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Compartmental distribution of striatal cell bodies expressing [Met]enkephalin-like immunoreactivity

(opioid peptides/striatum/basal ganglia/immunohistochemistry/neurotransmitters)

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ABSTRACT Striatal cell bodies and fibers expressing [Met]enkephalin ([Met]Enk)-like immunoreactivity were studied with two variants of the peroxidase-antiperoxidase method in normal primates and cats and in cats pretreated with colchicine. Strikingly different patterns of [Met]Enk-like immunoreactivity were observed, both in fiber and cell body immunostaining, depending on the technical protocols followed; no single histochemical protocol fully revealed the compartmentalization present. In the dorsal striatum, patches of [Met]Enk-positive neuropil, known to line up with the acetylcholinesterase-poor striatal zones called striosomes, appeared in sections treated by protocols favoring fiber immunostaining. In sections stained by procedures favoring perikaryal staining, the striosomes appeared as Enk-poor patches in a field of immunoreactive cells and neuropil. When cell-body staining was enhanced by pretreatment with colchicine, cells expressing [Met]Enk-like immunoreactivity appeared both in and out of striosomes, and the striosomal neuropil appeared Enk-rich. These results suggest that there are subtypes of Enk-positive neurons in the striatum, including a "colchicine-dependent subtype" in dorsal striosomes, and suggest that the Enk-positive striatal neuropil is also made up of different components. Immunospecificity of this dorsal striosomal system was further demonstrated by the finding that neurons expressing intense immunoreactivity to substance P and to dynorphin B were largely confined to striosomes.

There is convincing evidence from histochemical localization studies that a subset of the opioid peptides and opioid binding sites present in the caudate-putamen are concentrated in circumscribed tissue compartments called striosomes (1–5). In particular, immunostaining for [Met]enkephalin ([Met]Enk) appears in macroscopic patches or annuli embedded in regions having less immunoreactivity (1, 3); and a distinct patchiness of μ opioid binding sites has been demonstrated (4, 5). Both the [Met]Enk-positive patches (1) and the patches of heightened μ binding (5) have been shown to line up with acetylcholinesterase-poor zones visualized in serially adjoining sections. Therefore, the uneven peptide and binding-site distributions appear to reflect a common compartmental organization of the striatum, one followed also by several other neurotransmitter-related compounds and enzymes in the striatum (2, 3).

This modular striatal chemoarchitecture is of special interest because certain afferent and efferent connections of the caudate-putamen also observe a striosomal ordering (see refs. 2 and 3). Therefore, differential chemical gating of the pathways associated with the basal ganglia seems highly probable. Nonetheless, specific interpretation of the histochemical findings has been sharply limited because it has proven difficult to determine the cellular location of the ligand binding sites and immunoreactive elements making up the patch and nonpatch regions detected. For the ligand-binding material, adequate resolution is a major constraint, but for the immunohistochemistry, there are additional problems. It is often difficult to bring about simultaneously adequate staining of fibers and cell bodies (6, 7), and it is often necessary to block axonal transport with pharmacological agents such as colchicine in order to achieve maximal immunostaining of cell bodies (see ref. 7).

The study reported here was prompted by an unexpected observation made when we developed immunohistochemical protocols to favor perikaryal over fiber staining (8, 9): with the new protocols, the patches originally found to be [Met]-Enk-rich appeared to be [Met]Enk-poor. To follow up this observation we made systematic comparisons between patterns of perikaryal [Met]Enk-like immunoreactivity in the striatum and the distribution of immunoreactive fibers seen by means of protocols designed to demonstrate immunoreactivity in the neuropil. We further made preliminary tests of the effects of colchicine pretreatment on the generation of these patterns of cell-and-fiber immunostaining. Dramatic differences in the compartmental organization of striatal elements were observed under the different experimental conditions, raising general issues both for studies of the basal ganglia and for the use of immunohistochemistry to study the distribution of neuropeptides in the brain.

METHODS

Observations were made on the brains of 11 adult and adolescent (8–9.5 mo old) cats, two squirrel monkeys, and one marmoset. In 3 of the 11 adult cats, 0.25 mg of colchicine in 5 μl of 0.9% saline was injected into each lateral ventricle 24–48 hr before perfusion for a retrograde fast-blue study to be reported elsewhere. Brains were fixed by perfusion with 4% paraformaldehyde/3–5% sucrose/0.1 M sodium potassium phosphate buffer/0.9% saline (phosphate-buffered saline) or with 10% formalin/0.1 M sodium potassium phosphate buffer, pH 7.4. Frozen sections cut at 15 or 30 μm were processed by the peroxidase-antiperoxidase method (10) with protocols designed to reveal staining of striatal neuropil ("protocol A") or perikarya ("protocol B"). Series of sections were processed for [Met]Enk immunostaining and, in most cases, for substance P (SP) and dynorphin B immunostaining. Adjacent sets of sections were stained for acetylcholinesterase activity.

Protocol A, used in the initial demonstration of [Met]Enk-positive striosomes (1), included (*i*) a buffer wash, occasionally containing 0.2% Triton X-100; (*ii*) primary incubation in diluted antiserum for 1–5 days at 4°C; (*iii*) incubation in 1:50 IgG overnight at 4°C; and (*iv*) incubation in 1:50 peroxidase-antiperoxidase for 60 min at room temperature. All incuba-

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Abbreviations: [Met]Enk, [Met]enkephalin; SP, substance P.

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tion solutions contained 0.3% Triton X-100; some also contained 1% normal goat serum. Protocol B differed from protocol A in (*i*) including obligatory pretreatments with 10% MeOH/3% H_2O_2 and with Triton X-100 (usually 0.2% but occasionally 2%), (*ii*) in lacking Triton X-100 in the incubation steps but always including 1% normal goat serum and usually 1% conspecific serum, and (*iii*) in having IgG steps at 1:10 and peroxidase-antiperoxidase steps at 1:30 for 30 min at room temperature. For both protocols, each step was preceded and followed by buffer washes, usually 0.5 M Tris buffer containing 0.9% saline (Tris-buffered saline) but occasionally phosphate-buffered saline. General methods were carried out as described elsewhere (1).

Antiserum generated against [Met]Enk was provided by R. P. Elde (R153H, 1982–1984, usually diluted 1:600); antiserum against SP by R. Ho (G6, 1983–1984, diluted 1:250); and antiserum against dynorphin B by E. Weber (R2-4, diluted 1:100 to 1:500). Specificity of the immunohistochemical reactions was tested by (*i*) omitting antiserum from the primary incubation step, (*ii*) carrying out primary incubations in the presence of a saturating concentration of the respective synthetic peptide, and (*iii*) comparing the efficacy of homologous and heterologous peptides in preventing immunostaining.

RESULTS

Protocol-Dependent Patterns of Perikaryal and Fiber Immunostaining. In sections processed by protocol B for perikaryal immunoreactivity, there was pronounced heterogeneity in the distribution of medium-sized striatal neurons expressing [Met]Enk-like immunoreactivity both in the primates (Fig. 1) and in the cats (Fig. 2). Fields of intensely immunoreactive neurons appeared throughout the caudate nucleus and putamen, but these were interrupted by rounded and irregularly shaped zones in which most neurons were unstained or stained at background levels. In each species, the zones containing few immunoreactive cell bodies were typically characterized by a near-absence of immunostaining in the neuropil.

Fig. 2 contrasts such patterns of perikaryal immunostaining in the dorsal half of the cat's caudate nucleus with the distribution of Enk-rich patches of neuropil similar to those previously observed with protocol A (1). The section shown in Fig. 2*A* was stained for perikaryal [Met]Enk-like immunoreactivity by protocol B and illustrates parts of two [Met]-Enk-poor patches. The section shown in Fig. 2*C* was treated by the original protocol A. No immunoreactive cell bodies could be detected, but the striatal neuropil shows characteristic patches of [Met]Enk-positive granular reaction product lying in a background of lower immunoreactivity. Both patches of scant cell-body immunoreactivity such as that shown in Fig. 2*A* and patches of high neuropil immunoreactivity such as that shown in Fig. 2*C* lined up with acetylcholinesterase-poor striosomes seen in sections adjacent to them (see Fig. 2 *B* and *D*). These striosomal matches clearly imply that both the apparently [Met]Enk-poor patches revealed with protocol B and the apparently [Met]Enk-rich patches demonstrated with protocol A represented the same compartments. A direct neighboring-section demonstration of this coincidence is given in Fig. 2 *E* and *F*.

Immunostaining After Pretreatment with Colchicine. The patterns of immunostaining achieved with protocols A and B suggested that the striosomal patches, though innervated by a [Met]Enk-containing afferent system, might lack Enk-immunoreactive neurons. However, a second possibility was that [Met]Enk-containing cell bodies in fact were present in the striosomes (in addition to [Met]Enk-positive fibers), but that the immunoreactivity of these neurons was difficult to demonstrate compared to that of surrounding neurons. To help decide between these alternatives, we carried out preliminary experiments in which we pretreated cats with colchicine.

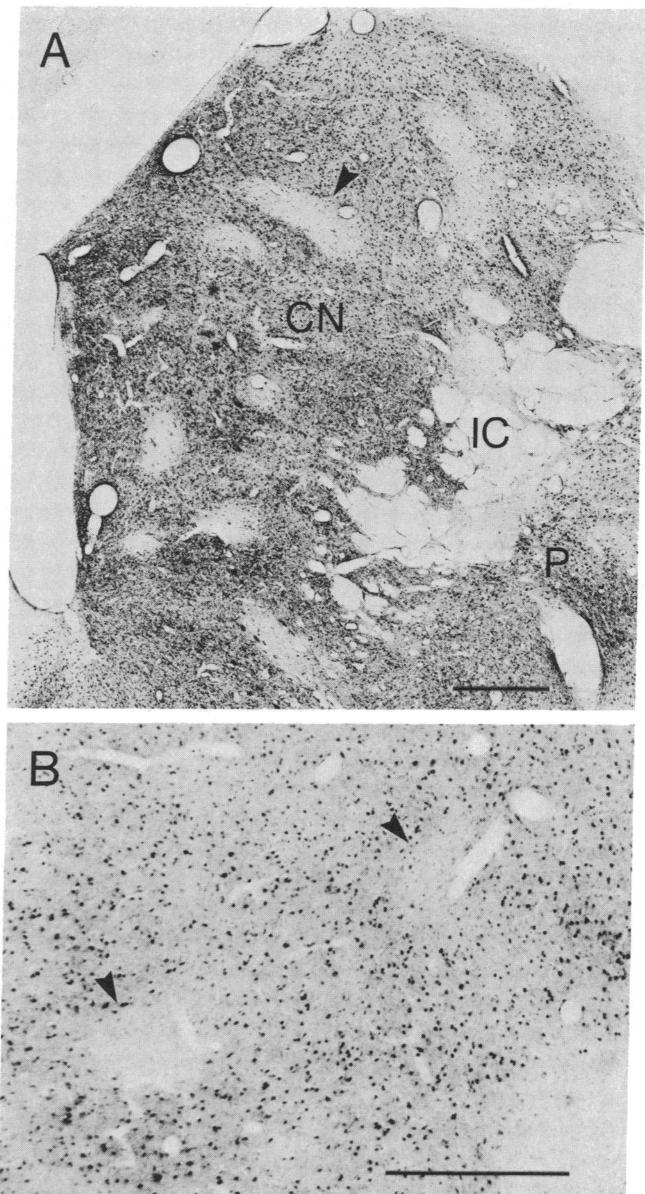

FIG. 1. Patches of low [Met]Enk immunostaining (pale zones, examples at arrowheads) detected in the striatum of marmoset (*A*) and of squirrel monkey (*B*) by protocol B favoring perikaryal [Met]-Enk-like immunoreactivity. CN, caudate nucleus; P, putamen; IC, internal capsule. (Bars in *A* and *B* = 0.5 mm.)

In protocol B sections from cats exposed to colchicine for 48 hr (Fig. 3), [Met]Enk-positive neurons appeared both inside and outside of patches of dense fiber immunostaining. In fact, without the patches of intense [Met]Enk-like immunoreactivity in the neuropil and the unreactive septa surrounding some of the patches, it would not have been possible to determine from the distribution of immunostaining where the striosomal patches lay. With 24-hr exposure, the patterns of cell-and-fiber immunostaining seemed intermediate between those observed in the untreated cats and in the cats receiving 48-hr exposures.

Topography. There were pronounced spatial gradients in the intensity of immunostaining for [Met]Enk in the striatum and also regional differences in the patch-patterns visible. These will be described elsewhere (see also refs. 1, 8 and 9),

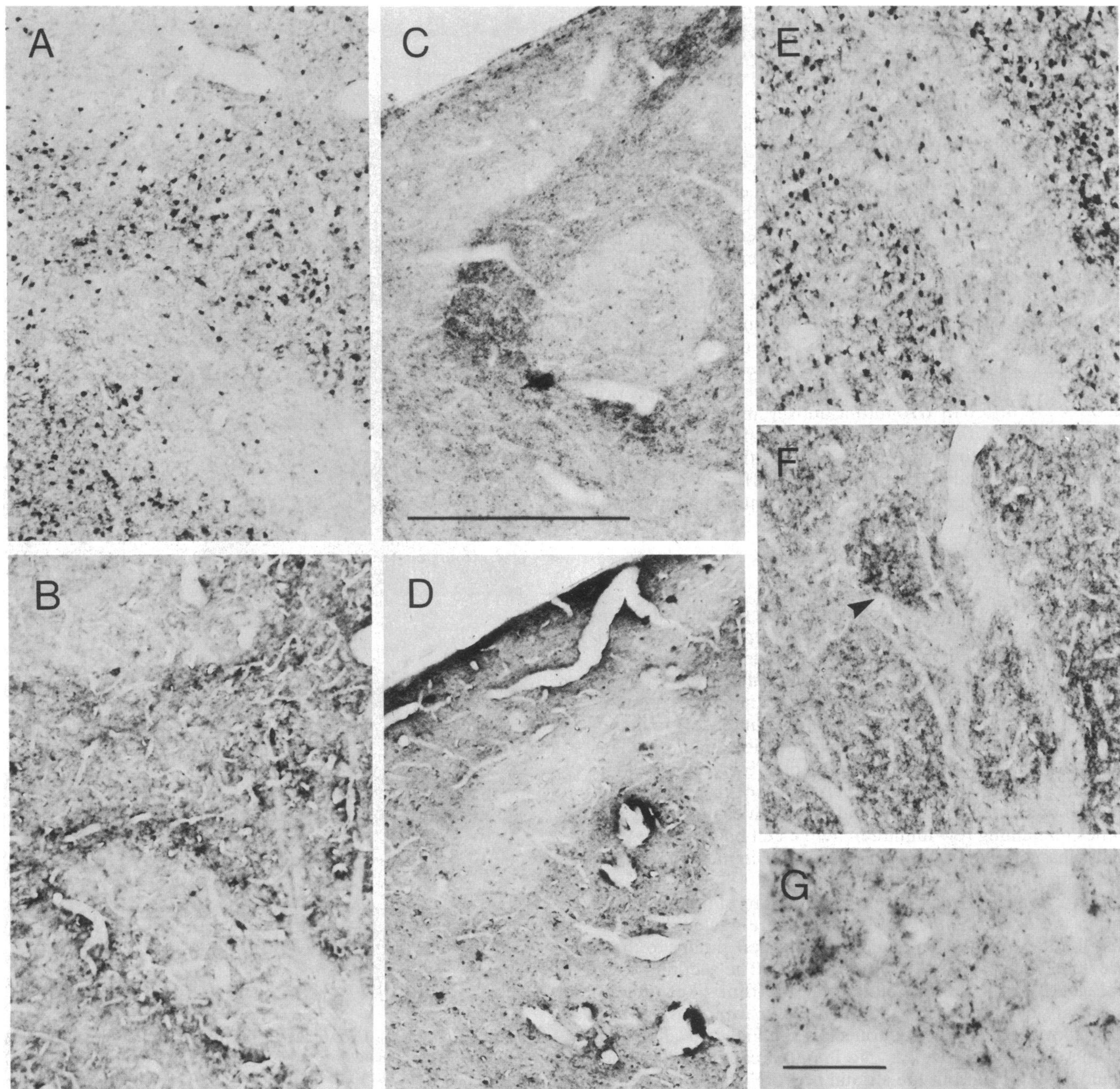

FIG. 2. Complementary striosomal patterns of [Met]Enk immunostaining produced by different immunohistochemical protocols in dorsal caudate nucleus of cat. (*A*) Patches of low cell body and neuropil [Met]Enk-like immunoreactivity in a field of immunostained cell bodies and neuropil demonstrated by use of protocol B. The Enk-poor patches match acetylcholinesterase-poor striosomes seen in the serially adjacent section (*B*). (*C*) Enk-positive patches (as originally described in ref. 1) detected by use of protocol A. These also match acetylcholinesterase-poor striosomes visualized in the serially adjoining section (*D*). (*E* and *F*) Near-serial sections showing that a single patch can appear Enk-poor with protocol B (*E*) and Enk-rich with protocol A (*F*). (*G*) View at high magnification of neuropil indicated by arrow in *F*. (Bars: *A*–*F* = 500 μm; G, 50 μm.)

but important general points include: (*i*) that [Met]Enk immunostaining was densest ventrally; (*ii*) that patches of low cell-body immunostaining were clearest in the caudate nucleus but were present also in the putamen; and (*iii*) that zones of low cell-body immunostaining (and matching immunoreactive fiber patches) were sharply defined in the dorsal part of the caudate nucleus in the cat, but that patterns were different and complex ventrally (see refs. 9 and 11). All examples illustrated for the cat are from this dorsal division.

Complementary Distributions of Perikarya Expressing Immunoreactivity to [Met]Enk and to SP and Dynorphin B. Parallel observations with protocol B in untreated cats demonstrated unequivocally that the patches of low cell body [Met]Enk-like immunoreactivity in the dorsal caudate nucleus were zones in which many neurons expressed intense SP-like immunoreactivity and dynorphin B-like immunoreactivity. Conversely, outside the striosomes, where [Met]Enk-positive neurons were readily demonstrable with protocol B, neurons expressing SP-like and dynorphin B-like immunoreactivity were rarely seen. SP-positive, dynorphin B-positive cell patches were also present in colchicine-pretreated brains. These contrasting patterns, illustrated in Fig. 4, emphasize that there is not a general difficulty in achieving perikaryal immunostaining in the dorsal striosomes.

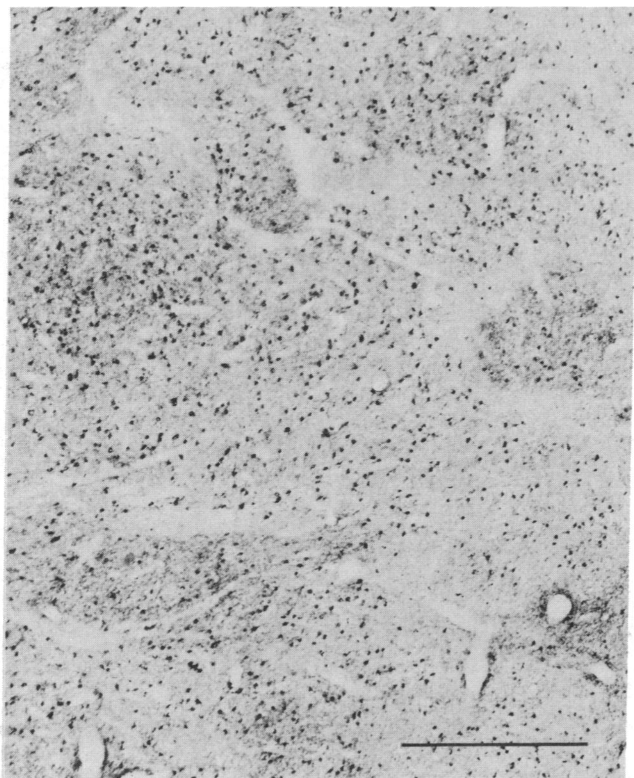

FIG. 3. Cell bodies expressing [Met]Enk-like immunoreactivity appear both inside and outside of striosomes in dorsal caudate nucleus of a cat pretreated with colchicine (0.5 mg) for 48 hr and processed by protocol B. Patches of dense neuropil staining mark striosomes, some of which are bordered by clear septa. (Bar = 0.5 mm.)

DISCUSSION

Immunohistochemistry has become a crucial link between studies of the biochemistry and the anatomy of the mammalian nervous system. The distributions of many neurotransmitters and neurotransmitter-related compounds, including neuropeptides, are now considered reproducible enough so that standard immunohistochemical reference maps are routinely constructed for the brain and spinal cord. There is growing evidence, however, at least for the hypothalamus, that the distributions of immunohistochemically observed peptides can be changed as a result of manipulating the functional activity of the peptidergic neurons in question (11, 12). It is further known, both for neuropeptides and for other substances, that different maps often must be constructed for immunoreactive nerve fibers and perikarya and, in particular, that intense perikaryal immunostaining of large numbers of neurons may require colchicine (7). The experiments described here demonstrate that, in addition to shifts in the numbers of neurons (or fibers) detected with a given antiserum, dramatic changes can be brought about in the relative distributions of immunoreactive cell bodies and immunoreactive fibers demonstrated in a given region, depending on the technical protocols followed.

The principal observations made in the brains not exposed to colchicine were that dorsal striosomes were striatal regions of dense [Met]Enk immunostaining when the elements stained were fiber processes and varicosities in the neuropil or simply a granular reaction product in neuropil (protocol A), but that these striosomes were regions of low [Met]Enk immunostaining when demonstrated with protocol B permitting immunostaining of cell bodies as well as neuropil. Both forms of immunostaining were blocked by absorption of the antiserum with synthetic [Met]Enk but not by absorption with synthetic SP or dynorphin B. The mechanisms underlying this differential detection of fiber and perikaryal [Met]-Enk-like immunoreactivity with the two protocols are not clear. The fact that the pretreatment steps seem critical confirms contrasting [Met]Enk immunostaining described by Arluison *et al.* (6) for sections from the rat striatum pretreated with H_2O_2 (for cell bodies) and Triton X-100 (for fibers and varicosities). The present findings differ, however, in that there was simultaneous immunostaining of cell bodies and neuropil with protocol B, in which both $MeOH/H_2O_2$ and Triton X-100 pretreatments were included.

Remarkably, the striatal neuropil was intensely immunoreactive in protocol A sections, but it was often nearly unstained in protocol B sections, even though the extrastriosomal neuropil (as well as extrastriosomal cell bodies) expressed [Met]Enk-like immunoreactivity. This suggests that the immunostaining of neuropil achieved with a single immunohistochemical protocol may not fully represent the immunoreactive elements present, even within a circumscribed region.

Although immunostaining of striatal cell bodies was in-

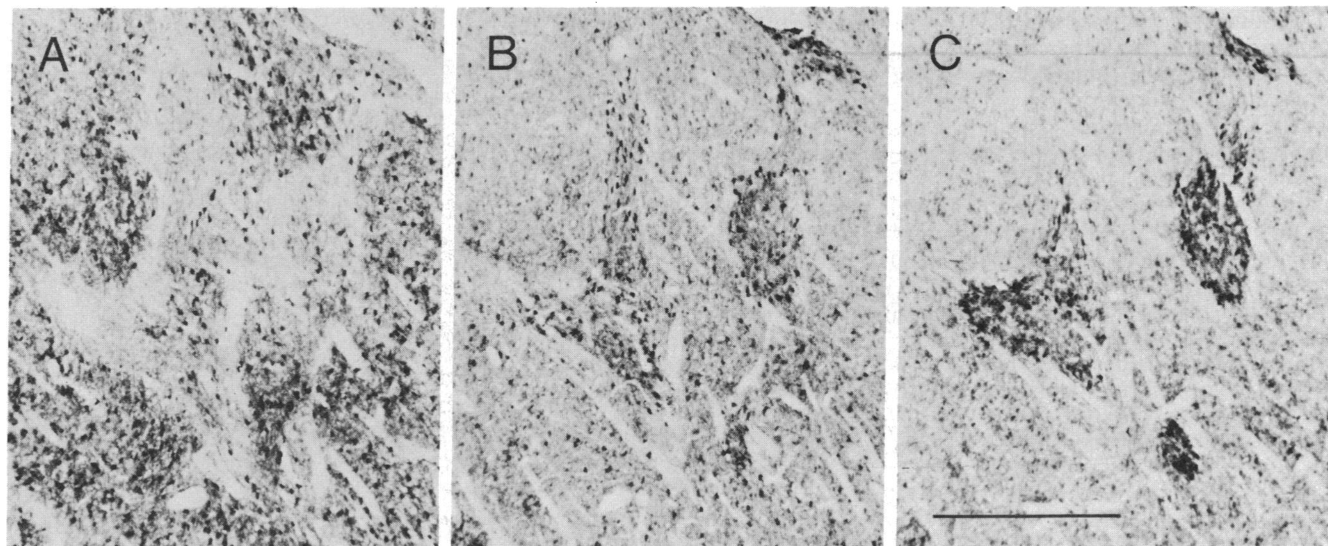

FIG. 4. Striosomal patches followed through three serially adjacent sections (*A–C*) processed consecutively by protocol B to show immunostaining for [Met]Enk (*A*), dynorphin B (*B*), and SP (*C*) in the dorsal caudate nucleus of adolescent cat processed without colchicine treatment. (Bar = 0.5 mm.)

tense in both primates and cats in sections treated by protocol B, the colchicine experiments in the cats suggest that it also was incomplete. The colchicine effect appeared to be time dependent and did not seem to result from nonspecific immunoreactivity of all striatal neurons because, in other sections from the same brains, intensely SP- and dynorphin B-positive neurons lay mainly in the dorsal striosomes, as they did in the untreated cats.

These findings strongly suggest that, even within a single local region of the brain, "[Met]Enk-positive neurons" may not consist of a single class of neurons but instead may comprise subtypes with distinct immunohistochemical properties. Specifically, the experiments suggest the existence of at least two types of [Met]Enk-positive neurons in the striatum: one, accounting for perikaryal [Met]Enk-like immunoreactivity appearing outside the striosomes; and a second, a "colchicine-dependent subtype," appearing prominently, but not necessarily exclusively, within the striosomes. No kittens have yet been exposed to colchicine, but it is interesting that low perikaryal [Met]Enk-like immunoreactivity of striosomal neurons is particularly striking in young kittens (8, 9).

It is not yet clear whether the colchicine induced qualitative or quantitative changes in immunogenicity in the colchicine-dependent neurons. The colchicine could have rendered another molecular form open to recognition by the antiserum. The peptide detected as a consequence could be unrelated to [Met]Enk except for a shared exposed antigenic site but would more likely be a precursor or a processed opioid peptide related to [Met]Enk. Noteworthy here is Williams and Dockray's report of perikaryal [Met]Enk-Arg^6-Phe^7 immunostaining in rat striatum (13).

If the antigen detected in the colchicine-dependent neurons were in fact [Met]Enk, then the change in perikaryal immunostaining could have resulted from an increase in their content of immunoreactive [Met]Enk by blockade of transport or degradation or by release of bound or otherwise unreactive [Met]Enk. An interesting possibility is that differences in the functional activity of the colchicine-dependent and extrastriosomal neurons might be responsible for their contrasting [Met]Enk immunostaining in the normal striatum as a result of differences in rates of peptide synthesis, processing, or transport (for example, proportional to their firing rates or to the lengths and branching patterns of their axons). If so, immunohistochemical methods may provide a means to gauge the activity of different subsets of neurons containing the same peptide.

The finding of subtypes of Enk-positive striatal neurons, including a colchicine-dependent subtype, is of interest for analysis of the basal ganglia, as these might have different morphological characteristics, patterns of neurotransmitter coexistence, or connections (see refs. 2 and 14–17). The fact that SP-positive and dynorphin B-positive neurons are selectively concentrated in dorsal striosomes, whereas the largest number of Enk-positive neurons lie outside these zones, is also noteworthy: Enk-like, dynorphin-like and SP-like peptides are the principle neuropeptides so far identified in the output pathways of the striatum and have nonmatching distributions in each of the main zones of termination of striatofugal fibers (18). Therefore, the modular arrangements of peptidergic neurons described here probably are related directly to the organization of biochemically specified subsets of striatal efferents (3).

The present observations also raise new questions about the origin of Enk-containing fibers in the striosomes and about the relationship between the prominent opioid receptor patches of the striatum (4, 5) and its [Met]Enk-immunoreactive elements. The opioid binding site clusters, by virtue of their alignment with acetylcholinesterase-poor striosomes (5), have been envisioned as corresponding to the Enk-rich striosomes originally demonstrated with a version of protocol A (1). This suggests a simple receptor-rich, peptide-rich match, but it is clear that the binding site clusters would be inversely related to sites of intense perikaryal and neuropil [Met]Enk-like immunoreactivity revealed by protocol B. Instances of apparent failure of matching between binding site distributions and the distributions of corresponding peptides have been reported for the striatum (19) and elsewhere and have become a general issue in localization studies. The findings reported here suggest that the concomitant use of different immunohistochemical protocols may help to resolve some of these discrepancies not only on strictly technical grounds but because, much as there are different receptor subtypes, there may be different subtypes of neurons containing a given peptide.

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