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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.

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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 (*p*H 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.

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The major finding is the difference between the number of double-labeled neurons seen in the raphe nuclei and in the SN (Fig. 1). At some levels of the DR, as many as half of the labeled cells were double-labeled with EB and DAPI-primuline. A smaller number of labeled cells, and consequently of double-labeled cells, was seen in the MR. At least a portion of these double-labeled DR and MR cells are presumably members of the serotonergic cell groups that project diffusely to the forebrain (*11*). On the other hand, in the SNC and the medially adjoining VTA, the populations of EB-labeled cells and DAPI-primuline–labeled cells were almost completely separate (Fig. 1). The DAPI-primuline–labeled cells often appeared in small clusters, alternating to a certain degree with groups of EB-labeled cells, across the medio-lateral extent of the SNC-VTA. In some but not all cases, a very small number of double-labeled cells could be seen. In all nine rats, the difference in double labeling between the SNC-VTA and the DR and MR, as well as the basic overall pattern of labeling, were the same.

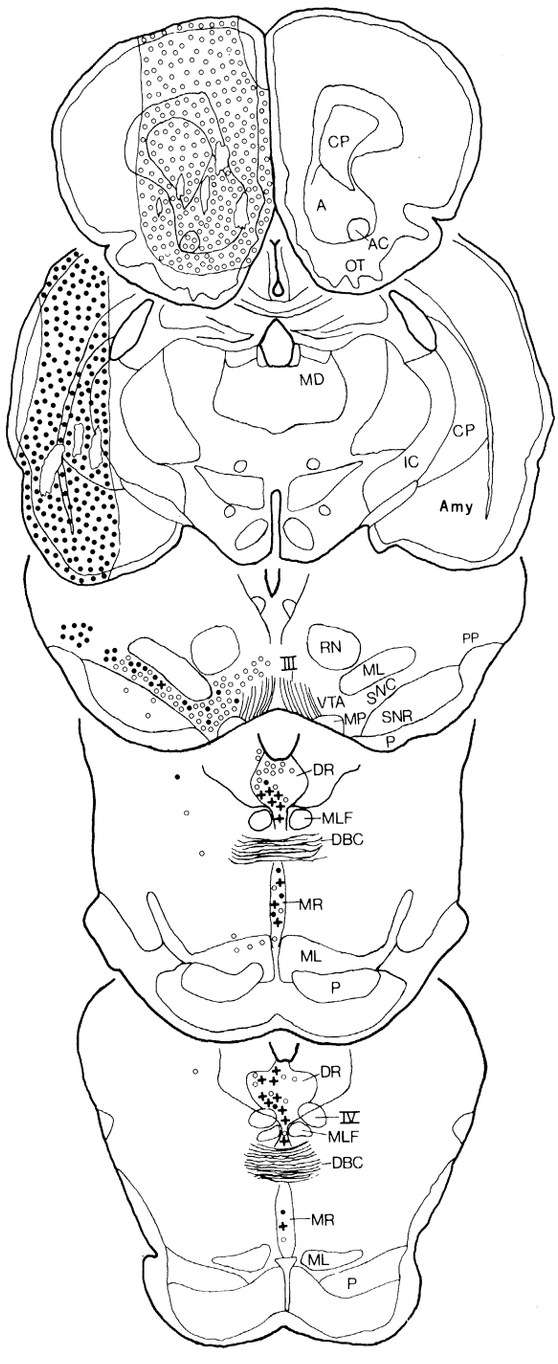

Fig. 1. Schematic distribution of brain stem cells with retrograde labeling by only EB (○), only DAPI-primuline (●), or with both EB and DAPI-primuline (+). The EB was injected into the anterior-medial forebrain (top section) and DAPI-primuline into the lateral-caudal forebrain (second section from the top). Abbreviations: *A*, accumbens; *AC*, anterior commissure; *Amy*, amygdala; *CP*, caudate-putamen; *DBC*, decussation of the brachium conjunctivum; *DR*, dorsal raphe; *IC*, internal capsule; *MD*, medio-dorsal thalamic nucleus; *ML*, medial lemniscus; *MLF*, medial longitudinal fasciculus; *MP*, mammillary peduncle; *MR*, median raphe; *OT*, olfactory tubercle; *P*, cerebral peduncle; *PP*, peripeduncular nucleus; *RN*, red nucleus; *SNC*, substantia nigra pars compacta; *SNR*, substantia nigra pars reticulata; *VTA*, ventral tegmental area; *III*, roots of the cranial nerve III; *IV*, trochlear nucleus.

In a second experimental group of five rats, EB was injected into the anterior-medial forebrain as described above; the lateral-caudal forebrain injections of DAPI-primuline were moved further caudal to avoid infringement upon the internal capsule. In these animals, the DAPI-primuline injections involved the ventral hippocampus, lateral parietal and temporal cortex, and parts of the entorhinal cortex. Despite this change in the injection site, many double-labeled cells were again present in the DR and MR. Only a very few DAPI-primuline cells were seen in the SNC-VTA, however, as might be expected with these more caudal DAPI-primuline injections (*8*, *12*). Although the injections in the first and second groups were large in order to maximize the probability of revealing double-labeled cells in the brain stem, double-labeled DR cells were also seen after considerably smaller injections of EB restricted to the caudate-putamen and of DAPI-primuline restricted primarily to the amygdala.

It has been suggested that some serotonergic fibers from the DR (*13*) terminate supra-ependymally in the cerebral ventricles (*14*). Therefore, in a control experiment, large injections of DAPI-primuline and EB were made directly into the ventricles at the level of the anterior thalamus, with some spread into the surrounding periventricular tissue occurring. These injections resulted in a faint labeling of only an extremely small number of cells in the caudal DR. Even this restricted labeling could conceivably arise from uptake by and transport from terminals in the periventricular tissue as much as from the supra-ependymal terminals. Thus, the serotonergic innervation of the cerebral ventricles (*14*) from the DR (*13*) appears to play little role in the results described above.

Our findings emphasize the widespread collateralization of the ascending raphe projections and the lack of such collateralization in the nigral projections. Nevertheless, some topographical organization can be seen in both brainstem cell groups with respect to the two injections shown in Fig. 1.

In the DR nucleus, moving from rostral to caudal, first only cells labeled with EB (from the anterior-medial forebrain injection) were seen. These cells appeared densely in the two portions of the DR nucleus: the ventromedial fountain (between the two medial longitudinal fasciculi) and the dorsomedial cluster (just beneath the aqueduct). More caudally, single cells double-labeled with both EB and DAPI-primuline (from the lateral-

caudal forebrain injection) appeared, first in the ventromedial fountain and then more caudally again in the dorsomedial cluster as well (Fig. 1). In the caudal end of the DR nucleus and further caudal in the B6 area (*15*), however, only DAPI-primuline labeled cells were seen (*9*).

In the lateral SNC at rostral levels (Fig. 1), separate groups of similar size of DAPI-primuline and EB-labeled cells were seen. In the medial SNC and VTA at these rostral levels, DAPI-primuline-labeled cells constituted small clusters surrounded by large numbers of EB-labeled cells. More caudally the distribution of EB-labeled cells remained roughly the same, but the DAPI-primuline-labeled cell clusters vanished laterally and thus became restricted to the medial SNC and VTA. At the caudal end of the SN, only EB-labeled neurons were present.

Previous reports have demonstrated that the groups of SNC-VTA cells projecting to different forebrain areas overlap (*8, 16, 17*), but also in several instances that SNC-VTA cells projecting to one forebrain area are different from those projecting to another (*8, 17, 18*). With a fluorescent retrograde double-labeling technique, we have demonstrated within individual animals, that the SNC-VTA cells innervating the lateral-caudal forebrain form interlocking cell clusters with those innervating the anterior-medial forebrain. This should be considered in light of a report that removing the striatum produces retrograde cell changes in clusters of SN cells interspersed with clusters of normal cells (*19*).

Different afferent inputs to certain brain regions may form mosaics of segregated terminal zones, for example, the ocular dominance columns in the visual cortex (*20*) and also the various cortical (*21*) and thalamic (*22*) terminal clusters in the monkey striatum. This is obviously reminiscent of the alternating efferent cell clusters in the SNC-VTA demonstrated in the present study. The interdigitation of cell clusters projecting to the anterior-medial versus lateral-caudal forebrain may allow both these forebrain areas to partake of information received from fiber systems terminating in different medio-lateral portions of the SNC-VTA.

The fluorescent retrograde double-labeling technique has also made it possible to demonstrate that, in contrast to cells of the SNC-VTA, a number of single raphe cells sends axon collaterals to two widely divergent forebrain areas. This result may explain why cells in one area of the DR are consistently labeled (in different animals) by horseradish peroxidase injections into different forebrain regions (*9*).

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Binocularity in Kittens Reared with Optically Induced Squint

Abstract. *The effects of conflicting visual images were studied without the confounding influences of oculomotor abnormalities: strabismus was simulated by rearing kittens with ophthalmic prisms before the eyes. After the animals had matured, the response properties of neurons in the visual cortex were studied. The proportion of binocularly excited neurons decreased; however, the extent of the ocular dominance alterations was related to the amount and direction of the prism-induced deviation.*

It has been consistently demonstrated that a nonconcomitant (paralytic) strabismus produced in kittens by surgically severing one or more of the extraocular muscles results in a severe loss of cortical binocularity (*1*). Depending on the direction of the deviation of the visual axes, a squint can also cause a loss in the spatial resolving capacity of lateral geniculate (LGN) neurons, a decrease in LGN cell size, and a functional loss in visual fields (*2*). Because fusion is usually impossible after such manipulations, it is tempting to attribute all of these alterations to the asymmetrical and conflicting visual inputs originating from the two eyes. In this respect, however, Maffei and Bisti (*3*) have recently shown that asymmetrical visual inputs to the cortex are not a necessary condition for the breakdown in binocularity that results from a surgically induced strabismus. Instead, their results indicate that asymmetrical ocular motility alone is sufficient to disrupt the binocular inputs to cortical neurons.

Since impaired ocular motility is a necessary consequence of surgically induced strabismus, it is not possible to evaluate completely the effects of conflicting visual inputs in such animals. Conflicting visual inputs can be produced without necessarily inducing oculomotor abnormalities by depriving one eye of form vision (for example, by unilateral occlusion). With this procedure, the deprived eye is put at a competitive disadvantage, and, as a result, the neurons in the visual cortex of the developing kitten become almost totally dominated by the normal eye (*4*). Therefore, with this type of manipulation, it is not possible to isolate the effects of conflicting visual inputs without one eye's being given a definite competitive advantage. Even when the occlusion is alternated

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