Dopamine synaptic complex with pyramidal neurons in primate cerebral cortex

(catecholamines/monoamines/cortical modulation/axospine synapses/rhesus monkey)

PATRICIA S. GOLDMAN-RAKIC*, CSABA LERANTH*†, S. MARK WILLIAMS*, NICOLE MONS‡,
AND MICHEL GEFFARD‡

*Section of Neuroanatomy and †Department of Obstetrics and Gynecology, Yale School of Medicine, New Haven, CT 06510; and ‡Institut de Biochemie
Cellular et Neurochemie du Centre National de la Recherche Scientifique, Bordeaux, France

ABSTRACT   Dopamine (DA)-containing projections to the cerebral cortex are considered to play an important role in cognitive processes. Using a recently developed monoclonal antiserum directed against DA and an antibody directed against tyrosine hydroxylase in combination with Golgi impregnation and electron microscopy, we have observed that DA and tyrosine hydroxylase afferents establish symmetric membrane specializations with the soma, dendritic shafts, and spines of identified pyramidal cells in the prefrontal, cingulate, and motor cortex of primates. The axospinous contacts invariably formed part of a synaptic complex in which the dendritic spine of a pyramidal neuron was the target of both a DA-positive symmetric *and* an unlabeled asymmetric bouton. This arrangement allows direct DA modulation of the overall excitability of cortical projection neurons by altering local spine responses to excitatory inputs.

Dopamine (DA) has been implicated in a wide range of cognitive and affective behaviors and its importance for diseases affecting thought processes, such as schizophrenia, has been repeatedly stressed (1). Although the highest brain concentrations of DA are found in the caudate nucleus, this neurotransmitter is also present in the cerebral cortex, particularly in areas like the prefrontal cortex (2, 3) that are involved in emotional and cognitive processing. In rodents (4) and nonhuman primates (5), experimental depletion of DA has, in fact, been shown to result in cognitive deficits.

A more complete understanding of DA's role in cortical function will require detailed knowledge about the cortical targets of DA afferents. The DA innervation of the primate prefrontal cortex has so far been studied only at the light microscopic level (6, 7). These studies reveal rich plexuses of DA-containing fibers in specific layers of the prefrontal cortex in monkeys (6, 7) and humans (8). However, definitive information on the postsynaptic structures innervated by these fibers is lacking. Accordingly, the goal of the present study was to provide ultrastructural data on the nature, distribution, and postsynaptic targets of dopaminergic boutons in the primate neocortex. We have used a recently available monoclonal antiserum directed against DA developed in one of our laboratories (9) as well as an antibody to its rate-limiting enzyme, tyrosine hydroxylase (TH) (10), and combined these methods with Golgi impregnation and electron microscopy (EM) to visualize DA synapses in the primate cortex.

MATERIALS AND METHODS

Five adult male rhesus monkey (*Macaca mulatta*) were deeply anesthetized and transcardially perfused using the

fixative of Van Eden *et al.* (11) for DA and that of Somogyi and Takagi (12) for TH. After perfusion, blocks of tissue were taken from the dorsal bank of the principal sulcus (Walker's area 46), the anterior cingulate gyrus (Brodmann's area 24), and the primary motor cortex (Brodmann's area 4) (see Fig. 1) and were then sectioned perpendicular to the pial surface on a vibratome or cryostat. The sections were stained with the ABC technique (13) using the Vectastain kit (Vector Laboratories). Dilutions of the primary antibodies were as follows: DA, 1:10,000 in phosphate buffer containing 0.1% sodium azide and 1% normal bovine serum; TH, 1:1500 diluted in phosphate buffer containing 0.1% sodium azide and 1% normal goat serum. Triton X-100 (0.3%) was added to the primary antibody to enhance penetration of the antibodies in sections for light microscopy, whereas a freeze-thaw technique was used for EM (14). After immunostaining, the sections for EM were postosmicated (30 min in 1% OsO_4), dehydrated in graded ethanols, and then flat embedded between aluminum foil and a coverslip. After 48 hr of curing at 56°C, the blocks were cut serially into ultrathin sections for correlated light and electron microscopic examination of the immunostained profiles. Serial ultrathin sections were obtained from six blocks of each area in each animal and were analyzed on a Philips CM10 electron microscope.

For the Golgi/EM analysis, we used the "sandwich" technique of Frotscher and Leranth (15) in which sections immunostained for TH were placed between small pieces of Parafilm and 10–12 sections were aligned, one on top of the other, to form a block. These sections were cut on a vibratome and immunostained as described above but were not freeze-thawed. The block was embedded in 5% agar, hardened, and then silver impregnated *en bloc*. The pairs of Parafilm-tissue sections were then separated and examined individually in the light microscope through a drop of 60% glycerol for detection of areas containing isolated, well-impregnated pyramidal cells. These neurons were drawn using a camera lucida, and, next, segments of dendrites that reached the surface of the section where the antibody could penetrate were cut serially at 3–4 μm.

RESULTS

Light microscopic analysis of DA-labeled vibratome sections and cryostat sections revealed staining of processes throughout the depth of the cortex in all three brain regions examined. In agreement with previous studies using a TH antiserum (6) or high-affinity uptake of [³H]DA (7), DA-immunoreactive fibers in prefrontal cortex were distributed in a bilaminar pattern across the cortical depth. They were most dense in the superficial layers (I, II, and IIIa), intermediate in concentration in the infragranular layers (V and VI), and

Abbreviations: DA, dopamine; TH, tyrosine hydroxylase; EM, electron microscopy.

lowest in layers IIIb and IV (Fig. 1). In the portions of cingulate and motor cortex examined, the superficial layers were also densely innervated but the bilaminar patterning was much less evident (not shown).

Electron microscopic study revealed numerous DA- and TH-immunoreactive boutons in the three areas examined. Without exception, they were of the symmetric type. Similar appearing DA- and TH-positive boutons were observed on cell somas, dendritic shafts, and spines (Fig. 2 *a-d*; Fig. 3 *b-d*). In addition, we discovered a triad of synaptic elements in which an immunopositive bouton and an unstained bouton invariably contacted the neck or head of the same spine (Fig. 2 *a-d*; Fig. 3*c*). The synaptic complexes formed by DA-positive boutons were indistinguishable from those formed by TH-positive boutons (compare Fig. 2 *a* and *b* with *c* and *d*). Whereas the DA- or TH-positive boutons consistently established symmetric synapses, the unstained boutons in the triadic complex always formed asymmetric membrane specializations of the

type traditionally associated with excitatory inputs—e.g., thalamo-cortical (16, 17) or cortico-cortical (16, 18) afferents. We almost never observed a DA-positive axospine synapse without encountering the unstained asymmetric member of the triadic complex in the same or nearby sections.

In order to identify the postsynaptic cell type contacted by catecholamine-containing terminals, we combined light and electron microscopic immunocytochemistry with Golgi impregnation as described. Since the high concentration of glutaraldehyde fixative required for DA immunostaining restricts the depth of antibody penetration in Golgi-impregnated material, our analysis had to be limited to TH, which is present in dopaminergic and noradrenergic terminals. However, it has been established that dopamine fibers contain a greater amount of TH than do noradrenergic fibers (19) and TH immunoreactivity remains in monkey prefrontal cortex after lesions of the noradrenergic locus coeruleus (6), indicating that TH labels predominantly dopamine fibers there.
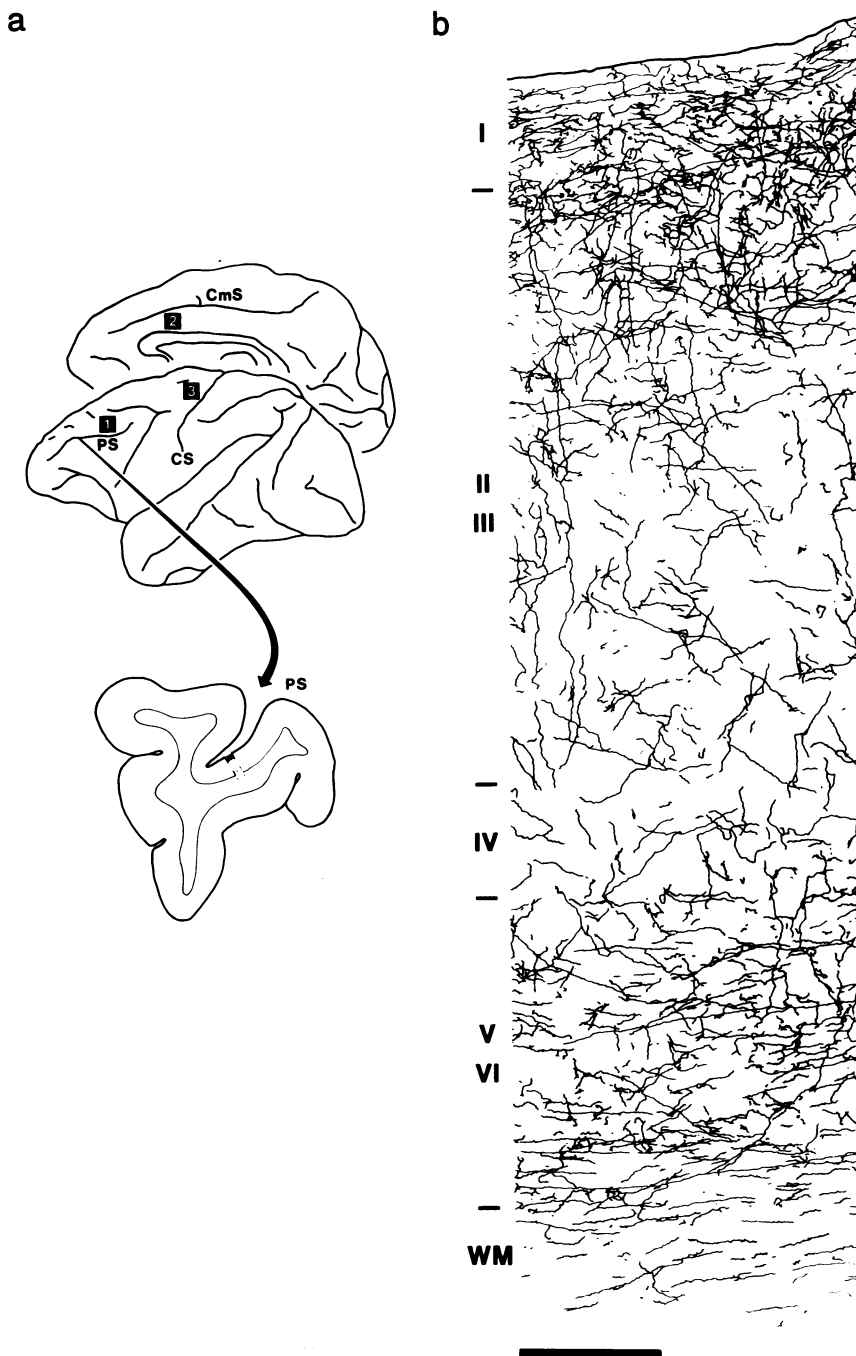
a

b

FIG. 1. (*a*) Brain diagrams of the cortex showing the approximate locations of blocks taken for light and electron microscopic analysis in this study. The coronal section through the prefontal cortex indicates the precise site of the drawing shown in *b*. (*b*) Camera lucida drawing of DA-immunopositive fibers in the principal sulcus of one rhesus monkey. Note the high density of fibers in layers I and II/IIIa, where the fibers appear to be running parallel to the cortical surface; there is a notable thinning of fiber density in the lower part of layer III and layer IV is virtually devoid of labeled fibers; in the deeper strata, the fiber density again increases and their intracortical distribution is a mixture of tangential, oblique, and vertical directions consistent with the pattern of basilar dendritic branches of their presumed targets in these layers. (Bar = 200 μm.)
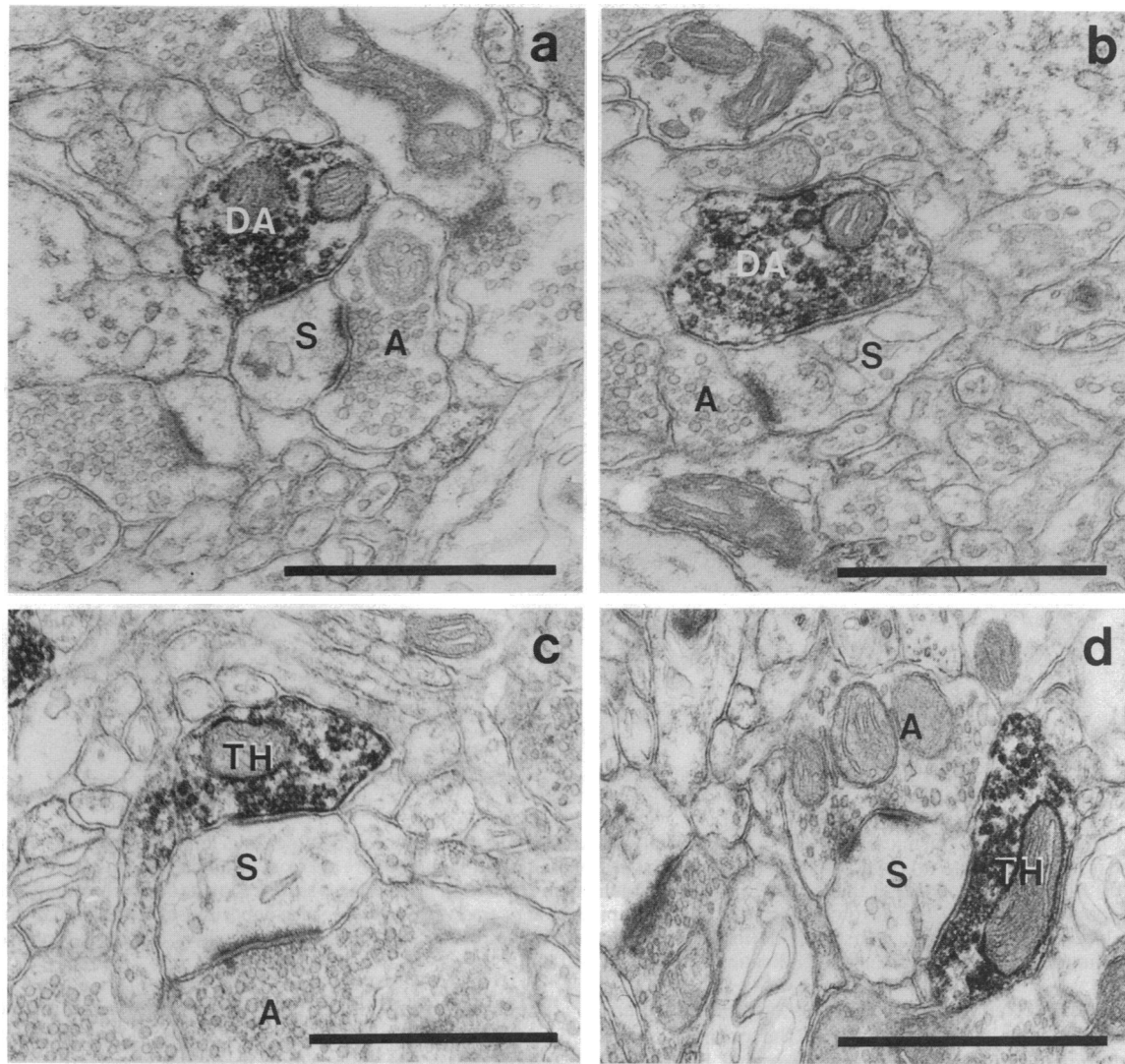
FIG. 2. Electron micrographs taken from layer II of the monkey prefrontal cortex immunostained for DA (*a* and *b*) or TH (*c* and *d*). The micrographs demonstrate some of the very characteristic and numerous synaptic triads of this cortical layer. In these synaptic triads the same spine (S) is postsynaptic to an immunopositive axon (TH or DA) and a nonimmunoreactive axon terminal (A). Triads with TH-positive axo-spinous synapses could not be differentiated from DA-positive triads in distribution, incidence, or ultrastructural criteria. The DA and the TH axons establish exclusively symmetric synaptic membrane specializations, whereas the nonimmunopositive axons in the synaptic triads form asymmetric synaptic connections. Although the DA- or TH-positive and the unstained axon components of the triad were often found in close apposition (see *a*, *b*, and *d*), there was no evidence of membrane specialization between them. Note the presence of the characteristic spine apparatus in all spines (S). (Bars = 1 μm.)

Fig. 3*a* shows an example of a Golgi-identified pyramidal neuron in layer III of prefrontal cortex. Examination of serial sections of one of its terminal apical dendrites revealed that TH-containing boutons formed symmetric synapses on the soma and dendritic shaft of this silver-impregnated pyramidal cell. Most important, the spines of pyramidal cells also formed synapses with TH-immunopositive boutons (Fig. 3 *c* and *d*). Finally, although the silver precipitate obscures membrane specializations to some degree, we were able to observe synaptic triads that were indistinguishable from those observed with the DA and TH antisera in our single-label material—i.e., the same Golgi-impregnated spine in contact with an immunopositive bouton forming a symmetric contact and an unstained bouton forming an asymmetric contact (Fig. 3 *c* and *d*).

DISCUSSION

The present study, an ultrastructural visualization of dopaminergic terminals in the primate cerebral cortex, presents evidence in the cortex of a synaptic arrangement in which the spines of pyramidal neurons are targets of DA afferents and another, as yet unspecified, but presumably excitatory input. These findings, which will be discussed in greater detail below, support the idea that DA terminals can directly alter the output of the cortex through their contacts with cortical efferent neurons.

Distribution and Mode of Termination of DA- and TH-Immunopositive Boutons. Numerous studies have shown a significant DA innervation of the cerebral cortex, particularly its prefrontal and anterior temporal areas (2, 3, 6–8, 11, 14). In previous studies of TH immunoreactivity, the DA innervation was described as bilaminar with higher fiber density in layers I and layers V and VI (6, 7). The present light microscopic observations of TH- and DA-labeled profiles support this general pattern of DA distribution in the cortex. Moreover, the DA innervation described in the present and previous studies matches the distribution of D1 and D2 receptors in the same areas (P.S.G.-R., D. W. Gallager, and M. S. Lidow, unpublished observations).
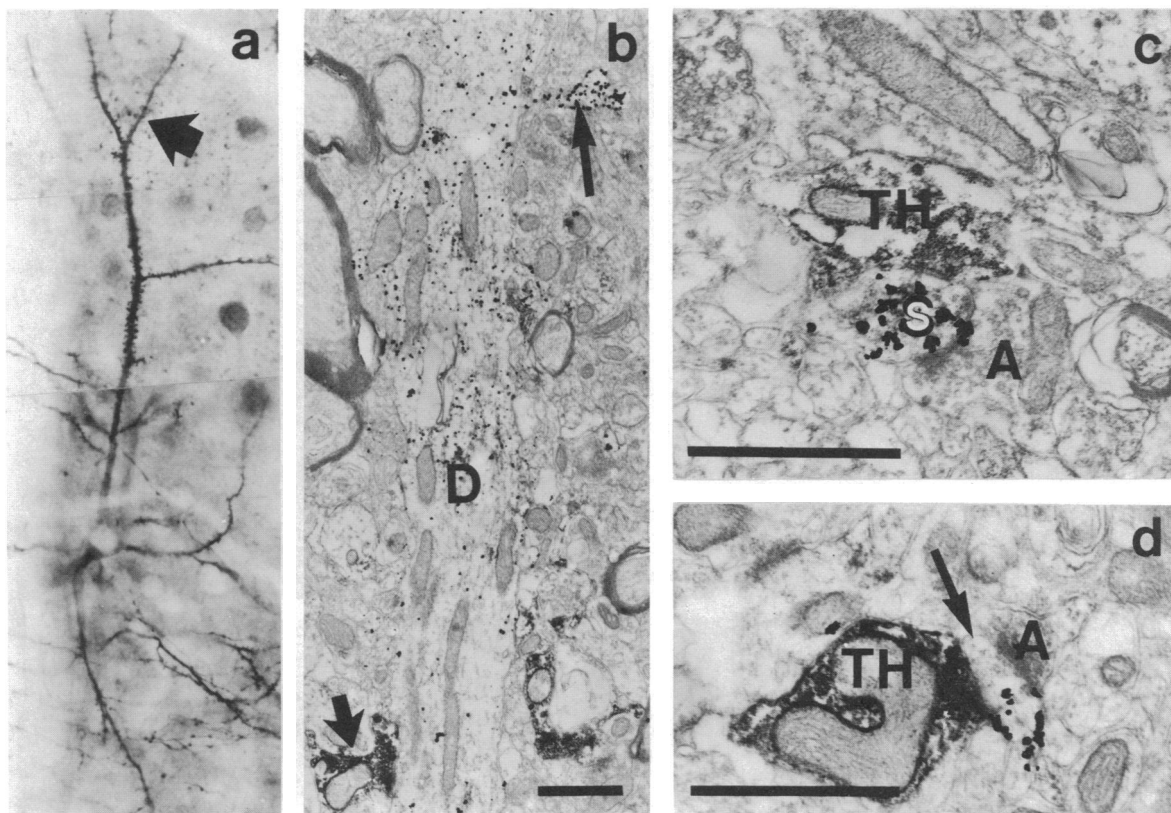
FIG. 3.   Light (*a*) and electron micrographs (*b–d*) demonstrate the results of an experiment combining Golgi impregnation and immunostaining for TH in the prefrontal cortex. (*a*) Golgi-impregnated pyramidal neuron. Ultrathin sections were cut from its apical dendrite (arrowhead) located in layer II. (*b*) Electron micrograph of this dendritic segment containing a spine (thin arrow); also shown is an axodendritic shaft synapse with a TH-immunoreactive bouton (thick arrow). (*c* and *d*) Additional spines (s in *c*; arrow in *d*) on this same dendritic segment taken from a different level of the block. These spines are postsynaptic to TH-immunoreactive boutons as well as to nonimmunoreactive axon terminals (A) that establish asymmetric contacts. (Original magnification, ×100; bars = 1 μm.)

Our electron microscopic observations on the nature and mode of termination of DA afferents in the primate cortex revealed numerous symmetric immunopositive boutons in apposition to postsynaptic membrane specializations in the same layers in which light microscopy revealed dense plex-uses of DA-immunoreactive fibers. The axospinous synaptic junctions, and especially their apparent symbiotic arrange-ment with an asymmetric, putatively excitatory, synapse on the same spine, were unexpected. However, the presence and invariant morphology of this tripartite synaptic complex in three anatomically and functionally distinct areas of the primate cortex indicate that this anatomical arrangement may be widespread and common to many cortical areas.

The symmetric synapses established between DA- and TH-positive boutons and the spines of cortical neurons are notable, given the generally low incidence of symmetric (putatively inhibitory) contacts on spines in the cortex [for review, see Colonnier (20)]. In addition, the conventional symmetric morphology of the DA and TH synapses observed in this study adds support to the growing evidence that monoamines in the cortex are released at specialized junc-tions (21, 22) as are other neurotransmitters. We wish to emphasize, however, that this finding does not rule out other synaptic arrangements involving DA in the cortex and, in fact, we have preliminary evidence that some DA terminals may synapse upon the somas of γ-aminobutyric acid (GABA) interneurons (C.L. and P.S.G.-R., unpublished observa-tions). Nor does it indicate that dopaminergic afferents are the only ones that form symmetric axospine synapses in the primate cerebral cortex. Glutamate decarboxylase- and pep-tide-containing symmetric inputs to spines have been ob-served in the cortex (23, 24) but the postsynaptic targets of

these afferents have thus far remained unidentified, and no evidence of a synaptic configuration involving an asymmetric bouton on the same spine has been reported.

Pyramidal Spines Are Targets of DA Afferents. The com-bined Golgi/EM immunohistochemical findings in this study provide strong evidence that the pyramidal neuron is one of the postsynaptic targets of DA afferents in the cerebral cortex. We observed DA- and TH-positive synpases on somas, on dendritic shafts, and also on spines of many large neurons that could only be pyramidal cells. More significant was the finding that DA synaptic contacts on dendritic spines were present in layer I, which is not known to contain any spiny interneurons (25). Further, spines are not uniformly distributed along the surface of pyramidal neurons but are most concentrated on apical dendrites that ascend into the upper layers, where a considerable number of DA synapses were found. In contrast, DA-positive axo-spinous contacts were sparse in layer IV of the principal sulcus, where the dopamine innervation is equally sparse and spiny stellate cells are virtually nonexistent (J. Lund, personal communi-cation). Finally, by combining immunohistochemistry with Golgi impregnation, we were able to directly demonstrate that TH-containing boutons terminate on the dendrites and spines of pyramidal cells identified by Golgi impregnation. Moreover, the TH-positive boutons were observed to form a three-way synaptic complex with an asymmetric synapse on the same argyrophilic spine head. These triads were identical with those observed in our single-label experiments.

Striatal DA Synapses. Our electron microscopic findings for DA and TH axo-spinous synapses in primate cortex bear a striking resemblance to the results of electron microscopic studies with TH in the caudate nucleus of rats. In monkeys,

as in rats, this structure has a high content of DA but contains very little norepinephrine (3). TH-immunopositive boutons in rat striatum, like those in the primate cortex, are invariably of the symmetric type and their most common targets are dendritic spines, although other axosomatic and axoshaft synapses are also present (26, 27). Moreover, the dendritic spines of the striatonigral projection neurons that receive input from TH-reactive boutons also receive input from an unstained asymmetric synaptic specialization. Freund *et al.* (26) suggested that an important function of DA in the caudate nucleus is to block the spread of excitatory signals at the level of the individual spine. The startling similarity in the targets and mode of termination of DA afferents in the cerebral cortex and caudate nucleus suggest a common principle of synaptic and functional organization in these two anatomically and functionally closely related structures. The similarity is the more impressive as TH boutons do not invariably form symmetric synapses in all brain regions. For example, in rat hippocampus, TH-positive boutons establish exclusively asymmetric synapses on the dendritic spines of hippocampal pyramidal neurons, whereas their contacts on GABAergic nonpyramidal neurons are symmetric (28).

DA Synaptic Complex: Mechanism of Cortical Modulation. The most commonly reported direct effect of DA on the spontaneous activity of prefrontal neurons is inhibition, and this effect can be produced by local microiontophoretic application of DA (29), by systemic administration of DA agonists (30), or by stimulated release consequent to electrical stimulation of the ventral tegmental area (VTA), where the cells of origin of the cortical DA projection lie (31, 32). Indeed, DA release produced by stimulation of brainstem VTA or iontophoretic application of DA can be shown to attenuate the excitatory response of rodent prefrontal neurons to thalamic stimulation (31) or to tail pinch (32). Similarly, DA applied to the prefrontal cortex of monkeys increases the signal-to-noise ratio in single neuronal activity recorded during a behavioral task when the animal was preparing to respond, whereas DA antagonists had the opposite effect (33). It is not known where or how these effects are mediated—i.e., via a direct effect on pyramidal neurons or via interneurons, or both. The present evidence that spines are the targets of symmetric DA-positive boutons and, furthermore, that an asymmetric bouton is also involved may provide some clues. Given that the spines of pyramidal cells in a variety of cortical areas receive a variety of excitatory synapses and can be the site of large EPSPs (34), it would appear that the DA terminal endings on spines are strategically placed within the cortical circuitry to alter local spine responses to excitatory inputs and, through these, make a contribution to the overall excitability of projection neurons in the cortex.

1. Creese, I., Burt, D. P. & Snyder, S.H. (1976) *Science* **192**, 481–483.
2. Thierry, A.-M., Stinus, L. & Glowinski, J. (1973) *Science* **182**, 499–501.
3. Brown, R. M., Crane, A. M. & Goldman, P. S. (1978) *Brain Res.* **124**, 133–150.
4. Simon, H., Stinus, L. & LeMoal, M. (1980) *Nature (London)* **286**, 150–151.
5. Brozoski, T. J., Brown, R. M., Rosvold, H. E. & Goldman, P. S. (1979) *Science* **205**, 929–931.
6. Lewis, D. A., Foote, S. L., Goldstein, M. & Morrison, J. H. (1988) *Brain Res.* **449**, 225–243.
7. Berger, B., Trottier, S., Verney, C., Gaspar, P. & Alvarez, C. (1988) *J. Comp. Neurol.* **273**, 99–119.
8. Gaspar, P., Berger, B., Febvret, A., Vigny, A. & Henry, J.-P. (1989) *J. Comp. Neurol.* **279**, 249–271.
9. Chagnaud, J. L., Mons, N., Tuffet, S., Grandier-Vazeilles, S. & Geffard, M. (1987) *J. Neurochem.* **49**, 487–494.
10. Pickel, V., Joh, T. & Reis, D. (1975) *Brain Res.* **85**, 295–300.
11. Van Eden, C. G., Hoornemann, E. M. D., Buijs, R. M., Matthussen, M. A. H., Geffard, M. & Uylings, H. B. J. (1987) *Neuroscience* **22**, 849–862.
12. Somogyi, P. & Takagi, H. (1982) *Neuroscience* **7**, 1779–1783.
13. Hsu, S. M., Raine, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **29**, 577–580.
14. Seguela, P., Watkins, K. C. & Descarries, L. (1988) *Brain Res.* **442**, 11–22.
15. Frotscher, M. & Leranth, C. (1988) *Histochemistry* **88**, 313–319.
16. Jones, E. G. & Powell, T. P. S. (1970) *Philos. Trans. R. Soc. Lond. B.* **257**, 45–62.
17. Peters, A. & Feldman, M. L. (1976) *J. Neurocytol.* **5**, 63–84.
18. Lund, J. S. & Lund, R. D. (1970) *Brain Res.* **17**, 25–45.
19. Hokfelt, T., Johansson, O., Fuxe, K., Goldstein, M. & Park, D. (1977) *Med. Biol.* **55**, 21–40.
20. Colonnier, M. (1981) in *The Organization of Cerebral Cortex*, ed. Schmitt, F. O. (MIT Press, Cambridge, MA), pp. 125–153.
21. Papadopoulos, G. C., Parnavelas, J. G. & Buijs, R. (1987) *Neurosci. Lett.* **76**, 275–279.
22. Molliver, M. E. & Kristt, D. A. (1975) *Neurosci. Lett.* **1**, 305–310.
23. Jones, E. G., DeFelipe, J., Hendry, S. H. C. & Maggio, J. E. (1988) *J. Neurosci.* **8**, 1206–1224.
24. Hendry, S. H. C., Houser, C. R., Jones, E. G. & Vaughn, J. E. (1983) *J. Neurocytol.* **12**, 639–660.
25. Marin-Padilla, M. (1984) in *Cerebral Cortex*, eds. Peters, A. & Jones, E. G. (Plenum, New York), Vol. 1, pp. 447–521.
26. Freund, T. F., Powell, J. F. & Smith, A. D. (1984) *Neuroscience* **13**, 1189–1215.
27. Pickel, V. M., Beckley, S., Joh, T. H. & Reis, D. J. (1981) *Brain Res.* **225**, 373–385.
28. Frotscher, M. & Leranth, C. (1987) *J. Comp. Neurol.* **243**, 58–70.
29. Bunney, B. S. & Agajanian, G. K. (1976) *Life Sci.* **19**, 1783–1792.
30. Peterson, S. L., St. Mary, J. S. & Harding, N. R. (1987) *Brain Res. Bull.* **18**, 723–729.
31. Ferron, A., Thierry, A.-M., Le Douarin, C. & Glowinski, J. (1984) *Brain Res.* **302**, 257–265.
32. Thierry, A.-M., Mantz, J., Milla, C. and Glowinski, J. (1988) in *Proceedings from the Conference on the Mesocorticolimbic Dopamine System*, eds. Kalivas, P. W. and Nemeroff, C. B. (New York Acad. Sci., New York), pp. 101–111.
33. Sawaguchi, T. & Matsumura, M. (1985) *Neurosci. Res.* **2**, 255–273.
34. Shepherd, G. (1974) *The Synaptic Organization of the Brain* (Oxford Univ. Press, Oxford, U.K.), pp. 321–328.