and the phorbol diester tumor promoters is not only very rapid but also affects 100 percent of the cell population. In contrast to the effects of other types of inducers of differentiation such as dimethyl sulfoxide in this and other cell systems, there are no nonresponsive, proliferating cells remaining in HL-60 cell cultures treated with effective tumor promoters.

GIOVANNI ROVERA
THOMAS G. O'BRIEN
LEILA DIAMOND

Wistar Institute of Anatomy
and Biology, Philadelphia,
Pennsylvania 19104

References and Notes

1. R. K. Boutwell, *CRC Crit. Rev. Toxicol.* **2**, 419 (1974); T. J. Slaga, A. Sivak, R. K. Boutwell, Eds., *Carcinogenesis—A Comprehensive Survey*, vol. 2, *Mechanisms of Tumor Promotion and Cocarcinogenesis* (Raven, New York, 1978); J. D. Scribner and R. Süss, *Int. Rev. Exp. Pathol.* **18**, 137 (1978).
2. I. B. Weinstein, M. Wigler, C. Pietropaolo, in *Origins of Human Cancer*, H. H. Hiatt, J. D. Watson, J. A. Winsten, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977), p. 751.
3. L. Diamond, T. G. O'Brien, G. Rovera, *Life Sci.* **23**, 1979 (1978).
4. G. Rovera, T. G. O'Brien, L. Diamond, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2894 (1977).
5. L. Diamond, T. G. O'Brien, G. Rovera, *Nature (London)* **269**, 247 (1977).
6. H. Yamasaki, E. Fibach, U. Nudel, I. B. Weinstein, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3451 (1977).
7. R. Cohen, M. Pacifici, N. Rubinstein, J. Biehl, H. Holtzer, *Nature (London)* **266**, 538 (1977); M. Pacifici and H. Holtzer, *Am. J. Anat.* **150**, 207 (1977); D. N. Ishii, E. Fibach, H. Yamasaki, I. B. Weinstein, *Science* **200**, 556 (1978); M. E. Lowe, M. Pacifici, H. Holtzer, *Cancer Res.* **38**, 2350 (1978); D. N. Ishii, *ibid.*, p. 3886.
8. I. B. Weinstein and M. Wigler, *Nature (London)* **270**, 659 (1977).
9. R. M. Miao, A. H. Fieldsteel, D. W. Fodge, *ibid.* **274**, 271 (1978).
10. S. J. Collins, R. C. Gallo, R. E. Gallagher, *ibid.* **270**, 347 (1977).
11. C. Friend, W. Scher, J. G. Holland, T. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 378 (1971).
12. S. J. Collins, F. W. Ruscetti, R. E. Gallagher, R. C. Gallo, *ibid.* **75**, 2458 (1978).
13. HL-60 cells were seeded at a density of 2×10^5 per milliliter into 60-mm petri dishes containing cover slips and were treated with TPA at a final concentration of $1.6 \times 10^{-7}M$. Tritiated thymidine (specific activity, 2 Ci/mmole; 0.1 μCi/ml) was added to the untreated control cultures at time zero and to the treated cultures 24 and 48 hours after the addition of TPA. Cytospin slide preparations of the cells in suspension and cover slip preparations of the adherent cells were processed for autoradiography by the procedure of V. J. Cristofalo and B. B. Scharf [*Exp. Cell Res.* **76**, 419 (1973)].
14. G. Rovera, D. Santoli, C. Damsky, *Proc. Natl. Acad. Sci. U.S.A.*, in press; A. Vorbrodt, P. Meo, G. Rovera, in preparation.
15. E. Hecker, in *Carcinogenesis—A Comprehensive Survey*, vol. 2, *Mechanisms of Tumor Promotion and Cocarcinogenesis*, T. J. Slaga, A. Sivak, R. K. Boutwell, Eds. (Raven, New York, 1978), p. 11; F. G. Bock and R. Burns, *J. Natl. Cancer Inst.* **30**, 393 (1963); C. Peraino, R. J. M. Fry, E. Staffeldt, W. E. Kisieleski, *Cancer Res.* **33**, 2701 (1973); R. M. Hicks, J. St. J. Wakefield, J. Chowaniec, *Chem. Biol. Interact.* **11**, 225 (1975).
16. J. A. Schwarz, A. Viaje, T. J. Slaga, S. H. Yuspa, H. Hennings, U. Lichti, *Chem. Biol. Interact.* **17**, 331 (1977).
17. S. Belman and W. Troll, *Cancer Res.* **32**, 450 (1972); W. Bollag, *Eur. J. Cancer* **8**, 689 (1972); A. K. Verma and R. K. Boutwell, *Cancer Res.* **37**, 2196 (1977); A. K. Verma, H. M. Rice, R. K. Boutwell, *Biochem. Biophys. Res. Commun.* **79**, 1160 (1977); M. Hozumi, M. Ogawa, T. Sugimura, T. Takeuchi, H. Umezawa, *Cancer Res.* **32**, 1725 (1972); W. Troll, A. Klassen, A. Janoff, *Science* **169**, 1211 (1970).
18. H. Yamasaki, E. Fibach, I. B. Weinstein, U. Nudel, R. A. Rifkind, P. A. Marks, in *Oncogenic Viruses and Host Cell Genes*, Y. Ikawa, Ed. (Academic Press, New York, in press).
19. P. E. Driedger and P. M. Blumberg, *Int. J. Cancer* **22**, 63 (1978).
20. M. Wigler, D. DeFeo, I. B. Weinstein, *Cancer Res.* **38**, 1434 (1978).
21. U. Lichti, T. J. Slaga, T. Ben, E. Patterson, H. Hennings, S. H. Yuspa, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3908 (1977).
22. S. M. Kupchan and R. L. Baxter, *Science* **187**, 652 (1975).
23. Supported in part by grants CA 21124, CA 08936, and CA 23413 from the National Cancer Institute and grant ES 01664 from the National Institute of Environmental Health Sciences. G.R. is a Scholar of the Leukemia Society of America. We thank R. Gallo for the HL-60 cells. Antipain and leupeptin were obtained through the United States-Japan Medical Science Program.

15 December 1978; revised 5 March 1979

Species-Specific Perceptual Processing of Vocal Sounds by Monkeys

Abstract. *Monkeys of four species were trained to discriminate between sets of natural tonal calls of Japanese macaques (*Macaca fuscata*) by the position of a frequency-inflection peak or by initial pitch. The Japanese macaques consistently performed best on peak position and the other species on pitch. The results imply special strategies for perceptual processing of vocal sounds and suggest parallels with human speech perception.*

In recent studies of the psychology and physiology of hearing, there is evidence of novel insights from the use of complex, biologically significant stimuli (*1*). Investigators are increasingly concerned that organisms may use special perceptual procedures in the processing of sounds with particular significance to species members, such as those used in social communication. The evolution of speech has evidently capitalized on particular attributes of the human auditory system, although the extent to which these are uniquely human is moot (*2*). We have undertaken to analyze how Japanese macaques (*Macaca fuscata*) perceive elements from their repertoire of communicative sounds to determine whether they have evolved predispositions for processing such signals. The aim is to discover basic commonalities and contrasts between speech perception and the perception of conspecific calls by monkeys. An earlier report described our discovery that Japanese macaques consistently exhibit right-ear dominance while engaged in the perceptual processing of these vocalizations (*3*). Here we explore whether these monkeys use other strategies characteristic of speech perception in the processing of their own vocalizations.

One task a human listener faces when decoding speech sounds is the extraction of the appropriate linguistic content in the face of variability introduced by differences in the speaker's age, sex, and individual vocal tract anatomy. This normalization process has been termed perceptual constancy (*4*). Recent data indicate that even prelinguistic human infants recognize essential differences between phonemic categories, which suggests that they perceive constant stimulus relationships among myriad acoustic cues (*5*, *6*). In this experiment we set out to determine whether Japanese macaques can also attend to particular, relevant acoustic dimensions even in the face of irrelevant yet prominent variability in other aspects of their vocalizations. In short, do these monkeys show perceptual constancy for their own communication sounds?

Green (*7*) has demonstrated the Japanese macaques' social usage of complex and subtle variations in acoustic structure. For example, one of the ten general classes of calls used by this species is called simply "coos." Although all coos sound similar to the human ear, being tonal and with extensive harmonic structure, seven subtypes can be distinguished on the basis of fine acoustic structure. On examining the situations in which they are used, each vocal subtype was found to be correlated with a different set of behavioral contexts, which suggested that each coo variant marked a particular and distinct social circumstance.

All coos are used in affinitive situations, often as part of contact solicitations. Two of the seven subtypes were used in our experiments. The coo type dubbed smooth early (SE) is generally uttered by calm, young individuals isolated from companions. A second coo variant, the smooth late (SL) is given by subordinate animals to more dominant troop members, predominantly by estrous females soliciting contact at the early stages of consort formation. Both types may be given by animals of all ages, and they appear to denote two different behavioral states. While these two coos tend to be uttered in different circumstances, they are similar in most acoustic dimensions. Green's diagnostic criterion for distinguishing them is the relative position of the peak frequency inflection. In SE's this occurs in the first two-thirds of the call, and in SL's in the final third (*3*).

We trained Japanese macaques and several other monkeys used as comparison species to perform an operant response to the playback of recorded calls. Classical methods of animal psychophysics were used to test the ability of the different species to discriminate between sets of coo vocalizations sorted along different acoustic dimensions (*8*). In one case the coos were arranged according to peak position, with SE's falling into one group and SL's into the second. In the second part of this experiment, the same coos were re-sorted on the basis, not of peak position, but of starting frequency. One group of stimuli had a low starting pitch (< 600 Hz), the other a high starting pitch (> 600 Hz). In this way two discrimination tasks were produced: one in which peak position was the target cue and pitch was an irrelevant, potentially distracting cue; and a second in which onset pitch was the target cue and peak position was irrelevant. Our objective was to determine whether frequency-peak position is a cue attended to preferentially by Japanese macaques, as predicted from Green's field study, and whether the comparison species would behave differently, using the simple target cue of initial frequency rather than the complex one of relative position of the frequency maximum. A similar method is used in experiments on speech perception to establish the relative salience of acoustic cues in speech sounds, contrasting, for example, linguistic cues with nonlinguistic properties such as pitch (*9*).

Table 1. Species differences in performance. The derivation of the index of task difficulty is explained in the text.

Species	Subject	Task	
		Peak	Pitch
	Experiment 1		
Macaca fuscata	120	1.0	3.6
Macaca fuscata	122	1.5	4.4
Macaca nemestrina	93	8.2	1.0
Macaca radiata	133	3.9	1.2
	Experiment 2		
Macaca fuscata	98	1.0	
Macaca fuscata	99	2.0	
Macaca fuscata	100	1.8	
Macaca nemestrina	35	4.1	
Macaca radiata	88	6.7	
Cercopithecus aethiops	58	7.1	

Subjects were trained to grasp a metal tube which initiated playback of audible stimuli through earphones (TDH-140) every 3 seconds. After one to four background stimuli had been presented, a target stimulus was inserted. A monkey's task was to detect the target stimulus as distinct from the background and to respond by releasing the contact tube within 2 seconds. Only the correct response was reinforced with a banana pellet. Failure to detect the target was not punished, but an erroneous response to a background stimulus produced a 5-second time-out.

In the peak discrimination task, the background stimuli were field-recorded exemplars from the SL class, and the target stimuli were from the SE class. In the pitch discrimination task, the background stimuli were low-pitched and the target stimuli high-pitched. Thus, in one test starting pitch was the critical cue and in the other, the position of the frequency peak. Each stimulus was electronically mixed with low-level wideband noise to mask noise in the field recordings. On a stimulus-to-stimulus basis, the sounds were delivered randomly to the left or right ear.

Four subjects—two Japanese macaques, one pig-tail macaque (*M. nemestrina*), and one bonnet macaque (*M. radiata*)—were trained first on one discrimination test, then on the other. The order of testing was counterbalanced across groups. In the initial stage of each test, subjects were required to discriminate between a single call from one group and one call from the other. No animals had any difficulty at this stage. New exemplars from the two classes were added each time the animals satisfied a set of a priori performance criteria (*10*).

Clear species differences in performance emerged as the total number of exemplars to be discriminated was increased (Fig. 1). If the discrimination task was based on the position of the peak in the call, Japanese macaques learned the task readily. However, if starting pitch was the relevant dimension, the Japanese macaques experienced much more difficulty. Mirror image results were obtained for the comparison species. Table 1 summarizes species differences in performance on the two discriminations. An index of task difficulty was derived by expressing the performance of each subject relative to that of the animal requiring the smallest cumulative number of sessions to complete the entire series for a particular task. Thus the entries in the table were obtained by dividing total sessions for completion by the number needed by the most proficient subject. The results confirm the impression conveyed by Fig. 1. Comparison species required far more sessions than the Japanese monkeys to complete the peak task and fewer sessions on the pitch task.

In a second experiment, six additional animals—three Japanese macaques, a pig-tail macaque, a bonnet macaque, and a vervet, *Cercopithecus aethiops*—were tested on the peak task in an attempt to replicate this finding of species differences in discrimination performance. These animals produced essentially corroborative results (Table 1) (*11*). All the Japanese animals found the peak task easier than did even the most proficient of the other species.

Since all calls were taken from field

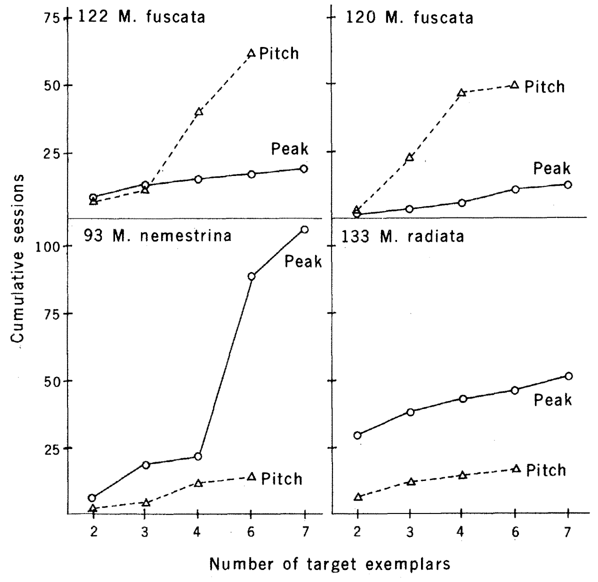

Fig. 1. Cumulative learning curves from the "pitch" and "peak" discrimination tasks for four monkeys. Each point represents the cumulative number of sessions necessary to meet the performance criteria and to advance beyond that stage of the problem. The total number of target exemplars discriminated at each stage is shown; they were discriminated from an equal number of background stimuli at each stage. The subjects on the left were tested on the pitch task first, those on the right, on the peak task.

recordings, they manifested all of the natural variability in acoustic dimensions inherent in these calls, in addition to those critical to the distinction between SE and SL coos. That is, although each call can be classified as an SE or SL on the basis of the position of the peak frequency, they also vary irregularly in duration, pitch, harmonic emphasis, structure, and so forth. Thus, one way of viewing these results is to consider the monkey's task as a problem in concept formation. In the peak discrimination task the subject is required to form concepts for both SE and SL and to classify background and target stimuli accordingly while ignoring "irrelevant" variations in other acoustic parameters, including pitch value. Similarly, in the pitch task the animals were required to form a high-pitch and low-pitch concept and to sort the calls on that basis while ignoring irrelevant variation in other parameters, including peak position. Hence, the apparent attentional preference of Japanese macaques for the peak cue facilitated their performance on the peak task but interfered with acquisition of the pitch task. The other monkey species evidently lack the attentional preference for peak, and treat pitch as the more salient feature, since their performance on the peak task was retarded relative to that on the pitch task.

The results demonstrate that the two acoustic cues manipulated in these experiments are not equipotential as discriminative stimuli for Japanese macaques and the other species tested. The attentional preference of the latter for starting pitch, probably because it is the simpler and acoustically more prominent of the two cues provided would be predicted if no special predispositions are brought to bear. The species-specific predisposition of Japanese macaques to discriminate groups of vocal sounds on the basis of the somewhat complex and subtle cue of frequency peak position parallels the evidence from field study suggesting that they use this cue for encoding information in their natural communicative repertoire.

The implication is that prior experience with these patterns of vocalization, either in the individual histories of the Japanese macaques we tested or in their evolutionary history, led to the adoption of special strategies for the perceptual processing of these calls. The results indicate that Japanese macaques attend selectively to the frequency peak cue in the face of irrelevant variation in other aspects of the calls. Further, when the task is changed, forcing subjects to shift attention to the starting pitch of the calls, Japanese monkeys become less proficient in the perceptual processing task, presumably because they continue to attend to the peak cue (*11*).

The pattern of results for the Japanese macaques is strikingly similar to the findings of human speech perception studies. Human infants, for example, recognize the essential differences between two phonemic categories in the face of irrelevant variation in other acoustic dimensions (*5*). Furthermore, infants are more efficient in forming acoustic categories based on phonemic contrasts than those based on pitch differences (*6*). These infant listeners thus seem to find some acoustic dimensions more salient than others in the processing of speech. In this sense we suggest that there are parallels between the cognitive strategies used by human and nonhuman primates in processing their own vocalizations. We have no ontogenetic information about the genesis of the special predispositions that Japanese macaques show in vocal perception, other than that subjects are wild-caught mature individuals raised in normal social groups, presumably with ample opportunity for experiencing natural usage of coo calls (*12*).

Another gap in our knowledge concerns the vocal usage of the other species. Vervet monkeys do not use coo calls (*13*). The other two macaque species' repertoires contain, as do all macaques, tonal clear calls or coo vocalizations with characteristics like those of SE's and SL's (*14*). Whether their social usage indicates that frequency peak position is useful for separating coo variants functionally, as it is in *M. fuscata*, remains to be determined (*15*). In either case, the ontogeny of usage remains unknown. Conceivably, the acoustic patterns of coos and their usage are culturally determined, with the divergence of performance between Japanese and other macaques in our experiments being consequent on histories of different individual experience. Alternatively, innate predispositions or some complex of innate perceptual tendencies that influence the learning process by which adult perceptual behavior is engendered may be involved. Such influences appear to characterize the ontogeny of human speech perception and the development of learned avian vocal perception (*16*). We hypothesize that such interplay of innate and learned perceptual predispositions is also involved in the ontogeny of species-specific vocal perception in nonhuman primates.

STEPHEN R. ZOLOTH*
Rockefeller University,
Field Research Center,
Millbrook, New York 12545
MICHAEL R. PETERSEN*
Department of Psychology,
Indiana University, Bloomington 47401
MICHAEL D. BEECHER
Department of Psychology,
University of Washington,
Seattle 98195
STEVEN GREEN
Department of Biology,
University of Miami,
Coral Gables, Florida 33124
PETER MARLER
Rockefeller University,
Field Research Center
DAVID B. MOODY
WILLIAM STEBBINS
Primate Laboratory,
Kresge Hearing Research Institute,
University of Michigan,
Ann Arbor 48109

References and Notes

1. T. Bullock, Ed., *Recognition of Complex Acoustic Signals* (Dahlem Konferenzen, Berlin, 1977), p. 1; F. Worden and R. Galambos, Eds., *Neurosci. Res. Program Bull.* **10** (1972).
2. J. Sinott, M. Beecher, D. Moody, W. Stebbins, *J. Acoust. Soc. Am.* **60**, 687 (1976); P. K. Kuhl and J. D. Miller, *Science* **190**, 69 (1975); P. Morse and C. Snowdon, *Percept. Psychophys.* **17**, 285 (1975); R. Waters and W. Wilson, *ibid.* **19**, 285 (1976); P. Kuhl and J. Miller, *J. Acoust. Soc. Am.* **63**, 905 (1978); C. Burdick and J. Miller, *ibid.* **58**, 415 (1975).
3. M. R. Petersen, M. D. Beecher, S. R. Zoloth, D. B. Moody, W. C. Stebbins, *Science* **202**, 324 (1978).
4. P. Kuhl, *Brain Behav. Evol.*, in press; D. Pisoni, *ibid.*, in press; D. Shankweiler, W. Strange, R. Verbrugge, in *Perceiving, Acting, and Comprehending: Toward an Ecological Psychology*, R. Shaw and J. Bransford, Eds. (Erlbaum, Hillsdale, N.J., 1976).
5. P. Eimas, in *Infant Perception: From Sensation to Cognition*, L. Cohen and P. Salapatek, Eds. (Academic Press, New York, 1975), p. 1; P. Kuhl and J. Miller, *J. Acoust. Soc. Am.* **58**, 56 (1975); J. Fodor, M. Garrett, S. Brill, *Percept. Psychophys.* **18**, 74 (1975); R. E. Eilers and F. D. Minifie, *J. Speech Hearing Res.* **18**, 158 (1975).
6. P. Kuhl, in *Hearing and Davis: Essays Honoring Hallowell Davis*, S. Hirsh, D. Eldridge, I. Hirsh, S. Silverman, Eds. (Washington Univ., St. Louis, 1976), p. 264.
7. S. Green, in *Primate Behavior*, vol. 4, *Developments in Field and Laboratory Research*, L. Rosenblum, Ed. (Academic Press, New York, 1975), p. 1.
8. M. Beecher, M. Petersen, S. Zoloth, D. Moody, W. Stebbins, *Brain Behav. Evol.*, in press; M. Petersen, thesis, University of Michigan (1978).
9. C. C. Wood, W. R. Goff, R. S. Day, *Science* **173**, 1248 (1971); S. Springer, *J. Exp. Psychol.* **101**, 159 (1973).
10. The criteria for adding stimuli were two successive sessions in which (i) the animal responded correctly to 90 percent of the total target stimuli, (ii) responded to at least 80 percent of the presentations of each target stimulus, and (iii) responded to no more than 10 percent of each nontarget stimulus. Since each animal received monaural stimulus presentations randomized across ears, these criteria were assayed in the "superior ear," that is, the ear that, on a given day, was most accurate in identifying target stimuli.
11. Analogous experiments with acoustic cues relevant to communication in the comparison species will be needed to determine if they exhibit perceptual specializations of their own.
12. T. Tanaka and S. Kotera, *Bull. Exp. Anim.* **22**, 471 (1973). Of the five animals used as comparison species, two were wild-caught (numbers 35 and 93, pig-tailed macaques), two were reared in seminatural social groups (numbers 88 and 133, bonnet macaques), and one was born and raised in the laboratory colony (number M58, the vervet). Variation in rearing environments of the comparison species had no systematic relationship to performance in either discrimination task that we could detect.
13. T. Struhsaker, in *Social Communication Among Primates*, S. Altmann, Ed. (Univ. of Chicago Press, Chicago, 1967).
14. R. Grimm, *J. Zool.* **152**, 361 (1967); T. Rowell, *Symp. Zool. Soc. London* **8**, 91 (1962); S. Green, *Z. Tierpsychol.* **38**, 304 (1975); M. Bertrand, *Bibl. Primatol.* **11** (1969).
15. For evidence that *M. arctoides* apparently utilizes a similar acoustic dimension, see R. Lillehei and C. Snowdon, *Behaviour* **65**, 270 (1978).
16. M. Bornstein, in *Psychological Development from Infancy*, M. Bornstein and W. Kessen, Eds. (Earlbaum, Hillside, N.J., 1978); P. Marler, in *The Nature of Life*, W. H. Heidcamp, Ed. (University Park Press, Baltimore, 1978).
17. These studies are a product of a unique collaboration between field biologists (from Rockefeller University) and experimental psychologists (from the University of Michigan) interested in characterizing the analytic mechanisms used in the processing of animal communication sounds. Special thanks to A. Liberman for many thought-provoking discussions, technical advice, and for making the facilities at Haskins Laboratories available. G. Kuhn and T. Halwes of Haskins provided expert advice concerning stimulus analysis and tape preparation. Supported by NSF grants BNS 77-19254 to M.D.B., W.C.S., and D.B.M. and BNS 75-19431 to P.M.; PHS grants MH 31386-01 to S.R.Z. and MH 24269 to S.G.; PHS training grant GM 1789-08 to Rockefeller University; NIH program project grant to Kresge Hearing Research Institute; National Institute of Child Health and Human Development grant NO1-HD-1-2420 to Haskins Laboratories; and PHS predoctoral fellowship MH 05993, a Sigma Xi grant-in-aid-of-research, and an H. H. Rackham dissertation grant to M.R.P.

* Send requests for reprints to S.R.Z. or M.R.P.

22 February 1979

Fluorescent Retrograde Double Labeling: Axonal Branching in the Ascending Raphe and Nigral Projections

Abstract. *Red fluorescent Evans blue and blue fluorescent DAPI-primuline were injected into the anterior-medial and lateral-caudal forebrains, respectively, of the same rats. Separate clusters of cells labeled by retrograde transport were observed in the substantia nigra, while in the dorsal raphe many cells were double-labeled. Thus, single raphe cells send divergent axon collaterals to widespread forebrain areas.*

Although monoamine cell groups make up only a small percentage of all brain stem neurons, they appear to account for a large portion of those projecting directly to the forebrain (*1*). The question arises whether single monoaminergic brain-stem cells each innervate a restricted forebrain area or instead give off multiple axon collaterals to various parts of the forebrain. We now report that single raphe cells send collaterals to widespread forebrain areas, whereas single substantia nigra (SN) cells have comparatively small projection sites.

Until recently, no simple, effective anatomical techniques existed for studying axonal branching (*2*). Although several retrograde double-labeling procedures for the tracing of axon collaterals have been described (*3*), they include autoradiographic procedures and are, therefore, time consuming. The demonstration that a variety of fluorescent substances are transported retrogradely in axons (*4, 5*) opened the possibility of investigating axonal branching by means of a simple retrograde double-labeling procedure. Thus, two substances (fluorescing different colors) may be injected into the two different axon termination sites of a group of neurons. If the two sites are supplied by collaterals of the same axons, both substances may be seen within the individual neuronal cell bodies after retrograde axonal transport (*2*).

In the first group of experiments, nine adult rats were each injected with Evans blue (EB), which fluoresces red, and a mixture of 4′,6-diamidino-2-phenylindol 2HCl (DAPI) and primuline, which fluoresces blue with gold granules (*2, 4*). A total of 0.5 μl of 10 percent EB (weight/volume) and 1 percent poly-L-ornithine (*2, 6*) in distilled water was injected in five needle penetrations into a large anterior-medial forebrain area comprising the frontal cortex, olfactory tubercle, and very anterior portions of the nucleus accumbens and caudate-putamen. On the same side of the brain (Fig. 1), a similar quantity of 2.5 percent DAPI:10 percent primuline (weight/volume) in distilled water was injected in the course of three needle penetrations into a large lateral-caudal forebrain area comprising the lateral parietal and temporal cortex, the amygdala, the caudal end of the caudate-putamen, and the lateral edge of the internal capsule. The structures involved in both the anterior-medial and lateral-caudal injections are known to receive projections from both the SN (*7, 8*) and the dorsal raphe (DR) nucleus (*9, 10*).

After surviving 3 to 5 days (*4*), the animals were anesthetized with Nembutal and perfused with saline followed by 10 percent formalin. The brains were kept overnight in cacodylate buffer (pH 7.2) containing 30 percent sucrose (weight/volume) and were then cut transversely into 30-μm-thick frozen sections, which were mounted on slides from distilled water and air dried. A fluorescence microscope (Leitz Ploempack) was used to examine the sections (*4*). Filters that provided 550-nm and 360-nm excitation lights were used to examine the red-fluorescing cells containing EB and the blue-fluorescing cells containing DAPI-primuline, respectively.

Labeled cells in the brain stem were observed primarily in substantia nigra pars compacta (SNC), ventral tegmental area (VTA), DR, and median raphe (MR) (Fig. 1). A small number of labeled cells was seen scattered in the tegmental reticular formation, but will not be discussed in detail. The DAPI-primuline-labeled cells were seen in the peripeduncular nucleus dorso-lateral to the SNC (Fig. 1) and in the medial geniculate body (not shown in Fig. 1). In addition, EB-labeled as well as DAPI-primuline-labeled cells, and indeed double-labeled cells, were sometimes seen in the locus coeruleus, but their labeling was very faint.

0036-8075/79/0525-0873$00.50/0