9. D. D. Heistad, M. L. Marcus, S. I. Said, P. M. Gross, *Am. J. Physiol.* **239**, H73 (1980).
10. S. P. Duckels and S. I. Said, *Eur. J. Pharmacol.* **78**, 371 (1982).
11. E. P. Wei, H. A. Kontos, S. I. Said, *Am. J. Physiol.* **239**, H765 (1980); J. McCulloch and L. Edvinsson, *ibid.* **238**, H449 (1980); D. A. Wilson *et al.*, *Circ. Res.* **48**, 138 (1981).
12. T. J-F. Lee, *Circ. Res.* **49**, 971 (1981); W. R. Hume and J. G. Waterson, *Blood Vessels* **15**, 348 (1978).
13. R. J. Winquist, C. Webb, D. Bohr, *Circ. Res.* **51**, 769 (1982).
14. J. Roth, M. Bendayan, L. Orci, *J. Histochem. Cytochem.* **26**, 1074 (1978).
15. ———, *ibid.* **28**, 55 (1980); J. Roth, M. Ravazzola, M. Bendayan, L. Orci, *Endocrinology* **108**, 247 (1981).
16. C. Yanaihara *et al.*, *Biomed. Res.* **1**, 449 (1980).
17. L. Probert, J. DeMey, J. M. Polak, *Nature (London)* **294**, 470 (1981).
18. O. Johansson and J. M. Lundberg, *Neuroscience* **69**, 847 (1981).
19. J. M. Lundberg, *Acta Physiol. Scand. Suppl.* **496** (1981).
20. Supported by NIH grants HL 27763 and BRSG S07RR0543, a grant-in-aid from the American Heart Association (83-1040), funds contributed in part by the American Heart Association–Illinois Affiliate, and funds from Southern Illinois University School of Medicine. We thank E. E. Daniel for allowing us to perform the protein A–gold technique in his laboratory, S. Sarwinski for her excellent technical assistance, L. Ragel for artwork, and S. Fluckiger for preparation of the manuscript.

16 November 1983; accepted 27 February 1984

Forebrain Lesions Disrupt Development But Not Maintenance of Song in Passerine Birds

Abstract. *The magnocellular nucleus of the anterior neostriatum is a forebrain nucleus of passerine birds that accumulates testosterone and makes monosynaptic connections with other telencephalic nuclei that control song production in adult birds. Lesions in the magnocellular nucleus disrupted song development in juvenile male zebra finches but did not affect maintenance of stable song patterns by adult birds. These results represent an instance in which lesions of a discrete brain region during only a restricted phase in the development of a learned behavior cause permanent impairment. Because cells of the magnocellular nucleus accumulate androgens these findings raise the possibility that this learning is mediated by hormones.*

Investigation of the neural basis of complex learned behaviors in vertebrates has been hampered by the difficulty of identifying discrete neural circuits for such behaviors: neural control of learning often seems to be broadly and diffusely represented in the brain (*1*). A salient exception to this problem is the highly localized system of hormone-sensitive central pathways that control learned vocal behavior in passerine birds (*2*). Previous investigations of this system have revealed the direct participation of discrete brain nuclei in song production by adult birds. We now report what we believe to be the first demonstration of the importance of a discrete forebrain nucleus for development of learned vocal patterns by young birds but not for production of stable song patterns by adults.

A young male zebra finch (*Poephila guttata*) learns to imitate the song of his father during a so-called "critical" or "sensitive" period of development (*3*). Song-related vocalizations are first produced at around 25 days of age; these early "subsong" vocalizations bear little resemblance to the bird's final song pattern, but vocal patterns become progressively more stereotyped between 50 and 90 days of age, and do not change thereafter. The stereotypical song patterns of adult zebra finches do not change even if birds are deprived of auditory feedback (by deafening) and of feedback from the vocal organ [by severing afferent fibers traveling from the vocal organ to the brain (*4*)]. Conversely, the song patterns of young birds are completely disrupted after deafening (*4*) and may also be affected by eliminating feedback from the vocal organ (*5*).

The system of interconnected brain nuclei that controls adult song production in passerine birds is shown in Fig. 1. Neurons in the caudal nucleus of the ventral hyperstriatum (HVc) project directly onto the robust nucleus of the archistriatum (RA). Axons of RA cells leave the telencephalon and synapse on the hypoglossal motor neurons (nXIIts) that innervate the vocal organ (syrinx). Bilateral lesions of either the HVc or the RA severely disrupt vocal behavior in adult songbirds (*2*). The magnocellular nucleus of the anterior neostriatum (MAN) projects directly onto both the HVc and the RA (*2*). Song (and possibly song learning) are androgen-dependent behaviors (*6*), and cells in the MAN, HVc, RA, and nXIIts accumulate testosterone or its metabolites (*7*).

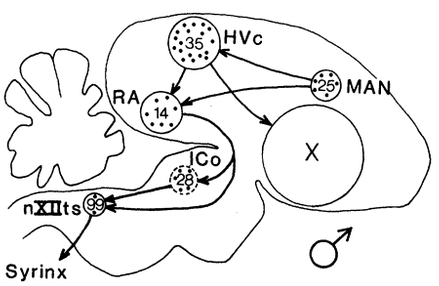

Fig. 1. Schematic drawing of the sagittal view of the neural network involved with song control in male passerine birds. Evidence for direct participation in song control has previously been reported only for telencephalic nuclei HVc and RA (*2*). Dots indicate nuclei containing androgen-accumulating cells; numbers are percentages of cells labeled within a given nucleus (*7*). Abbreviations: *HVc*, caudal nucleus of ventral hyperstriatum; *RA*, robust nucleus of archistriatum; *nXIIts*, tracheosyringeal portion of hypoglossal nucleus; MAN, magnocellular nucleus of the anterior neostriatum; *ICo*, intercollicular nucleus; *X*, area X.

The MAN has traditionally been defined as part of the song system, although nothing is known of its function. The purpose of our study was to determine the role of the MAN in the development of learned vocal behavior by juvenile male zebra finches, the production of stable song patterns by adult males, or both.

Twenty male zebra finches ranging in age from 35 to > 90 days received bilateral lesions aimed at the MAN. Birds 50 days of age and older were recorded while singing before undergoing surgery. Electrolytic lesions were produced under anesthesia (Equithesin), with monopolar stainless-steel insulated electrodes to pass anodal d-c current of 90 to 100 μA for 60 seconds. The song patterns of all birds were recorded postoperatively at approximately 2-week intervals until the birds were at least 90 days old, at which time they were killed with an overdose of anesthetic. Their brains were fixed, embedded, sectioned at 40 μm, and stained with thionin. The use of a microprojector to examine the sections allowed the exact location of the lesion to be verified. Song recordings were analyzed with a sound spectrograph (Kay Elemetrics, model 7800).

Birds with complete bilateral lesions in the MAN made when they were between 35 and 50 days old (n = 6) produced severely abnormal vocalizations until they were killed (Fig. 2). Their "songs" usually consisted of one or two highly abnormal notes, often produced at very low amplitude. Their notes lacked the frequency modulations characteristic of normal zebra finch song and were produced in extremely long bouts of singing which lacked normal phrasing. These abnormal song patterns appeared when birds were first recorded after surgery (typically 48 to 72 hours). In contrast, birds with lesions that missed all or most of the MAN (n = 5) showed normal song development (Fig. 2) (*8*). Their final song patterns consisted of short stereotyped phrases including approximately five

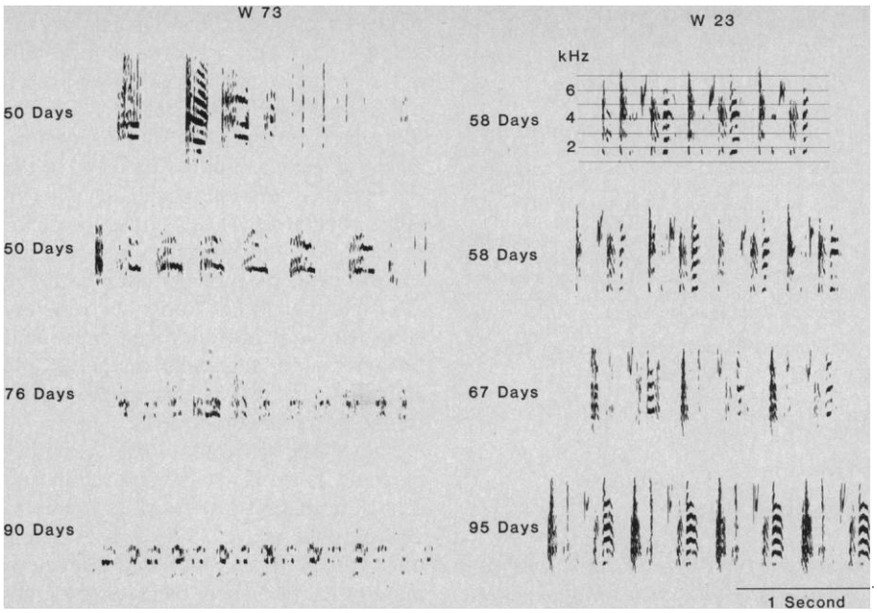

Fig. 2. Spectrographs showing abnormal song development in a bird with a complete bilateral lesion of the MAN made at 35 days (left) and normal song development in a bird with a control lesion made at 38 days (right).

notes; individual notes were highly frequency modulated, contained evenly spaced harmonics, or both.

Birds that received complete bilateral lesions in MAN between 55 and 65 days (n = 9) were disrupted in proportion to their individual song development: those in which no recognizable song pattern had yet emerged immediately produced highly abnormal vocalizations, whereas those in which a recognizable song pattern had developed showed no immediate disruption. These latter birds, however, tended to simplify their songs gradually or display slight abnormalities by the time they were 90 days old. Adult birds (≥ 90 days) with complete bilateral lesions in the MAN (n = 3) produced completely normal songs at as long as 5 weeks after surgery.

These results represent a unique instance, to our knowledge, in which lesions of a discrete brain region during only a restricted phase in the development of a learned behavior cause permanent impairment (*9*). In a broad sense, therefore, further study of this effect promises to enhance our understanding of neural mechanisms underlying learning and development (*10*).

The disruptive effect of MAN lesions can logically be attributed to one of two alternative mechanisms. One possibility is that lesions in the MAN may exert their effect by interfering with normal neural development in the HVc and RA, since such lesions deprive cells in those nuclei of a major source of afferent input. However, examination of these brains does not support this idea. For example, the volume of the RA does not vary between birds with complete MAN lesions (and abnormal songs) and those with lesions that missed all or most of MAN (*11*). Alternatively, these results may indicate that functions important for vocal learning are being carried out in the MAN. This possibility is particularly intriguing because MAN cells accumulate androgens (*7*). Song production in adult zebra finches depends on androgens (*6*). The presence or absence of gonadal steroids about the time of hatching determines sexual differentiation of the song system; Gurney (*12*) demonstrated that estrogen was effective in masculinizing the song nuclei of female zebra finch chicks. These findings raise the possibility that there are developmental changes in hormone binding in the MAN (and other nuclei) that may be related to song learning. Further experiments are needed to determine whether there are morphological or biochemical correlates of vocal learning in the MAN which are hormone dependent.

These results are also significant in that the time course of the effectiveness of lesions parallels the development of song as a motor pattern. One of the central questions concerning vocal learning has centered on how an auditory memory ("template") of song is transformed into a corresponding motor pattern. That deafening disrupts song in juvenile, but not adult, birds has been interpreted as evidence for a sensitive period for auditory-motor learning of song (*4*). The results presented here suggest that the MAN may play an important role in the formation of such an auditory-motor transcription. One possibility is that the MAN acts as a conduit for auditory information relevant to song-related feedback. However, there is no neuroanatomical evidence that the MAN receives any auditory inputs (*2*). Furthermore, emergence of a motor pattern of song in zebra finches does not signify an end to the need for auditory feedback, since deafening continues to exert a deleterious effect on song behavior up until zebra finches are approximately 85 to 90 days old (*4*). Thus, the time course of song development as a motor pattern and the effectiveness of MAN lesions appear to be different from the time course of dependence on auditory feedback. This pattern of results may indicate that functions important for motor learning of song are being carried out in the MAN during a restricted period of development (although the MAN is clearly not on the main motor output path in adulthood). Thereafter, vocal development either becomes independent of these functions, or they are somehow "transferred" to another area of the brain.

SARAH W. BOTTJER
ELIZABETH A. MIESNER
ARTHUR P. ARNOLD
Department of Psychology and Brain Research Institute, University of California, Los Angeles 90024

References and Notes

1. K. S. Lashley, in *Physiological Mechanisms in Animal Behavior* (Society for Experimental Biology, Academic Press, Cambridge, 1950), pp. 454–482.
2. F. Nottebohm, in *Prog. Psychobiol. Physiol. Psychol.* **9**, 85 (1980); ———, T. M. Stokes, C. M. Leonard, *J. Comp. Neurol.* **165**, 457 (1976); J. McCasland and M. Konishi, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7815 (1981); M. E. Gurney, *J. Neurosci.* **1**, 658 (1981); D. B. Kelley and F. Nottebohm, *J. Comp. Neurol.* **183**, 455 (1979); F. Nottebohm, D. B. Kelley, J. A. Paton, *ibid.* **207**, 344 (1982).
3. K. Immelmann, in *Bird Vocalizations*, R. A. Hinde, Ed. (Cambridge Univ. Press, Cambridge, 1969), pp. 61–74. Zebra finches that are acoustically isolated at ≥ 40 days of age are able to produce a good copy of their father's song.
4. M. Konishi, *Z. Tierpsychol.* **22**, 770 (1965); F. Nottebohm, *Ibis* **110**, 549 (1968); P. H. Price, *J. Comp. Physiol. Psychol.* **93**, 260 (1979); S. W. Bottjer and A. P. Arnold, *J. Neurosci.*, in press.
5. A. P. Arnold and S. W. Bottjer, *Soc. Neurosci. Abstr.* **9**, 537 (1983).
6. E. Prove, *J. Ornithol.* **115**, 338 (1974); A. P. Arnold, *J. Exp. Zool.* **191**, 261 (1975); *ibid.*, p. 309; F. Nottebohm, *Ibis* **111**, 386 (1969).
7. A. P. Arnold, F. Nottebohm, D. W. Pfaff, *J. Comp. Neurol.* **165**, 487 (1976); A. P. Arnold and A. Saltiel, *Science* **205**, 702 (1979).
8. Because the MAN and area X are situated close to each other, it seemed possible that lesions in the MAN might interfere with axons projecting from HVc to area X. However, neuroanatomical tracing studies do not support this idea. We have used both anterograde (silver degeneration) and retrograde (horseradish peroxidase) techniques to show that fibers leave the HVc and follow the ventricle, ultimately entering area X from its medial side without traversing the MAN. Moreover, bilateral lesion of the lamina medullaris dorsalis immediately posterior to MAN and posterodorsal to area X in a 40-day-old bird had no effect on song. However, it seems likely that area X may also play a role in song development, since its cells receive a direct projection from the HVc.
9. Similarly, McCabe *et al.* have shown that lesions in a restricted region of the chick brain—

the intermediate and medial part of the hyperstriatum ventrale—completely prevent the acquisition of a learned preference prior to imprinting, but result in only a reduced preference (impaired retention) afterward [B. J. McCabe, J. Cipolla-Neto, G. Horn, P. P. G. Bateson, *Neurosci. Lett. Suppl.* **3**, S381 (1979); *Exp. Brain Res.* **48**, 13 (1982)].

10. Our results stand in contrast to a result frequently obtained in similar studies—namely, that adult lesions disrupt a variety of behaviors more than do early (perinatal) ones [G. E. Schneider, *Neuropsychologia* **17**, 557 (1979)].
11. The volume of RA was 0.368 mm (n = 5; range, 0.28 to 0.51) and 0.436 mm (n = 5; range, 0.41 to 0.46) in birds with lesions of the MAN and control lesions, respectively (U = 5, $P > 0.10$). Of course, MAN lesions may exert subtle effects on RA or HVc neurons (such as on dendritic morphology) that gross volume measurements do not detect.
12. M. E. Gurney and M. Konishi, *Science* **208**, 1380 (1980).
13. Supported by PHS grants NS18392 to S.W.B. and NS19645 to A.P.A. and by NSF grant BNS 80-06798 to A.P.A.

1 February 1984; accepted 15 March 1984

Decreased Oxidation of Labeled Glucose by Dissociated Brain Cells in the Presence of Fetal Bovine Serum

Abstract. *The effect of serum on the rate of substrate oxidation by dissociated brain cells in vitro was examined. At a serum protein concentration of approximately 0.55 milligram per milliliter, oxidation of [6-^{14}C]glucose to $^{14}CO_2$ was decreased more than 50 percent. Oxidation of [3-^{14}C]-3-hydroxybutyrate and [U-^{14}C]glutamine was decreased much less. Serum from cows, rats, horses, and humans produced similar effects, as did serum from young and old animals and from both sexes. The effect on [6-^{14}C]glucose oxidation was proportional to serum protein concentration, and significant inhibitory activity was obtained with dialyzed serum. Heating (80°C for 10 minutes) significantly reduced the inhibitory activity. These results suggest the presence of a factor in serum that can preferentially decrease glucose oxidation. Such a factor would have profound implications for metabolic regulation in vivo and for studies of cells in vitro in which serum is included in the growth medium.*

It is an accepted practice to add serum (5 to 20 percent) to most tissue culture systems, including media used for neuronal and glial cell cultures (*1, 2*). This practice is based on the idea that serum is required to support the proliferation and survival of cells in culture (*3, 4*). However, the value of including serum in cell culture media has been questioned (*5, 6*), mainly because of the variability of the components in serum. This has led to the identification of an increasing number of "growth factors" required for the maintenance of neuronal cells in culture (*7–10*).

One rationale for using defined media is that every cell type may need a specific microenvironment for survival and growth (*6, 11*). Kaufman and Barrett (*12*) recently identified a serum fraction that supported long-term survival of dissociated rat neurons more reliably than unfractionated serum and suggested that some serum fractions may be toxic to nerve cells in culture. The implication is that a variety of factors in serum may affect several different aspects of the cell. Most investigators have described the effects of serum or isolated factors in terms of cell viability, proliferation, or survival; relatively few have studied the effects of serum on substrate oxidation (*13*). We (*14–16*) and others (*17–19*) have investigated the metabolic characteristics of brain cells in culture. Most of the studies concerned the nature of nutrients required by these cells and possible differences in the capacities of various cell types to use different substrates. Since serum produces a variety of effects on the proliferation and survival of cells in culture, we attempted to examine the effect of serum on the rate of substrate oxidation. We report that the addition of serum causes a much greater decrease in the oxidation of labeled glucose by dissociated brain cells than in the oxidation of other substrates.

Dissociated brain cells were prepared from adult albino Wistar rats (200 to 250 g) (*14–16*). After rapid removal of the brain, the tissue was immersed in 0.9 percent NaCl, cut into small pieces, incubated with 0.2 percent trypsin, washed three times, and further dissociated by gentle titration. The suspension was centrifuged at 600 rev/min for 5 minutes and the supernatant was removed. Cells were resuspended in 0.9 percent NaCl and filtered through Nitex. Trypan blue exclusion and lactate dehydrogenase leakage revealed that cell viability exceeded 90 percent.

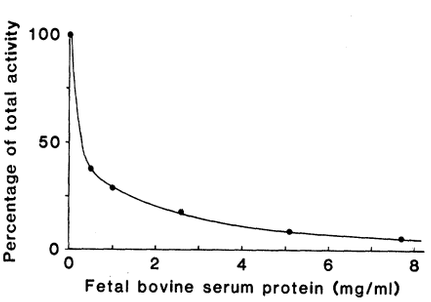

Fig. 1. The effect of increasing fetal bovine serum protein concentrations on the rate of [6-^{14}C]glucose oxidation by dissociated brain cells. Oxidation rates are expressed as percentages of the control value (2.12 nmole/hour per milligram of protein). Each point represents the average of triplicate determinations with variations less than 10 percent. The specific activity of [6-^{14}C]glucose was 296,000 dpm/μmole.

Measurement of ^{14}C-labeled substrate oxidation by dissociated brain cells was performed as previously described (*14*). In these experiments $^{14}CO_2$ produced was trapped on Hyamine hydroxide–saturated filter paper in hanging center wells and counted in a liquid scintillation spectrometer. Previous studies revealed that the rate of $^{14}CO_2$ production is linear for up to 2 hours and is proportional to protein concentration. Cell protein concentrations were maintained between 0.75 and 1.5 mg per sample. The specific activity of [6-^{14}C]glucose was 0.1 μCi/μmole; similar specific activities were used for other labeled substrates.

Addition of 10 percent rat serum greatly reduced the rate of $^{14}CO_2$ production from [6-^{14}C]glucose (Table 1). Similar results were obtained with horse, human, and bovine serum. The inhibitory activity was not affected by freezing for up to 3 weeks and appeared to be stable at 4°C for more than 72 hours. The activity was present in a commercial preparation of dialyzed fetal calf serum and in rat serum samples that had been dialyzed for 18 hours, although the amount of inhibitory activity was considerably reduced in these preparations.

Figure 1 shows the inhibitory effect of fetal bovine serum on [6-^{14}C]glucose oxidation to $^{14}CO_2$ by dissociated brain cells as a function of serum protein concentration. At a relatively low serum protein concentration (0.5 mg/ml), the rate of oxidation was decreased more than 50 percent. With increasing concentrations of serum, the rate decreased exponentially. A considerable amount of the activity was lost when fetal bovine serum was heated (80°C for 10 minutes).

Figure 2 shows the effects of rat serum on the oxidation of several different substrates. Serum had its most pronounced effects on [6-^{14}C]glucose oxidation, decreasing the rate > 75 percent at a serum protein concentration of 2.6 mg/ml. In contrast, an equivalent amount of serum caused only a 35 percent decrease in [U-^{14}C]glutamine oxidation and a < 20 percent decrease in [3-^{14}C]-3-hydroxybutyrate oxidation. Increasing the serum concentration fourfold resulted in a relatively small increment in the inhibition of the latter two substrates. However, the rate of [6-^{14}C]glucose oxidation was decreased to < 10 percent of the control value.