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Immunocytochemical localization of GABAergic neurones at the electron microscopical level

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Summary

Antibodies prepared to purified brain glutamic acid decarboxylase (GAD), the synthesizing enzyme for the neurotransmitter, γ -aminobutyric acid (GABA), have been utilized with an unlabelled antibody method to localize GABAergic neurones in both light and electron microscopic preparations. A modification of Sternberger's peroxidase-antiperoxidase (PAP) complex is used to localize the site of anti-GAD binding, and the PAP complex is visualized with diaminobenzidine and H_2O_2 . The reaction product is visible in both the light and electron microscopes. The ability to localize and identify labelled profiles in the electron microscope provides more functional information than light microscopical preparations. For example, the GAD-positive reaction product occurs mostly in association with synaptic vesicles within axon terminals, and this localization indicates the importance of GAD for the packaging and storage of GABA. The somata and dendrites of neurones giving rise to these terminals are visualized in colchicine-injected material. The GABAergic neurones form axo-somatic, axo-dendritic, axo-axonal and dendro-dendritic synapses in various regions of the rat central nervous system. Pre-treatments of animals with anterograde degeneration have shown the significance of some of the GABAergic terminals that form axo-axonal synapses in the spinal cord.

In many brain regions, such as the cerebral cortex, hippocampus and olfactory bulb, virtually all of the GABAergic synapses are derived from local circuit neurones. In other regions such as the cerebellum and neostriatum, the GABAergic terminals are derived from both local circuit neurones and the local axon collaterals of projection neurones that have their somata within these regions. A third type of configuration of GABAergic terminals occurs in the globus pallidus and substantia nigra where these terminals are derived from distant brain regions, axon collaterals of projection neurones and from local circuit neurones. Together, these results indicate the complex organization of the GABAergic system of the brain that has been vividly revealed with electron microscopical immunocytochemistry.

Introduction

The challenge of immunocytochemical methods is to localize biochemicals to intracellular sites with the use of antibodies that recognize antigenic sites of these

molecules. In many studies of the nervous system immunocytochemistry has revealed new functions for neurones in various brain regions (Hökfelt *et al.*, 1975, 1976, 1977, 1978; Swanson & Hartman, 1975; Pickel *et al.*, 1976; McGeer *et al.*, 1978; Roberts, 1980). Most of these studies were limited to the light microscopical level where a major shortcoming occurs in deciding the significance of labelled processes and fibres that are not continuous with a cell body. Just as the electron microscope expanded the knowledge of the central nervous system (C.N.S.) from the time of its first application with the confirmation of the synapse in 1955 (Palay & Palade, 1955), the use of ultrastructural immunocytochemical preparations provides improved resolution for the localization of molecules in the brain and spinal cord.

The following article details the techniques that we have utilized for electron microscopical immunocytochemistry. The present discussion will focus on the methods used to localize the GABAergic neurones in electron microscopical preparations of the nervous system. A more comprehensive account of immunocytochemical methods for the localization of neurotransmitter-related proteins and peptides for both light and electron microscopy appears elsewhere (Vaughn *et al.*, 1981). The completeness of that account is beyond the aims of this article which will emphasize the variables involved with ultrastructural immunocytochemistry in different regions of the nervous system.

In these studies (McLaughlin *et al.*, 1974, 1975; Ribak *et al.*, 1976, 1977, 1978, 1979a, b, 1980; Ribak, 1978), the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD), was used to demonstrate GABAergic neurones. This enzyme regulates the rate-limiting step for the formation of GABA by decarboxylating glutamic acid. The presence of GAD within a neurone indicates that the neurotransmitter, GABA, is made by this neurone and, therefore, could be used for effecting neurotransmission. Since the regional activity of GAD in the C.N.S. is highly correlated with the concentration of GABA, it is likely that GAD is a good marker for GABAergic neurones (Fahn, 1976; Fonnum & Walberg, 1973; Kuriyama *et al.*, 1966). Furthermore, since the morphological characteristics and distribution of axon terminals that stain by GAD immunocytochemistry correlate with results obtained from morphological, biochemical, physiological and pharmacological studies of GABAergic neurones in many brain regions (see Vaughn *et al.*, 1981), the presence of GAD is diagnostic of GABAergic synaptic terminals. Thus, it is virtually certain that immunocytochemical studies of GAD identify GABAergic neurones in the C.N.S.

Materials and methods

Animals

Adult Sprague-Dawley rats were used most commonly in these experiments. However, adequate results were obtained with other species, including rabbits and monkeys (*Macaca mulatta* and *M. fascicularis*).

Experimental pre-treatments

When immunocytochemical studies are combined with other methods, various experimental pre-treatments are used to provide the desired preparations. Among these pre-treatments are (1)

anterograde degeneration for the identification of a known afferent population of terminals (Barber *et al.*, 1978), (2) colchicine injections for the identification of the somata and dendrites of GABAergic neurones (Ribak, 1978; Ribak *et al.*, 1978, 1979b), (3) alumina gel applications for the creation of epileptic foci in neocortex (Ribak *et al.*, 1979a) and (4) the interruption of GABAergic axons that project to a brain region for the study of GABAergic terminals that are intrinsic to that region (Ribak *et al.*, 1980). Only two of these pre-treatments have been used in conjunction with electron microscopical studies of immunocytochemical preparations, the anterograde degeneration and the colchicine injections.

For anterograde degeneration, dorsal roots that enter the spinal cord are visualized through the dura of anaesthetized rats and are crushed into pieces with fine forceps proximal to the ganglia. These animals are allowed to survive for 24, 36 or 48 h, and these survival times have produced adequate numbers of degenerating terminals (Barber *et al.*, 1978).

For colchicine injections, colchicine is injected directly into the brain region to be studied from a 10 μ l Hamilton syringe that is mounted on a micromanipulator. The tip of the microsyringe is stereotaxically placed into the desired brain region of the anaesthetized animal and 2–5 μ l of colchicine (10 μ g/ μ l saline; Sigma, St. Louis, Missouri) are injected over a 10 min period. Following the completion of the injection, the syringe is kept in place for another 5 min before removing it from the brain. Colchicine is concentrated in the region surrounding the injection site by this method. Thus, if neuronal cell bodies in this location are synthesizing GAD, it can be detected within somata and dendrites following this type of colchicine pre-treatment. However, this method has two disadvantages: its effect is limited to neurones in the vicinity of the injection and it produces a lesion and disrupts the neuropile in the brain region being studied. Recently, injections of colchicine have been made into the lateral ventricle (Vaughn *et al.*, 1981). It has been possible to enhance somal and dendritic staining in a large number of regions throughout the forebrain and brain stem in the same animal using this intraventricular method of colchicine treatment. However, since it is possible that adequate concentrations of colchicine will not reach every region of the C.N.S. following intraventricular injection, a lack of somal staining in a region of interest should be verified by the use of the local injection method described above. Colchicine-treated rats are sacrificed by intracardiac perfusions following a 24–48 h post-injection survival time.

Fixation procedures

First, the rats are anaesthetized by intraperitoneal injections of chloral hydrate (35% 0.15 ml/100 g body weight). Then a tracheotomy is performed with an assistant administering a gas mixture to keep blood oxygen levels high and to maintain dilation of blood vessels (5% CO₂ and 95% O₂). At this time, the rat is placed on its back on the perfusion table, and its forelegs and tail are loosely taped to the table's surface. The thoracic cavity is opened carefully with blunt scissors while the assistant controls respiration to avoid damage to the lungs. After clamping the mammary arteries near the neck, the ventral rib cage is removed to provide a working area around the heart. Then, the left ventricle of the heart is injected with 0.5 ml of a solution of 1% sodium nitrite with 50 units of heparin to dilate the vasculature and prevent clotting. While this solution is circulating for 2 min, the assistant continues to respire the rat until the time the fixative solution is perfused.

The fixative solution (see below for its composition) is driven by regulated air pressure through an IV-drip tubing and a 15 gauge cannula through the apex of the heart. For better perfusions of regions in the brain as opposed to the spinal cord, the dorsal aorta is clamped in the lower thorax prior to this step. The initial flow of fixative through the cannula should be rapid so that it forms a steady stream to flush the arteries and perfuse the small vessels. Immediately after the start of flow, the right atrium is excised to provide an outflow for the blood and fixative. After the initial washout, the fixative (500 ml for a 250 g rat) is perfused at a drip rate of 1–3 drops/s for about 15 min. Then the rat is placed in a plastic bag in the refrigerator overnight to allow further fixation.

Brains are dissected from the crania the following day, and specimens are stored in 4% paraformaldehyde in 0.12 M buffer until it is time to cut sections.

The perfusion solution used in our laboratory contains 4% paraformaldehyde, 0.1–0.4% glutaraldehyde and 0.002% CaCl_2 in 0.12 M Millonig's (1961) phosphate buffer at pH 7.2. Varying amounts of glutaraldehyde are used for each brain region analysed because higher amounts of glutaraldehyde provide better ultrastructural preservation but at the same time they decrease the antigenicity of GAD. Therefore, a series of animals, each fixed with a different concentration of glutaraldehyde, is used for every initial study of a new brain region.

Tissue sectioning

Specimens of a particular brain region are blocked from the brain and are sectioned on either a Sorvall TC-2 tissue sectioner or the Oxford Vibratome. The Sorvall instrument is limited in that it can only accommodate small tissue blocks that have a height less than the 3 mm edge of the razor blade. However, this instrument can cut sections rapidly at thicknesses that range from 50 to 200 μm . In contrast, the vibratome is slower for cutting sections but is able to section a much larger block face. All sections are collected in fresh buffer at 4° C before processing for GAD immunocytochemistry.

Immunocytochemical procedure

The immunocytochemical procedure employed for tissue used in electron microscopy was similar to that previously described (Barber & Saito, 1976; McLaughlin *et al.*, 1974, 1975; Wood *et al.*, 1976; Ribak *et al.*, 1976, 1977, 1978; Vaughn *et al.*, 1981). Briefly, sections are incubated in normal rat serum for 1 h and then rinsed in phosphate buffer before being incubated for 1 h in either rabbit anti-GAD serum or control rabbit serum. Following a 2.5 h buffer wash, the sections are incubated 1 h in goat anti-rabbit serum (Antibodies Inc, Davis, California). Sections are then washed in buffer for 2.5 h, incubated in a peroxidase–antiperoxidase Fab complex (Slemmon *et al.*, 1980) for 1 h and washed again in buffer for 2.5 h before being reacted with 3,3'-diaminobenzidine-4HCl (Sigma, St. Louis, Missouri) and H_2O_2 for 30 min. Following the immunocytochemical reactions, the sections are washed for 30 min in buffer. The selected brain regions are dissected from the sections, post-fixed in OsO_4 , *en bloc* stained in aqueous uranyl acetate, dehydrated and plastic embedded.

Sometimes the immunocytochemical reagents do not penetrate deeply into the tissue sections and such specimens need to have microfractures induced by freezing and thawing. Briefly, the sections are rinsed for 1 h in buffer and placed into a 30% cryoprotectant sucrose solution at 4° C for 24 h. The sections are held in aluminum foil and rapidly frozen in either liquid Freon or liquid propane cooled by liquid nitrogen. After being frozen, the sections are thawed by placing them in buffer at 4° C. These sections can be stored in 4° C buffer for several weeks without damaging the tissue or the antigenicity. Another manner used to improve penetration of staining is the sectioning of a brain region in different planes (coronal, sagittal and horizontal). With this approach, the optimal orientation will have a maximum number of neurones in contact with the cut surfaces.

Ultra-thin sectioning

Before ultra-thin sections are obtained from a block, the intensity of staining is verified in 1 μm semi-thin sections in the light microscope. Due to the limited penetration of immunochemical reagents, only a few semi-thin sections should be cut before taking ultra-thin sections. These latter sections are cut with a diamond knife, and ribbons of sections are picked up on Formvar-coated slot grids to allow for an examination of the entire section and to follow stained profiles in a series of sections. The use of serial sections requires more time in the examination of immunocytochemical preparations but it provides better examples of stained profiles than random

sections mounted on mesh grids. For example, a GAD-positive terminal may not show nice parallel membranes in one section, but the adjacent section may show the same terminal forming a clearly defined synapse. Sometimes, the use of serial sections is mandated in regions of the brain where stained profiles are involved in complex serial synaptic relationships that must be reconstructed from a number of sections (see Ribak *et al.* 1977). Thus, it is important to utilize serial sections routinely for ultrastructural immunocytochemical studies.

The staining of ultra-thin sections of the C.N.S. with uranyl acetate and lead citrate greatly facilitates a comprehensive analysis of the neuropile in electron micrographs of immunocytochemical preparations. However, it is advisable to omit such staining from some sections so that one can determine whether certain cellular profiles are made dense by immunocytochemical staining or by the routine heavy atom staining. It must be emphasized that heavy atom staining cannot be omitted altogether because it is necessary to use OsO₄ to stabilize and enhance the DAB polymer formed by the action of peroxidase. Immunocytochemical controls obviously play a primary role in determining if the staining of a profile is due to specific immunoperoxidase reaction product. Therefore, sections of control tissue should also be examined both with and without staining in uranyl acetate and lead citrate.

Results

The following account reviews the immunocytochemical localization of GAD in electron microscopical preparations from various regions in the brain. These results are presented in order to illustrate the various technical modifications and improvements which were needed to describe the role that these GAD-positive neurones had in the local synaptic circuitry. In this manner, these ultrastructural results are able to show more information than that obtained from light microscopical preparations which only provide the location of positive cell bodies and/or the distribution of stained fibres, whether they be axons or dendrites. Emphasis in this presentation of results will be placed on the importance of pre-treatments of animals for the elucidation of synaptic connections.

Substantia nigra

Of all the regions studied in the brain and spinal cord, the substantia nigra was found to have the largest amount of GAD-positive reaction product per volume of tissue. In light microscopical preparations, the GAD-positive staining appeared as continuous processes with large varicosities that either encircled nonstained areas or formed parallel chains (Fig. 1). The significance of this staining pattern was revealed by the electron microscopical analysis.

Our initial ultrastructural observations on the substantia nigra showed that using 0.1% glutaraldehyde in the fixative solution did not produce adequate ultrastructural preservation. However, the GAD-positive reaction product was localized to specific axon terminals in these preparations thereby confirming that the GAD in this tissue was antigenic. Although previous immunocytochemical studies of GAD localization in the cerebellum, retina and spinal cord have successfully employed 0.1% glutaraldehyde in the fixative (McLaughlin *et al.*, 1974, 1975; Wood *et al.*, 1976), some parts of the C.N.S.

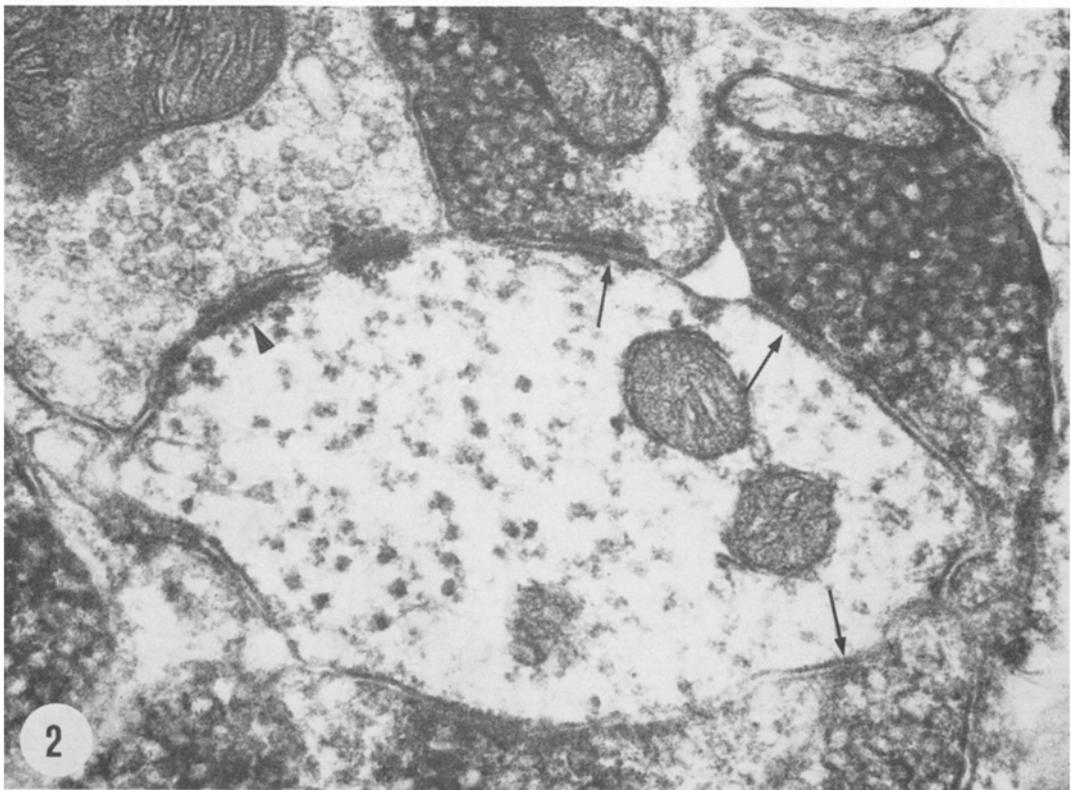
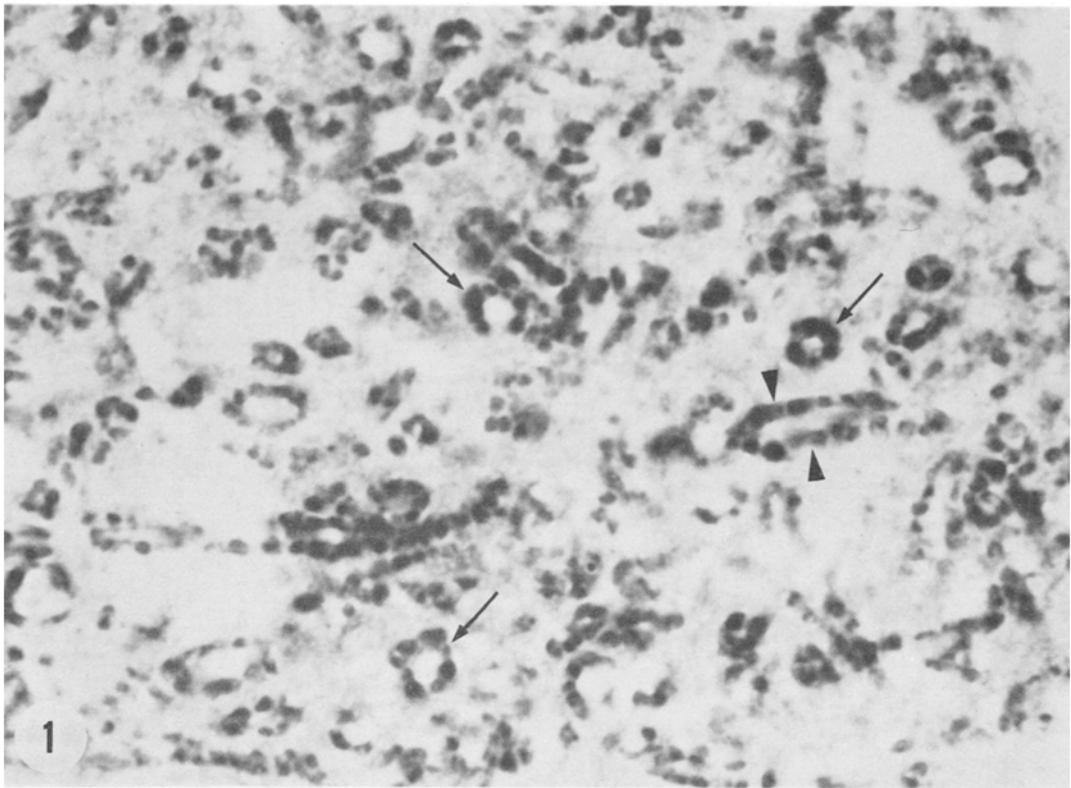
require greater concentrations of glutaraldehyde than this in order to achieve adequate ultrastructural preservation. In order to correct this defect without adversely affecting the specificity of the immunocytochemistry, a series of experiments was carried out using graded concentrations of glutaraldehyde 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 staining and ultrastructural preservation (Figs. 2–4). These latter preparations show dendrites surrounded by axon terminals that contain GAD-positive reaction product (Fig. 2). This distribution coincides with the location of the rings of GAD-positive staining in the light microscopical preparations.

A majority of GAD-positive axon terminals form symmetrical synaptic junctions with the dendritic profiles, although it is not uncommon to observe GAD-positive terminals forming asymmetrical synaptic junctions. An estimate of the relative proportions of GAD-positive terminals that form symmetrical and asymmetrical 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 symmetrical synaptic junctions while approximately 15% formed asymmetrical 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. McLaughlin *et al.*, 1975).

In accord with the light microscopical distribution of GAD-positive puncta, some GAD-positive axon terminals also were observed to be pre-synaptic to somata. These GAD-positive terminals all formed symmetrical synaptic junctions and were not nearly so numerous as those that were pre-synaptic to dendrites. In control sections of the substantia nigra, no GAD-positive reaction product was observed in axon terminals (Fig. 5). The results of this study were consistent with biochemical, pharmacological and physiological data which previously had indicated that neurones of the neostriatum and globus pallidus could exert a GABA-mediated post-synaptic inhibition on the neurones of the substantia nigra.

Fig. 1. Semi-thin (1 μm) section of the pars reticulata from a slice incubated in anti-GAD serum. Rings of GAD-positive staining (arrows) enclose unstained areas that are shown to be dendrites in Fig. 2. Also shown are parallel GAD-positive chains of staining (arrowheads) that indicate GAD-positive terminals are apposed to longitudinally sectioned dendrites. $\times 2000$.

Fig. 2. Electron micrograph of substantia nigra incubated in anti-GAD serum showing axon terminals filled with GAD-positive reaction product. These terminals form symmetrical synapses (arrows) with a dendritic shaft. The unstained terminal contains round synaptic vesicles and forms an asymmetrical synapse (arrowhead) with the dendritic shaft. $\times 67\ 000$. Figs. 1 and 2 are reproduced with permission from Ribak *et al.* (1976).



Olfactory bulb

In semi-thin, 1 μm sections of the olfactory bulb, GAD-positive reaction product occurred in the perikaryal cytoplasm of cells in the glomerular and granule cell layers. In addition, GAD-positive reaction product was distributed throughout all cell layers in the form of punctate structures (Figs. 6–8). The sizes of the puncta were similar to those in the cerebellum and spinal cord which have been shown to correspond to synaptic terminals containing GAD-positive reaction product (McLaughlin, 1974, 1975; Wood *et al.*, 1976). However, the highest density of these puncta was found in the glomerular and external plexiform layers where few axon terminals form synapses because most synapses are made by dendrites in those layers (see Shepherd, 1972, for a review).

Observations made with the electron microscope confirmed the presence of GAD-positive reaction product within the somatic and dendritic cytoplasm of granule and periglomerular cells. In addition, the cell types of origin of the GAD-positive puncta in the external plexiform and glomerular layers were identified in electron microscopical preparations using a combination of both random and serial sections.

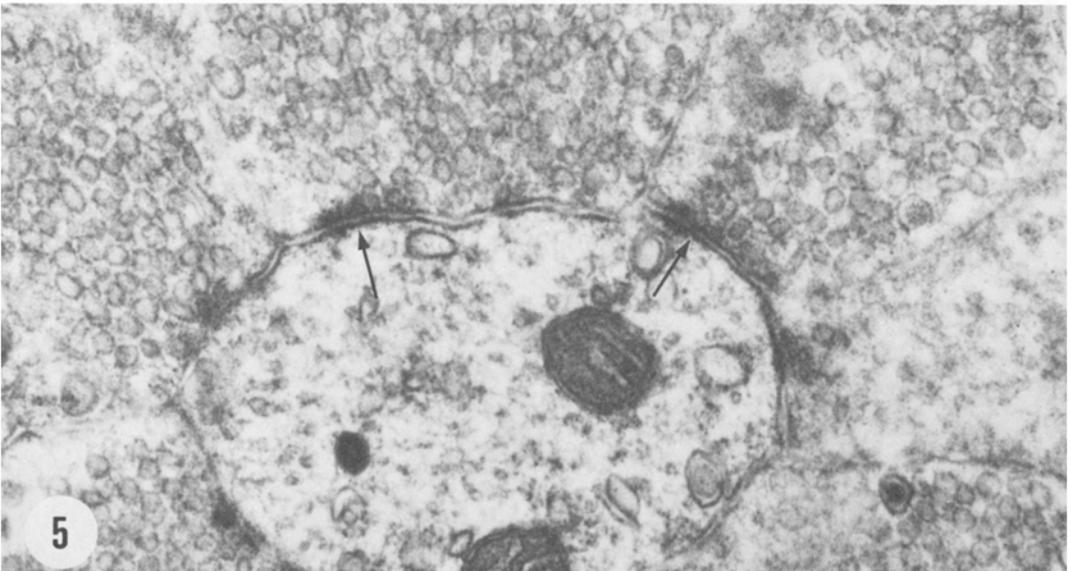
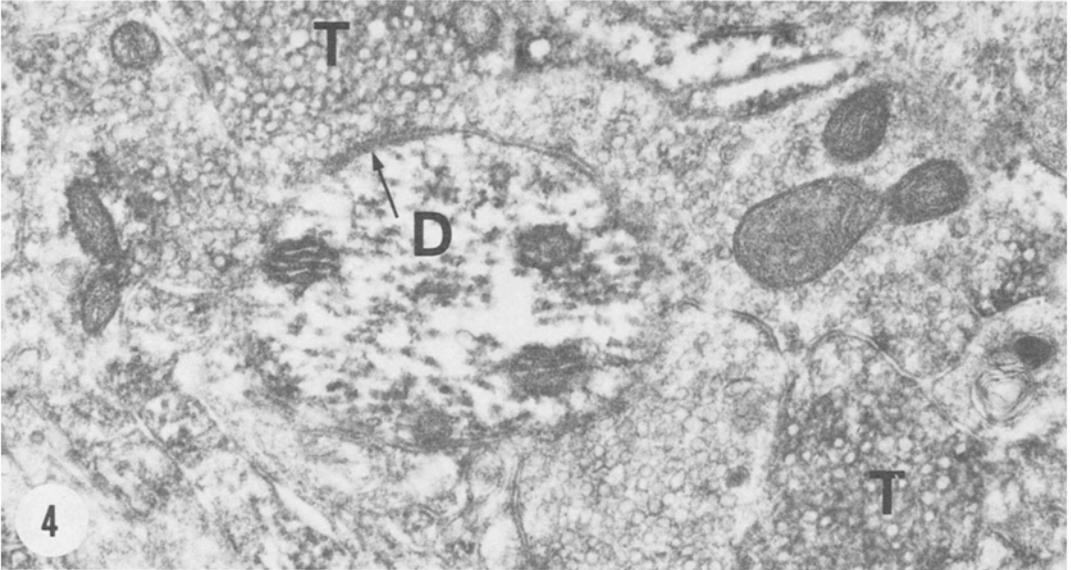
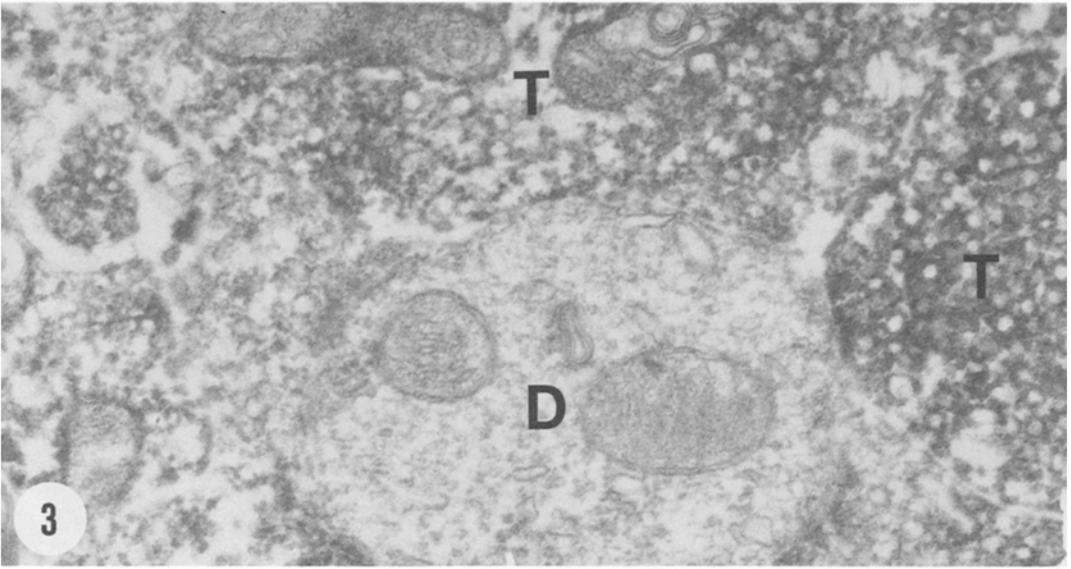
Most of the granule cells in the granule cell layer contained reaction product in their somata and proximal dendrites. The highest concentration of somal reaction product occurred upon the surfaces of the cisternae and small vesicles of the Golgi apparatus and along the surfaces of mitochondria and microtubules. In contrast, the somata and dendrites of the short-axon cells of the granule cell layer and of the mitral cells did not exhibit reaction product in specimens incubated in anti-GAD serum.

The dendrites of granule cells ascend into the external plexiform layer where they bear a number of spine-like structures, or gemmules, upon their surfaces. GAD-positive reaction product was observed within these dendrites and gemmules which contained flattened synaptic vesicles and formed reciprocal synaptic junctions with the shafts of

Fig. 3. Electron micrograph of substantia nigra incubated in anti-GAD serum from a specimen perfused with 0.1% glutaraldehyde in the fixative solution. GAD-positive reaction product appears dense in two axon terminals (T) adjacent to an unstained dendrite (D). No synaptic specializations are observed because the membranes were not preserved by the fixative. Another sign of poor fixation is the disruption of membranes within the mitochondria. $\times 60\ 000$.

Fig. 4. Electron micrograph of substantia nigra incubated in anti-GAD serum from a specimen perfused with 1.0% glutaraldehyde in the fixative solution. The number of terminals with dense reaction product (T) is reduced but one of the GAD-positive terminals is shown to form a symmetrical synapse (arrow) with a dendrite (D). Although better fixation was obtained with this preparation, non-specific reaction product was found in dendrites (D) that lack staining in preparations with lower glutaraldehyde concentrations (Figs. 2, 3). $\times 30\ 000$.

Fig. 5. Electron micrograph of substantia nigra incubated in control rabbit serum from a specimen perfused with 0.4% glutaraldehyde in the fixative solution. All of the terminals lack reaction product, although two of them form symmetrical synapses (arrows) with the dendrite. $\times 61\ 000$. Reproduced with permission from Ribak *et al.* (1976).



mitral cell dendrites. GAD-positive reaction product was concentrated in the part of gemmule where the synaptic vesicles were clustered (the granule-to-mitral synapse), and was absent from the area of the gemmule where the mitral-to-granule synapse occurs. The mitral-to-granule synapse had no GAD-positive reaction product associated with the round synaptic vesicles aggregated at the presynaptic membrane of the mitral dendrite. These findings in the external plexiform layer emphasize the importance of ultrastructural immunocytochemistry because such synaptic relationships are not possible to observe or infer at the light microscopical level.

The somata and dendrites of many periglomerular cells were stained with GAD-positive reaction product that had a similar intracellular distribution to that found within the somata of the granule cells, that is, the highest concentration of reaction product occurred around the cisternae and vesicles of the Golgi apparatus. The GAD-positive periglomerular cells also showed reaction product in their proximal dendrites which entered the glomerular neuropile. In addition, the distal dendrites and gemmules of these cells were identified with serial sections (Figs. 9, 10) which showed them entering into reciprocal synaptic relationships with mitral/tufted dendrites and also involved in serial synaptic relationships. These findings support our contention that serial sections are very useful for immunocytochemical studies. Furthermore, our data that show GAD

Figs. 6–8. Semi-thin ($1\ \mu\text{m}$) sections of various layers of the olfactory bulb from a tissue slice incubated in anti-GAD serum.

Fig. 6. Somal and dendritic staining in a periglomerular cell (p) in the glomerular layer (GL). The GAD-positive dendrite (arrowhead) is directed toward a glomerulus which contains many GAD-positive puncta (arrows). $\times 2000$.

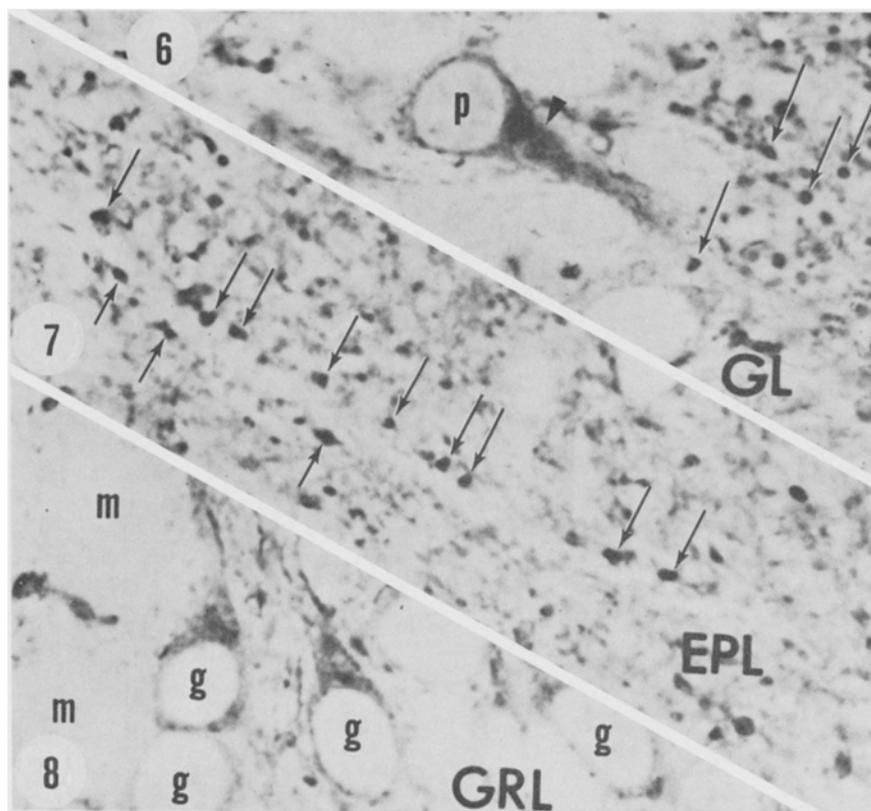
Fig. 7. GAD-positive puncta (arrows) lined up adjacent to a longitudinally sectioned, mitral cell secondary dendrite in the external plexiform layer (EPL). $\times 2000$.

Fig. 8. Reaction product within the somata and dendrites of granule cells (g) in the granule cell layer (GRL). The adjacent somata of mitral cells (m) do not exhibit this staining. $\times 2000$.

Figs. 9 and 10. Electron micrographs from serial sections of a glomerulus in olfactory bulb incubated in anti-GAD serum. These micrographs show a serial synaptic arrangement involving a periglomerular cell dendrite.

Fig. 9. A periglomerular cell dendritic shaft (d_1) containing synaptic vesicles, mitochondria and GAD-positive reaction product. This dendrite (d_1) is presynaptic to a mitral/tufted dendritic shaft (m/t) and to two other dendritic shafts of unknown origin (d_2 and d_3). The arrows indicate the polarity of each synapse. $\times 35\ 000$.

Fig. 10. An adjacent section showing the same GAD-positive periglomerular cell dendritic shaft (d_1) as in Fig. 7. In this section, the GAD-positive dendrite (d_1) appears to be post-synaptic to an axon terminal (t) that is probably derived from a centrifugal fibre. Also, d_1 is shown to be presynaptic to the same small dendritic profile (d_3) as in Fig. 7. Since this periglomerular cell dendrite is presynaptic to three dendritic profiles and post-synaptic to both an axon terminal and, as determined in another part of the series, the mitral/tufted dendrite (m/t), this dendrite then forms part of a serial synaptic arrangement. $\times 35\ 000$. Figs. 6–10 are reproduced with permission from Ribak *et al.* (1977).



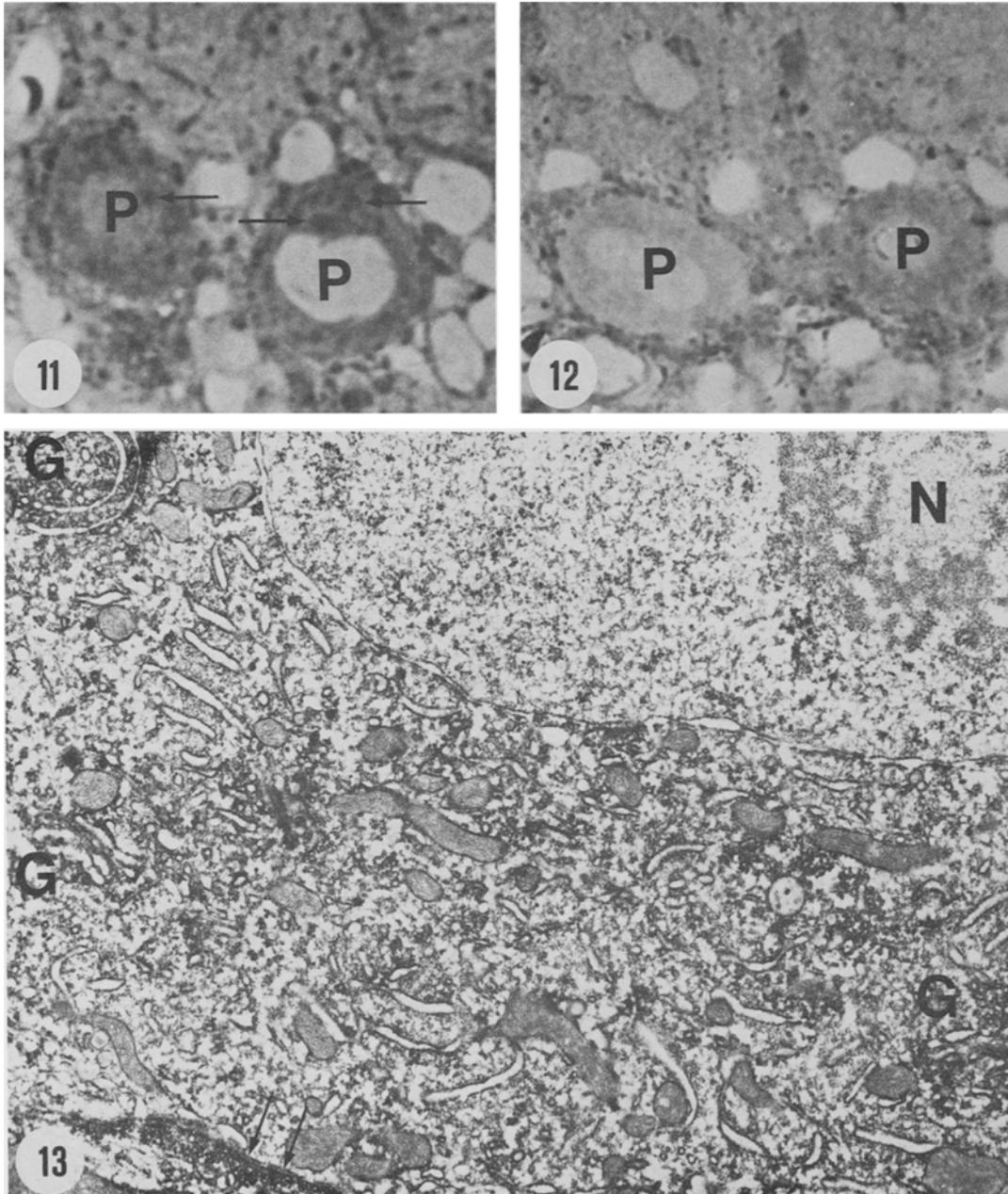


Fig. 11. Semi-thin ($1\ \mu\text{m}$) section of colchicine-injected cerebellum incubated in anti-GAD serum. The GAD-positive reaction product appears as indistinct clumps (arrows) in the somata of Purkinje cells (P). These clumps may correspond to the sites of the Golgi complex since this organelle is associated with concentrated GAD-positive reaction product (see Fig. 13). $\times 1100$.

Fig. 12. Semi-thin ($1\ \mu\text{m}$) section of non-injected cerebellum incubated in anti-GAD serum. The Purkinje somata (P) lack GAD-positive reaction product. $\times 1100$.

within both granule and periglomerular neurones strongly support suggestions based on physiological and pharmacological studies that these inhibitory local circuit neurones use GABA as their neurotransmitter.

Cerebellum

The findings in the olfactory bulb were the first to demonstrate the somata and dendrites of GABAergic neurones with immunocytochemical methods and proved to be important for the identification of the somata of GABAergic neurones in the cerebellum and subsequent brain regions that were studied. Thus, GAD within somata and dendrites in the olfactory bulb was in an antigenic form similar to that found previously in axon terminals, and the reason that somal GAD was not present in these other studies (McLaughlin *et al.*, 1974, 1975; Ribak *et al.*, 1976) was due to very low GAD concentrations. The suggested cause for this low amount of GAD within somata found in these other brain regions was that these somata were from neurones which had exclusively presynaptic axons. Therefore, rapid axonal transport of newly synthesized GAD from somata to axon terminals in these neurones probably prevented the accumulation of detectable concentrations of GAD-positive reaction product within their somata and dendrites. The following results in the cerebellum demonstrate the usefulness of colchicine pre-treatment for the inhibition of axoplasmic transport and the subsequent GAD staining of neuronal somata and dendrites.

In light microscopical preparations of colchicine-injected cerebellar cortex, GAD-positive reaction product was found in puncta distributed throughout the layers of the cerebellar cortex in the pattern described previously (McLaughlin *et al.*, 1974). In addition, the proximal dendrites and somata of Purkinje (Figs. 11, 12) and Golgi II neurones and the somata of basket and stellate cells contained detectable accumulations of GAD-positive reaction product. The reaction product appeared in the cytoplasm of the somata and dendrites but was not present within the nuclei, and in all cases, the majority of the GAD-positive somata were located within 100–500 μm of the injection site.

Electron microscopical preparations of colchicine-injected cerebellar specimens that were incubated in anti-GAD serum contained reaction product in certain pre-synaptic terminals as described previously (McLaughlin *et al.*, 1974). These preparations also contained GAD-positive reaction product within the somata and dendrites of Purkinje, stellate, basket and Golgi II neurones. The distribution of the reaction product within somata and dendrites of these neurones was similar to that observed in the somata and dendrites of the granule and periglomerular cells of the olfactory bulb. The highest concentration of reaction product occurred adjacent to the cisternae and vesicles of the

Fig. 13. Electron micrograph of a Purkinje soma from a colchicine-injected cerebellum incubated in anti-GAD serum. The electron-opaque, GAD-positive reaction product appears to be most concentrated around the cisternae of the Golgi apparatus (G). Neither the nucleus nor the nucleolus (N) contain GAD-positive reaction product. A GAD-positive axon terminal of a basket neurone forms a synaptic junction (arrows) with the Purkinje soma. $\times 17\,500$.

Golgi apparatus (Fig. 13). These findings indicate that the Golgi apparatus may be a way-station for newly synthesized GAD prior to its axoplasmic transport to presynaptic terminals.

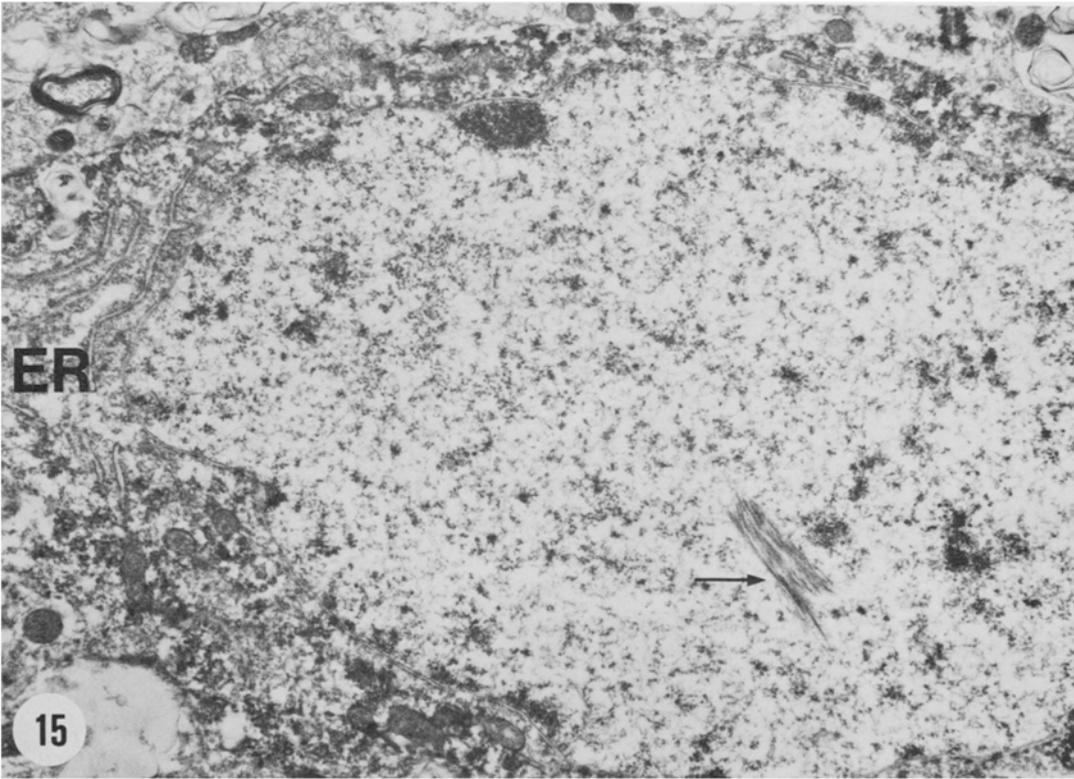
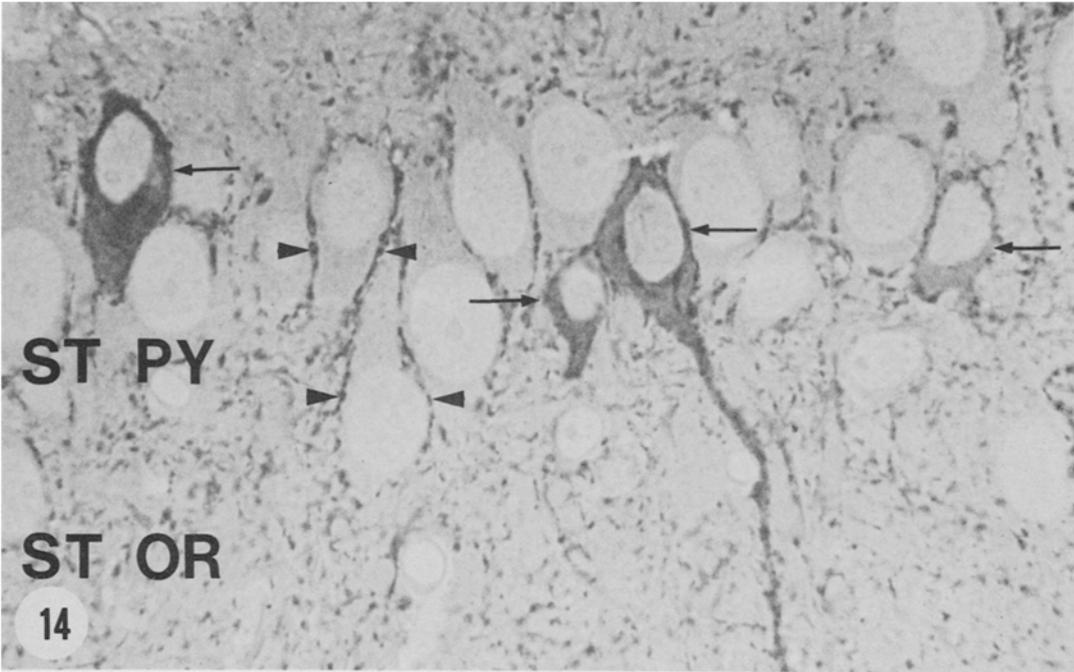
The results of this study indicate that the use of colchicine produces detectable concentrations of GAD in the somata and dendrites of neurones which previously had detectable concentrations limited to their axon terminals. Thus, the four neuronal types previously described to have GAD within axon terminals, that is, Purkinje, Golgi II, stellate and basket neurones, were shown to contain GAD within their cell bodies following colchicine injections into the cerebellum. These findings are consistent with physiological and pharmacological data which show that: (1) Purkinje cells project out of the cerebellar cortex to inhibit neurones in Deiters nucleus and the deep cerebellar nuclei monosynaptically; (2) basket cells make inhibitory local circuit synapses with the somata, axon hillocks and initial axon segments of Purkinje cells; (3) stellate cells form inhibitory local circuit synapses with the dendrites of Purkinje cells; and (4) Golgi II cells make inhibitory local circuit connections with dendrites of granule cells.

Hippocampus

Colchicine-injected preparations were utilized in this study to show the somata and dendrites of the GABAergic neurones in the hippocampus. The results of a previous study had shown that the GAD-positive puncta were distributed throughout the hippocampus and that some of these puncta formed pericellular baskets around the somata of the hippocampal projection neurones, pyramidal and granule cells (Barber & Saito, 1976). The results obtained with colchicine-injected specimens (Ribak *et al.*, 1978) showed the location of the GABAergic, hippocampal neurones including the basket cells that distribute their axon terminals around pyramidal and granule cell somata (Fig. 14). Thus, GAD-positive reaction product was found in the somata of (1) polygonal cells in st. oriens, (2) horizontal cells at the border with the alveus, (3) pyramidal basket cells in st. pyramidale, (4) horizontal and other short-axon cells in st. radiatum and lacunosum, (5) pyramidal basket cells and other short-axon cells in the polymorph layer of the dentate gyrus, and (6) short-axon cells in the molecular layer of

Fig. 14. Semi-thin ($1\ \mu\text{m}$) section from a colchicine-injected Ammon's horn incubated in anti-GAD serum. The GAD-positive somata of presumptive pyramidal basket neurones (arrows) are interspersed in the pyramidal layer (ST PY) with the unstained somata of hippocampal pyramids. One of the GAD-positive somata has a stained dendrite extending into stratum oriens (ST OR). Numerous GAD-containing puncta (arrowheads) that probably correspond to basket axon terminals surround the pyramidal neurones. Many dense, GAD-positive puncta are also present in strata radiatum and oriens. $\times 900$.

Fig. 15. A GAD-positive neuronal soma located in stratum oriens. The opaque GAD-positive reaction product is sparsely scattered throughout the perikaryal cytoplasm but it is absent from the region of granular endoplasmic reticulum (ER). The nucleus contains an intranuclear rod (arrow). $\times 12\ 000$. Figs. 11–15 are reproduced with permission from Ribak *et al.* (1978).



the dentate gyrus. The somata of pyramidal and granule cells did not contain GAD-positive reaction product.

Electron microscopical preparations of specimens from colchicine-injected hippocampus that were incubated in anti-GAD serum, contained reaction product in axon terminals, somata and dendrites. Some of the GAD-positive axon terminals formed symmetrical synaptic junctions with pyramidal and granule cell somata and their dendritic shafts. The GAD-positive terminals forming axo-somatic synapses corresponded in location to the basket cell endings as described previously (Blackstad & Flood, 1963). The location of GAD-positive somata in electron microscopical preparations coincided with their distribution in light microscopical preparations. The distribution of reaction product within GAD-positive somata of basket and other short-axon neurones was similar to the observations in the neurones of the cerebellar cortex (Fig. 15 and cf. Fig. 13). Electron microscopical examination confirmed the light microscopical observations that reaction product was absent from pyramidal and granule cell somata and from all neurones of the hippocampus in sections incubated with control serum. These results indicate that the GABAergic neurones in the hippocampus are basket and other short-axon neurones (Cajal, 1911; Lorente de Nó, 1934).

Spinal cord

The localization of GAD in the spinal cord with immunocytochemical methods demonstrated GAD-positive axon terminals throughout the grey matter with high concentrations in the dorsal horn laminae I–III (McLaughlin *et al.*, 1975). Electron microscopical preparations revealed that GAD-positive terminals were presynaptic to dendrites, cell bodies, and other axon terminals. The axo-axonal synapses were most numerous in laminae II and III but those terminals forming axo-axonal synapses in the ventral horn were presynaptic to larger axon terminals, which were in turn presynaptic to motor neurone somata. GAD-positive synaptic terminals were also presynaptic to large and small dendrites and motor neurone somata in the ventral horn. The observation of GAD-positive terminals presynaptic to dendrites and cell bodies in both dorsal and ventral horns was compatible with the evidence suggesting that GABA terminals may mediate post-synaptic inhibition of spinal interneurons and of motor neurones. However, the significance of the terminals that form axo-axonal synapses could not be determined in these normal preparations.

The use of anterograde degeneration as an experimental pre-treatment provided preparations that aided in the understanding of the synaptic circuitry of the GABAergic axo-axonal synapses (Barber *et al.*, 1978). Multiple dorsal rhizotomies were performed unilaterally at lumbar levels L1–L4 in adult rats, and the spinal cords were examined 24–48 h later. Large numbers of degenerating terminals were observed in the ipsilateral substantia gelatinosa, but not contralaterally. In contrast, the distribution of GAD-positive reaction product appeared to be normal on both sides of the cord. In electron microscopical preparations, primary afferent terminals were found in various states of degeneration on the side of the interruption of the dorsal roots, and GAD-positive axon

terminals were found to be pre-synaptic to degenerating primary afferent terminals in the substantia gelatinosa. These data indicate that the presynaptic inhibition of primary afferents is mediated by axo-axonal synapses formed between GABA-releasing local circuits, neurones and primary sensory neurones. Furthermore, the various synaptic relationships of GAD-positive terminals in the dorsal horn of the rat spinal cord has led to reasonable hypotheses about how release of GABA from these terminals could participate in such presynaptically modulated phenomena as primary afferent depolarization, the dorsal root reflex, and primary afferent hyperpolarization (Barber *et al.*, 1978).

Cerebral cortex

The localization of GAD to the GABAergic neurones in the neocortex utilized specimens that were pre-treated with colchicine injections. This extensive electron microscopical analysis was made in the rat visual cortex (Ribak, 1978) but results in other cortical areas in the rat (Ribak, 1978) and in the monkey (Ribak *et al.*, 1979a) indicate that similar conclusions can be made for neocortex in general. Briefly, GAD-positive reaction product was observed in somata, proximal dendrites and axon terminals of non-pyramidal neurones in all cortical layers, including the immediately subjacent white matter. In electron microscopical preparations, GAD-positive axon terminals formed symmetrical synaptic junctions most commonly with dendritic shafts and somata of all types of cortical neurones, and less frequently with dendritic spines and initial axon segments of pyramidal neurones. The concentration of GAD-positive terminals adjacent to the pyramidal neurones of layers III and V indicated that the pericellular basket plexus derived from short-axon stellate neurones (Cajal, 1911; Marin-Padilla, 1969) was composed of GABAergic inhibitory terminals. The intracellular distribution of GAD-positive reaction product within the somata of stellate neurones was similar to that observed in other brain regions. In addition, these GAD-positive stellate neurones had characteristics similar to those described by others for cortical aspiny and sparsely spiny stellate neurones (for example, Peters & Fairén, 1978). These features included a mixture of asymmetrical and symmetrical synapses upon their somata and dendritic shafts, dendrites of small diameter that lacked spines, and terminals that formed symmetrical synapses (Figs. 16, 17). These findings in combination with physiological and pharmacological data indicate that these local circuit neurones tonically mediate GABAergic inhibition in the neocortex.

In a subsequent study of epileptic cerebral cortex in monkeys, experiments were planned to test whether a decrease in the number of inhibitory axon terminals could lead to seizure activity of pyramidal neurones (Ribak *et al.*, 1979a). Electron microscopical analyses revealed that the GAD-positive punctate structures in light microscopical preparations were equivalent to GAD-positive axon terminals that formed symmetrical synaptic junctions. A comparison was made of the number of GAD-positive puncta in non-epileptic sensorimotor cortex with those found in epileptic cortex from monkeys treated with alumina gel. The results showed a significant decrease of GABAergic

terminals at sites of seizure foci and, therefore, suggested that such a decrease could be responsible for the observed epileptic activity of cortical pyramidal neurones.

Corpus striatum

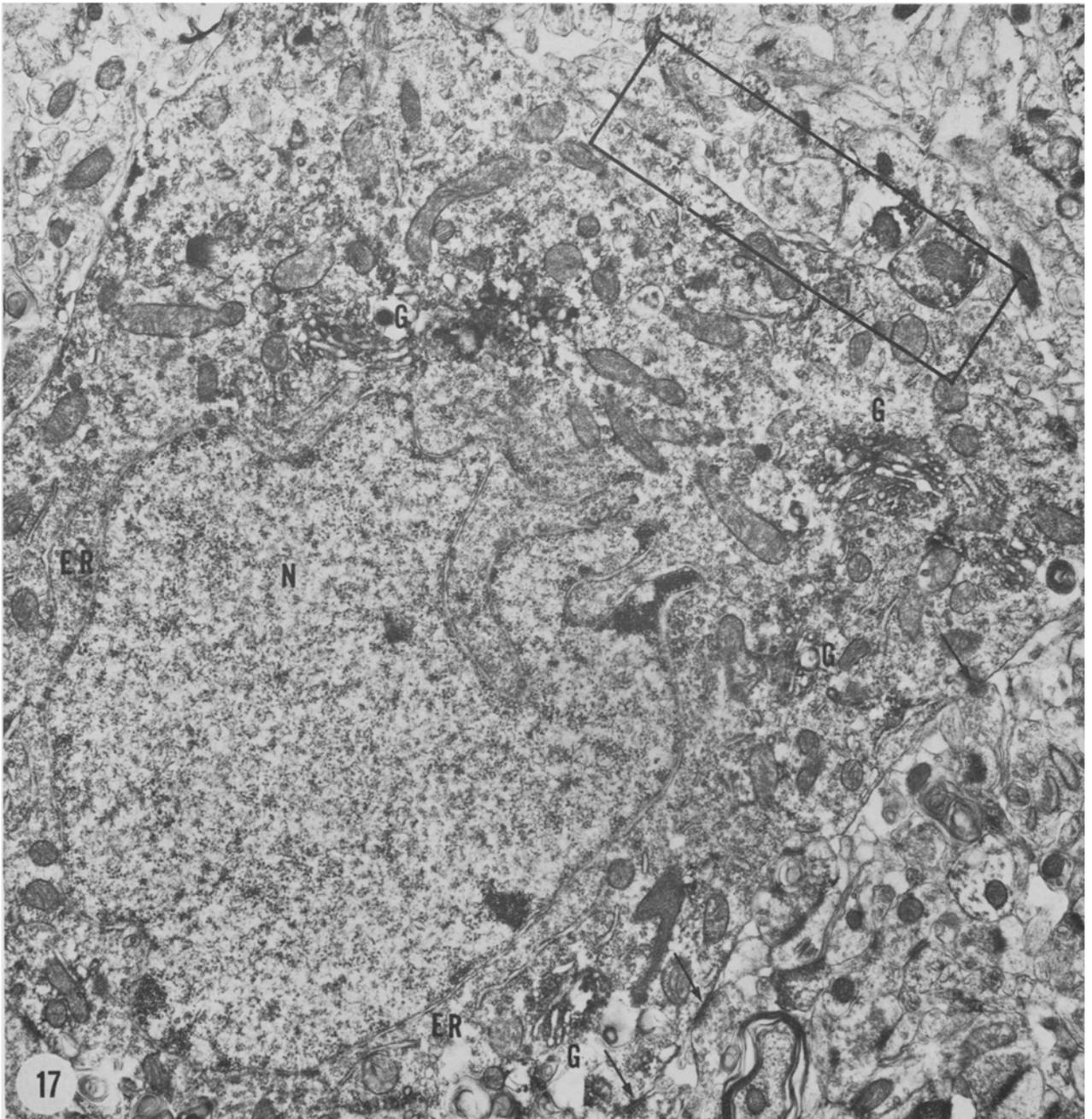
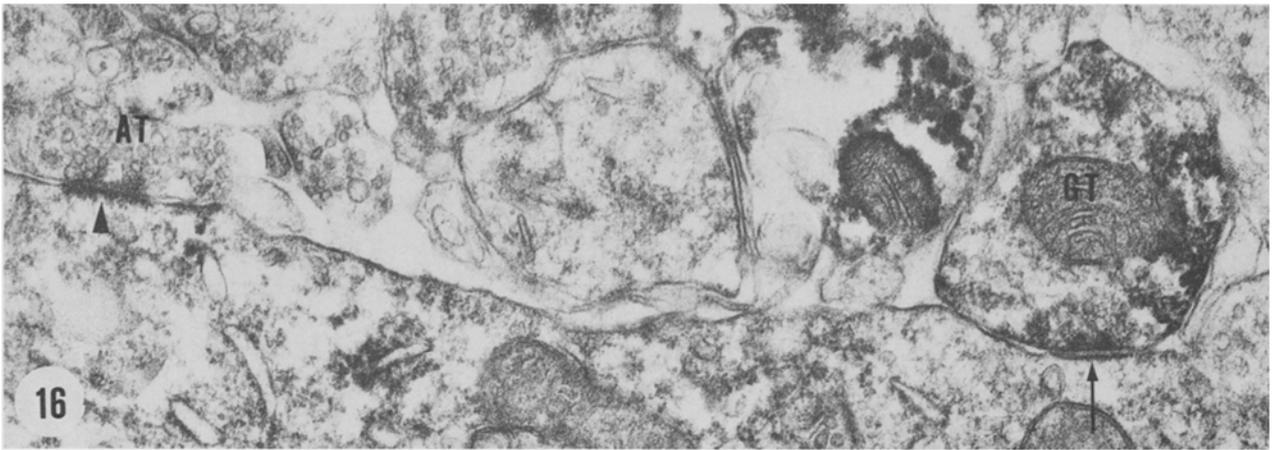
An analysis of the GAD-containing neurones in the rat neostriatum, pallidum and entopeduncular nucleus was made to investigate the sources of the GABAergic projection to the substantia nigra (Ribak *et al.*, 1979b). A large amount of GAD-positive reaction product was observed in both the pallidum and entopeduncular nucleus in light microscopical preparations and was localized ultrastructurally to axon terminals that surrounded dendrites and large somata. In the neostriatum the relative numbers of GAD-positive axon terminals per unit area were substantially less than in the pallidum. GAD-positive terminals predominantly formed symmetrical synapses with somata, dendrites and spines, but a small number of them formed asymmetrical synapses with either dendrites or spines. The presence of GAD within these terminals is consistent with results of other investigations which have indicated that the striatopallidal and striatoentopeduncular pathways as well as neostriatal local circuit neurones and/or collaterals from neostriatal projection neurones, use GABA as a neurotransmitter.

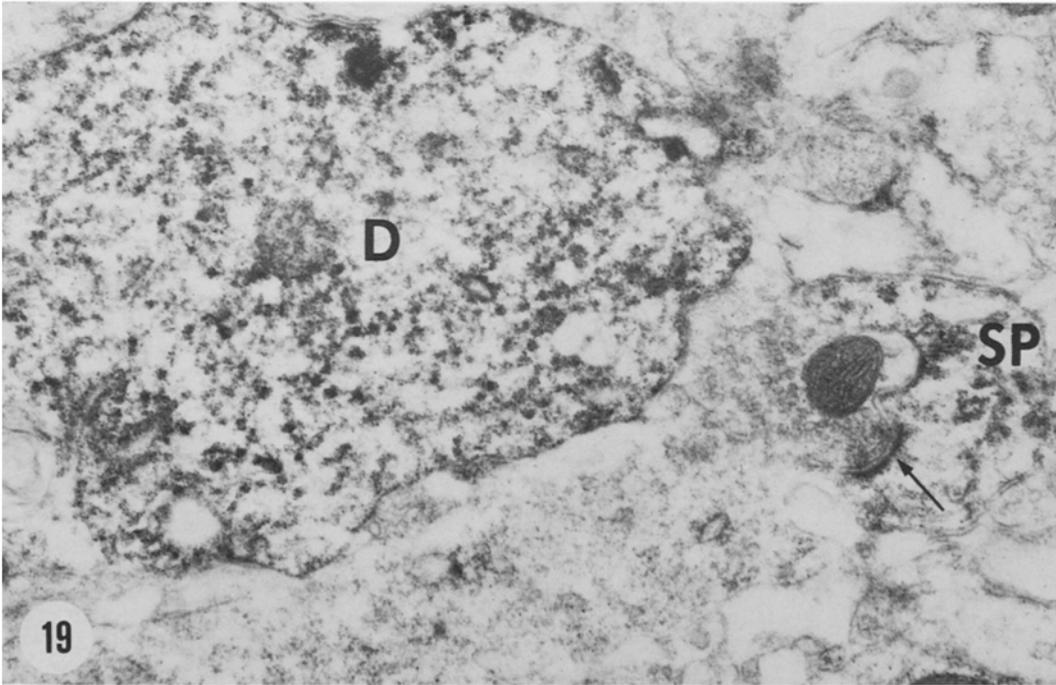
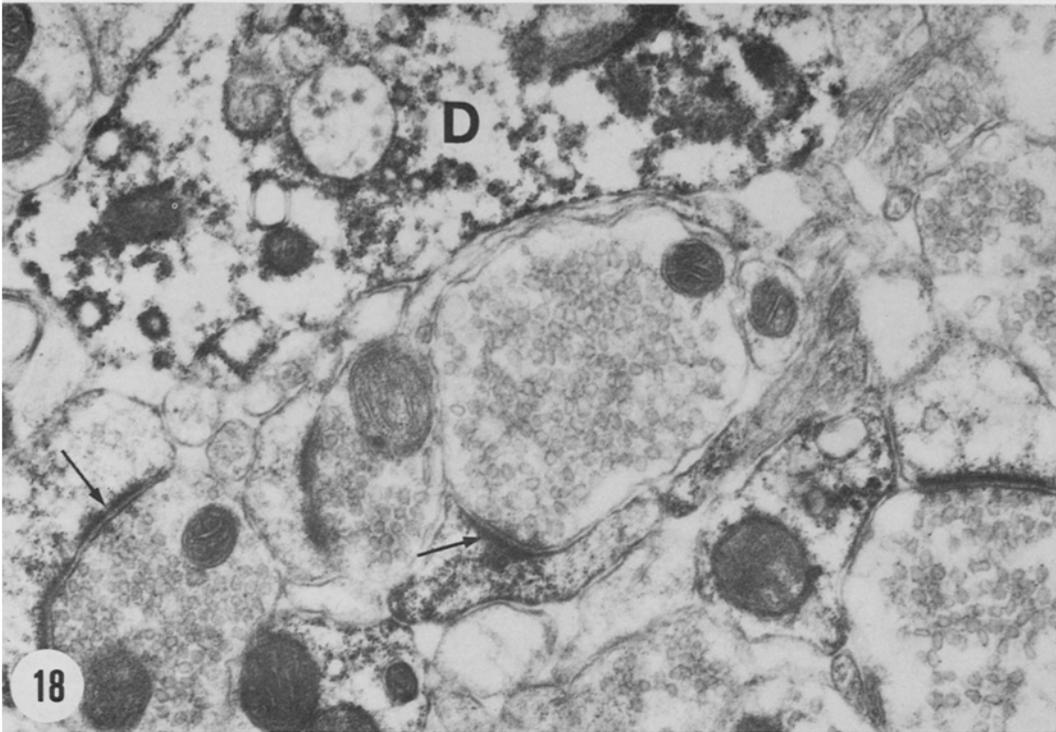
GAD-positive reaction product was also localized within the somata and dendrites of neostriatal and pallidal neurones in colchicine-injected preparations. The GAD-positive somata in the pallidum were medium-sized neurones and since such cells project to the substantia nigra, our results are in agreement with those from other studies which demonstrate a GABAergic, pallidonigral pathway (Hattori *et al.*, 1975). In the neostriatum, GAD-positive somata were identified light microscopically as medium-sized neurones with either round or fusiform shapes. Electron microscopical examinations also showed GAD-positive reaction product within the perikaryal and dendritic cytoplasm of these neurones, as well as in dendritic spines (Figs. 18, 19). These findings are in accord with the results of studies which have indicated that medium-sized, spinous

Figs. 16 and 17. Electron micrographs of a GAD-positive soma that was identified as an aspiny stellate cell in layer IV of visual cortex.

Fig. 16. A higher magnification of a portion of this soma (indicated by the box in Fig. 17) which shows an example of the two types of axo-somatic synaptic junctions located on cortical GAD-positive somata. One axon terminal (GT) contains GAD-positive reaction product and forms a symmetric synaptic junction (arrow). The other axon terminal (AT) lack reaction product and forms an asymmetrical synaptic junction (arrowhead). $\times 46\ 000$.

Fig. 17. This large cortical soma is shown to contain an infolded nucleus (N), scattered cisternae of granular endoplasmic reticulum (ER), and cisternae of Golgi complex (G) that have GAD-positive reaction product concentrated around them. In addition to the synaptic junctions shown in Fig. 14, this soma forms asymmetrical synaptic junctions (arrows) with axon terminals which lack reaction product. $\times 15\ 000$. Figs. 16 and 17 are reproduced with permission from Ribak (1978).





neurones of the neostriatum give rise to a GABAergic striatonigral pathway (for example, Kitai, *et al.*, 1979).

Discussion

The use of immunocytochemistry at the electron microscopical level has revealed the complex synaptic configurations of GABAergic neurones in various regions of the brain and spinal cord. The types of synapses that involved GAD-positive, vesicle-containing profiles included axo-somatic, axo-dendritic, axo-axonal and dendro-dendritic synapses. The exclusive use of light microscopical immunocytochemical preparations would have failed to demonstrate this variety of synaptic arrangements. Therefore, electron microscopical immunocytochemistry provides the resolution for determining the possible functional significance of labelled structures in the C.N.S.

Experimental pre-treatment of specimens to be used for immunocytochemical studies can add more functional relationships than that provided by normal preparations. The use of colchicine injections has identified the somata and dendritic shafts of GABAergic neurones. In addition, the use of anterograde degeneration has aided in describing the functional variety of GABAergic synapses in the dorsal horn of the spinal cord (Barber *et al.*, 1978). Other experimental pre-treatments have been useful for light microscopical studies (Ribak *et al.*, 1979a, 1980). Finally, the importance of serial sections for immunocytochemical studies has been shown, especially in the study of the olfactory bulb.

Neuroanatomical implications

The results of our studies of GABAergic neurones have indicated three different configurations for the sources of GABAergic axon terminals in various brain regions. In many brain regions such as the cerebral cortex, hippocampus and olfactory bulb, it appears that all of the synapses made by GABAergic terminals are derived from local circuit neurones (Fig. 20). The other axon terminals in these brain regions do not appear to utilize GABA as their neurotransmitter.

A more complex configuration for GABAergic terminals occurs in brain regions such as the neostriatum and the cerebellum. In these cases, GABAergic terminals are derived

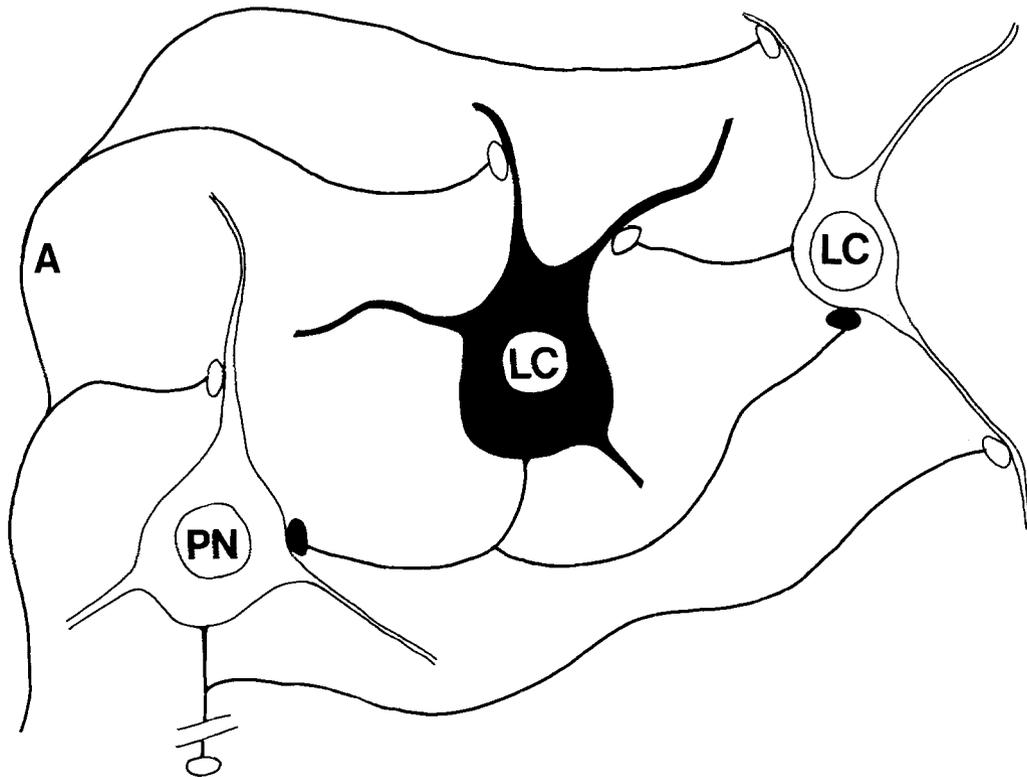
Figs. 18 and 19. Electron micrographs of colchicine-injected neostriatum incubated in anti-GAD serum show labelled dendrites of medium-sized spinous neurones.

Fig. 18. GAD-positive reaction product in a dendrite (D) and in two dendritic spines which form distinct asymmetrical synapses (arrows) with GAD-negative axon terminals. $\times 40\ 000$.

Fig. 19. A large dendritic profile (D) and a neighbouring dendritic spine (SP) which both contain GAD-positive product. The spine (SP) forms an asymmetrical synapse (arrow) with a GAD-negative axon terminal. $\times 35\ 000$. Figs. 18 and 19 are reproduced with permission from Ribak *et al.* (1979).

from both local circuit neurones and the axon collaterals of projection neurones (Fig. 21). Many afferent projections to these brain regions are known to contain neurotransmitters other than GABA. Furthermore, other physiological data indicate that the cerebellum and neostriatum contain excitatory local circuit neurones in addition to the inhibitory GABAergic cells.

A third configuration of GABAergic terminals occurs in the substantia nigra and globus pallidus where these terminals are derived from: (1) local circuit neurones, (2) axon collaterals of projection neurones, and (3) neurones in distant brain regions that send afferent projections (Fig. 22). This last configuration is clearly the most complex of the three. The exact purpose for three different sources of GABAergic synapses is unclear. However, for the substantia nigra, other projection neurones with axon collat-



Figs. 20–22. Diagrams of the three different configurations for the sources of GABAergic axon terminals in the C.N.S.

Fig. 20 represents the most simple of the configurations. All GABAergic axon terminals (filled) are derived from local circuit neurones (darkened LC), whereas terminals derived from (1) the afferents (A) to this brain region, (2) other types of local circuit neurones (clear LC), and (3) axon collaterals of projection neurones (PN) do not utilize GABA. An example of this configuration is the neocortex where GABAergic aspiny stellate cells inhibit spinous stellate and pyramidal cells.

erals are known to utilize a different neurotransmitter, dopamine. Thus, even in brain regions where the GABAergic terminals represent a large majority of all terminals, the existence of other chemically identified terminals has also been shown.

Our immunocytochemical studies of GABAergic neurones have illustrated the many different varieties of neurones that utilize the same neurotransmitter. This finding stands in contrast to the noradrenergic neurones of the brain that are found in the locus coeruleus (Swanson & Hartman, 1975). These neurones are classified exclusively as projection neurones because they exert their major influence in brain regions distant from the locus coeruleus. On the other hand, the GABAergic neurones may be either projection or local circuit neurones as discussed above. This fact is not widely recognized because GABAergic inhibitory neurones were always thought to be exclusively local circuit neurones. However, our results and the results of others have indicated that GABAergic neurones project axons into the striatonigral, striatopallidal, pallidonigral,

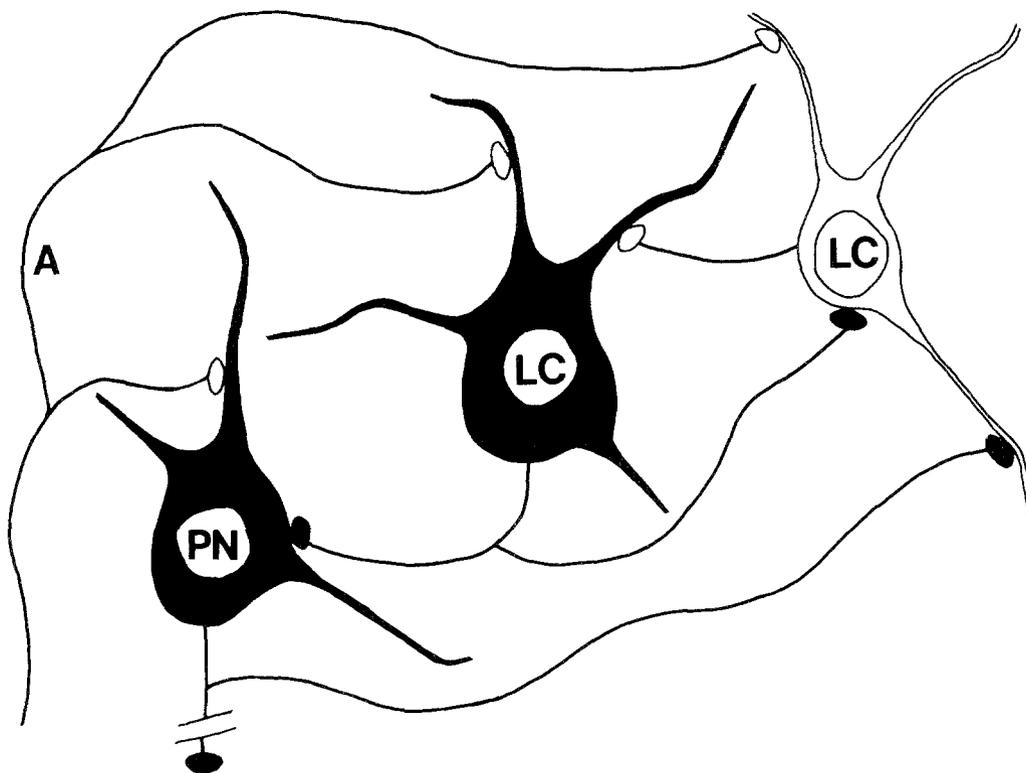


Fig. 21 represents another configuration for GABAergic terminals in a brain region. In this instance, GABAergic terminals arise from local circuit neurones (darkened LC) and from axon collaterals of projection neurones (PN). An example of this configuration occurs in the neostriatum where GABAergic medium-sized spinous cells that project to the substantia nigra have many local axon collaterals. Cholinergic local circuit neurones (clear LC) and glutamatergic corticostriatal afferents (A) lack GABA.

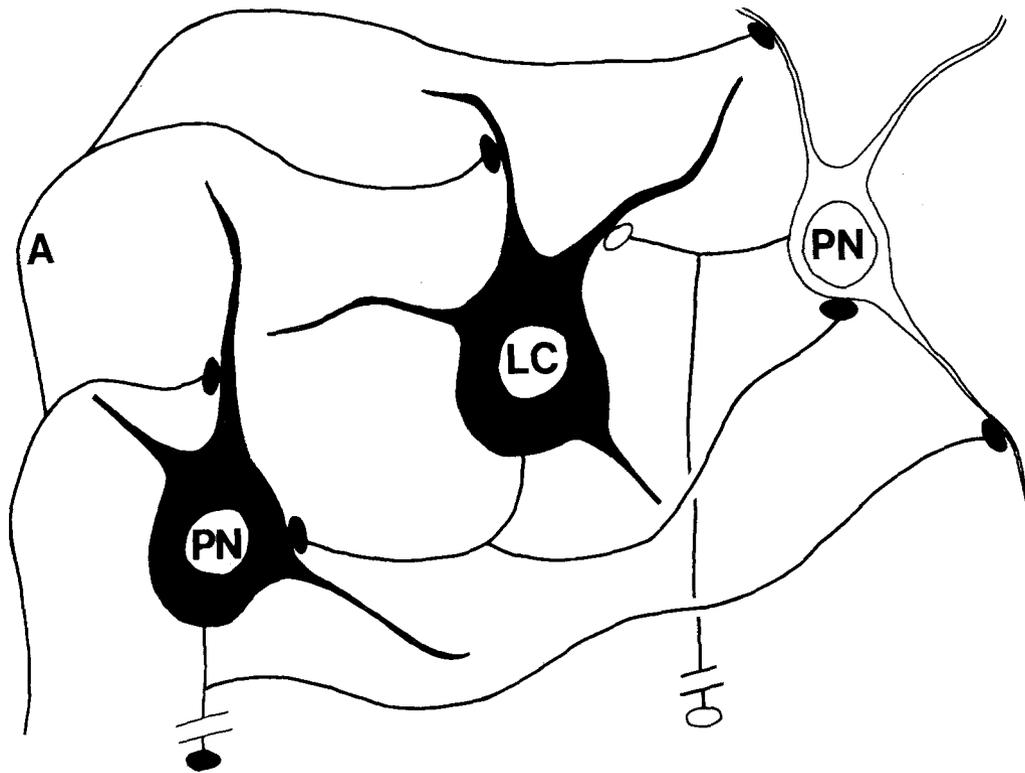


Fig. 22 shows a third configuration for GABAergic axon terminals. This local circuitry illustrates the situation in the substantia nigra where striatonigral and pallidonigral afferents (A), local circuit neurons (LC) and axon collaterals of projection neurons (filled PN) give rise to GABAergic terminals. The dopaminergic projection neurone (clear PN) also contributes to the local circuitry. Figs. 20–22 were prepared with the assistance of H. Dowling.

vestibulo-ocular and cerebellar-vestibular pathways (for example, Ito, 1976). Therefore, GABAergic neurones can clearly be either projection neurones or local circuit neurones.

The use of these two terms for describing neurones in the C.N.S. is not as accurate as we desire at times. Our immunocytochemical studies of GABAergic neurones in the neostriatum have illustrated a shortcoming with the use of these terms. Briefly, our results and the results of other investigators (Kitai *et al.*, 1979) indicate that the GABAergic neurones in the neostriatum are medium-sized, spiny cells which have projections to the globus pallidus and substantia nigra as well as many local connections or axon collaterals in the vicinity of their somata. Therefore, these neurones may function as either projection neurones or local circuit neurones depending on the experimental conditions. For example, recent data obtained from isolated neostriatum (Hassler, 1979) indicate that the local axon collaterals of GABAergic projection neurones do not

degenerate following the interruption of their long projection axons*. The persistence of these axon collaterals suggests that projection neurones may be transformed into local circuit neurones. Indeed, almost all projection neurones that have local axon collaterals have this potential to function as local circuit neurones. However, other projection neurones, such as those cells in the thalamus that project to the cerebral cortex, display retrograde degeneration after the interruption of their axonal projections to the cortex (Ralston, 1971). Results from Golgi studies indicate that these cells have only a sparse local axon ramification. Therefore, the number and extent of local axon collaterals for projection neurones have functional implications in various experimental and disease conditions. For these reasons, the term projection neurone perhaps should no longer be used. Instead, neurones previously classified to this type should be categorized into two proposed subclassifications: projection neurones with either profuse axon collaterals (PAC projection neurones) or those with sparse axon collaterals (SAC projection neurones). The use of these two terms for particular projection neurones must rely upon experimental data that show persistence of axons after isolation as well as Golgi data that indicate an axon's local configuration. The purpose of these two proposed terms is to indicate that, even though projection neurones are not local circuit neurones (LCNs), some projection neurones, such as those in the neostriatum (PAC type), can provide many local axon collaterals. Therefore, PAC projection neurones should be analysed along with LCNs to obtain an understanding of the local circuitry of a brain region.

Future technical advances

The use of immunocytochemistry for the localization of neurotransmitter enzymes and peptides at the electron microscopical level provides new insights into the chemical functioning of neurones in the brain. However, technical advances are needed to relate these insights to the neuroanatomical connections of these chemically specified neurones. The use of immunocytochemistry in combination with retrograde tracing studies could provide valuable information in regard to chemically specified projections. Histochemical methods for other neurotransmitters could also be used with immunocytochemical preparations for demonstrating multiple neurotransmitters. A third method that could be used in concert with immunocytochemistry is the Golgi–electron microscopical (EM) method (Fairén *et al.*, 1977). This latter method allows for the identification of all processes of a single neurone at the ultrastructural level. Although we have identified GABAergic neurones in certain brain regions by utilizing colchicine-injected preparations and serial section analysis, the combined use of immunocytochemistry and Golgi–EM methods could provide a more rapid manner for this identification. At the present time, many technical problems need to be solved

*Hassler (1979) has shown that the axon terminals that form symmetric synapses persist after isolation of the neostriatum. Terminals which form this type of synapse have been shown to contain GAD, the marker for GABA. Furthermore, when the neostriatum is separated by a hemitranssection from the substantia nigra, the distribution of GAD in the neostriatum does not appear to change, although there is a severe loss in the substantia nigra (Ribak *et al.*, 1980).

for these future advances. However, the use of immunocytochemical electron microscopy in combination with these other experimental techniques could provide valuable contributions for the understanding of brain function.

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