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IMMUNOCYTOCHEMICAL LOCALIZATION OF GLUTAMATE DECARBOXYLASE IN RAT SUBSTANTIA NIGRA*

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SUMMARY

L-Glutamate decarboxylase (GAD, EC 4.1.1.15), the enzyme which catalyzes the α -decarboxylation of L-glutamate to form γ -aminobutyric acid (GABA), was localized both light and electron microscopically in rat substantia nigra by an immunoperoxidase method. Large amounts of GAD-positive reaction product were seen throughout the substantia nigra in light microscopic preparations, and it appeared to be localized in punctate structures that were apposed to dendrites and somata. Electron microscopic studies revealed that most of the axon terminals in the substantia nigra were filled with GAD-positive reaction product and formed both axodendritic and axosomatic synapses. Many dendrites were extensively surrounded by GAD-positive terminals which most commonly formed symmetric synaptic junctions, although some formed asymmetric synaptic junctions.

The results of this investigation are consistent with biochemical, pharmacological and physiological data which have indicated that neurons of the neostriatum and globus pallidus exert a GABA-mediated, postsynaptic inhibition upon the neurons of the substantia nigra. These findings provide another example in the vertebrate central nervous system where Golgi I projection neurons are inhibitory and use GABA as their neurotransmitter.

INTRODUCTION

The substantia nigra contains high levels of both γ -aminobutyric acid (GABA) and its synthesizing enzyme, glutamate decarboxylase (GAD, EC 4.1.1.15)^{5,6,22}. Recent experimental studies have suggested that most of the GAD and GABA

* A preliminary report of this work was presented at the 55th Annual Meeting of the Association for Research on Nervous and Mental Diseases on the subject of the Basal Ganglia.

within the substantia nigra is contained in the axon terminals of the neurons that give rise to the striatonigral^{8,13,15,31} and the pallidonigral^{17,18} pathways. In these studies, parts of the basal ganglia were ablated or the striatonigral pathway was interrupted, and consequently the quantities of GAD and GABA in the substantia nigra were observed to decrease substantially. Electron microscopic studies of degeneration following ablation of the neostriatum in rats¹¹, cats^{10,14} and monkeys²⁹ have demonstrated the location, morphology and relative number of axon terminals in the substantia nigra which are associated with the striatonigral pathway. Also, the location and morphology of axon terminals arising from the pallidonigral pathway in rats have been described in electron microscopic autoradiographs following an injection of tritiated leucine into the globus pallidus¹². In addition, physiological studies have shown that stimulation of the caudate nucleus produces an inhibition of neuronal firing in the substantia nigra which is blocked by picrotoxin^{25,35} and which can be mimicked by microiontophoretic application of GABA into the substantia nigra⁷.

Thus, evidence is accumulating from a number of investigative approaches that the axons of the striatonigral and pallidonigral pathways monosynaptically inhibit neurons within the substantia nigra and that this postsynaptic inhibition is mediated by GABA. However, ultrastructural evidence is lacking that GABA is localized within axon terminals arising from the striatonigral and pallidonigral pathways. Although there is no method for demonstrating the location of endogenous GABA with ultrastructural techniques, it is possible to localize the GABA synthetic enzyme, GAD, at the synaptic level using immunocytochemical methods^{19,20,34}. Since the regional activity of GAD in the central nervous system is highly correlated with the concentration of GABA^{5,9,16} and since GAD has been demonstrated immunocytochemically only within specific axon terminals which are probably GABAergic^{19,20,28,34}, it is likely that the presence of GAD is diagnostic of GABAergic synaptic terminals. Thus, an immunocytochemical localization of GAD in the substantia nigra should provide information that can be correlated with the results of other investigators who have studied the synaptic relationships of striatal and pallidal axons within the substantia nigra^{10-12,14,29}. Such a correlation should provide a strong indication as to whether or not striatonigral and pallidonigral axon terminals possess the enzyme required for GABA synthesis.

METHODS AND MATERIALS

Tissue preparation

Adult Sprague-Dawley rats were anesthetized by intraperitoneal injections of chloral hydrate and were then fixed via intracardiac perfusions²⁴ with a solution containing 4.0% paraformaldehyde, 0.002% CaCl₂, and varying concentrations of glutaraldehyde (Polysciences, Washington, Pa.) in 0.12 M Millonig's phosphate buffer²¹, at pH 7.2 and 37 °C. Animals were perfused with fixatives containing the following concentrations of glutaraldehyde: 0.1, 0.2, 0.4, 1.0 and 5.0%. Brains were dissected from the cranium the following day, and the brain stems were hemisected

midsagittally so that specimens from each animal could be used for both light and electron microscopy. The hemisected brain stems used for light microscopy were immersed overnight in a cryoprotectant 30% sucrose solution. Following rapid freezing, transverse frozen sections of these specimens were cut at 40 μm on a cryostat¹. The matching hemisected brain stems to be used for electron microscopy were cut transversely at 150 μm using a Sorvall TC-2 tissue sectioner. Sections containing the substantia nigra were selected and placed in the phosphate buffer²¹.

Immunocytochemical procedure

The immunocytochemical procedure employed for tissue used in both light and electron microscopy was similar to that previously described^{1,19,20,34}, except that a peroxidase-antiperoxidase procedure modified from that of Sternberger³⁰ was used instead of a peroxidase-conjugated goat antirabbit IgG procedure. Briefly, sections were incubated in normal rat serum for 30 min and then rinsed in phosphate buffer before being incubated for 30 min in either rabbit anti-GAD serum or control rabbit serum. Following a 2.5 h buffer wash, the sections were incubated 30 min in goat antirabbit serum (Antibodies, Inc., Davis, Calif.). Sections were then washed in buffer for 2.5 h, incubated in a peroxidase-antiperoxidase Fab complex² for 30 min, and washed again in buffer for 2.5 h before being reacted with 3,3'-diaminobenzidine-4 HCl (Sigma, St. Louis, Mo.) and H_2O_2 as described previously^{1,19,20,34}. Following the immunocytochemical reactions, the sections for light microscopy were poststained 30 sec in 0.1% OsO_4 and mounted on glass slides. The sections for electron microscopy were incubated in the immunocytochemical reagents twice as long as those for light microscopy. Blocks of tissue containing the substantia nigra were dissected from these sections and they were postfixed in OsO_4 , en bloc stained in aqueous uranyl acetate, dehydrated and embedded as described previously^{19,20,34}.

OBSERVATIONS

Although previous immunocytochemical studies of GAD localization in the cerebellum, retina and spinal cord have successfully employed 0.1% glutaraldehyde in the fixative^{19,20,34}, some parts of the central nervous system require greater concentrations of glutaraldehyde than this in order to achieve adequate ultrastructural preservation. The initial experiments on the substantia nigra using 0.1% glutaraldehyde gave a specific localization of the GAD-positive reaction product but did not produce adequate ultrastructural preservation. In order to correct this defect without adversely affecting the specificity of the immunocytochemistry, a series of experiments was carried out using graded concentrations of glutaraldehyde in the perfusing solution. Briefly, specific GAD-positive reactions were obtained in specimens perfused with solutions containing 0.1%, 0.2% and 0.4% glutaraldehyde. However, the specificity of the immunocytochemistry was deficient in tissue obtained from animals perfused with fixatives containing 1.0% and 5.0% glutaraldehyde. Specimens perfused with a fixative that contained 4.0% paraformaldehyde and 0.4% glutaraldehyde produced the most acceptable compromise between specific immunocytochemical

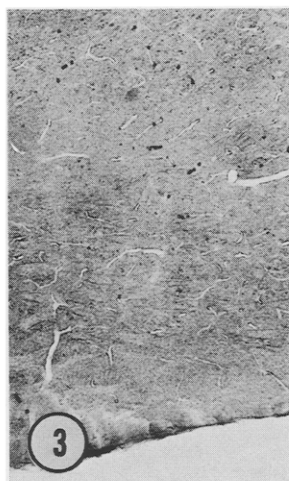
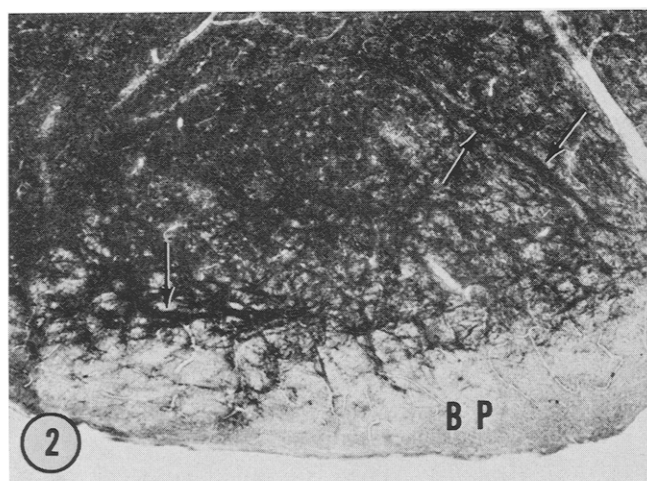
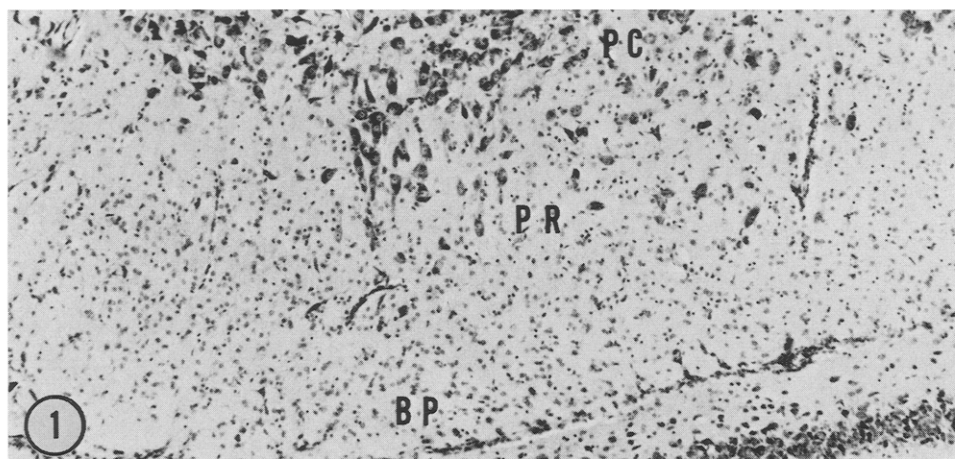


Fig. 1. Nissl-stained, paraffin section of the rat substantia nigra showing the cell-rich pars compacta (PC) and the cell-sparse, pars reticulata (PR). The basis pedunculus (BP) is subjacent to the pars reticulata. $\times 75$.

Fig. 2. Frozen section of the substantia nigra incubated in anti-GAD serum showing a dense deposition of reaction product in both the pars compacta and pars reticulata. Within the pars reticulata, there are dendritic-like processes that appear to be covered by the GAD-positive reaction product (arrows). The basis pedunculus (BP) is free of reaction product except for an occasional GAD-positive outlining of dendritic-like processes emanating from the pars reticulata. $\times 75$.

Fig. 3. A frozen section of substantia nigra incubated in control rabbit serum lacks specific staining. The dark spherical structures are red blood cells which are stained because they possess endogenous peroxidase-like activity. $\times 75$.

staining and ultrastructural preservation. Therefore, all of the results to be presented in the following sections were obtained from these specimens.

Light microscopy of GAD localization in the substantia nigra

The rat substantia nigra is subdivided into a dorsal portion, the pars compacta, and a larger ventral portion, the pars reticulata, that is located adjacent to the basis

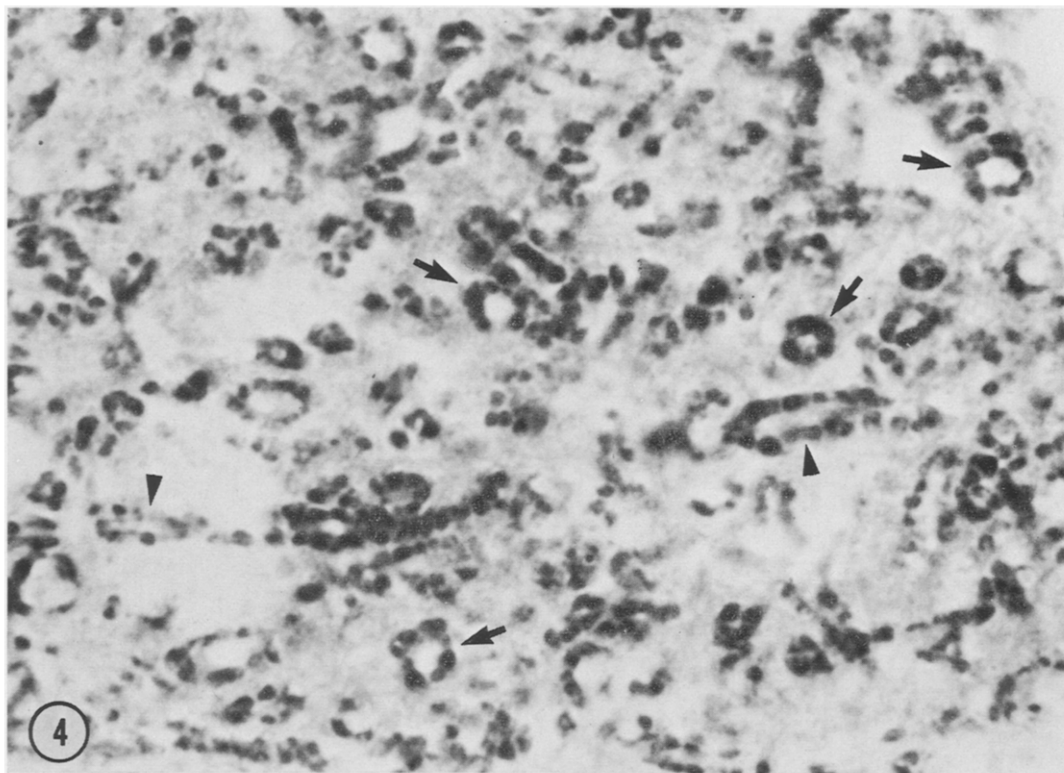


Fig. 4. Semithin ($1\ \mu\text{m}$) section of the pars reticulata from a slice incubated in anti-GAD serum. The transversely sectioned dendrites are encircled by GAD-positive puncta (arrows). Also shown are obliquely and longitudinally sectioned dendrites that have GAD-positive puncta along their surfaces (arrowheads). $\times 2000$.

pedunculus (Fig. 1). The pars compacta contains most of the neuronal somata of the substantia nigra, and these somata send many of their dendrites ventrally into the pars reticulata^{3,27,29}.

At low magnifications, frozen sections of substantia nigra incubated in anti-GAD serum showed a large amount of reaction product (Fig. 2). The pars compacta was almost completely blackened by reaction product except for sites occupied by neuronal somata. The deposition of reaction product was not so ubiquitous in the pars reticulata, where non-stained neuropil was interspersed with stained neuropil in such a way as to produce a reticular pattern. This pattern is reminiscent of that observed in Golgi preparations where the dendrites of nigral neurons are ensheathed by a plexus of afferent fibers²⁹. Frozen sections of substantia nigra that were incubated in the control serum showed no specific staining (Fig. 3).

At high magnifications, the GAD-positive reaction product appeared as dense, punctate structures sharply defined against a relatively clear background in both the $40\ \mu\text{m}$ frozen sections and the semithin ($1\ \mu\text{m}$) plastic sections taken from blocks of tissue processed for electron microscopy. Previous studies have shown that the punctate appearance of GAD-positive reaction product is due to its concentration in certain

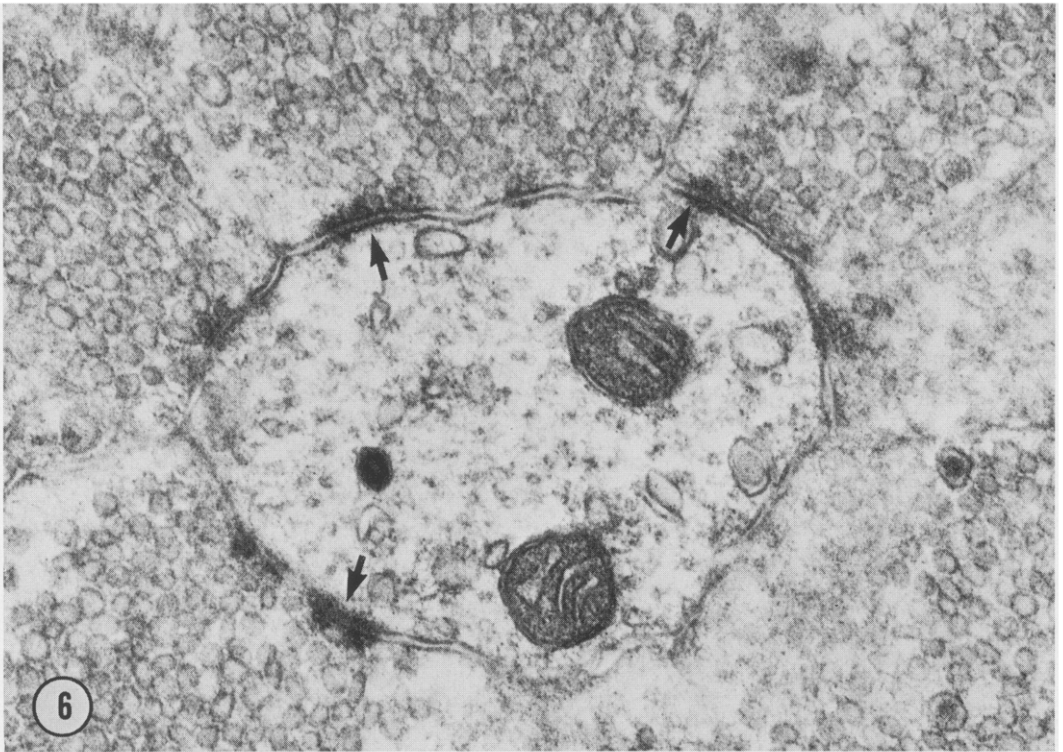
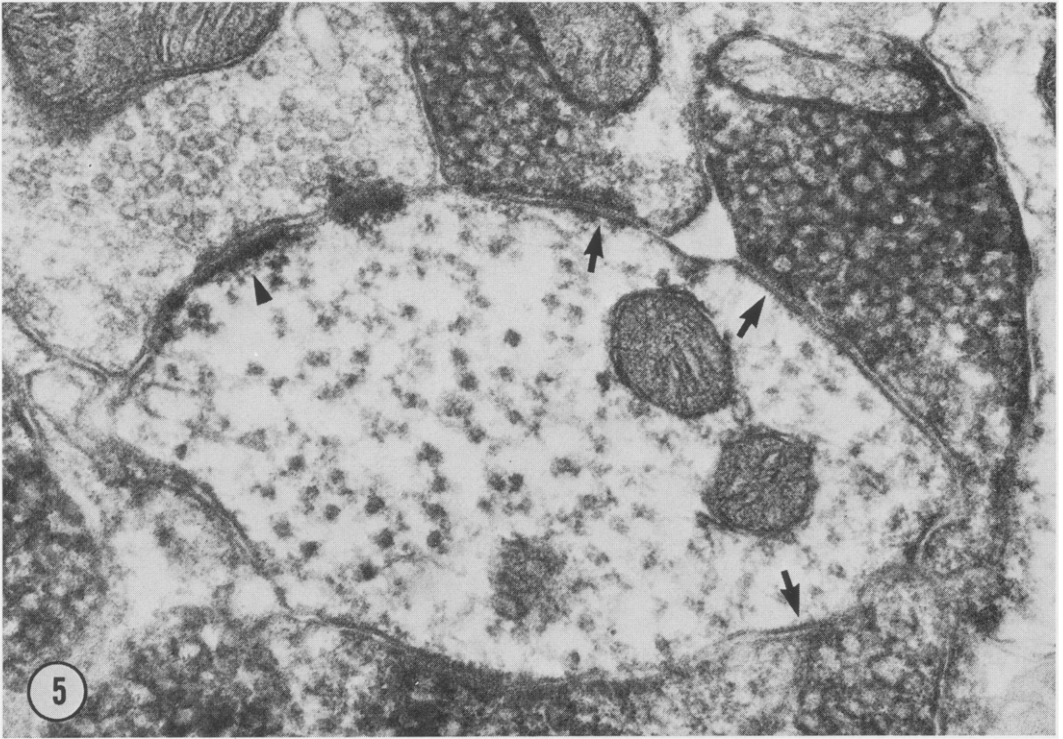


Fig. 5. Electron micrograph of substantia nigra incubated in anti-GAD serum showing axon terminals filled with GAD-positive reaction product. These terminals form symmetric synapses (arrows) with a dendritic shaft. The unstained terminal contains round synaptic vesicles and forms an asymmetric synapse (arrowhead) with the dendritic shaft. $\times 67,000$.

Fig. 6. Electron micrograph of substantia nigra incubated in control rabbit serum. In contrast to Fig. 5, all of the axon terminals lack reaction product although they form symmetric synapses (arrows) with the dendritic shaft. $\times 61,000$.

axon terminals^{1,20,34}. In Fig. 4, GAD-positive puncta are shown in close apposition to dendritic profiles. In most instances, transversely sectioned dendritic profiles were completely encircled with GAD-positive puncta. Longitudinally and obliquely sectioned dendritic profiles also showed numerous puncta adjacent to their surfaces. In addition, GAD-positive puncta were found to be perisomatic in the pars compacta.

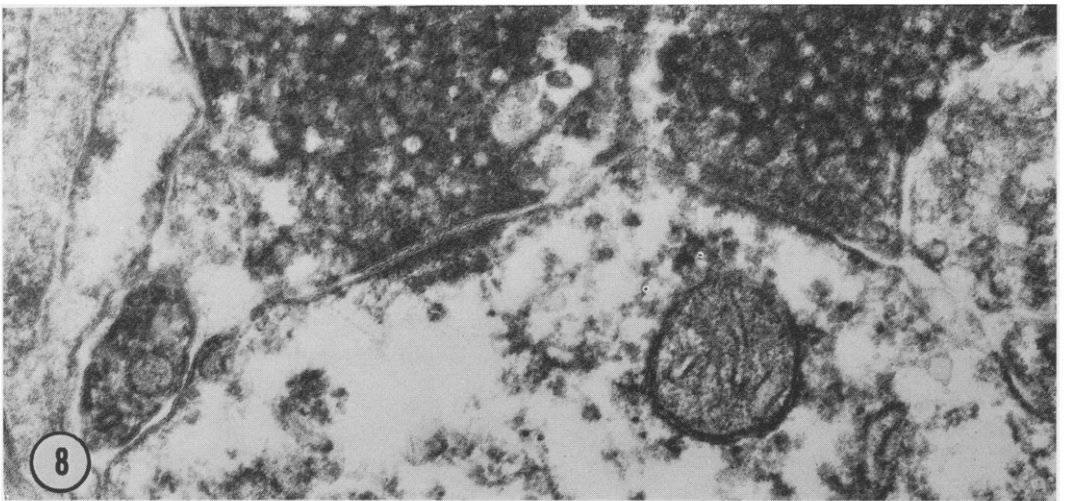
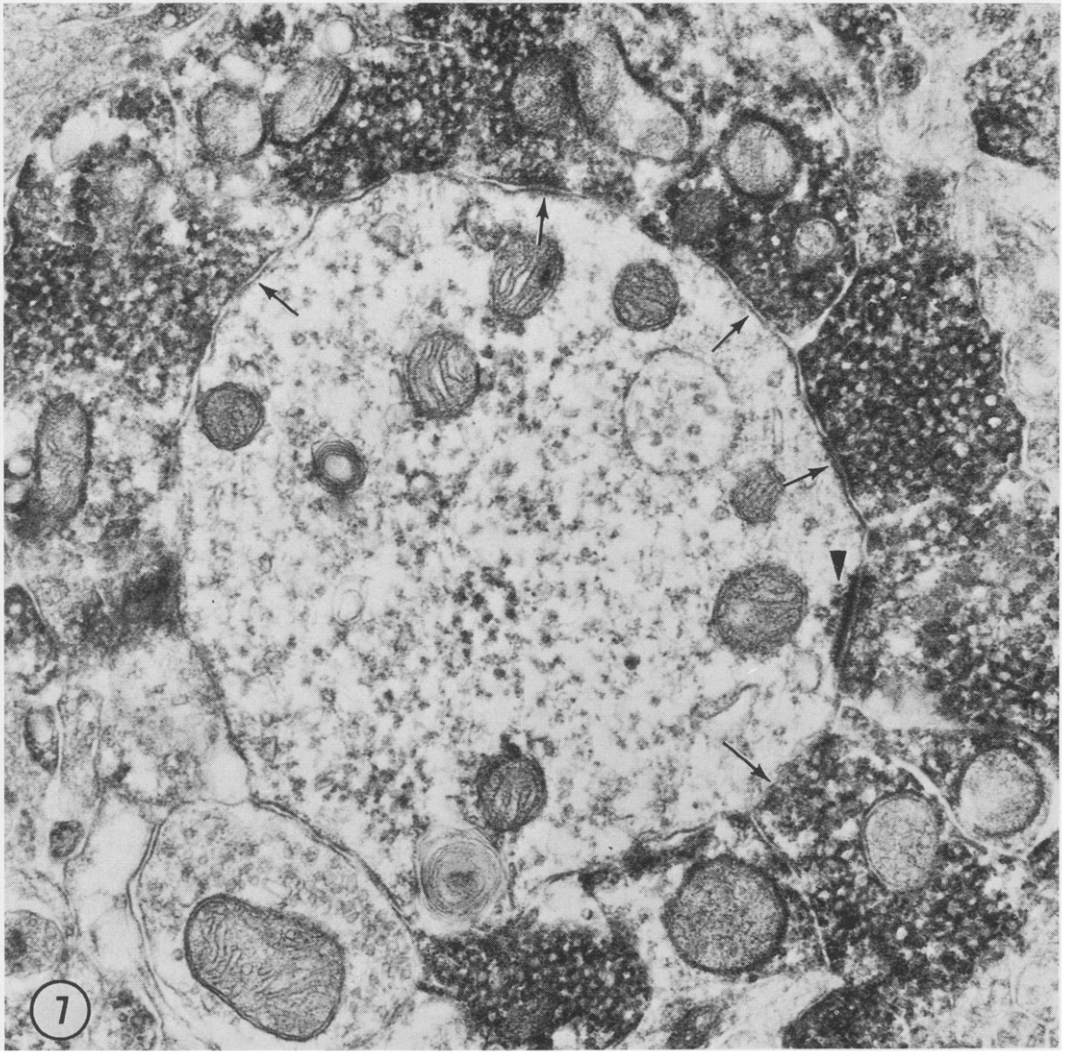
Electron microscopy of GAD localization in the substantia nigra

In the pars reticulata, dendritic shafts have been shown to be surrounded by axon terminals which form primarily symmetric synaptic junctions and contain pleomorphic synaptic vesicles¹¹ (also see Fig. 6). A similar observation has been reported in ultrastructural studies of the substantia nigra in rabbits³³, cats²⁷ and monkeys²⁹. In thin sections of nigral slices incubated in anti-GAD serum, dendrites were surrounded by axon terminals containing GAD-positive reaction product (Figs. 5 and 7). This distribution coincides with the location of many of the GAD-positive puncta in the light microscopic preparations.

A majority of these GAD-positive axon terminals form symmetric synaptic junctions with the dendritic profiles, although it is not uncommon to observe GAD-positive terminals forming asymmetric synaptic junctions. For example, in Fig. 7, there are 10 axon terminals synapsing with a dendritic shaft and 8 of these contain GAD-positive reaction product. One of the GAD-positive terminals forms an asymmetric synaptic junction exhibiting a subjunctional dense body²³. Fig. 8 shows a higher magnification of two GAD-positive terminals, one of which forms an asymmetric synaptic junction that also has an associated subjunctional dense body. However, GAD-positive terminals forming asymmetric synaptic junctions also were observed without an associated subjunctional dense body. An estimate of the relative proportions of GAD-positive terminals that form symmetric and asymmetric synaptic junctions was obtained by counting and categorizing 60 different GAD-positive terminals in random electron micrographs of the substantia nigra. The obtained data showed that about 85% of the GAD-positive terminals formed symmetric synaptic junctions while approximately 15% formed asymmetric synaptic junctions. These findings further emphasize the need for caution when speculating about functional aspects of synapses solely on the basis of their ultrastructural characteristics (cf. ref. 19).

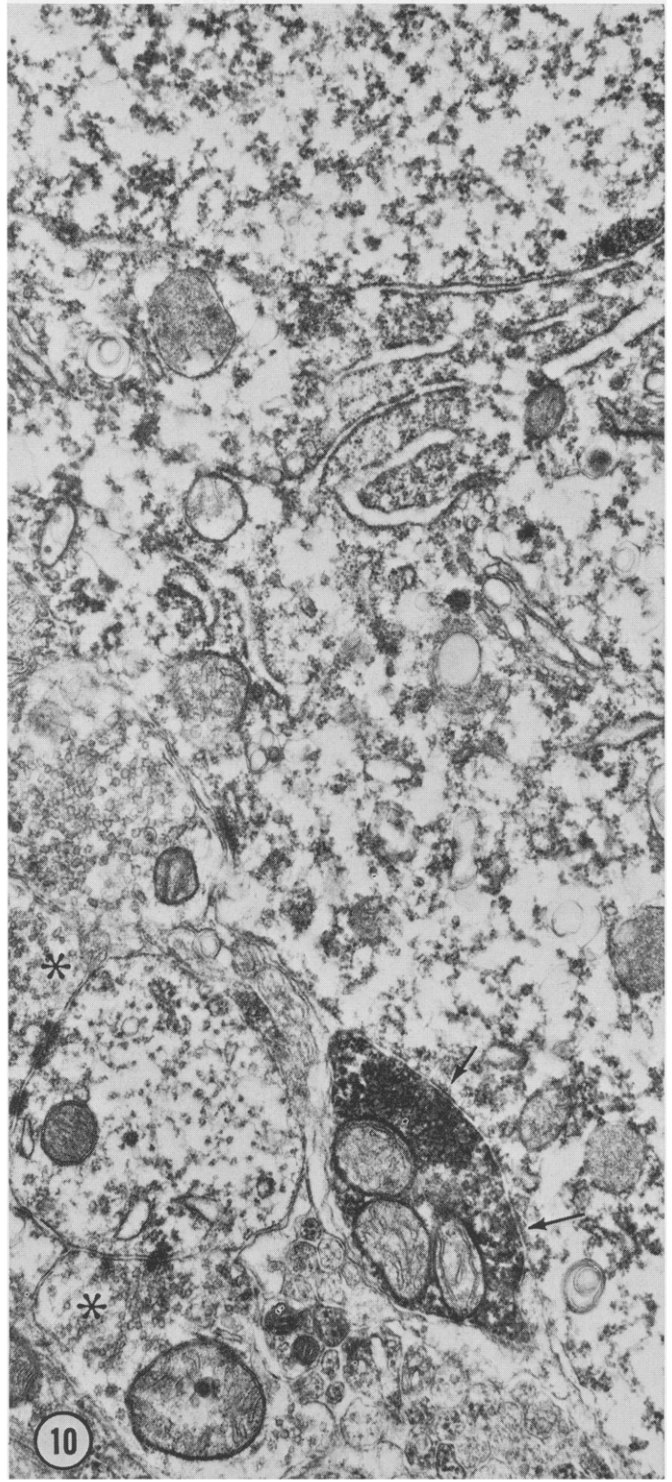
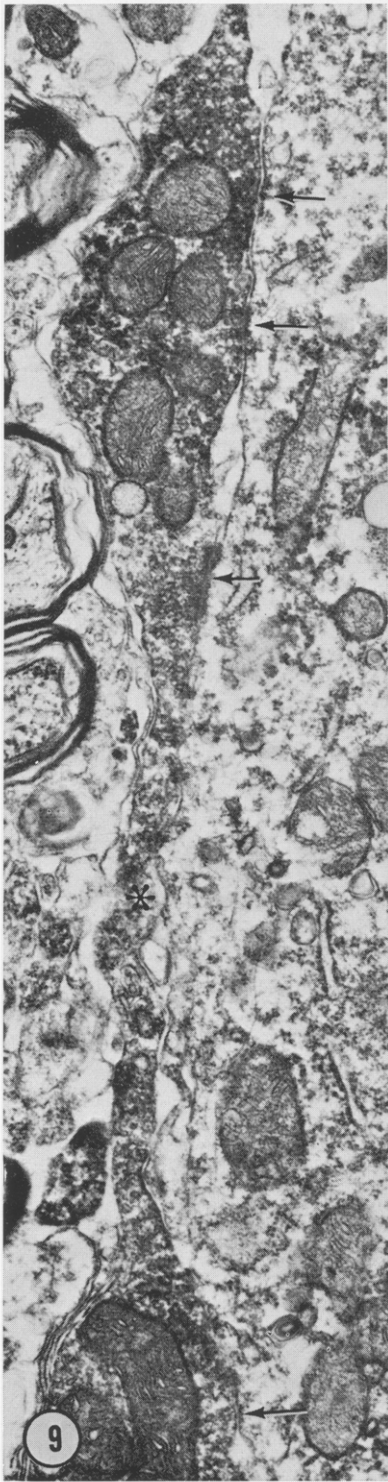
In accord with the light microscopic distribution of GAD-positive puncta, some GAD-positive axon terminals also were observed to be presynaptic to somata (Figs. 9 and 10). These GAD-positive terminals all formed symmetric synaptic junctions and were not nearly so numerous as those that were presynaptic to dendrites. Fig. 9 shows an axosomatic synaptic junction formed by a GAD-positive 'bouton en passant'. This bouton is entirely filled with reaction product, including the thin, unmyelinated 'interterminal' part of the axon which also contains some synaptic vesicles. A similar staining of many transversely sectioned, unmyelinated axons was also observed, and such profiles may represent preterminal axons.

In control sections of the substantia nigra, no GAD-positive reaction product was observed in axon terminals (e.g., Fig. 6) or within any other components of the



Figs. 7 and 8. Electron micrographs of substantia nigra sections that were incubated in anti-GAD serum. Note the large number of GAD-positive axon terminals that synapse (arrows) with the transversely sectioned dendrite in Fig. 7. One of these GAD-positive terminals forms an asymmetric synaptic junction which exhibits a subjunctional dense body (arrowhead). $\times 41,000$.

Fig. 8 shows a higher magnification of another field where two GAD-positive terminals synapse with a dendritic shaft. The synaptic junction formed by the terminal on the left is asymmetric while that of the terminal on the right is symmetric. $\times 68,000$.



Figs. 9 and 10. GAD-positive terminals forming axosomatic synapses. Fig. 9 shows a GAD-positive 'bouton en passant' which forms symmetric synaptic junctions (arrows) with an adjacent soma. Note that GAD-positive reaction product fills the 'interterminal' (asterisk) — as well as the terminal — part of the axon. $\times 30,000$. In Fig. 10, there is a GAD-positive axon terminal (arrows) forming a symmetric synaptic junction with a cell body of a nigral neuron. Note that the two axon terminals forming asymmetric synaptic junctions with an adjacent dendritic shaft are not GAD-positive (asterisks). $\times 25,000$.

neuropil. Furthermore, sections incubated in anti-GAD serum exhibited no specific staining within nigral neurons. This lack of staining for GAD was anticipated since there is evidence that another neurotransmitter candidate, dopamine, is concentrated within the neurons of the substantia nigra^{3,4,33}.

DISCUSSION

The results of this study demonstrate that a large proportion of the axon terminals in the substantia nigra contain the enzyme (GAD) required for GABA synthesis. It is likely that many of these GAD-positive terminals arise from the striatonigral or pallidonigral pathways because their relative numbers, locations and ultrastructural features all correspond to those terminals described in experimental morphological studies of these pathways^{10-12,14,29}. For example, GAD-positive terminals and axon terminals arising from the striatonigral and pallidonigral pathways both account for a substantial proportion of the axon terminals within the substantia nigra. Also, both groups of terminals (i.e., GAD-positive ones and those arising from the neostriatum and the globus pallidus) appear to form the vast majority of axodendritic and axosomatic synapses in the substantia nigra. Finally, both groups of terminals most commonly form symmetric synaptic junctions, although both GAD-positive terminals and striatonigral terminals also form some asymmetric synaptic junctions with dendritic shafts. Thus, on a correlational basis, it is likely that many of the GAD-positive axon terminals observed in this study of the substantia nigra arise from neurons located in the neostriatum and the globus pallidus.

This conclusion is consistent with those biochemical studies which have suggested that most of the GAD and GABA within the substantia nigra is probably contained in the axon terminals of the neurons that give rise to the striatonigral^{8,13,15,31} and the pallidonigral^{17,18} pathways. Furthermore, it is also consistent with the physiological and pharmacological evidence that striatonigral axons exert a GABA-mediated, monosynaptic inhibition upon neurons of the substantia nigra^{7,25,32,35}.

Generally, GABAergic inhibition has been considered to be a function of local circuit neurons²⁶. Now there is evidence for two exceptions to this generalization; namely, the Purkinje cells of the cerebellum²⁰ and the neurons in the neostriatum and globus pallidus that project to the substantia nigra. Thus, it may be that projection neurons play a larger role in GABAergic inhibition within vertebrate central nervous systems than was thought previously.

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