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Chemospecificity of ontogenetic units in the striatum: Demonstration by combining [^{3}H]thymidine neuronography and histochemical staining

(brain development/neurogenesis/acetylcholinesterase/basal ganglia)

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ABSTRACT **Neurons being generated in the striatum of 10 fetal cats were pulse labeled by injection of [^{3}H]thymidine directly into the maternal uterus at times ranging between the 22nd and 30th days (E22–E30) of the 65-day gestational period. Many of the striatal neurons labeled during this interval were found, at adolescence, to form 100- to 600-μm-wide cell clusters in the caudate nucleus. In E24–E30 specimens, we compared the distributions of these cell clusters with the locations of patches of low acetylcholinesterase activity and high enkephalin immunoreactivity (the "striosomes") visualized in serially adjoining sections. We found precise matches between most of the cell clusters and the acetylcholinesterase-poor enkephalin-rich zones, regardless of the embryonic age at which exposure to the [^{3}H]thymidine had occurred. We conclude that the histochemically distinct striosomal patchworks observed in the acetylcholinesterase and enkephalin preparations correspond to ontogenetic units of the striatum.**

Anatomical studies in the past 10 years have established the existence of histochemically distinct units in the mammalian striatum but it is perplexing how the units visualized in the developing striatum relate to those of the adult. On the one hand, processes containing dopamine and acetylcholinesterase appear in patches in cross sections through the fetal striatum but are broadly or at least more homogeneously distributed in the adult (1–6). On the other hand, a striking histochemical patterning remains in the adult striatum in the form of patches of low acetylcholinesterase activity called "striosomes" (7, 8), and these have been shown to correspond to regions of high opiate peptide immunoreactivity (9, 10) and opiate receptor binding (11) and also to be correlated with mosaic patterns of both afferent (11–13) and efferent (14) connections of the striatum. It is important to learn how the patches of the fetal striatum relate to the striosomal patches seen in the adult and to determine whether the patterns are related mainly to intrinsic or to extrinsic influences acting on striatal tissue during development. There is already evidence from [^{3}H]thymidine studies that one form of compartmentalization of the mature striatum may derive at least in part from the grouping together of neurons generated at similar times during development: neurons made radioactive by injection of [^{3}H]thymidine during fetal life have been found in clusters in the mature striatum in both mouse (15) and monkey (16). In the present study on the cat, we attempted to determine the relationship between such ontogenically defined cell clusters in the striatum and the striosomal compartments defined on the basis of their histochemistry.

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MATERIALS AND METHODS

[^{3}H]Thymidine [New England Nuclear; 250 μCi (1 Ci = 3.7 $\times$ 10^{10} becquerels) in 0.25 ml of sterile water] was injected directly into each embryonic chamber of the uterus in nine pregnant cats mated 22–30 days before surgery (17). Cats from the injected litters were born at gestational ages of 65–68 days and were perfused when 2–6 months old with 10% formol/saline or 4% paraformaldehyde/0.1 M phosphate buffer/0.9% saline. The group studied included single animals exposed at embryonic days E22, E23, E24, and E27 and two animals exposed on each of the embryonic days E26, E29, and E30. Frozen 15-μm (and occasional 30-μm) transverse sections through the head of the caudate nucleus were prepared for autoradiography (exposure times, 10–32 weeks) and serially adjoining sections were processed for acetylcholinesterase histochemistry or for [Met]enkephalin-like immunoreactivity by peroxidase–antiperoxidase immunohistochemistry using methods described in detail elsewhere (10, 13). The distributions of [^{3}H]thymidine-labeled neurons visualized in the caudate nucleus under dark-field illumination were plotted and compared with the locations of zones of low acetylcholinesterase activity or high enkephalin-like immunoreactivity charted from immediately adjoining sections.

RESULTS

In every brain studied, there were conspicuous clusters of heavily labeled neurons in the caudate nucleus and smaller numbers of labeled cells scattered outside the clusters. As reported by Brand and Rakic (16) for the primate, the labeled neurons in the clusters were mainly of medium size (the "small" neurons of the traditional large/small terminology). Many of the heavily labeled cells that lay outside the clusters were also of medium size. Some heavily labeled large neurons were seen at all embryonic ages studied but they were most conspicuous in the animals injected earliest. They often appeared singly or in association with labeled medium-sized cells but did not seem to form aggregates with one another.

The clusters of labeled medium-sized neurons varied in width from about 100 to 600 μm and had cross-sectional shapes ranging from simple circles and ellipses to more complex branched forms. Some were arranged in long streamers stretching across as much as two-thirds of the width of the caudate nucleus. The number of labeled neurons in the clusters ranged from a few to >100. Although many of the neurons in a given cluster appeared heavily labeled, there were also lightly labeled neurons present, as well as a few neurons that appeared unlabeled when viewed in the plane of the nucleus. Such clusters

most likely contained many cells that underwent their final division within the first few hours after the "pulse" injection but must also have contained some cells that continued to divide. The unlabeled neurons in the clusters could represent the product of many such divisions or neurons that were not in the proliferative pool at the time of the injection (18).

There were remarkably precise matches between these [^{3}H]thymidine-labeled cell clusters and the acetylcholinesterase-poor or enkephalin-rich zones visible in adjoining sections in corresponding parts of the caudate nucleus. This was true regardless of the age of the animal at the time of its exposure to [^{3}H]thymidine over the period E24–E30.

Examples of the correspondences between the acetylcholinesterase-poor zones and the labeled cell clusters are shown for E26 and E29 specimens in Figs. 1 and 2. The sections in Fig. 1 (E26) were taken from the caudal part of the head of the caudate nucleus, where the simple patterns in the acetylcholinesterase stain make it easy to see the correspondences. There are three densely labeled cell clusters, two having obvious enzyme-poor matches and the third (most ventral) having a match that is more difficult to see. Fig. 2 shows a more rostral level of the caudate nucleus from a brain exposed to [^{3}H]thymidine on E29. Although the labeling of cell clusters along the medial part of the caudate nucleus is not strong, there are many matches evident and, in fact, close inspection shows that every acetylcholinesterase-poor zone has a correspondent in the autoradiogram. There are also many strongly labeled neurons not aggregated in clusters. As in the E26 pair shown in Fig. 1, these tend to be most numerous along the lateral edge of the caudate nucleus.

An example of the opiate–thymidine cluster correspondence is shown in Fig. 3, in which a section from an E27 brain processed for [Met]enkephalin immunoreactivity is illustrated together with an autoradiogram of a serially adjoining section. Each cluster of labeled cells in the autoradiogram corresponds to an enkephalin-rich patch. This pair of sections also illustrates a finding common to all but the E30 brains, namely, that the medial part of the caudate nucleus was relatively weakly labeled in the thymidine sections despite the presence of enkephalin (or acetylcholinesterase) figures in this medial region.

Although the cytoarchitecture of the caudate nucleus is by no means uniform (19–21), patches such as those easily seen in the [^{3}H]thymidine, acetylcholinesterase, and enkephalin sections are not as obvious in the Nissl stain. It is therefore important to stress that hints of the thymidine labeling patterns were routinely visible in the cytoarchitecture and there were sometimes striking matches between aggregates of cells seen in the Nissl stain and thymidine autoradiography. In Fig. 4, for example, the U shape formed by the narrow strip of labeled cells can be detected in the Nissl-stained section as a thin rim of spindle-shaped cell bodies wrapped around a fairly prominent circular cluster of more darkly stained neurons. A second example, showing a prominent Nissl-stained cell cluster with thymidine and enkephalin correspondents, is marked by the arrow in Fig. 3.

DISCUSSION

The main finding of this study is that clusters of medium-sized striatal neurons generated during the interval of embryonic development from E24–E30 come to lie in regions that, in the adult, are histochemically and immunohistochemically distinct and that correspond to the acetylcholinesterase-poor opiate-rich compartments called striosomes. The match between the striosomes and the pulse-labeled cell clusters occurred in every animal for which we prepared serially adjoining sets of autoradiograms and histochemical slides.

Cellular Aggregates in the Striatum. The correspondence between the striosomes and the clusters of [^{3}H]thymidine-positive neurons firmly establishes the striosomal unit as a macroscopic compartment related to the organization of perikarya intrinsic to the striatum. Aside from its developmental implications, this is an important conclusion because both the acetylcholinesterase histochemistry originally used to demonstrate the striosomes (7, 8) and the enkephalin immunohistochemistry (9, 10) have tended to mark the neuropil in a diffuse way without adequately revealing neural cell bodies. The only direct experimental evidence for a strict relation of the striosomes to constellations of specialized striatal perikarya is our previous

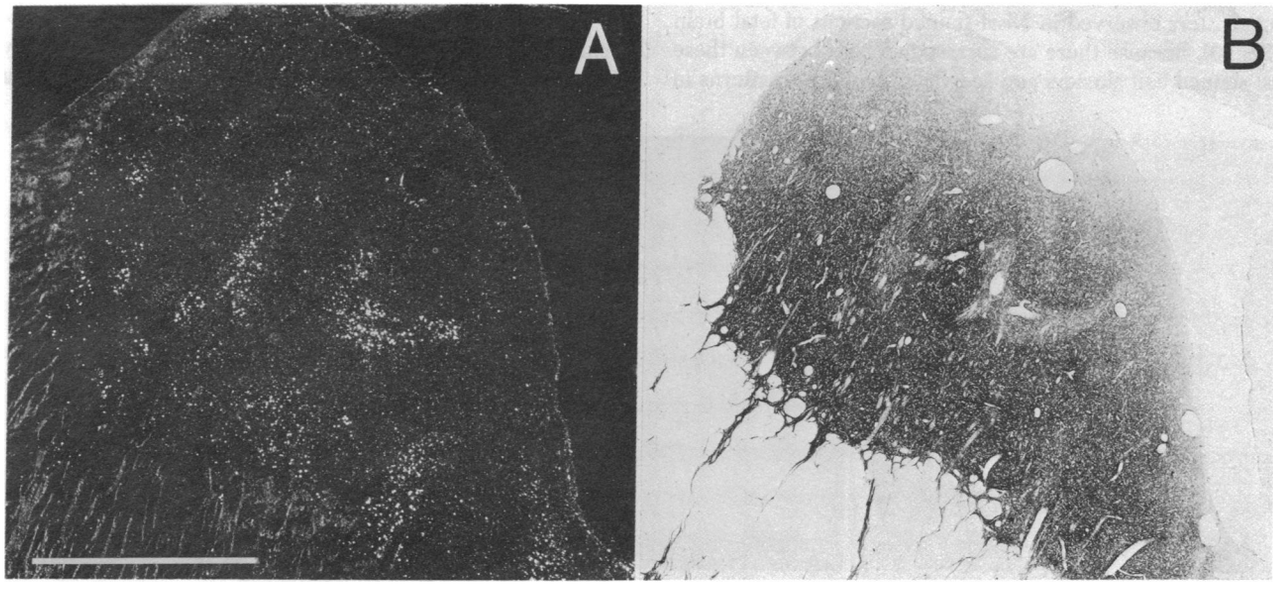

FIG. 1. Serially adjoining transverse sections through the caudate nucleus of a cat exposed to [^{3}H]thymidine at E26 showing the matching macroscopic patterns visible after autoradiography (*A*, dark-field photograph) and after acetylcholinesterase staining (*B*, light-field photograph). Bar = 2 mm.

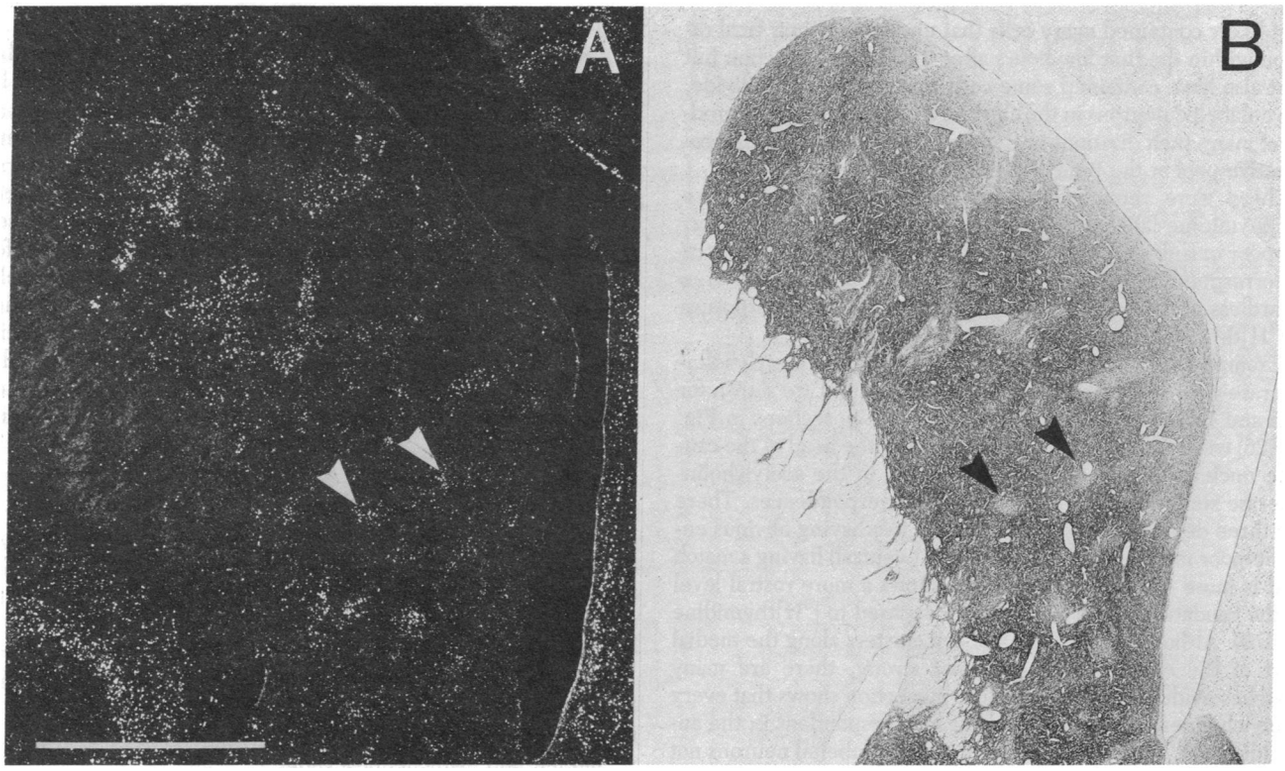

FIG. 2. Demonstration of spatial correspondence between radioactively labeled cell clusters and acetylcholinesterase-poor zones (striosomes) visible in serially adjoining transverse sections through the caudate nucleus of a cat exposed to [^{3}H]thymidine at E29. (*A*) Dark-field photograph of autoradiogram. (*B*) Light-field photograph. Note that even the small patches match ►. Bar = 2 mm.

finding that striatal cells labeled by retrograde transport after injection of horseradish peroxidase into the pallidum or substantia nigra form mosaics in which zones of least labeling are aligned with the acetylcholinesterase-poor zones visible in neighboring sections (14). By extension, the present observation that cell groupings related to the thymidine clusters can be seen in the Nissl-stained sections (Figs. 3 and 4) provides further support (cf. refs. 19–23). It will be interesting to see whether these cell clusters are related to the even more prominent clusters observed in Nissl-stained sections of fetal brain (6, 22, 23), because there are correspondences between these Nissl-stained cell clusters and acetylcholinesterase patterns in the kitten fetus (ref. 22; unpublished data) and between such Nissl-stained clusters and figures formed by corticostriatal fibers in the monkey fetus (23).

Striosomes as Ontogenetic Units. We were amazed to find that, over the entire 7-day span covered by our correlational study, the striosomes and [^{3}H]thymidine-labeled cell clusters were closely matched. First, we knew from previous work on the cat that the acetylcholinesterase-poor zones visible in single cross sections are actually parts of highly branched labyrinths running through much of the caudate nucleus (7, 10). The conclusion seemed almost inescapable that, day after day, many of the neurons born during this time came to inhabit the same

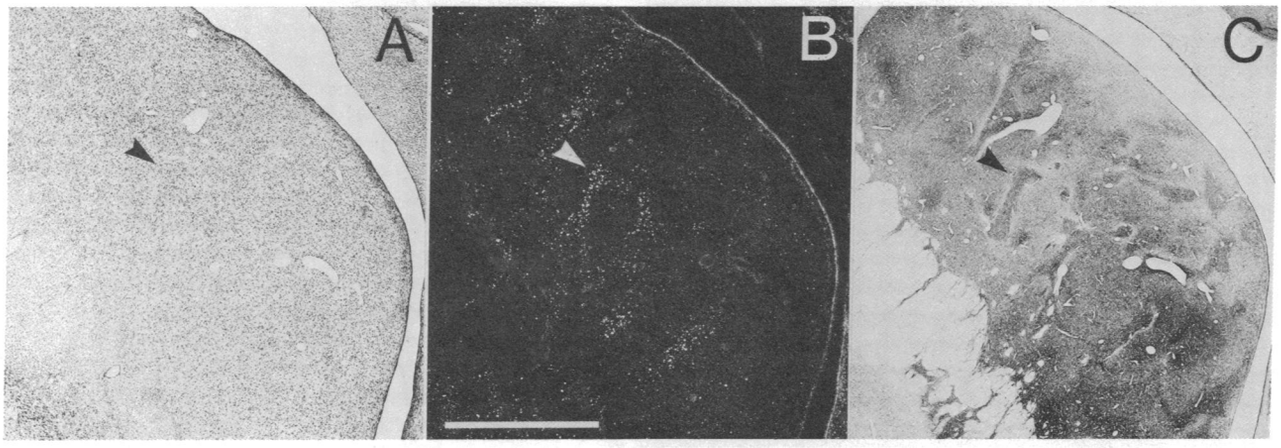

FIG. 3. Comparison of clusters of [^{3}H]thymidine-labeled neurons in the caudate nucleus of an E27 specimen (*B*, dark-field photograph) with patterns in the same section stained for Nissl substance and viewed under light-field optics (*A*) and in a serially adjoining section processed for [Met]enkephalin-like immunoreactivity (*C*). Note correspondence of patch at ►. Bar = 2 mm.

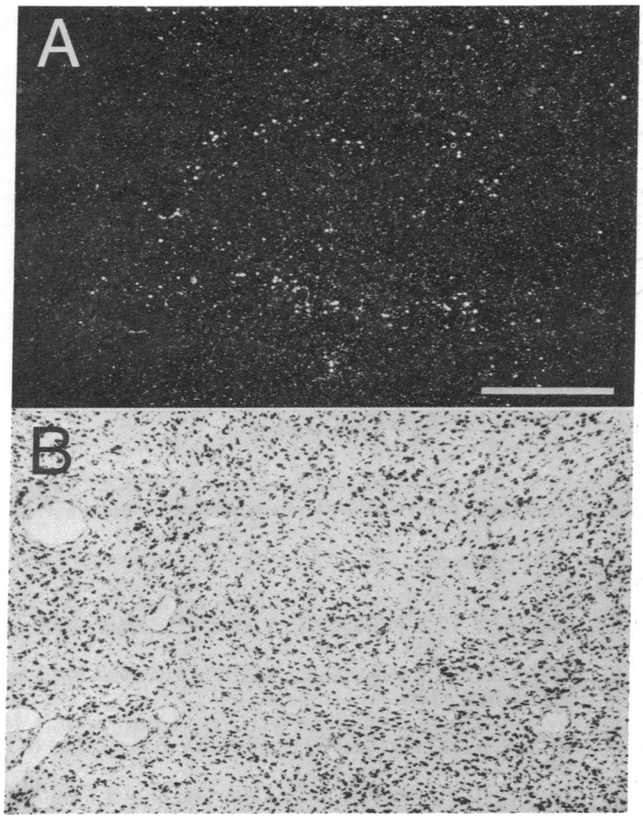

FIG. 4. Matched dark-field and light-field photographs of the same field of neurons in the caudate nucleus of an E30 specimen illustrating a horseshoe-shaped form visible in a [^{3}H]thymidine autoradiogram (*A*) and its correspondent in the Nissl stain (*B*) formed by a swirl of striatal neurons surrounding a central darkly stained cell cluster. Compare with Fig. 3. Bar = 0.5 mm.

striosomal networks and only them. This was all the more remarkable in view of the fact that the acetylcholinesterase-poor striosomes probably comprise not much more than 20% of the volume of the caudate nucleus in the cat (10).

One possibility raised by this finding is that the histochemically defined striosomes are ontogenetic units of the striatum, analogous, for example, to a single layer of the cortex (or to a subset of layers) by virtue of containing aggregations of neurons born during a particular span within a larger time window of ontogenesis (24, 25). Our sample of brains is small and we cannot yet say what the full time-window range of striatal ontogenesis is in the cat nor whether labeling of neurons outside the striosomes would be more common at earlier or at later injection times than those we used. However, Brand and Rakic (16) have found in the monkey that clustering of [^{3}H]thymidine-labeled cells occurs independently of when the exposure to [^{3}H]thymidine occurs during striatal neurogenesis. This suggests that, as we study animals injected at earlier and later times, we may be able to determine whether there are clusters of striatal neurons with different birthdays corresponding to other histochemically distinct striatal compartments (for example, certain substance P-positive zones described in ref. 10).

Crucial to an argument relating striosomes to ontogenetic units is the completeness of the matches between the striosomes and the clusters of [^{3}H]thymidine-labeled neurons. If the entire labyrinthine complex of striosomes comprised one ontogenetic unit, every acetylcholinesterase-poor zone in every section should be matched by a labeled cluster in the thymidine material. In the E24–E30 series described here, there were typically matches between most of the prominent labeled cell clusters and the striosomes visible in serially adjoining acetylcholinesterase or enkephalin sections. It was striking, however, that, in most of the [^{3}H]thymidine brains, labeled cell clusters were rare in the medial part of the caudate nucleus and, if present, tended to have weakly labeled cells. The only exceptions to this situation were in the two E30 specimens, in which medial labeling was considerably more prominent.

Possible Mechanisms of Development. The conclusion that striatal cells with common birth dates come ultimately to lie within striosomal compartments implies that, despite the apparent randomness in the distribution of cogenerated cell clusters, these clusters are in fact systematically ordered. How the cogenerated neurons come to take their final positions thus becomes a question requiring knowledge of the development of the striosomes themselves and also information about when, following their final divisions, the cells born at a given time come to aggregate in relation to the striosomes. Aside from the development of the mosaic patterns, there is the further question of how the patterns of [^{3}H]thymidine labeling we have observed can be understood in terms of the extremely sharp developmental gradients seen in fetal cat brains in the disposition of acetylcholinesterase (26, 27) and tyrosine hydroxylase immunoreactivity (28). In the caudate nucleus at E35, for example, acetylcholinesterase is restricted to a thin homogeneous band next to the internal capsule; there are hints of inhomogeneities in this lateral zone by E37 and well-formed patches are present (at least rostrally) by E39. Throughout this time and through the next decade of development, the medial part of the caudate nucleus (and the ganglionic eminence itself) is virtually devoid of acetylcholinesterase staining and, even later, there is still a razor-sharp line between the enlarging mosaic-filled lateral part and the diminishing medial unstained part of the nucleus. In both the acetylcholinesterase and tyrosine hydroxylase material, we could not help noticing the similarity between these gradient patterns—at least the tyrosine hydroxylase patterns reflecting dopaminergic afferents—and the mediolateral gradient present in many of the thymidine-labeled brains of the present series. However, no simple gradient scheme of striatal development has been observed in previous studies (15, 16) and our own series is still too limited to let us judge whether, in the cat, different mediolateral districts of the caudate nucleus would be labeled at different gestational times.

As for the striosomal mosaics themselves, Fig. 5 illustrates three distinct possibilities for how such patterns might develop. The *migratory trail* formulation (Fig. 5*A*) suggests that the striosomes visible in the adult are actually traces of the migratory trails of newly generated striatal neurons and allows for sequential onsets of migration for neurons having similar birthdays. The initial migratory trails need not be so tortuous as the highly branched striosomal labyrinths of the adult, because secondary distortions or rearrangements could be expected to occur as the brain develops. The *preferred target* scheme (Fig. 5*B*) is more compatible with the notion that neurons follow migratory routes unrelated to the striosomes but requires that the neurons terminate their migrations (or have their migrations terminated) when their paths intersect some striosomal marker. According to this formulation, there would be no need for programming sequential migrations, as a variety of timing schedules could lead to the same result so long as the striosomal marker remained, in some way, capable of triggering the cessation of migration. The notion of *selective stabilization* (ref. 29; Fig. 5*C*) suggests that the striosomal compartmentalization may be a secondary result of the preferential development of most of the neurons inside the striosomes at the expense of commonly dated neurons outside the striosomes.

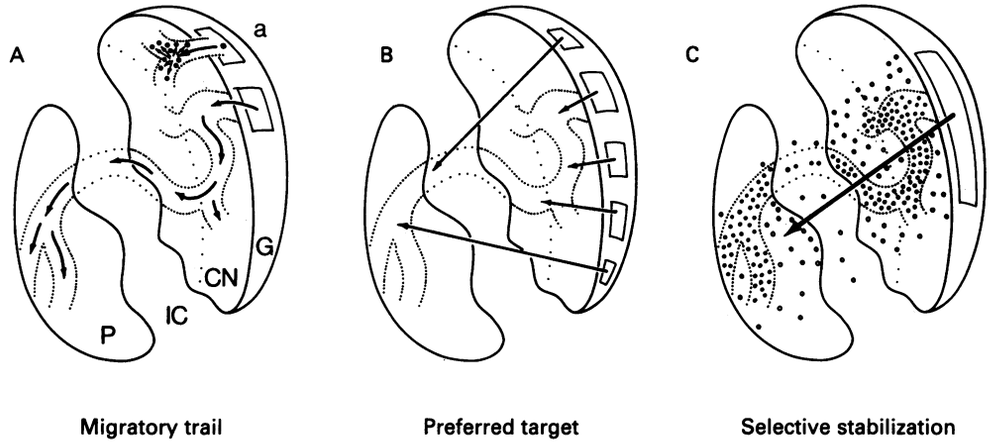

FIG. 5. Hypothetical modes of migration of striatal neurons that could account for the finding that neurons pulse labeled at E24–E30 come to lie in the striosomal labyrinths. The mechanism of clonal expansion from ganglionic eminence or from pioneer cells is indicated at point a. P, putamen; IC, internal capsule; CN, caudate nucleus; G, ganglionic eminence.

These three possibilities are formulated on the assumption that, once cells leave the ganglionic eminence, they do not undergo further division and that accordingly the striosomes are formed by properties of the cells themselves (30), including a property of mutual attraction, or by properties exogenous to the cells that collect or select them. It is important to emphasize, however, that each formulation is also compatible with the possibility (point a in Fig. 5A) that migrations are limited and that either much of the movement of neurons into the striatum is by a mechanism of clonal expansion or a small number of "pioneer" cells migrate out and seed the future striosomal matrix with cells still capable of dividing. The fact that many of the [^{3}H]thymidine cell clusters we saw contained a mixture of heavily labeled, lightly labeled, and unlabeled neurons is consistent with the idea of clonal expansion if it is assumed that not all of the original pioneer cells undergo further division. What is clearly needed now is a coordinated autoradiographic and histochemical study of fetal brains collected at closely spaced intervals following initial exposure to [^{3}H]thymidine.

Note Added in Proof. Since submission of this article, we have learned that Goldman-Rakic (31) has demonstrated clustering of neurons in Nissl-stained material of adult monkey striatum and that Brand has reported on striatal ontogenesis (32).

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1. Tennyson, V. M., Barrett, R. E., Cohen, G., Côté, L., Heikkila, R. & Mytilineou, C. (1972) *Brain Res.* **46**, 251–285.
2. Nobin, A. & Björklund, A. (1973) *Acta Physiol. Scand.* Suppl. 388, **88**, 1–40.
3. Olson, L., Boréus, L. O. & Seiger, Å. (1973) *Z. Anat. Entwicklungs gesch.* **139**, 259–282.
4. Graybiel, A. M., Pickel, V. M., Joh, T. H., Reis, D. J. & Ragsdale, C. W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5871–5875.
5. Butcher, L. L. & Hodge, G. K. (1976) *Brain Res.* **106**, 223–240.
6. Graybiel, A. M. & Ragsdale, C. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1214–1218.
7. Graybiel, A. M. & Ragsdale, C. W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5723–5726.
8. Graybiel, A. M. & Ragsdale, C. W. (1978) *Neurosci. Abstr.* **4**, 44.
9. Graybiel, A. M., Ragsdale, C. W., Yoneoka, E. S. & Elde, R. P. (1980) *Neurosci. Abstr.* **6**, 342.
10. Graybiel, A. M., Ragsdale, C. W., Yoneoka, E. S. & Elde, R. P. (1981) *Neuroscience* **6**, 377–397.
11. Herkenham, M. & Pert, C. B. (1981) *Nature (London)* **291**, 415–418.
12. Ragsdale, C. W. & Graybiel, A. M. (1979) *Neurosci. Abstr.* **4**, 78.
13. Ragsdale, C. W. & Graybiel, A. M. (1981) *Brain Res.* **208**, 259–266.
14. Graybiel, A. M., Ragsdale, C. W. & Moon Edley, S. (1979) *Exp. Brain Res.* **34**, 189–195.
15. Angevine, J. B., Jr., & McConnell, J. A. (1974) *Anat. Rec.* **178**, 300.
16. Brand, S. & Rakic, P. (1979) *Neuroscience* **4**, 767–778.
17. Hickey, T. L. & Cox, N. R. (1979) *Neurosci. Abstr.* **5**, 788.
18. Sidman, R. L. (1970) in *Contemporary Research Methods in Neuroanatomy*, eds. Nauta, W. J. H. & Ebbeson, S. O. E. (Springer, New York), pp. 252–274.
19. Papez, J. W. (1929) *Comparative Neurology* (Crowell, New York), p. 319.
20. Mensah, P. L. (1977) *Brain Res.* **137**, 53–66.
21. Mensah, P. L. (1977) *Neurosci. Abstr.* **3**, 42.
22. Graybiel, A. M. (1981) in *Cytochemical Methods in Neuroanatomy*, eds. Palay, S. & Chan-Palay, V. (Liss, New York), in press.
23. Goldman-Rakic, P. S. (1981) *J. Neurosci.* **1**, 721–735.
24. Angevine, J. B., Jr. & Sidman, R. L. (1961) *Nature (London)* **192**, 766–768.
25. Rakic, P. (1974) *Science* **183**, 425–427.
26. Graybiel, A. M. & Ragsdale, C. W. (1979) in *Development and Chemical Specificity of Neurons*, eds. Cuenod, M., Kreutzberg, G. W. & Bloom, F. E. (Elsevier, Amsterdam), pp. 239–283.
27. Ragsdale, C. W. & Graybiel, A. M. (1979) *Neuro-Sci. Lett. Suppl.* **3**, S26.
28. Graybiel, A. M., Pickel, V. M., Joh, T. H., Reis, D. J. & Elde, R. P. (1981) *Neuro-Sci. Lett. Suppl.* **7**, S476.
29. Changeux, J.-P. & Danchin, A. (1976) *Nature (London)* **264**, 705–712.
30. Singer, M., Nordlander, R. H. & Egar, M. (1979) *J. Comp. Neurol.* **185**, 1–22.
31. Goldman-Rakic, P. S. (1982) *J. Comp. Neurol.*, in press.
32. Brand, S. (1981) *Neurosci. Abstr.* **7**, 850.