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A Immunocytochemical Analysis of Methionine Enkephalin, Substance P and Glutamic Acid Decarboxy.... Within Neostriatal Projection Neurons: A Light and Electron Microscopic Study

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RONALD HOWARD BRADLEY

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A Immunocytochemical Analysis of Methionine Enkephalin, Substance P and Glutamic Acid Decarboxylase Within Neostriatal Projection Neurons: A Light and Electron Microscopic Study

by

Ronald Howard Bradley

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ABSTRACT

A Immunocytochemical Analysis of Methionine Enkephalin, Substance P and Glutamic Acid Decarboxylase Within Neostriatal Projection Neurons: A Light and Electron Microscopic Study

by

Ronald Howard Bradley

The aim of the present study was to analyze the putative neurotransmitters, GABA, methionine enkephalin, leucine enkephalin and substance P, contained within neostriatal projection neurons. Analysis was carried out at both the light and electron microscopy levels. Projection neurons were identified by retrograde uptake of wheat germ agglutinin, n-[acetyl-^{3}H] following stereotaxic injections into either substantia nigra or globus pallidus.

Detection of neurotransmitters within neostriatal neurons was accomplished by immunocytochemical methods. Determination of projection neuron and neurotransmitter was accomplished by a combined double label immunofluorescence method and a retrograde labeling technique. Immunocytochemical analysis at the electron microscopy level was accomplished by a modified avidin-biotin technique.

Results at the light level suggest that at least some medium-sized projection neurons immunocytochemically reacted with either glutamate decarboxylase (GABA marker), methionine enkephalin, leucine enkephalin or substance P. It was additionally found that some single neostriatal neurons reacted both to leucine enkephalin-GAD or methionine enkephalin-GAD. Comparison of immunocytochemically localized GAD and

γ-aminobutyric acid transaminase (GABA-T) revealed that these two enzymes are not contained within the same population of neostriatal neurons. GAD was consistently localized within medium-sized projection neurons. GABA-T was observed in medium-sized cells and also a population of large neostriatal neurons.

Immunocytochemical localization of GAD, methionine enkephalin and substance P was observed in neostriatal neurons possessing ultrastructural characteristics of medium spiny neurons. Immunoreactivity was seen localized within somatal cytoplasm specifically on ribosomes, rough endoplasmic reticulum and Golgi vesicles. Additionally, these neurotransmitters (GAD, Met-enk or SP) were localized within the cytoplasm of dendrites, spines of dendrites and pre-synaptic axonal terminals. Ultrastructural localization of GAD and GABA-T revealed these enzymes are found in different neurons. GAD immunoreactivity was only seen in medium spiny neurons whereas GABA-T was seen in medium-spiny neurons and medium aspiny neurons. Additionally, GABA-T was only seen in post-synaptic structures.

The majority of the terminals labeled for met-enk or GAD appeared to make symmetrical contacts. These synapses were formed with either labeled dendrites, labeled spines or unlabeled processes. Axoaxonic labeled synapses were not observed. Substance P antisera stained a preponderance of asymmetrical synapses with labeled and unlabeled dendrites and spines of dendrites. Somatic and axoaxonic labeled synapses were not observed.

These findings support that methionine enkephalin, GABA or substance P containing neostriatal neurons contribute to the intrinsic collateralization within striatum as well as contribute to striatopallidal and striatonigral circuitry.

To my loving wife, Susan and to my parents. These people have made everything possible and bearable.

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Chapter 1. Introduction

INTRODUCTION

There have been numerous immunocytochemical studies exploring the localization of putative neurotransmitters within the neostriatum of the rat. These reports have been controversial in respect to whether these putative neurotransmitter candidates are localized within projection neurons of the neostriatum. The aim of the present study was to investigate the neostriatal projection neurons and localize their putative neurotransmitters, methionine enkephalin, substance P and GABA. Projection neurons were identified by retrograde uptake of wheat germ agglutinin, N-[acetyl-^{3}H] following stereotaxic injections into either substantia nigra or globus pallidus. Detection of neurotransmitters with neostriatal neurons was accomplished by immunocytochemical methods. At the light level, determination of projection neuron and neurotransmitter was accomplished by a combined double label immunofluorescence method and retrograde labeling technique. Ultrastructural characteristics of immunocytochemically identified neostriatal neurons were analysed by both the conventional PAP method as well as a newer avidin-biotin technique.

A. Neostriatal Intrinsic Organization

1. Light Microscopic Studies

Neostriatal neurons in general fall into three size classifications: Large neurons with somatic diameters of 20 to 50 μm; medium sized neurons with somatic diameters of 10 to 20 μm; and small neurons with somatic diameters less than 10 μm. The relatively few numbers of large neurons present exhibit a large cytoplasm to nucleus ratio and the nucleus is variable in shape. The cytoplasm of these large neurons contain numerous organelles visible in Nissl stained preparations. The medium sized neurons are the most numerous in the neostriatum and the majority contain a pale, smooth, round and unindented nucleus. The small neurons which are rarely found have a deeply invaginated nucleus and a thin rim of cytoplasm (Ramon y Cajal, 1911; Kemp and Powell, 1971; Fox and Rafols, 1976; Pasik et al., 1979; Chang, 1981).

Modern Golgi investigations have examined the cat caudate nucleus (Kemp and Powell, 1971) and the monkey neostriatum (Fox and Rafols, 1976 for a review). These investigations described a large number of medium spiny neurons possessing extensive local axon collateralization suggesting that many of these medium-sized neurons were intrinsic in their function within neostriatum. The studies of Pasik et al., (1976) and DiFiglia et al., (1976) indicated that the most frequently Golgi impregnated neostriatal neuron was that of the medium spiny type. Earlier retrograde HRP studies concluded that the majority of neostriatal projection neurons were the medium-sized type (Grofova, 1975; Bunny and Aghajanian, 1976). Several Golgi studies have examined neuronal cell types in neostriatum (Dimova et al., 1980; Danner and Pfister, 1979; Lu and Brown, 1977; Mensah and Deadwyler, 1974). These investigators have described 5 to 8 different cell types within neostriatum. The nomenclature used by these investigators has resulted in confusion and misunderstanding by other investigators. However, Chang (1981) offers an alternative, simplified classification for the rat neostriatum which is more thorough due to the correlation between intracellular HRP, retrograde HRP and Golgi-toned studies. A brief description is given below.

The large type I neuron possesses a fusiform-shaped soma (somatic area > 300 sq. μm) and an absence of somatic spines. These neurons have poorly branched dendrites and are similar to the giant neurons described by Ramon y Cajal (1911) in the human. Similar reports have been made in the cat (Kemp and Powell, 1971), the monkey (Fox et al., 1971) and the cat (Danner and Pfister, 1979). These cells resemble the "giant spiny II neurons" reported in the monkey (DiFiglia et al., 1976; Pasik et al., 1976). These large neurons are considered to be cholinergic or cholinesterase-containing interneurons (Butcher and Bilesikjian, 1975; Kimura et al., 1980). Evidence of whether these large type I neurons are projection neurons has not yet been entirely resolved.

The large type II neuron possesses spines on both somata and proximal dendrites. The presence of somatic and dendritic spines indicated to Chang (1981) a functional difference between the large type I and large type II neurons. These large type II neurons are similar to the "Spiny II neurons" described in the monkey (DiFiglia et al., 1976; Pasik et al., 1976). These neurons are found to possess a myelinated axon and may be a projection neuron (Chang, 1981).

The type I medium neuron is the most frequently impregnated neostriatal neuron. This medium spiny cell type has been previously identified in Golgi (Pasik et al., 1976; Somogyi and Smith, 1979) and intracellular HRP studies in the rat (Preston et al., 1980; Wilson and Groves, 1980; Van der Maelen et al., 1979) and the cat (Kitai et al., 1976; Kocsis and Kitai, 1977; Kocsis et al., 1976; Kitai et al., 1978). These neurons possess smooth, round and unindented nuclei (somatic area 1 100-300 sq. μm). The proximal dendrites have a smooth appearance and the distal dendrites are heavily laden with spines (Chang, 1981; Wilson and Groves, 1980; Chang et al., 1982). These neurons have been shown to exhibit extensive local axon collateralizations (Kocsis et al., 1976; Preston et al., 1980; Wilson and Groves, 1980; Bishop et al., 1982).

The efferent axons of these type I medium neurons were found to make two distinct projections after giving off local collaterals within striatum.

The main axons of the cells in the first group were unbranched in their trajectory toward globus pallidus and gave little collateralization within globus pallidus and were found to continue caudally at least as far as the internal capsule. The second group had main axons which branched into two or three primary branches within striatum. The main axons entered globus pallidus where they immediately gave rise to a very distinct spatial arborization. The axon then continued to the caudal part of globus pallidus and gave another spatial arborization. Chang (1981) concluded that despite their identical somatodendritic morphology, these represented two separate populations of neostriatal projection neurons.

The type II neuron was characterized by somatic spines. The somatic spines were sparsely distributed over the surface of the soma and can be missed if serial sections are not observed (Chang, 1981). The dendrites are sparsely covered with spines over their entire length. The nucleus is deeply indented. These cells are similar to the "medium Spiny II neuron" described by DiFiglia (1976) and Pasik et al., (1976) and the aspiny cells with somatic spines as described by Fox (1974).

The <u>type III medium</u> neuron morphologically resembled the type I medium neurons except that they possess little or no dendritic spines (Chang, 1981). These neurons are similar to the type II or type IV neuron described by Dimova et al., 1980) in the rat; the medium smooth cell of Kemp and Powell, 1971) in the cat and (the Aspiny III of DiFiglia et al., 1976). These cells similarly have local axon collaterals within striatum and are therefore considered to have a local integrative function (Chang, 1981; DiFiglia et al., 1976; Kemp and Powell, 1971).

The <u>type IV medium</u> neuron possessed a nucleus with a shallow indentation and a smooth somata. These neurons are distinguished from the type III medium cell by the presence of profusely branching dendrites.

This type of dendritic arrangement has been previously in the monkey by Fox (1971) as the "spidery aspiny neuron." The axon of these cells become myelinated 25 μm from the soma (Chang, 1981). The type III and type IV medium neurons described by Chang (1981) have been considered by others to be one class of neuron and has been classically described as the medium aspiny neuron of the neostriatum. The basic difference as described above is that the type IV medium neuron has more dendrites than that of the type III medium neuron.

The type V medium neurons are characterized by a smooth soma and aspinous dendrites. The type V medium neurons have extensive radiating dendritic fields (up to 250 μm from the soma) which have shorter branching patterns (120 μm in the cat and 150 μm in the monkey). These cannot be easily differentiated from those neurons described by Kemp and Powell (1971; cat) and the Aspiny I neurons described by DiFiglia (1976; monkey). Recent reports have described neurons with radiating, beaded dendrites in the monkey (Rafols and Fox, 1979) and the rat (Dimova et al., 1980) possessing somatic and dendritic morphologies similar to this cell type.

The small type neuron was shown by Chang (1981) using the Golgi method to possess a defined axon which arborized locally within the dendritic field. The soma (somatic area = < 100 sq. μm) of these neurons contain spines and a deeply invaginated nuclear envelope.

2. Ultrastructural Characteristics of Rat Neostriatal Neurons

The sources of major afferent to the striatum originate in the mesencephalic raphe, cerebral cortex, thalamus and substantia nigra. These synaptic inputs have been identified as asymmetrical in configuration (Kemp and Powell, 1971; Chung et al., 1977; Hassler et al., 1978; Hattori et al., 1973). The symmetrical synapses observed within the striatum are believed to be derived from neostriatal interneurons and from the local axon collaterals of neostriatal efferent neurons (Chang, 1981; Wilson and Groves, 1980; Kemp and Powell, 1971; DiFiglia et al., 1980).

The two types of large neurons described by Chang (1981) are similar to those reported in other electron microscopic studies (Kemp and Powell, 1971; Pasik et al., 1976; DiFiglia et al., 1976). These neurons possess a deeply invaginated nucleus, dense bodies, numerous free ribosomes, rough endoplasmic reticulum, perinuclear Golgi complexes and numerous mitochondria. Type I large neurons lack somatic spines whereas the type II large neurons possess somatic spines. Additionally, the type II large neurons receive axosomatic synapses. The dendrites of these large neurons receive asymmetrical synapses containing medium to small-sized vesicles. Many of Chang's (1981) large neurons (type I and II) possessed myelinated axons suggesting that they are projection neurons (Fox et al., 1971). Studies using retrograde transport of HRP (Grofova, 1978) or virus particles (Bak et al., 1978) have shown that large neurons labeled following injections into SN indicating that some of these large neurons were projection neurons.

The type I medium neurons were found by Chang (1981) and Wilson and Groves (1980) to be the only medium type neuron possessing a smooth, round, unindented nucleus. These type I medium neurons are ultrastructurally very similar to the projection neurons retrogradely filled with HRP following injections into the globus pallidus (Chang, 1981). Recently, Somogyi and Smith (1979) using a combined retrograde HRP and Golgi-gold toned technique demonstrated that medium spiny neurons project to the substantia nigra. Intracellular HRP studies have identified the medium spiny type neurons as projecting to globus pallidus (Preston et al., 1980; Chang, 1981). The cytoplasm of these neurons contained numerous ribosomes and mitochondria. Less frequently found were Golgi complexes, rough endoplasmic reticulum, and smooth endoplasmic reticulum. Numerous microtubules were observed scattered throughout the cytoplasm. Similar observations have been made by numerous investigators (Chang, 1981; Wilson and Groves, 1980; DiFiglia et al., 1976; Kemp and Powell, 1971). Additionally, an occasional dense body and cilium were observed (Chang, 1981; Rafols and Fox, 1976). Axosomatic synapses were always seen and were symmetrical. The primary dendrites were found receiving symmetrical synapses on the shafts of distal dendrites. Asymmetrical synapses were restricted to the dendritic spines. The terminal configuration was that of an asymmetrical synapse and thus originated from afferents to neostriatum. The axons of these medium type I neurons made symmetrical contacts within striatum (Wilson and Groves, 1980; Bishop et al., 1982; Chang, 1981) and globus pallidus.

The type II medium neuron was characterized by a deeply invaginated nuclear envelope. Additionally, these nuclei possessed an intranuclear rod (Dimova et al., 1980; Chang, 1981). The cytoplasmic constituents are similar to those of type I medium neurons. Numerous axosomatic synapses are found on the smooth somatic surface and the characteristic somatic spines. The dendritic shafts of these neurons received more axodendritic synapses than those seen in the type I medium neuron (Chang, 1981).

The type III medium neuron contained shallow nuclear indentations. Dimova et al., (1980) suggested that these neurons contain intranuclear rods. The cytoplasm contained scarce profiles of rough endoplasmic reticulum and Golgi complexes which is similar to the type I medium neuron (Chang, 1981; Kemp and Powell, 1971).

The type IV medium neuron possessed a shallow nucleus and a similar distribution of cytoplasmic organelles as described for the type III medium neuron. The major difference between this cell type and that of the type I medium neuron is the lack of dendritic spines (Chang, 1981).

The type V medium neuron was characterized by the presence of a large indented nucleus. The cytoplasm contained a well-developed Golgi complex and numerous arrays of rough endoplasmic reticulum. The dendrites lack spines and are presumably similar to those aspiny neurons described by DiFiglia (1980). The synapses formed by this type V medium neuron were symmetrical. These synapses were observed on dendritic shafts (axodendritic) and neighboring somata (axosomatic).

The small neurons within the rat neostriatum have a deeply indented nuclear envelope and a thin rim of cytoplasm. Axosomatic synapses could be identified. The dendrites lacked spines. The axons as seen at the light level arise from the dendrites (Chang, 1981). As mentioned earlier, these neurons are rare.

A summary of the cytological features of neostriatal neurons has shown the most prominent and probably the only neostriatal projection neuron is that of the type I medium cell type or the medium spiny neuron. This cell type has the characteristics of possessing a smooth, round and unindented nucleus. There is a thin rim of cytoplasm around the nucleus and the dendrites are heavily laden with spines. Since this type I medium neuron or more commonly called the medium spiny neuron, is easily recognized by the criteria established by Chang (1981) and Wilson and Groves (1980), I shall follow these criteria for their identification at the light and electron microscopic level of immunocytochemically labeled neostriatal neurons. Additionally, using the criteria for the other cell types described by Chang (1981), I will be able to differentiate these neurons from the type I medium spiny neuron.

B. Neostriatal Efferent Pathways

The major neostriatal efferent targets are well documented as the globus pallidus (GP) and the substantia nigra (SN). The following is a brief review of current knowledge of these pathways.

The striato-pallidal pathway has been shown to be topographically organized in mediolateral and dorsoventral and rostrocaudal directions (Wilson and Phelan, 1982; Szabo, 1962; Szabo, 1970; Szabo, 1981). These striato-pallidal axons are known to be thinly myelinated and to make symmetrical synapses within the pallidum (Fox and Rafols, 1975; Kemp, 1970). Recent evidence by Preston et al., (1980) has indicated that neostriatal projection neurons give rise to axon collaterals within GP before they enter the internal capsule. These projection neurons were found by Chang (1981) to consist of two classes of neurons as described above.

The striatonigral pathway terminates mainly in the pars reticulata and in reverse topographical organization as observed in the neostriatum (Grofova, 1979; Domesick, 1977; Bunney and Aghajanian, 1976; Grofova and Rinvik, 1970; Schwyn and Fox, 1974). These striatonigral fibers possess a distinct mediolateral distribution within the substantia nigra. The general pattern consists of the most ventral regions of neostriatum terminating dorsally within SN and dorsal regions of neostriatum terminating in the ventral regions of SN. Striatonigral fibers terminate as symmetrical type synapses within SN, similar to those made by striato-pallidal fibers within pallidum (Kemp, 1970; Grofova and Rinvik, 1970; Ribak et al., 1976; Chang, 1981).

C. Wheat Germ Agglutinin Utilized as a Axonal Tracer

In a recent report by Steindler and Bradley (1983), wheat germ agglutinin, N-[acetyl-^{3}H] was found to be an efficient and sensitive retrograde marker. Injections of very small quantities of WGA were shown to generate significantly high concentrations of the retrograde marker within projection neurons. Previous reports (Steindler and Deniau, 1980; Steindler, 1982) have shown this axonal tracing method (WGA) to involve a minimal amount of fibers of passage at the injection site. The power of this axonal tracing technique is the ability to localize the WGA, N-[acetyl-^{3}H] by either autoradiography or by immunocytochemical methods. This enables the identification of the projection neuron and to simultaneously identify the putative neurotransmitter localized within the same projection neuron (Lechan et al., 1981; Steindler and Bradley, 1983; Gros et al., 1977).

Recent biochemical evidence by Margolis (1981) has suggested that there are two different populations of wheat germ agglutinin that are transported. One population retrogradely transports while another population is anterogradely transported. This view prompted a biochemical investigation of the commercially available radiolabeled derivative of WGA, N-[acetyl-^{3}H] from New England Nuclear, Inc. Analysis of the native and the affinity purified derivative, WGA preparations were needed to determine if there was indeed two fractions of WGA, an 18,000 dalton protein and a set of smaller fragments less than 12,000 daltons. In a previous report Margolis (1981) utilized ^{125}I-labeled WGA and found by biochemical analysis that there were two fractions of WGA. The first fraction was the radiolabeled WGA and was determined only to be transported in an anterograde direction. It was suggested that a nonspecific fraction was being transported in a retrograde fashion.

Biochemical analysis of WGA, N-[acetyl-^{3}H] (Steindler and Bradley, 1983) has shown that the presence of either a dimeric form in one SDS-PAGE or a monomer form in another PAGE system which was dependent upon the SDS concentrations.

D. Neurotransmitter Candidates Within the Neostriatum

Neurotransmitter candidates must meet certain criteria before being accepted as definitive neurotransmitters. The presynaptic criteria are as follows: 1) the neuron must contain the necessary enzyme for the production of the neurotransmitter candidate; 2) the neurotransmitter must be localized within synaptic vesicles or stored in the presynaptic terminal; and 3) the neurotransmitter must be released when either chemical or electrical stimulation is applied to the neuron (Mulder, 1982; Erulker, 1978).

At present a necessary first step to show these neurotransmitters within neurons is by either immunocytochemical analysis or biochemical-lesion studies and these have been the major methods of localizing these neurotransmitter candidates within neostriatum. The following is a brief review of the neurotransmitter candidates and their origin and distribution within striatum.

Acetylcholinesterase has been shown by immunohistochemical techniques (Butcher and Bilezikjian, 1975; Butcher and Butcher, 1974), cholineacetyl-transferase localization (Hattori and McGeer, 1974; Kimura et al., 1980; Mao et al., 1977) and biochemical isolation techniques to be localized within striatum (Fonnum et al., 1978; Fonnum and Walaas, 1979). The acetylcholine striatal neurons are thought to be of the large type and are probably the interneurons within neostriatum.

Cholecystokinin has been isolated by biochemical methods from neostriatum and the exact location within a distinct neuronal type has not been resolved yet (Robberecht et al., 1978).

Dopamine has been localized within striatum by various biochemical and histological techniques. Dopamine has been well documented to originate from the nigrostriatal pathway (Fallon and Moore, 1978; Fibiger et al., 1972; Fuxe, 1965; Giorguieff et al., 1978; Hattori et al., 1973; McGeer et al., 1971; Ross and Reis, 1974; Seeman, 1981; Ungerstedt et al., 1982 for a review; Verserg et al., 1976). Evidence by Giorguieff et al., (1978) has shown GABA to cause the release of dopamine within neostriatal slice preparations. It was also thought that dopamine is deficient in Parkinson's disease and alleviation of the symptoms are caused by administration of the precursor to dopamine, namely L-DOPA (Birkmayer and Hornykiewicz, 1962; Carlsson, 1958). Ungerstedt and Arbuthnott (1970; 1971) concluded from animal experiments that by lesioning the nigrostriatal pathway they found aberrant animal behavior and it was worsened by the administration of amphetamines. It was the work of Iversen (1977) that suggested that the nigrostriatal system was involved in motor arousal while the dopamine containing mesolimbic areas are involved with the motivational arousal (Rondrup and Munkvad, 1967).

Glutamate within striatum is thought to originate from the corticostriatal pathway (see Cotman et al., 1981). The major isolation techniques utilized so far have either been biochemical (Divac et al., 1977; Fagg and Lane, 1979; Fonnum et al., 1978; Kravitz, 1967; McGeer et al., 1977; Storm-Mathisen, 1977; Walaas, 1981) or pharmacological (Nistri and Constanti, 1979). Recent advances into production of an antibody against glutamate will allow for more precise localization within striatum (Storm-Mathisen et al., 1983).

Norepinephrine has been localized within the neostriatum by biochemical methods (Fuxe, 1965; McGeer et al., 1971; Versteeg et al., 1976) and pharmacological analysis (Aghajanian and Bloom, 1967). The exact origin of the noradenalin is not known nor is the function.

Neurotensin has recently been biochemically analyzed by Leeman (1982) and immunocytochemically localized within the striatum. Earlier works isolated a neurotensin-like compound biochemically from the neostriatum (Uhl and Snyder, 1967). It has also been shown by immunohistochemical techniques that neostriatum contains neurotensin-like reactivity (Kobayashi et al., 1977). The exact effect and location within striatum has yet to be resolved.

Serotonin (5-hydroxytrytamine) has been localized within striatum by biochemical techniques (Carlsson, 1958) and more conventionally by a histochemical technique (Amin et al., 1954; Bobillier et al., 1976; Fuxe and Johnson, 1974; Ternaux et al., 1977; van der Kooy, 1979). Evidence was shown the dorsal raphe to be the source of serotonergic input to the striatum (Bobillier et al., 1976). The electrophysiological evidence of the effect of serotonin within neostriatum was shown by these investigators to be excitatory (Park et al., 1982; Vander Maelen et al., 1979).

Somatostatin has been localized within neostriatum by immunohistochemical methods and the exact effect has not been resolved yet (DiFiglia and Aronin, 1982; Kobayashi et al., 1977).

Vasoactive intestinal peptide (VIP) has also been localized within neostriatum and a possible mechanism of action is not known (Fuxe et al., 1977; Giachetti et al., 1977). VIP has been localized only on labeled terminals within striatum so far. The exact origin of these terminals has not been resolved.

The next three neurotransmitter candidates have received the most attention and are the basis for my studies. These are the 1) enkephalins (methionine or leucine), 2) substance P; and 3) glutamate decarboxylase (a GABA marker). Descriptions of these neurotransmitter candidates and their localizations are detailed below.

E. Immunocytochemistry: Application to the Enkephalins, Substance P and GAD

Any immunocytochemical study has to consider the variables involved for determination of antigenic determinants within any cell or within plasma membrane bound compartments. In recent reviews of neuroimmunocytochemical investigations of the localization of neurotransmitters (Pool et al., 1982; Moriaty, 1973; Sternberger, 1974), the importance of the specificity of the antibody for the antigen has been emphasized. Several laborious tests must be performed before the initial analysis of tissue localization. The first is to determine whether the antibody does indeed form a precipitant band with the antigen or tissue. The second is to determine whether the rabbit anti-neurotransmitter forms a precipitant band with the rabbit peroxidase-anti-peroxidase utilized in the intensification of the antigenic site. Finally, one must confirm that the rabbit peroxidase-anti-peroxidase does not absorb to the tissue in a random fashion (i.e. non-specific binding). If there is any non-specific binding occurring in the tissue preparations, the study becomes invalid.

Another important aspect of immunocytochemistry is the intensification of the antigenic site. The most widely adopted method is that of Sternberger (1974) utilizing the unlabeled antibody enzyme technique. It has become apparent from recent investigations and our own personal usage of the PAP technique, that there are numerous variances in results obtained with the PAP method. In the present study, I shall compare the PAP method with that of the new avidin:biotin technique as outlined by Hsu et al., (1981). The problem that is encountered with the PAP method in neuroimmunocytochemical investigations could be the relative size of the PAP molecule, which reaches a million daltons. The other limiting factor of the PAP method may be the impenetrability of the 3,3'diaminobenzidine within the vibratome sections. These variabilities will be examined in this study.

The neostriatum (caudate-putamen) contains the highest concentration of enkephalin, an opiod pentapeptide, within the central nervous system (Buijs, 1982; Elde et al., 1976; Osborne et al., 1978). The enkephalins, which are related to the morphine-line pentapeptides, have been found in substantial quantities within the neostriatum (Swaab, 1982) as well as localization of opiate receptors within neostriatum (Herkenham and Pert, 1980; 1981). Herkenham has recently demonstrated the existence of opiate receptors within striatum that are sensitive to the enkephalin antagonist, naloxone. Recently, a report on the immunohistochemical localization of leucine enkephalin in monkey basal ganglia (DiFiglia et al., 1981) localized immunoreactivity within medium-sized neurons with ultrastructural characteristics similar to type I medium (spiny) neurons. Evidence for a neurotransmitter role of enkephalin has been suggested by the _in vitro_ release of enkephalin in caudate slice preparation after K^+ and veratridine application (Henderson et al., 1978). Additionally, met-enkephalin inhibits spontaneous activity of caudate neurons and their late phase responses to cortical stimulation (Fry and Zieglgansberger, 1979). An earlier work on non-colchicine treated animals was shown to exhibit met-enkephalin immunoreactivity within medium spiny neurons

(Pickel et al., 1980).

Substance P was found to be in highest concentrations within the substantia nigra (Brownstein et al., 1976). As mentioned earlier, the striatonigral pathway has been well documented (see Grofova, 1979 for review) and it has been postulated that these striatonigral neurons utilize substance P as a neurotransmitter (DiFiglia et al., 1982). Substance P has been iontophoretically applied to the substantia nigra and found to cause an excitatory state within nigra (Fry and Zieglgansberger, 1979; Zieglgansberger, 1982). The exact mechanism for the synthesis of substance has not yet been resolved. It has been shown that substance P can be released from striatal slices by capsaicin (Gamse et al., 1979). Thus, it has fulfilled one criteria of a neurotransmitter candidate. The second is the localization within terminals or neurons of origin. This criterion was recently analyzed by DiFiglia et al., (1982) by immunohistochemical methods in substantia nigra. They were able to show the localization within asymmetrical synapses within the SN neuropil. A recent report by Bolam et al., (1983) has shown substance P to be localized within medium aspiny and medium spiny neurons. The terminals possessing immunoreactivity formed symmetrical synapses. Previous reports on substance P localization has been either by biochemical lesion studies (Gale et al., 1977; Hong et al., 1977) or immunohistochemical methods (Amin et al., 1954; Brownstein et al., 1976; Cuello et al., 1977; Cuello and Kanzawa, 1978; Hokfelt et al., 1977; Jessell et al., 1978; Pelletier et al., 1977; Pickel et al., 1979; Scully et al., 1978; Walker et al., 1976). There has been no direct visualization of the projection neuron and its putative neurotransmitter localized within the same neuron.

The neostriatum has been shown to contain gamma aminobutyric acid and its synthesizing enzyme, glutamic acid decarboxylase (GAD). Perry et al., (1973) have shown a decrease in GABA concentrations in Huntington's Chorea patients, thus it has become extremely important to understand GABA's function within the normal neostriatum. It is of interest that these Huntington patients are treated with either haloperidol or by the phenothiazines (blocks dopamine receptors) and reserpine (dopamine depleter). The most widely prescribed anxiolytic drug in America, the benzodiazepines, potentiate the effects of GABA (Haefely et al., 1978). The exact mechanism is not yet known. Biochemical lesion studies have identified neostriatum as the source of GABA to the globus pallidus and substantia nigra (Fonnum et al., 1978; Fonnum et al., 1974; Gale et al., 1977; Kim et al., 1971; Storm-Mathisen, 1977). The majority of the cellular localization studies have employed immunocytochemical localizations of GAD (Ribak et al., 1976; Ribak et al., 1977; Ribak et al., 1978; Ribak et al., 1979) or a histochemical localization of the digestive enzyme for GABA, GABA-transaminase (Hattori et al., 1973; McGeer and McGeer, 1975; McLaughlin et al., 1974; Vincent et al., 1982). The most recent development has been the production of an antibody directly against GABA itself (Storm-Mathisen et al., 1983). A recent study by Bolam et al., (1983) utilized direct injections of ^{3}H-GABA into striatum combined with Golgi-gold toning to show that the neostriatal neurons with GABA are of the medium aspiny type which have been considered as interneurons. It was the work of Ribak et al., (1979) that showed GAD immunoreactivity within medium-sized neurons and dendritic spines. Ribak et al., (1979) suggested that these medium-sized neostriatal neurons were characteristic of the medium spiny.

Chapter 2: Material and Methods

A. Labeling of Neostriatal Projection Neurons

In order to retrogradely label neostriatal projection neurons, WGA, N-[acety-^{3}H] was stereotaxically injected into substantia nigra or globus pallidus (Figs. 1 and 2).

A total of 26 male Sprague-Dawley rats weighing 250-300 grams were anesthetized with nembutol (50 mg/kg) administered intraperitoneally and placed into a stereotaxic holder (David Kopf Instruments). Following small hole dorsal craniotomy, pressure injections (.2 μl) of wheat germ agglutinin, N-[acetyl-^{3}H] (New England Nuclear) were stereotaxically placed into either substantia nigra or globus pallidus using a glass pipette attached to a Hamilton syringe. After 48 hours post-injection period, colchicine (5 μl, 10 μg/ml) was injected stereotaxically into the contralateral ventricle. The animals were allowed to survive an additional 48 hours before being processed for light microscopy.

The animals were perfused intracardially with 50 ml of a microtubule stabilizing buffer (MSB) containing lidocaine (1 ml/100 ml buffer) and heparin (25,000 units/100 ml buffer) for 10 minutes followed by the fixative (Bradley et al., 1978). The MSB is known to be effective as a biochemical isolation buffer for brain microtubules (Shelanski et al., 1973) and consists of 0.1 mM-MES (2[N-morpholino]-ethane sulfonic acid), 1 mM EGTA (ethylene glycol-bis-[beta-amino-ethyl-ether]N, N'-tetra-acetic acid), 1 mM GTP (guanosine 5-triphosphate) and 0.1 mM $MgCl_2$. This buffer is extremely effective in preserving neurotransmitters within synaptic vesicles due to the EGTA, $MgCl_2$ and GTP (Mulder, 1982). Additionally, antigenic preservation is maintained by lowered aldehyde concentrations in the fixative (Sternberger, 1973).

The fixative consisted of 1% glutaraldehyde and 1% paraformaldehyde in MSB (pH 6.9(at 37^{o}C. A total of 800 mls was perfused through each

Figure 1 Schematic representation of the stereotaxic injections and the colchicine treatment schedule.

INJECT RAT BRAIN WITH COLD or HOT WHEAT GERM AGGLUTININ
(SUBSTANTIA NIGRA or GLOBUS PALLIDUS)

↓

WAIT FOR 48 HOURS

↓

INJECT COLCHICINE INTO CONTRALATERAL VENTRICLE

↓

WAIT FOR 48 HOURS

↓

PERFUSE ANIMAL WITH 1% GLUT.,1% PARAFORM. IN MES BUFFER

↓

HEMI-SECTION BRAIN AND EMBED IN GLYCOL METHACRYLATE OR
VIBRATOME SECTION OR FROZEN SECTION

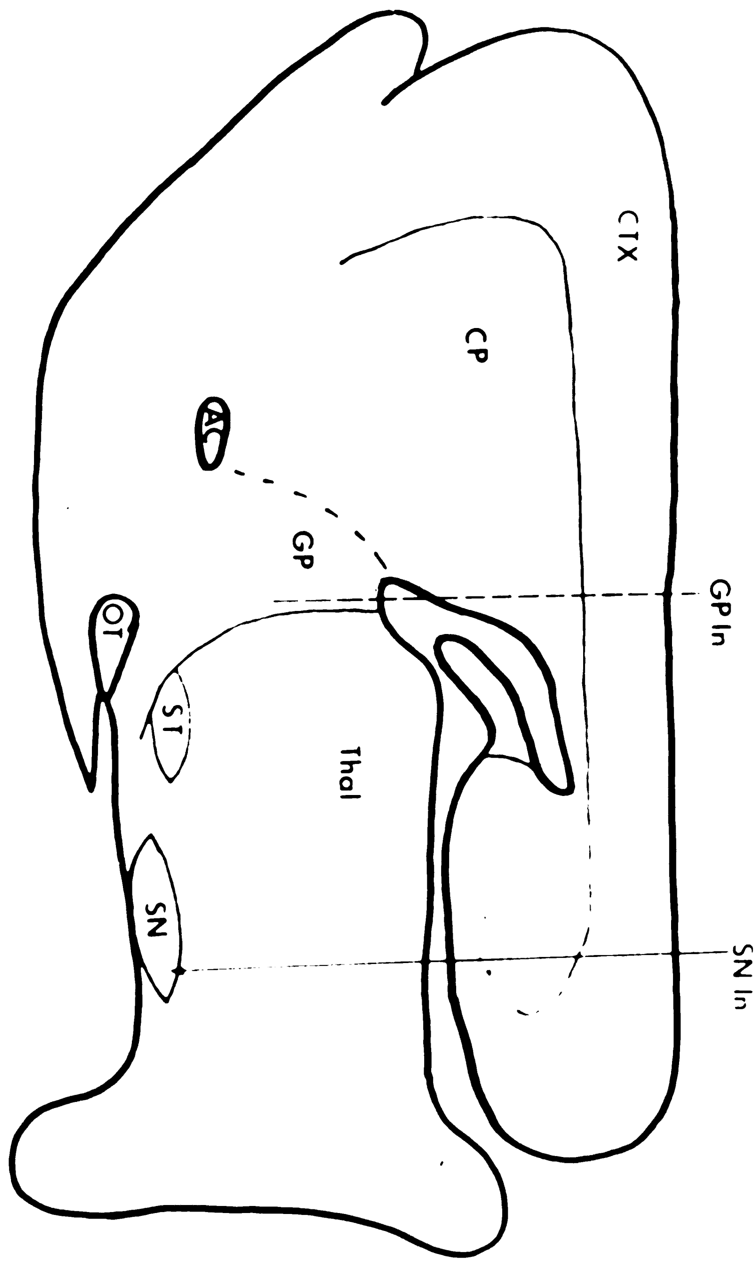
↓

INCUBATE SECTIONS FOR PAP OR IMMUNOFLUORESCENCE

1

Figure 2 Diagramatic representation of the rat brain where the stereotaxic injection of WGA is into globus pallidus (GPIn) and substantia nigra (SNIn). CTX (cortex); CP (caudate-putamen); GP (globus pallidus); Thal (thalamus); ST (subthalamus); AC (anterior commissure); OT (optic tract); SN (substantia nigra).

2



animal. The brains were removed from the skull, immersed for an additional hour in the same fixative and extensively washed in MSB. The brains were then either hemisectioned and embedded in glycol-methacrylate or sectioned on a vibratome at a thickness of 60 μm in MSB.

1. Colchicine treatments

Recent studies on the localization of CNS neurotransmitters using immunocytochemical techniques have employed colchicine injections into the lateral ventricles in order to increase neurotransmitter content within the somata of neurons (Ribak et al., 1979 and Hokfelt et al., 1977). These colchicine injections have not always allowed preservation of neuronal morphology or its antigenic determinants (i.e. cellular enzymes). In part, this may be due to the buffers routinely utilized and their aldehyde concentrations. In this study we chose to employ a new buffer (MSB) and lower the aldehyde concentrations used in fixative of colchicine treated animals.

Two white Sprague-Dawley rats for colchicine treatment and two white Sprague-Dawley rats untreated were utilized for comparison. Five μls of colchicine (10 μg/ml) was stereotaxically injected into both lateral ventricles over a period of 25 minutes. After 48 hours post-injection period, the rats (untreated and colchicine treated) were perfused, via the left ventricle of the heart, with 300 mls of fixative (1% paraformaldehyde, 1% glutaraldehyde in a microtubule stabilizing buffer-MSB). Brains were removed from the skull and placed in the fixative for an additional hour. A block of tissue containing striatum was excised from the brain, washed with MSB and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 hour at $4^{o}C$. The blocked striatal tissue was dehydrated in graded ethanols, rinsed twice in propylene oxide (15 min.) and embedded in Epon-Araldite. Ultrathin sections (80 nm thick) were cut on an LKB-V ultramicrotome and stained in lead citrate and viewed on a JEOL 100CX electron microscope.

B. Antibody Purification and Analysis

Antibodies to methionine enkephalin, leucine enkephalin and substance P were bought from Immunonuclear, Inc. and subsequently purified so as to contain only immunoglobulins (Bradley et al., 1978). Specificity of the antibodies was tested by immunoelectrophoresis, immunodiffusion (Ouchterlony) or rocket gel immunoelectrophoresis. After purification, each antibody was precipitated only with its specific antigen (Sigma) (e.g. - anti-methionine enkephalin precipitated only with methionine enkephalin). Antibodies to glutamic acid decarboxylase (GAD) and gamma aminobutyric acid transaminase (GT) were obtained from Dr. J.-Y. Wu (Baylor College of Medicine, Texas). Rat brain neostriatal synaptosomal preparations were used in testing GAD and GT antibody specificity. Immunoelectrophoresis of GAD and GT antibodies with brain homogenate from white Sprague-Dawley rats resulted in a single precipitant band indicating cross reactivity with the rats utilized in our experiments.

Immunocytochemical localization of WGA was accomplished by a rabbit antibody to WGA purified to an IgG fraction (a gracious gift of Dr. Myron Lyon, Wayne State University). All antisera used in these experiments were free of complement and albumin (Bradley et al., 1978).

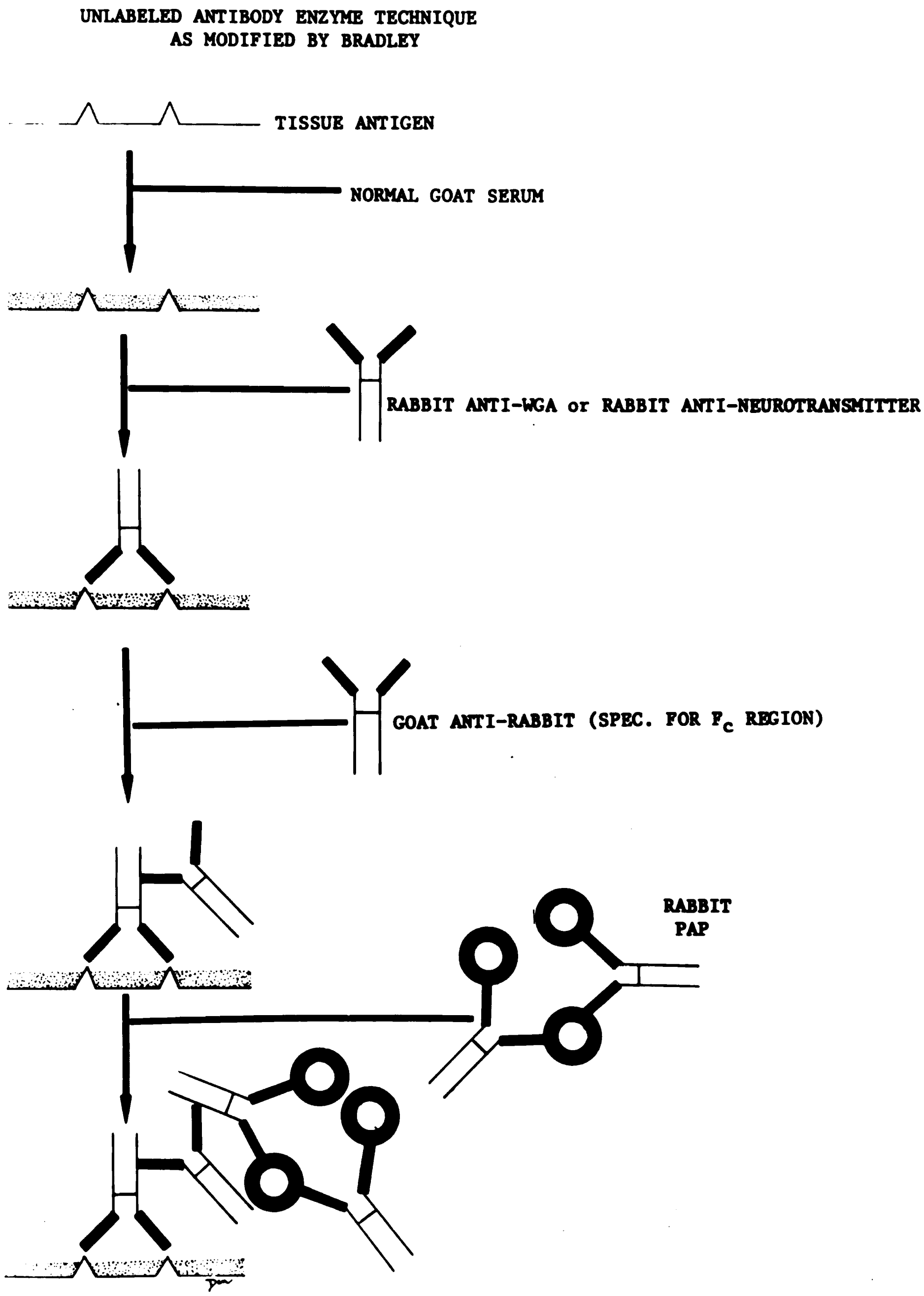
C. Immunocytochemical Procedures - Glycol Methacrylate Sections

Glycol methacrylate sections (4-6 μm thick) of striatum were employed for use in identification of projection neurons and this putative neurotransmitters. Glycol methacrylate sections, mounted on cleaned glass slides, were initially incubated in 10% normal goat serum for 15 minutes

(4^oC) to mask all low affinity binding sites. The goat serum was drawn off and primary antiserum (WGA, ME, LE, GAD, GT or SP), 1:500 dilution in fresh 0.05 M tris buffered saline at pH 7.2 (TBS) was then applied for a period of 12-36 hours at 4^oC. Sections were extensively washed and 30% goat-anti-rabbit (specific for the rabbit F_c portion of the IgG molecule) conjugated to fluorescein was incubated for 2 hours at 4^oC. The sections were washed three times in TBS, rinsed three times with 1% goat-anti-rabbit, washed again with TBS and re-incubated with purified rabbit anti-WGA for 6 hours at 4^oC. The sections were washed with TBS three times and incubated 2 hours with 30% goat-anti-rabbit in TBS. Finally, the sections were coverslipped and viewed in a Leitz Orthoplan light microscope with appropriate excitation radiation and filters (fluorescence: blue radiation with BG38, BG12, TK495 and K495 filters; rhodamine: green radiation with 560, BG36, TK580 and K580 filters). Several GM sections were double labeled for the co-existence of two neurotransmitters within the same neuron. GM sections were incubated in GAD antiserum for 12 hours at 4^oC. The sections were washed with TBS, incubated with goat-anti-rabbit conjugated to fluorescein for 4 hours and washed extensively with TBS (seven times - 8 min. each). The sections were rinsed three times with unconjugated goat-anti-rabbit, incubated with 10% normal goat serum (10 min.), drained and incubated with either methionine or leucine enkephalin for 4 hours. Sections were then washed in TBS, incubated with goat-anti-rabbit conjugated to rhodamine for 2 hours and then washed in TBS. Sections were then coverslipped and viewed in a Leitz Orthoplan with appropriate radiation and filters (see above for details).

A series of several GM sections were alternatively incubated with a 1:1,000 dilution of primary antiserum (WGA, ME, LE, GAD, GT or SP) for

Figure 3 Diagramatic representation of the unlabeled antibody enzyme technique as modified by Bradley (1978). Tissue is incubated with normal goat serum to mask the low affinity binding sites. Tissue is then incubated with rabbit anti-neurotransmitter or anti-WGA. Tissue is then washed, incubated with goat-anti-rabbit (specific for the IgG molecule and the F_c region), washed and incubated with rabbit-peroxidase-anti-eproxidase (PAP). This reaction is then visualized with 3,3' diaminobenzidine.


UNLABELED ANTIBODY ENZYME TECHNIQUE
AS MODIFIED BY BRADLEY
TISSUE ANTIGEN
NORMAL GOAT SERUM
RABBIT ANTI-WGA or RABBIT ANTI-NEUROTRANSMITTER
GOAT ANTI-RABBIT (SPEC. FOR F_c REGION)
RABBIT
PAP

3

more precise morphological identifications of cell types. Sections were washed with TBS, incubated with a 1:20 dilution of goat-anti-rabbit (specific for F_c region on the rabbit IgG molecule) for 4 hours at 4^oC and washed three times with TBS. Sections were then incubated with rabbit-peroxidase-anti-peroxidase (1:50) for 2 hours at 4^oC, washed in 0.05 M tris (pH 7.2) and incubated with 3,3'-diaminobenzidine (DAB, 25 mg/ml buffer) for 10 minutes (Fig. 3). Sections were then incubated with fresh DAB containing 0.01% hydrogen peroxide for 15 minutes, washed with tris buffer three times and post-fixed in 0.1% osmium tetroxide in distilled water (Bradley et al., 1978). Sections were then coverslipped and viewed unstained.

Vibratome sections (40-60 μm thick) were incubated in the modified peroxidase-anti-peroxidase (PAP) technique as outlined above for GM morphological slide preparations.

Immunocytochemical methods were performed on brains where the injection site was visualized. One brain was serially vibratome sectioned (50 μm) for localization of the globus pallidus injection site. These sections were run for immunocytochemical localization of WGA (see above for details) and then subsequently dipped in NTB2, nuclear emulsion (Kodak). These sections were exposed for 6 weeks and then developed. One brain was sectioned for substantia nigra injection site at 6 μm (GM sections) and dipped in nuclear emulsion and allowed to expose for 6 weeks. These sections were then developed and counterstained. Tissue sections utilized for double labeling (projection neuron-transmitter) were selected from a suitable injection site case. These brains were observed in a Leitz Orthoplan light microscope.

antigen.

Sections were rinsed with three washes of fresh TBS (21 min.) and incubated with biotinylated goat-anti-rabbit (1:100) for 2 hrs. at $4^{o}C$ with constant agitation. Sections were again rinsed with fresh TBS (3X - 21 min.) and re-incubated with avidin:biotinylated horseradish peroxidase complex (Hsu et al., 1981) (Vector Lab., Inc.) for 3 hrs at $4^{o}C$ with constant agitation (Fig. 4). Sections were rinsed three times (7 min. each) with fresh tris (0.05 M) and incubated with 3'3-diaminobenzidine (0.25 mg/ml) in fresh 0.05 M tris (pH 7.2) for 15 min. at $4^{o}C$ with constant agitation. The DAB solution was decanted and fresh DAB was added containing 0.01% hydrogen peroxide for 20 min. at $4^{o}C$ with agitation. DAB-H_2O_2 solution was drawn off and the sections were rinsed three times with tris buffer. The tris buffer was drawn off and replaced with 0.1 M cacodylate buffer (pH 7.2) for 20 min. The cacodylate buffer was then replaced with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at $4^{o}C$. The sections were rinsed with cacodylate buffer, followed by a rinse in physiological saline (0.85% NaCl-aqueous) and en bloc stained with 0.25% uranyl acetate in physiological saline, dehydrated in graded ethanols and pure propylene oxide (2 changes). Sections were embedded in Epon-Araldite and cut on a LKB-V ultramicrotome with a diamond knife. Sections were cut to a thickness of 80 nm and viewed unstained in a JEOL-100CX electron microscope at 60 KV.

Control sections consisted of either precipitation of the antibody with excess antigen or substitution of normal rabbit serum for the primary antibody. These sections were run parallel to the previously described sections in order to check for the specificity of the avidin-biotin complex technique as well as the antibody sensitivity and specificity.

D. Immunocytochemical Procedures: Ultrastructural Techniques

1. Animals

Eight white Sprague-Dawley rats weighing 250-300 grams were used. Animals were anesthetized with nembutol (50 mg/kg) administered intraperitoneally and placed in a stereotaxic holder (David Kopf Instruments). After performing a small dorsal craniotomy, 5 μl of colchicine (10 μg/ml) was stereotaxically injected into each lateral ventricle. After 48 hours the animals were deeply anesthetized with nembutol (75 mg/kg) and perfused intracardially with 50 ml of a microtubule stabilizing buffer (MSB) containing lidocaine (1 ml/100 ml buffer) and heparin (25,000 units/100 ml buffer) for 10 minutes followed by fixative.

As mentioned earlier, MSB has a unique stabilizing affect on ultrastructural preservation and details of this buffer are outlined above. The fixative consisted of 1% glutaraldehyde and 1% paraformaldehyde in MSB (pH 6.9) at 37^{o}C. Brains were removed from the skull, hemisectioned and immersed in MSB. The hemi-brains were sectioned on a vibratome (50 μm thick) and stored in MSB.

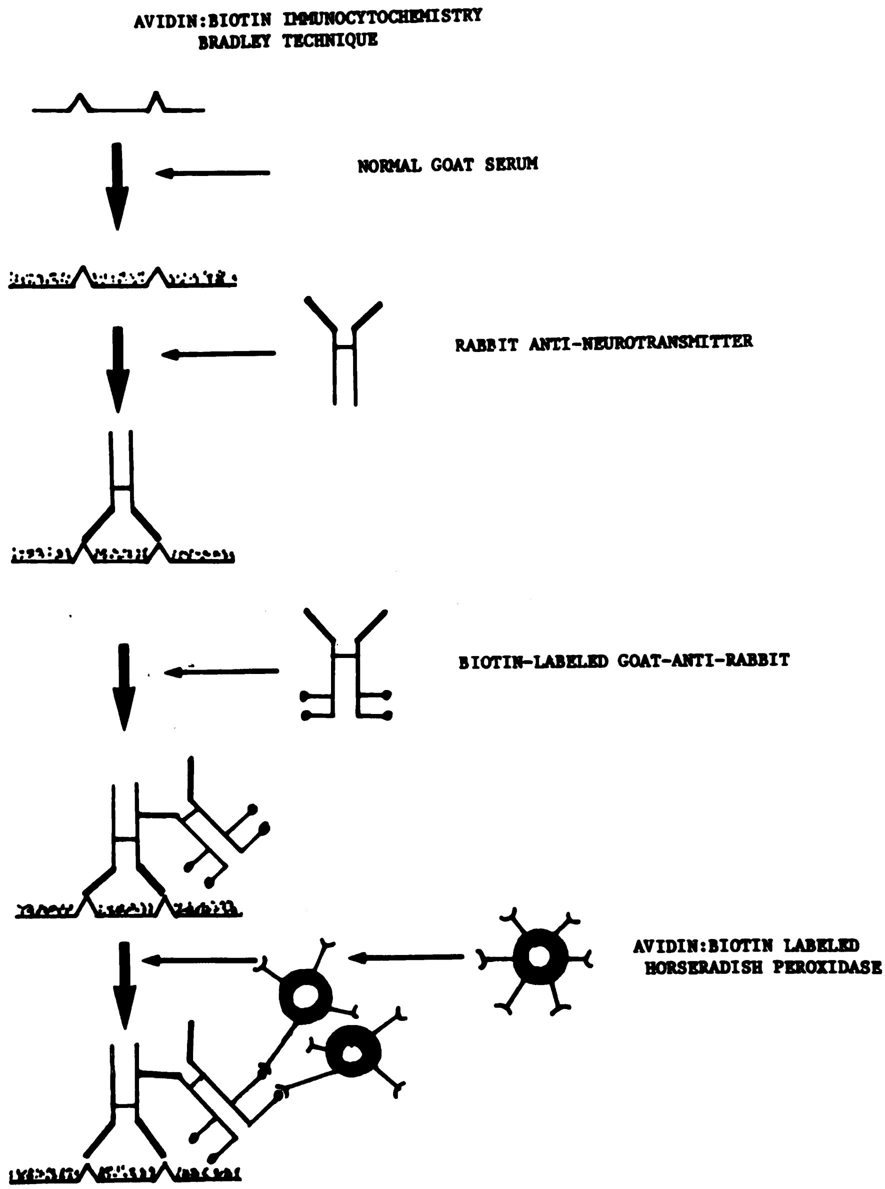
2. Vibratome Section Analysis

Vibratome sections were incubated with 10% normal goat serum for 15 min. to mask low affinity binding sites. Sera were drawn off and sections incubated 12-36 hrs. with purified rabbit anti-neurotransmitter (met-enk, SP or GAD) diluted 1:500 in fresh 0.05 M tris buffered saline (TBS) at pH 7.2 and at 4^{o}C. The purification and specificity of these antibodies has been previously discussed. In summary, anti-met-enk was found to react only with met-enk and not with either leucine enkephalin or substance P. GAD and SP antibodies only reacted with their specific antigen.

Sections were rinsed with three washes of fresh TBS (21 min.) and incubated with biotinylated goat-anti-rabbit (1:100) for 2 hrs. at $4^{\circ}C$ with constant agitation. Sections were again rinsed with fresh TBS (3X - 21 min.) and re-incubated with avidin:biotinylated horseradish peroxidase complex (Hsu et al., 1981) (Vector Lab., Inc.) for 3 hrs at $4^{\circ}C$ with constant agitation (Fig. 4). Sections were rinsed three times (7 min. each) with fresh tris (0.05 M) and incubated with 3'3-diaminobenzidine (0.25 mg/ml) in fresh 0.05 M tris (pH 7.2) for 15 min. at $4^{\circ}C$ with constant agitation. The DAB solution was decanted and fresh DAB was added containing 0.01% hydrogen peroxide for 20 min. at $4^{\circ}C$ with agitation. DAB-H_2O_2 solution was drawn off and the sections were rinsed three times with tris buffer. The tris buffer was drawn off and replaced with 0.1 M cacodylate buffer (pH 7.2) for 20 min. The cacodylate buffer was then replaced with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at $4^{\circ}C$. The sections were rinsed with cacodylate buffer, followed by a rinse in physiological saline (0.85% NaCl-aqueous) and en bloc stained with 0.25% uranyl acetate in physiological saline, dehydrated in graded ethanols and pure propylene oxide (2 changes). Sections were embedded in Epon-Araldite and cut on a LKB-V ultramicrotome with a diamond knife. Sections were cut to a thickness of 80 nm and viewed unstained in a JEOL-100CX electron microscope at 60 KV.

Control sections consisted of either precipitation of the antibody with excess antigen or substitution of normal rabbit serum for the primary antibody. These sections were run parallel to the previously described sections in order to check for the specificity of the avidin-biotin complex technique as well as the antibody sensitivity and specificity.

Figure 4 Diagramatic representation of the Avidin:biotin complex immunoperoxidase technique. Modifications were made in the staining times and the visualization of the peroxidase.


AVIDIN:BIOTIN IMMUNOCYTOCHEMISTRY
BRADLEY TECHNIQUE
NORMAL GOAT SERUM
RABBIT ANTI-NEUROTRANSMITTER
BIOTIN-LABELED GOAT-ANTI-RABBIT
AVIDIN:BIOTIN LABELED
HORSERADISH PEROXIDASE

4

E. Wheat Germ Agglutinin: Biochemical Analysis

Biochemical analysis of wheat germ agglutinin (WGA) was performed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using two distinct and separate methods. The two SDS-PAGE methods used a discontinuous system with reduced concentrations of SDS as outlined by Neville (1971) and Laemmli (1970). SDS-PAGE analysis was performed on the native WGA (obtained from New England Nuclear, same batch used to prepare the acetylated and radiolabeled acetylated preparations), column purified WGA, [acetyl-^{3}H]-N-acetylated (0.8 mCi/ml, 55 Ci/mmol, in Tris buffer pH 7-0) (herin referred to as WGA, N-[acetyl-^{3}H]) and a 2X concentrated WGA, N-[acetyl-^{3}H] preparation. Following acetylation, the WGA was purified by affinity chromatography in a N-acetylglucosamine-Sepharose column and then extensively dialyzed against phosphate buffer by New England Nuclear. Molecular weight standards were used for comparison and consisted of the following: Cytochrome C (12,300 daltons); Lysozyme (14,300 daltons); Beta-lactoglobulin (18,400 daltons); Alpha-chimotrypsinogen (25,700 daltons) and ovalbumin (43,000 daltons). These molecular weight standards were obtained from Bethesda Research Laboratories. There were certain modifications applied to the SDS-PAGE discontinuous systems. The Neville system consisted of 0.1% SDS and 11% polyacrylamide in the running gel, using a sample buffer (pH 8.8) containing 1% SDS in .01 M tris-acetate buffer with 0.001% EDTA and 0.1% 2-mercaptoethanol. The Laemmli system consisted of 0.1% SDS and 13% polyacrylamide in the running gel using a sample buffer (6.8) containing 2% SDS in 0.0625 M tris base with 10% glycerol and 5% 2-mercaptoethanol. Both systems had 3% polyacrylamide in the stacking gel. The two systems were compared for their isolation characteristics of the WGA polypeptide. These two systems were chosen for their low molecular weight isolation characteristics and differ in the results obtained through use of dissimilar concentrations of SDS, polyacrylamide and reducer. Fluorography was also performed on the two SDS-PAGE systems for the detection of WGA, N-[acetyl-^{3}H] bands and comparison with the Coomassie brilliant blue staining patterns. The wet gels were immersed in EN^{3}HANCE (New England Nuclear) and reacted with continuous agitation for one hour. The EN^{3}HANCE was then poured off and precipitated with distilled water for one hour. The gels were dried, overlaid with X-ray film (Kodak X-Omat XAR-5), and exposed for 1.5 hrs - 6 days at which time the films were then developed in D-19 for 5 min. Finally, freshly run polyacrylamide gels were cut into 2 mm sections and incubated in 10% hydrogen peroxide overnight to dissolve the gels, and then counted in a Beckman LS 7,000 Scintillation Counter. The counts from both concentrated and nonconcentrated WGA, N-[acetyl-^{3}H] preparations were plotted and compared to controls (vials containing samples of cold and hot acetyl-WGA) and background.

Chapter 3: Results

A. Colchicine Treatment vs. Normal Morphology

It was necessary in the present ultrastructural immunocytochemical study to evaluate the effects of colchicine on normal morphology. The reason for such a study is that the neostriatal tissue undergoes numerous buffer washes, incubations with antiserum that are foreign to the striatal tissue and changes in the osmolarity of the buffers. It therefore became imperative to observe the ultrastructual characteristics of the neostriatal neuropil and the neurons' response to just the colchicine treatment.

It was generally observed that the neuropil was occasionally swollen and synaptic boutons appeared disrupted (Figs. 5 and 7). Random samples from different regions of striatum were observed for the effect of colchicine. It was seen that the most widely affected cell type was those of the glial cells. The normal morphology of the untreated rat neostriatum in comparison (utilizing the MSB and low aldehyde concentrations) was morphologically intact and exhibited excellent morphological preservation (Figs. 6 and 9).

The effect of colchicine on the cytoplasm of neurons was noted to have an effect on several organelles and membrane bound structures. It was observed that there was an increase in the number of coated vesicles, a disruption in the Golgi apparatus and a noticeable increase in the number of multivesicular bodies (Fig. 8). Observations near the lateral ventricle (dorsal striatum) were seen to include an increase in the number of astrocytes, swelling in the axons and dendrites and an apparent disruption in the synaptic boutons (Fig. 10). It is not shown here but there was an apparent influx of neutrophils within the striatal neuropil presumably for cellular repair.

Figure 5 Low magnification of colchicine treated rat neostriatum. Numerous swollen profiles are seen within the neuropil (arrow). A swollen synaptic terminal is seen synapsing onto a neuron (curved arrow). Nucleus (N). Bar = 2μm.

Figure 6 A neostriatal neuron from a untreated rat fixed with the MSB and low aldehyde is shown to exhibit good morphological preservation. A terminal (arrow) is seen synapsing onto a medium-sized neuron(N). The cytoplasm contains numerous ribosomes, few Golgi profiles (arrowhead) and mitochondria. Bar = 2 μm.

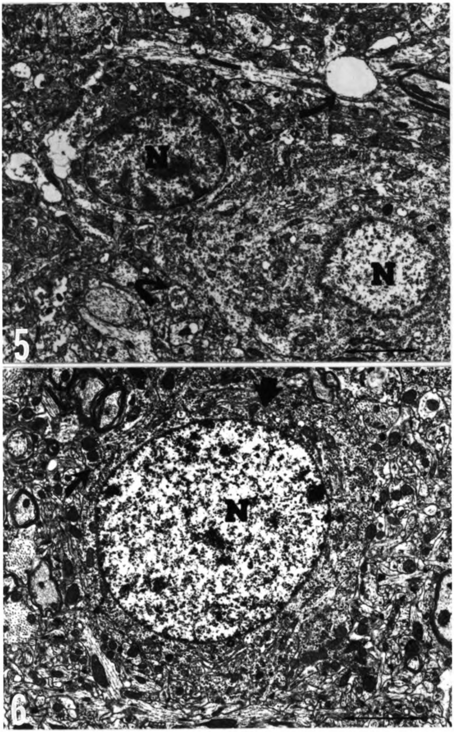

N
N
5
N
6

Figure 7 This micrograph is of a colchicine treated rat revealing a neostriatal neuron (N) containing swollen and disrupted Golgi complexes (curved arrows). The neuropil appears swollen (arrow). Bar = 1μm.

Figure 8 The cytoplasm of a colchicine treated neostriatal neuron is seen to contain multivesicular bodies (MVB), swollen Golgi complex (arrow) and numerous coated vesicles (curved arrow). Bar = 1μm.

Figure 9 Neuropil from a untreated neostriatum is seen to contain a spine (arrow) emerging from a dendrite (D). The endothelial cell on the capillary (cl) is observed to be intact. Mitochondria (M). Bar = 1μm.

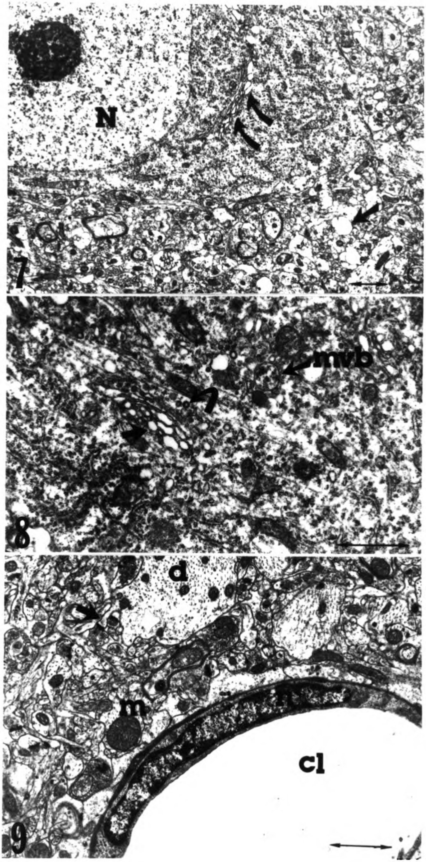
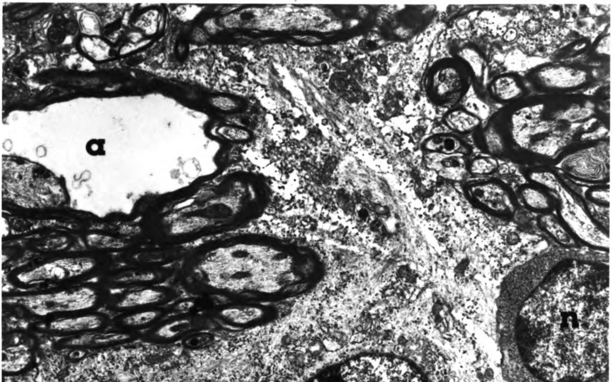

N
7
mvb
8
d
m
n
cl
9

Figure 10 This micrograph is taken from the dorsal striatum adjacent to the ventricular injection site and reveals that the neuropil lacks organization and continuity. Swelling has occurred within a myelinated axon (a). Remnants of a neuron (n) and an oligodendrocyte (n) can be seen. Bar = 2 um.


a
n
N
10

In contrast, the untreated rat neostriatum was found to exhibit a clear resolution of neuronal appearance (fig. 6) and an excellent preservation of the neuropil (fig. 9). Observations of the capillaries within the untreated rat neostriatum exhibited the endothelial cells to be intact without any noticeable changes in their plasma membranes or cytoplasmic constituents (fig. 9).

B. Wheat Germ Agglutinin

The results from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of wheat germ agglutinin (WGA) and radiolabeled derivatives of the lectin are shown in Fig. 11. In lanes 1 through 5 are results obtained employing the Laemmli discontinuous SDS-PAGE which was modified to contain 13% polyacrylamide. In lane 1 are the molecular weight standards. Lane 2 contains the native WGA which reveals several major bands. One band is around 20,000 daltons, another is 18,000 daltons, and there appear to be multiple bands between 18,000 and 14,000 daltons. The banding pattern of WGA appears broader than that of the molecular weight standards seen in lane 1 possibly due to glycosylation of these proteins as well as an intentional overloading of the gels in order to look for the presence of low molecular weight species and rule out contamination while examining the homogeneity of the lectin preparations. In lane 3 is cold N-acetylated WGA (made from the native WGA seen in lane 2 using the acetic anhydride method and affinity-purified in a N-acetylglucosamine Sepharose column at New England Nuclear) which was obtained to check the column purification as well as for use in comparison with the radiolabeled derivative. A major band is visible in lane 3 at 18,000 daltons which can also be seen in lane 4 which shows Coomassie blue staining of the affinity-purified radiolabeled derivative (i.e. WGA, N-[acetyl-^{3}H], and in addition, there appear to be a few faintly stained minor bands above 18,000 daltons in both lanes. The fluorograph from lane 4 is shown in 5 which, in addition to showing inseparable bands at and above 18,000 daltons, reveals the presence of a band at approximately 36,000 daltons. Clearly there are no other polypeptides which appear to have migrated below 18,000 daltons in both Coomassie blue stained gels and fluorographs from these gels of WGA, N-[acetyl-^{3}H].

We also decided to employ a second discontinuous SDS-PAGE system similar to that described by Neville (1971) that yielded better separation of polypeptides between 18,000 and 43,000 daltons when compared to the previously described system (compare molecular weight standards shown in lane 8 to those of lane 1 where a poorer separation between 18,000 and 12,000 daltons can also be seen with the Neville system). In lane 6 are the same molecular weight standards seen in lane 1. Lane 7 again shows the Coomassie blue stained image from the native WGA. The appearance of banding here is quite different from that seen using the Laemmli system (lane 2). In addition to a band of 18,000 daltons, and rather than the presence of multiple bands below this value as seen in the Laemmli system, the Neville gel of the native WGA reveals numerous bands between 18,000 and 43,000 daltons as well as bands above this. Lane 8 is from a Coomassie blue stained gel of affinity-purified WGA, N-[acetyl-^{3}H] that shows a major band at 18,000 daltons and a lighter stained band at approximately 36,000 daltons, and fluorography of this gel (lane 9) again reveals major bands at 18,000 and 36,000 daltons, with some labeling (though not in the form of discrete bands) between these bands and above. Lanes 8 and 9 are therefore quite similar to lanes 4 and 5 with an apparent increased resolution due to better separation between 18,000 and 43,000 daltons with the Neville system, and both systems fail to reveal any banding patterns of the affinity-purified preparations below 18,000. It should also be noted that two times concentrated samples of WGA, N-[acetyl-^{3}H] yielded the same banding patterns as seen in lanes 4, 5, 8 and 9, although the bands were broader due to an extreme overloading of the gels, with again no apparent polypeptides that have migrated below

18,000 daltons.

The possibility that some polypeptides below 30,000 daltons might have been eluted from the gels during fixation, staining, or washing was examined by way of performing scintillation counting on fresh gels run identical to those depicted in Fig. 11. Figure 12 is a pair of graphs from two of these gels run on a sample of affinity-purified WGA, N-[acetyl-^{3}H] using the Neville system, and can be compared with the fluorograph in lane 9 of Fig. 11. A background reading of 1500 counts per minute was obtained from a sample of cold acetyl-WGA. Measurements performed on adjacent lanes containing samples that were stained or processed for fluorography revealed the presence of a major peak (greater than 4×10^6 cpm) at 18,000 daltons and a relatively smaller peak near 36,000 daltons (approximately 775,000 cpm) (see Fig. 12). In addition, considerabley smaller peaks are visible near 12,000 daltons (approximately 98,000 cpm at its maximal height) and above 43,000 daltons (approximately 238,000 cpm). The plot of counts from a gel of two times concentrated WGA (right graph), N-[acetyl-^{3}H] is similar to that shown in Fig. 12a. These values obtained from scintillation counting appear rather compatible with the fluorographic image shown in lane 9 of Fig. 11, with the exception of the very small peak near 12,000 daltons which was not detectable in the stained gels or their accompanying fluorographs. (These results have been reported in a paper by Steindler and Bradley, 1983).

C. Antibody Purification

All antiserum utilized in this study were purified to an immunoglobulin fraction and were free of albumin and complement. The antibodies employed were analyzed for specificity as well as their

Figure 11 Lane 1 through 7 are Laemmli SDS-PAGE system and Lane 8 through 13 are Neville SDS-PAGE system.

Lane 1 is the molecular weight standards which consist of the following animal polypeptides as mentioned in the material and methods: Cytochrome C-12,3000; Lysozyme-14,300; Beta-lactoglobulin-18,400; Alpha-chemotrypsinogen-25,700; and Ovalbumin-43,000 daltons. Lanes 2 and 9 are the native wheat germ agglutinin, non-column purified. Lane 3 is the column purified WGA-n-(acetylated). Lanes 4 and 10 are WGA, n-(acetyl-^{3}H) and lane 5 and 11 are the corresponding fluorographs. Lanes 6 and 12 are 2X concentrated. Lanes 7 and 13 are the corresponding fluorographs.

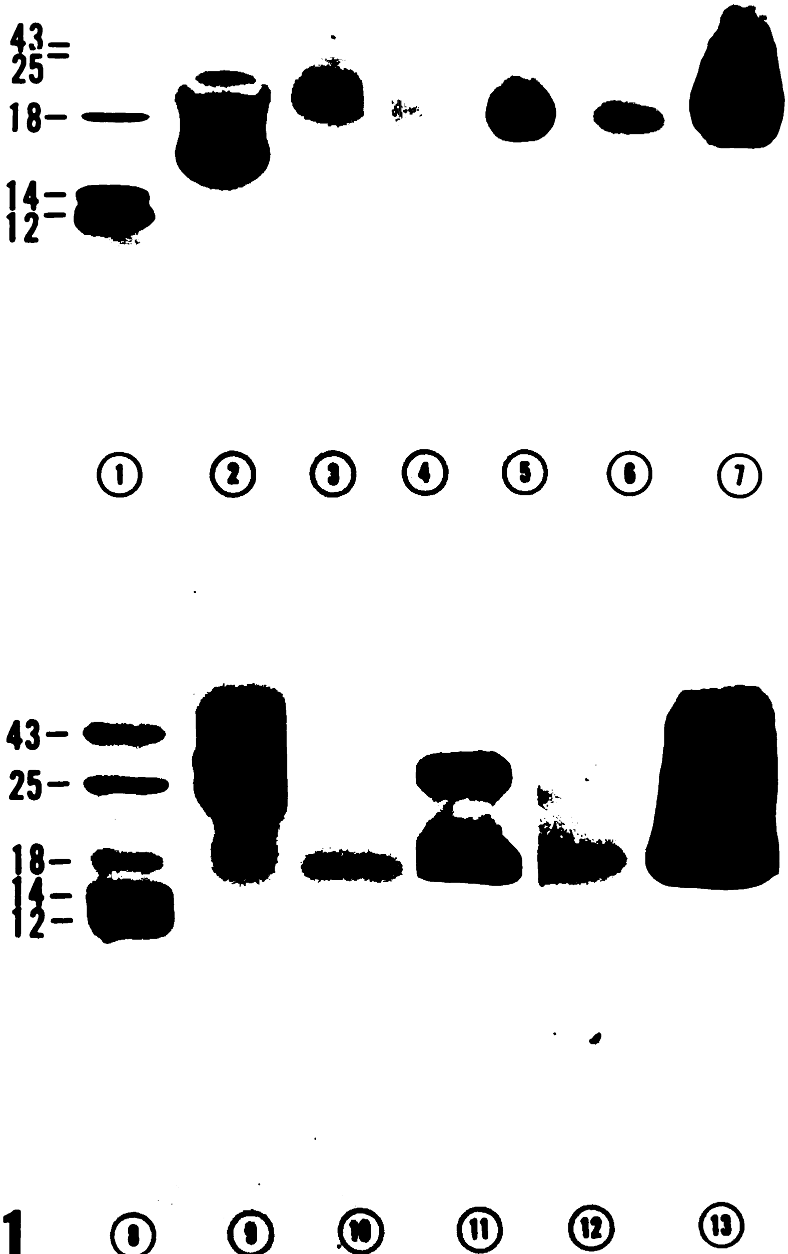


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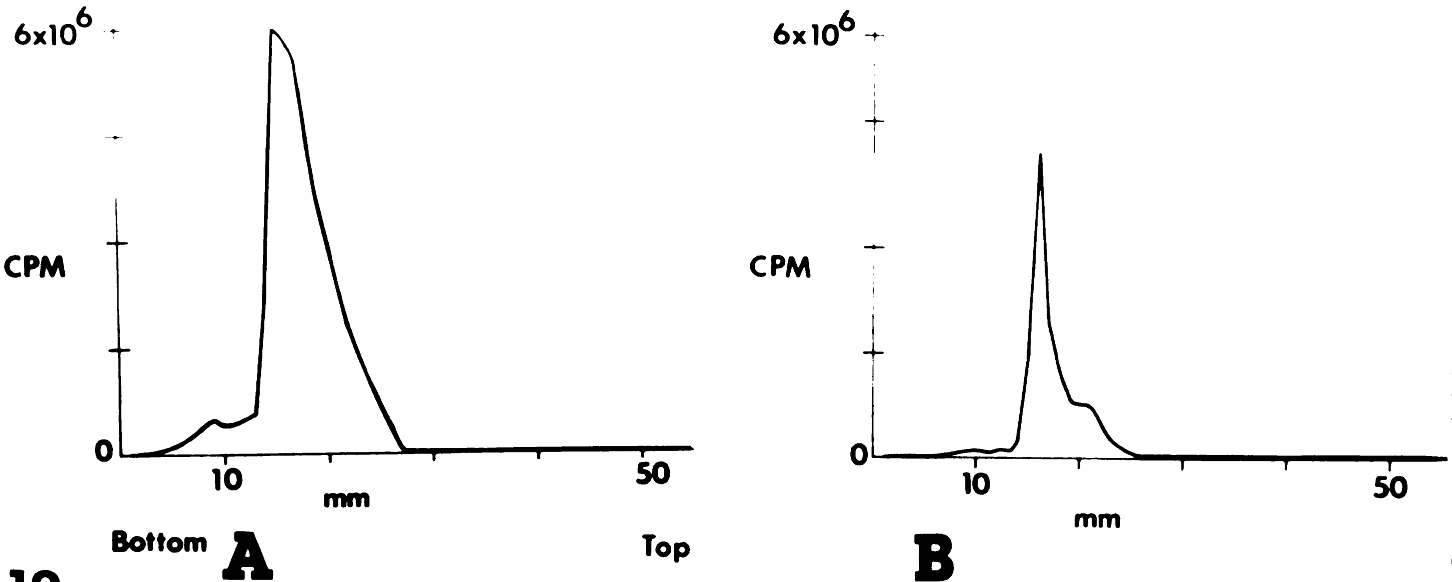
Figure 12 Graphs A and B are representations of the scintillation counting from the Neville SDS-PAGE of concentrated and non-concentrated wheat germ agglutinin, n-(acetyl-^{3}H). The SDS-PAGE gels were cut from the migrating dye front (bottom) towards the Top of the gel. The cut gels were 2 mm. sections and counted respectively.



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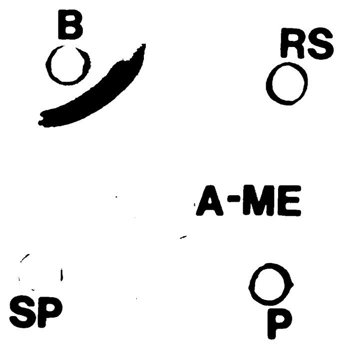
sensitivity to rat brain homogenates. Initial analysis of the rabbit anti-methionine enkephalin (ME) antiserum revealed a double precipitant band when incubated with rat brain homogenates identified by immunodiffusion-Ouchterlony (Fig. 13). However, the rabbit anti-ME was not observed to cross react with either substance P, rabbit-peroxidase - anti-peroxidase or normal rabbit serum. It was believed that the anti-ME sera contained additional antibodies to leucine enkephalin, a common contaminate in Immunonuclear,Inc. antibody. The rabbit anti-ME sera were then absorbed with 10 ug/ml leucine enkephalin, spun down, and the supernatant re-analyzed with immunoelectrophoresis (Fig. 14). This purified rabbit anti-ME sera exhibited only one precipitant band with rat brain homogenate. Rat brain homogenate was additonally found not to cross react with normal sheep serum.

Analysis of the rabbit anti-substance P showed specificity for substance P and exhibited no cross reactivity to either methionine enkephalin, normal sheep serum or normal rabbit serum (Fig. 15).

GAD and GABA-T antisera obtained from Dr. Wu's lab were analyzed for rat brain cross reactivity. Rabbit anti-GAD sera was shown to cross react with white Spraque-Dawley rat brain homogenates (Fig. 16) and no cross reactivity to either methionine enkephalin or normal rabbit serum was shown. Rabbit anti-substance P sera showed partial cross reactivity with rat brain homogenates, indicating no cross reactivity with the GAD antisera. Analysis of rabbit anti-gamma aminobutyric acid transaminase (GABA-T) sera showed a cross reactivity with whole brain, striatum, and striatum plus globus pallidus homogenates (fig. 17). Rabbit anti-GABA-T sera did not cross react with normal rabbit serum.

Figure 13 Ouchterlony characterization of rabbit anti-methionine enkephalin: A-ME (1:100 rabbit anti-methionine enkephalin) is located in the center well. B(rat brain homogenate); RS (10% normal rabbit serum); SP (substance P-10 ug/ml) and P (1:50 dilution of rabbit peroxidase anti-peroxidase).

Figure 14 Immunoelectrophoresis Gel of absorbed rabbit anti-methionine enkephalin (anti-ME is in the center well). SS (1:20 normal sheep serum), B (rat brain homogenate).



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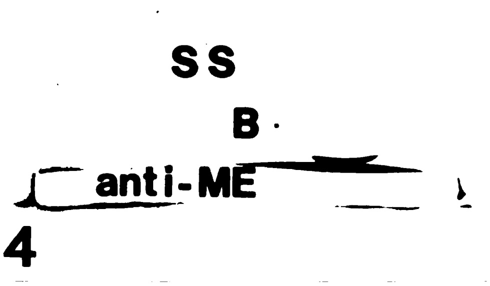

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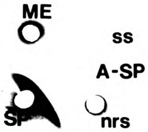
Figure 15 Ouchterlony gel immunodiffusion of rabbit anti-substance P (A-SP); ME (10 μg/ml methionine enkephalin); ss (10% normal sheep serum); nrs (10% normal rabbit serum) and SP (10 μg/ml substance P).

Figure 16 Ouchterlony gel immunodiffusion of rabbit anti-glutamic acid decarboxylase (A-GAD); ME (10 μg/ml methionine enkephalin); B (rat brain homogenate); SP (1:1000 rabbit anti-substance P); nrs (normal rabbit serum).

Figure 17 Ouchterlony gel immunodiffusion of rabbit anti-gamma aminobutryic acid transaminase (GT); WB (whole rat brain homogenate); S (striatal homogenate); S-GP (rat striatal and globus pallidus homogenate); nrs (10% normal rabbit serum).

Figure 18 Ouchterlony gel immunodiffusion of rabbit anti-leucine enkephalin (A-LE); ME (10 μg/ml methionine enkephalin); nrs (normal rabbit serum); LE (leucine enkephalin-10 μg/ml); SP (10 μg/ml substance P).



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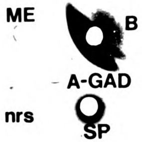

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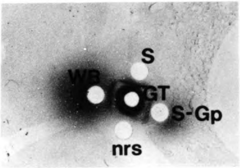

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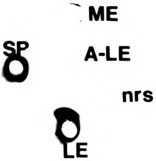

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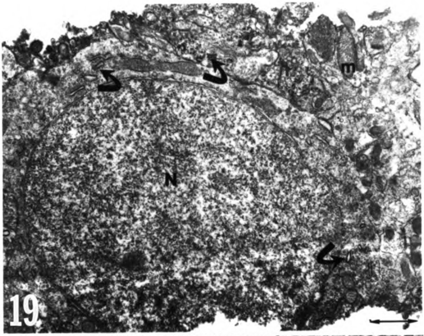
Figure 19 This is a cross section of 10 μm vibratome section of rat neostriatum incubated with rabbit anti-GAD and visualized with rabbit PAP. Curved arrows are shown to exhibit a labeled rough endoplasmic reticulum. Nucleus (N); Mitochrondria (M). Bar = 1 μm.

Figure 20 This vibratome section (60 μm) is of rat neostriatum that was incubated with rabbit-anti-GAD and visualized with the avidin:biotin complex immunoperoxidase technique. Nucleus (N); Mitochrondria (M). Bar = 1 μm.


N
m
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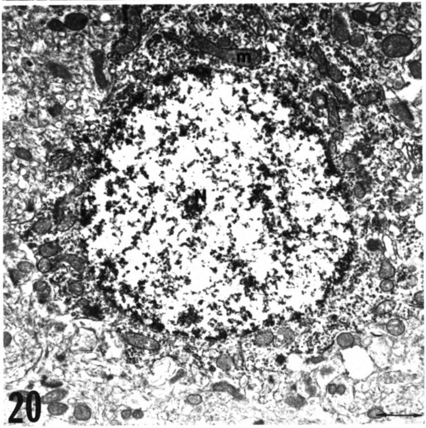
m
N
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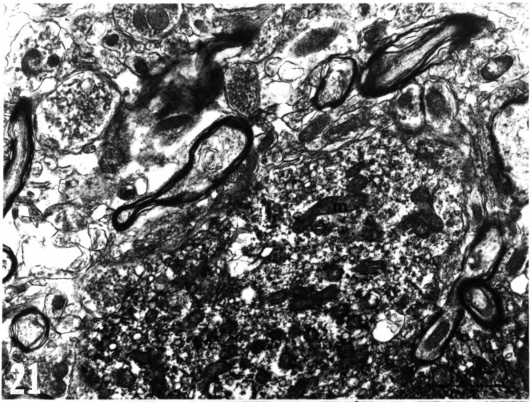
Figure 21 Vibratome section (30 um) was incubated with 1:500 rabbit anti-GAD revealing a labeled dendrite visualized with rabbit PAP. Mitochrondria (M). Bar = 1 um.

Figure 22 This vibratome section (60 um) was incubated with 1:500 rabbit anti-GAD and visualized with the avidin:biotin complex immunoperoxidase technique. Arrows indicate specific localizations within the dendrite. Arrowheads show labeled dendritic profile which is in the striatal neuropil. Bar = 1 um.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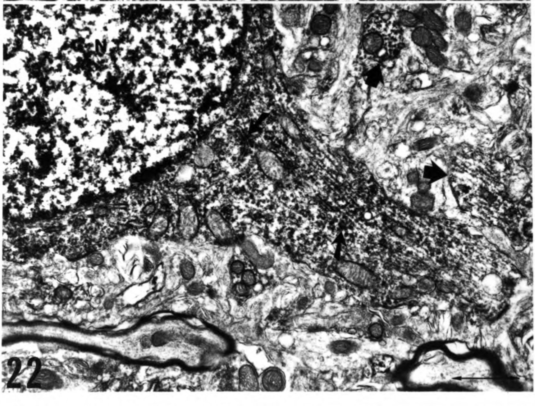
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N

Rabbit anti-leucine enkephalin sera were absorbed with 10 ug/ml of methionine enkephalin and analyzed with immunodiffusion (Fig.18). Rabbit anti-LE sera were shown to cross react with leucine enkephalin and not with substance P, normal rabbit serum or methionine enkephalin.

D. Peroxidase-Anti-Peroxidase vs. Avidin:Biotin Immunocytochemistry

Initial experiments employing PAP gave inconsistent results as well as spurious, random staining for putative neurotransmitters. Vibratome sections were analyzed for the depth of penetration of antibody and the PAP or avidin:biotin complex method.

Cross sections of vibratome sections were viewed in the electron microscope. Localization of the enzyme GAD was chosen for the comparitive analysis of penetration properties. The use of PAP for the intensification of GAD resulted in a limited penetration of the PAP (less than 1.5um)into the cytoplasm of the neuron (Fig. 19). As can be seen by this micrograph, this vibratome section thickness was rather thin (less than 15 um). The overall PAP labeling of the GAD antigens appeared cloudy and was not specfically localized within the cytolasm. However, analysis of the avidin:biotin complexes yeilded an entirely different picture. Penetration obtained with the avidin:biotin method was seen to go as deep as 25 um within a 60 um vibratoem section (Fig. 20). Immunocytochemical labeling of GAD utilizing avidin:biotin method was distinctly localized on the ribosomes and rough endoplasmic reticulum within neostriatal neurons. Proximal dendrites labeled for GAD were found to contain labeling on ribosomes and within the cytoplasm (Fig. 22). In contrast, GAD labeling with PAP molecules were found to be non-specifically localized throughout the cytoplasm of dendrites (Fig. 21).

Therefore, due to the better penetration of labeling obtained with the avidin:biotin method, further analysis of neurotransmitters at the ultrastructual level were visualized with the avidin:biotin method.

E. Light Microscopic Analysis: Neostriatal Projection Neurons and their Putative Neurotransmitters

1. Neuroanatomical Analysis of Wheat Germ Agglutinin Labeling

Labeling of neostriatal prjection neurons was accomplished by stereotaxic injections of wheat germ agglutinin (WGA) into either substantia nigra (Fig. 23) or globus pallidus (Fig. 27). Autoradiographic identification of these injection sites showed that regions adjacent to either the substantia nigra (Fig. 24) or the globus pallidus (Fig. 28) injection sites contained very little concentrations of the radiolabeled WGA. An additional method for analyzing the injection site and the labeled neostriatal neurons containing the WGA antigen was to employ an immunocytochemical method. It was found that the neostriatal neurons could be seen to have the WGA antigen within their cytoplasm located in dense granules. To further confirm that the radiolabel was indeed located within these neurons, a combined double label technique was employed. The vibratome sections had been immunocytochemically identified for the WGA antigen. Therefore, the sections were mounted on glass slides and dipped in NBT2 nuclear emulsion and exposed. This autoradiographic identification revealed that the neostriatal neurons that contained immunoperoxidase also had silver grains above the cells (Fig. 29).

Observations were made of hippocampus to discern any labeleing and to control for the injection into globus pallidus. Hippocampal neurons were not autoradiographically labeled. Observations of cortex showed an occasional cerebral cortical neruon to be labeled. Observations of substantia nigra after a globus pallidus injection of WGA revealed several neurons labeled and the subthalamus also revealed neuronal labeling. Observations and samples were only made on the ipsilateral side as that of the WGA injection into either substantia nigra or globus pallidus.

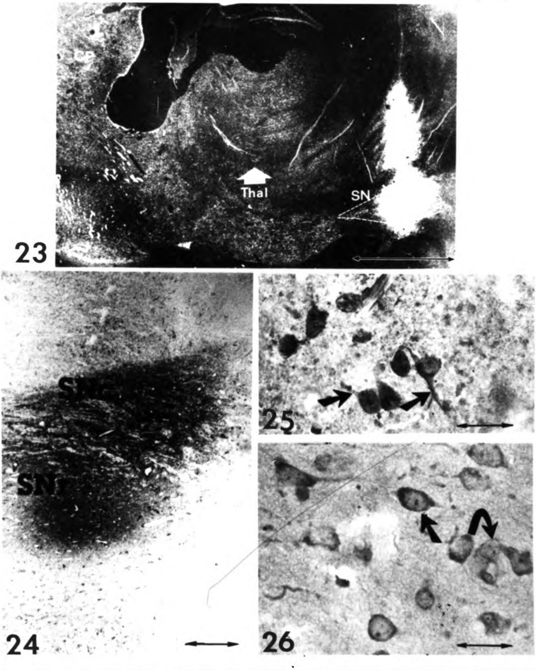
Observations of neuronal labeling were made after the substantia nigra injection of WGA and the injection site was localized with anti-WGA and autoradiography (Fig. 24). Neostriatal neurons were labeled throughout neostriatum and cerebral cortex was shown to have only a background silver grain content. The substantia nigra injection was seen also to label the subthalamus with numerous silver grains over neurons. The labeling within the substantia nigra was seen in neurons and scattered throughout the neuropil (Fig. 25). The small pressure injections of WGA did not appear to destroy the cellular intregrity of the substantia nigra.

Figure 23 A 4μm glycol methacrylate darkfield autoradiographic section exposed for the localization of wheat germ agglutinin, N-(acetyl-^{3}H) which was stereotaxically injected into substantia nigra (SN). Thalamus (Thal); Caudate-putamen (CP). Bar = 2mm.

Figure 24 Higher magnification of the injection site as seen in brightfield. Autoradiographic labeling is seen in substantia nigra pars compacta (SNc) and substantia nigra pars reticulata (SNr). Bar = 200μm.

Figure 25 This vibratome section was incubated with purified rabbit anti-WGA revealing numerous labeled neurons and their dendrites (arrows) after a substantia nigra injection. Bar = 20μm.

Figure 26 A 4μm glycol methacrylate section was incubated with rabbit anti-WGA and visualized with avidin: biotin technique. The straight arrows depicit a labeled neuron whereas the curved arrows show an unlabeled neuron. Bar = 20μm.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Thal
SN
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25
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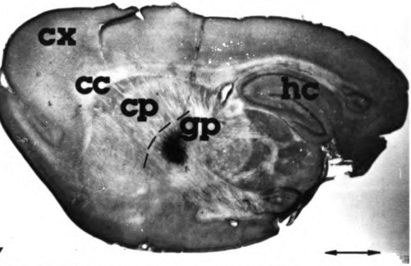
Figure 27 A vibratome section (60μm) of the globus pallidus injection site of WGA. The WGA was visualized by incubating the section with rabbit anti-WGA and intensified with the avidin:biotin immunoperoxidase method. Cortec (Cx); corpus callosum (CC); caudate-putamen (CP); globus pallidus (GP) and hippocampus (hs). Bar = 2mm.

Figure 28 Vibratome section of globus pallidus injection site which is exposed for autoradiography identification and was incubated with anti-WGA to identify the neostriatal projection neurons. This section is therefore, autoradiographically and immunocytochemically labeled. Caudate-putamen (CP) and globus pallidus (GP). Bar = 200μm.

Figure 29 Autoradiographic (a) and immunocytochemically (g) identification of WGA is seen within a single neostriatal neuron. Bar = 10μm.



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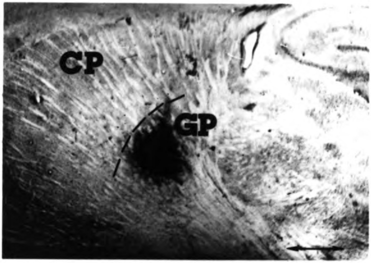

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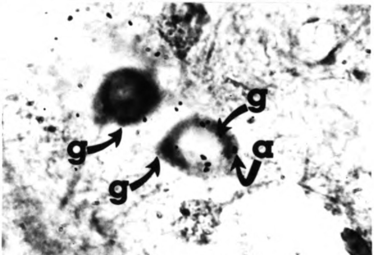

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2. Neuroimmunocytochemistry of Putative Neurotransmitters

Vibratome sections were utilized for examining the distribution of immunocytochemically identified neurons within the rat brain and for the determination of somatic sizes. Additionally, glycol methacrylate sections were used for examining the morphological characteristics of neostriatal neurons identified for putative neurotransmitter.

Control sections were performed by incubating vibratome sections with either normal rabbit serum (10%) or antibody absorbed with the antibody's specific antigen (Substance P plus anti-substance P). These control sections showed a lack of any somatic staining anywhere within the rat brain after osmium intensification (Fig. 30). The myelinated fiber fascicles are observed to be stained by the osmium.

Observations of the sections incubated with GABA-T antisera revealed numerous different cell types stained throughout the rat brain (Fig. 31). The cells labeled within the striatum appear to be large neurons, medium-sized and a small class of neurons. Different sized neurons could also be seen in cerebral cortex, globus pallidus, substantia nigra and subthalamus. The corpus callosum exhibited cellular staining in a small punctate type of cell.

Rabbit anti-substance P was shown to label cells within striatum that were medium-sized. These cells were observed to be the heaviest stained in the rostral region of striatum while other regions exhibited staining to a lesser degree (Fig. 32). Substance P was localized in fibers within the substantia nigra and the caudal region of the globus pallidus. The pontine reticular formation showed several neurons to be stained as well as fibers.

Glutamic acid decarboxylase (GAD) antisera was seen to stain the greatest number of medium-sized neurons in the ventrocaudal regions (Fig. 34). The dorsal region of striatum contained GAD positive neurons but to a lesser degree (Fig. 33). GAD positive neurons within striatum were seen in clusters of 4-7. The heaviest concentration of GAD positive medium-sized neurons was seen clustered along the caudal border of striatum adjacent to globus pallidus. GAD positive neurons were seen scattered throughout the cortex but was not specifically localized in any specific layer or region. Staining within the globus pallidus was rarely if ever observed. GAD positive neurons and numerous fibers were observed in the substantia nigra pars reticulata. GAD immunoreactivity was also seen localized within the hippocampus. The staining within neurons was seen to be mainly in the cytoplasm of the somata and occasionally dendritic profiles. GAD was observed in the cerebellum.

Methionine enkephalin immunoreactive cell bodies were observed to be the heaviest in the ventral striatum and regions of striatum adjacent to the globus pallidus (Fig. 35). The cell bodies were those of the medium-sized neurons and they appeared to be in clusters scattered throughout striatum. Methionine enkephalin fibers were mainly confined to the rostral regions of globus pallidus with less fiber staining observed in the caudal regions. Several large neurons were seen stained within the globus pallidus. Fiber staining in the substantia nigra was relatively small in comparison to striatum or rostral globus pallidus. The methionine enkephalin immunostaining in the medium-sized neurons observed above was mainly in the cytoplasm perinuclear orientated and occasionally the dendrites were labeled.

Observations at a higher magnification of immunopositive neurons within the neostriatum are reported below. These observations were made on vibratome sections (60 um) and 4 um glycol methacrylate (GM) sections for an overall characterization of the neuronal morphology.

GAD immunoreactive medium-sized neurons (10-15um) are seen in clusters of five in this vibratome section from the ventral region of striatum (Fig.36). Several dendritic profiles are seen labeled coming off the main soma of immunopositive somata. Numerous immunopositive fibers are observed adjacent in the neuropil. GM sections stained for GAD exhibit staining in the cytoplasm in a perinuclear fashion (Fig.37). Numerous cellular profiles are seen to be not stained for GAD scattered around immunopositive neurons. The GAD immunopositive neurons possess a smooth, round, unindented and pale nuclei. The nucleoli appear to be positive for GAD.

GABA-T immunopositive neurons are seen scattered throughout the striatum. A vibratome section from the rostral region of the neostriatum is shown to exhibit several sizes of immunopositive neurons (Fig. 38). Large, medium and small neurons appear to be immunopositive and possess an immunopositive rim of cytoplasm. The background staining within the striatum is seen to be considerably heavier than any other neurotransmitter localized. Dendrites are not stained for GABA-T. A GM section reveals immunoreactivity within a large neuron possessing a variable rim of cytoplasm (Fig.39). Several other neuronal profiles are seen to lack any GABA-T staining.

Vibratome sections that were incubated with rabbit anti-methionine enkephalin revealed immunoreativity within medium-sized neurons (Fig. 40). These neurons possess a thin rim of immunopositive cytoplasm and staining within dendrites was occasionally seen. Numerous immunopositive fibers were scattered throughout the neuropil. A glycol methacrylate section stained for ME is shown to exhibit a medium-sized neuron with a thin rim of immunopositive cytoplasm (Fig. 41). The dendrites are observed here to contain immunoreactivity.

Substance P immunoreactivity was seen in vibratome sections to be localized within medium-sized neurons. These medium-sized neurons were seen to possess a round nucleus and a thin rim of immunoreactivity in the cytoplasm. Numerous substance P immunopositive fibers are seen scattered throughout the neuropil. Incubation of substance P with a GM section is shown to reveal a medium-sized neuron with a thin rim of immunopositive cytoplasm. The nucleus is found to be round and pale. Several dendrites are observed to contain immunoreactivity (Fig. 43) which are seen to be projecting away from the immunoreactive soma.

3. Double Label: Projection Neuron/Neurotransmitter

Double label immunofluorescent staining was performed on glycol methacrylate sections for the identification of the neostriatal projection neuron and their putative neurotransmitter. The neurotransmitter was visualized with fluorescein and the identification of the retrogradely transported WGA was visualized with rhodamine.

Substance P positive neurons from the rostral striatum (Fig. 44) were shown to also contain the WGA antigen (Fig. 45) following a globus pallidus injection of WGA. However, not all substance P positive neurons (Fig. 46) were shown to contain the WGA antigen following a substantia nigra injection (Fig. 47). The neuropil shows fiber staining especially in the fiber bundles within striatum (Figs. 46 and 47). The immunofluorescent staining patterns of the immunopositive neuron clouds the nuclear configuration. Sometimes, one can make out the dendritic profiles of the neurons that are stained (Fig. 44).

Rabbit anti-methionine enkephalin is seen to stain numerous neostriatal neurons from the caudal region of striatum (Fig.48). Observation of the WGA localization reveals that the same population of neurons is also stained (Fig. 49). The WGA injection site was into globus pallidus. The background staining pattern is a little heavier than that of other neurotransmitters. When a glycol methacrylate section was incubated with anti-GAD (Fig. 50) and re-incubated with rabbit anti-methionine enkephalin (Fig. 51), staining of a single neostriatal neuron can be seen. The glycol methacrylate section is from the caudal region of striatum near the globus pallidus border.

Glycol methacrylate sections of ventrocaudal region of striatum incubated with rabbit anti-GAD (Fig. 52) and re-incubated with anti-WGA reveals a different population of neurons that is stained.

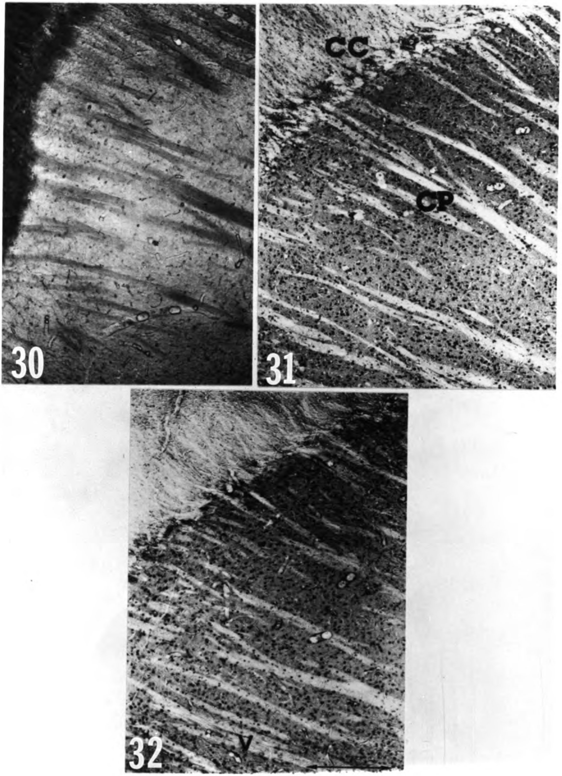
The injection of WGA was into substantia nigra and the striatonigral neurons (Fig. 53) that are labeled for the WGA antigen do not all contain the enzyme GAD. This area is near large fibers that are passing through striatum and show some degree of staining. Another section of caudal striatum incubated for GAD reveals four different neurons that are positive (Fig. 54) and when the same section was re-incubated for leucine enkephalin it now only reveals that three of these neurons contain the peptide (Fig. 55).

A summary of all the combined immunostaining patterns from PAP and immunofluorescent experiments are diagramatically represented in Figures 56 and 57. It was generally found that the substance P neuronal distribution was the heaviest in the rostral and dorsal regions of the rat neostriatum. There was some degree of substance P immunoreactivity with the substantia nigra. The majority of this staining was in the fibers with an occasional neuron stained. The distribution of methionine enkephalin immunoreactivity was localized in the caudal region of the striatum with a heavy fiber staining in the rostral region of globus pallidus. The medial regions of striatum appeared to contain the heaviest patterns of immunoreactivity for methionine enkephalin. GAD appeared to have a similiar pattern of distribution to that of methionine enkephalin. However, there appeared to be a clustering of GAD immunoreactive neurons in the caudal region of striatum adjacent to the globus pallidus. GAD immunoreactive neurons were also seen in the subthalamus and the substantia nigra reticulata. Double labeling for the putative neurotransmitters was seen in the caudal regions of striatum (either GAD plus LE or GAD plus ME).

Figure 30 A control section (60μm). Normal rabbit serum (10%) was substituted for the primary antibody and treated the same.

Figure 31 This vibratome section (60μm) was incubated with 1:500 rabbit anti-GABA-transaminase was visualized with the avidin:biotin technique. Corpus callosum (cc) and Caudate-putamen (CP).

Figure 32 This vibratome section (60μm) was incubated with 1:500 rabbit anti-substance P and was visualized with the avidin:biotin technique. Ventral striatum (V). Bar = 200μm and is the same for figs. 30, 31 and 32.


30
31
CC
CP
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V

Figure 33 Vibratome section (60 um) of dorsal striatum was incubated with 1:500 rabbit anti-GAD and was visualized with avidin:biotin technique. Corpus callosum (cc). Bar = 200 μm.

Figure 34 Higher magnification of a vibratome section (60 um) from ventral striatum which was incubated with 1:500 rabbit anti-GAD. Caudate-putamen (CP). Bar = 200 μm.

Figure 35 Low magnification light micrograph of a vibratome section (60 um) which was incubated with 1:500 rabbit anti-methionine enkephalin and was visualized with PAP. Ventral striatum (V). Bar = 200 μm.


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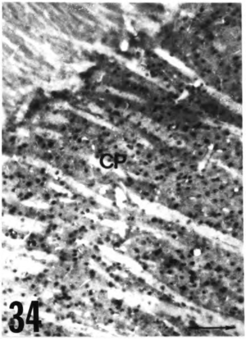
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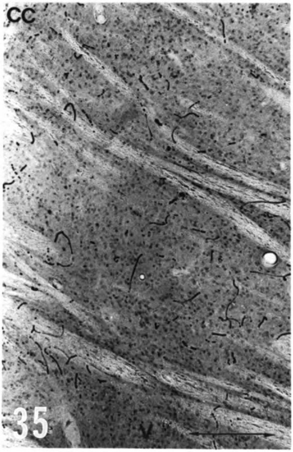
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Figure 36 A vibratome section (60 um) of ventral striatum which is exhibiting GAD immunoreactivity within neostriatal neurons. Dendrites are seen labeled (arrows). Bar = 20 μm.

Figure 37 A 4 um glycol methacrylate section of striatum was incubated with 1:500 rabbit anti-GAD. Several medium-sized neurons are seen labeled (curved arrows). Unlabeled neurons are seen scattered throughout the neuropil (arrowheads). Bar = 20 μm.

Figure 38 This vibratome section (60 μm) of rostral neostriatum was incubated with 1:500 rabbit anti-GABA-T and reveals labeled neurons. Large neurons (arrowhead) and medium sized neurons (curved arrow) are seen labeled. Bar = 20 μm.

Figure 39 Glycol methacrylate section (4 um) of neostriatum was incubated with rabbit anti-GABA-T and reveals a labeled large neuron (arrowhead) and a unlabeled cell (arrow). Bar = 20 μm.

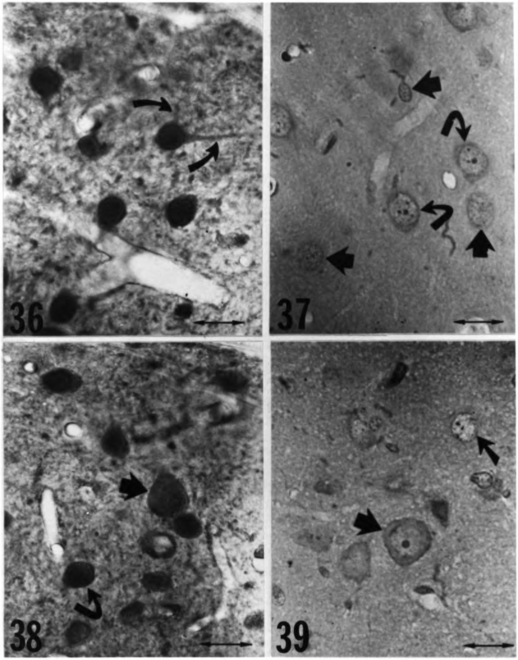

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Figure 40 A vibratome section of rostral striatum was incubated with 1:500 rabbit anti-substance P which shows labeling in numerous neurons. Bar = 20 μm.

Figure 41 A glycol methacrylate section of striatum was incubated with 1:500 rabbit anti-substance P and visualized with PAP. Arrows show dendrites of this labeled noestriatal neuron. Bar = 10 μm.

Figure 42 Vibratome section of the ventrocaudal region of striatum which is showing methionine enkephalin positive neurons. Bar = 20 μm.

Figure 43 Glycol methacrylate section of the ventrocaudal region of striatum and is showing a labeled neostriatal neuron for methionine enkephalin localization. Arrows show the dendrites of this neuron. Bar = 10 μm.

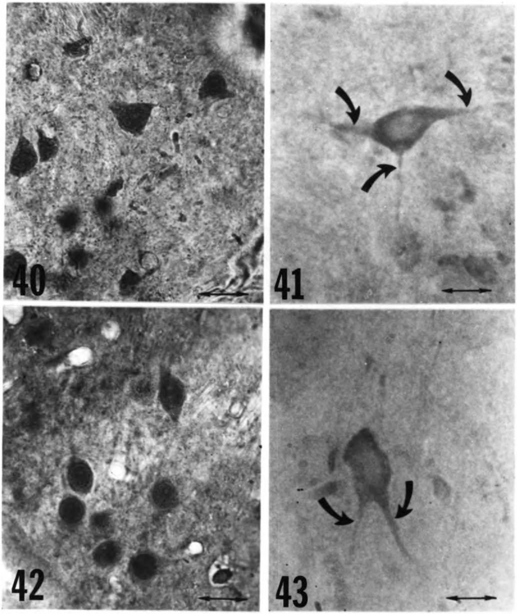
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Figure 44	This glycol methacrylate section of rostral striatum was incubated with 1:100 rabbit anti-substance P and visualized with goat-anti rabbit conjugated to fluorescein. Bar = 20 μm.
Figure 45	Same section as in fig. 44 but it is re-incubated with 1:100 rabbit anti-WGA and visualized with goat-anti-rabbit conjugated to rhodamine. These neurons labeled with for WGA represent projection neurons, i.e.-striatopallidal neurons
Figure 46	A glycol methacrylate section that was incubated with 1:100 rabbit anti-substance P. Bar = 20 μm.
Figure 47	Same section as in fig. 46 which was re-incubated with rabbit anti-WGA and visualized with goat-anti-rabbit-rhodamine after a substantia nigra injection of WGA. Arrowhead shows a neostriatal neuron which does not label for WGA.

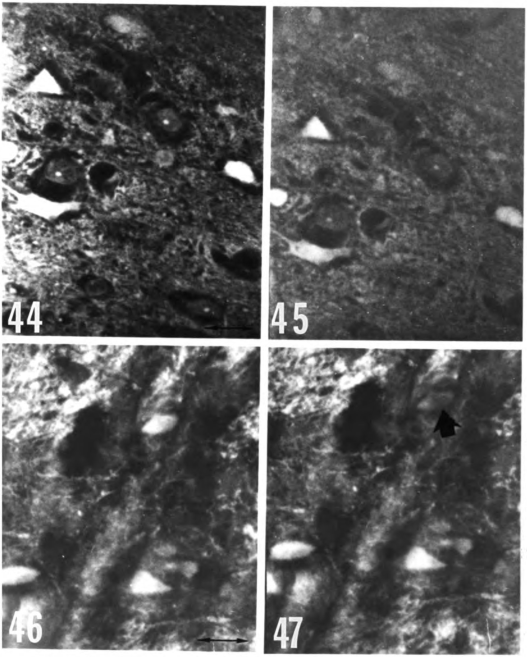
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Figure 48 A glycol methacrylate section of ventral stritaum which is revealing several methionine enkephalin positive neurons as visualized with fluorescene.

Figure 49 Same section as fig. 48 which was re-incubated with goat-anti-rabbit-rhodamine. WGA was injected into globus pallidus. Bar = 20 µm.

Figure 50 This glycol methacrylate section was incubated with 1:100 rabbit anti-methionine enkephalin and visualized with goat-anti-rabbit-rhodamine. A single neuron is seen labeled in the upper right hand corner.

Figure 51 Same section as fig. 50 which was re-incubated with 1:100 rabbit anti-GAD and visualized with goat-anti-rabbit-rhodamine. Bar = 20 µm.

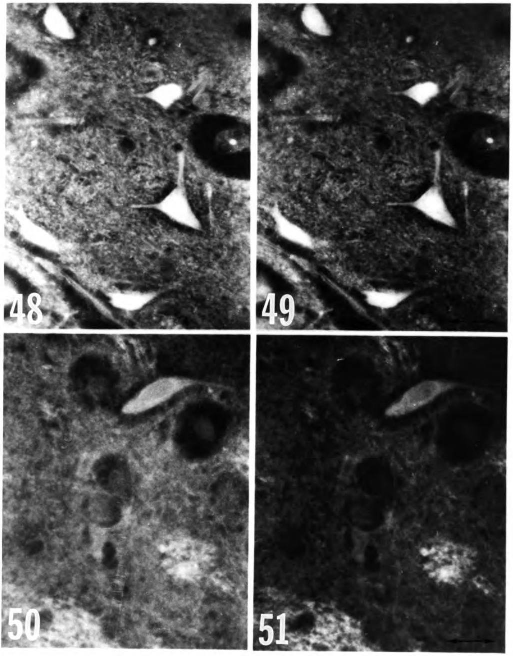
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Figure 52 Glycol methacrylate section of ventrocaudal striatum is seen to reveal GAD positive cells as visualized with goat-anti-rabbit-fluorescene to identify the rabbit anti-GAD. Arrow shows a lack of staining in this cell. Bar = 20 μm.

Figure 53 Same section as fig. 52. which was re-incubated with 1:100 rabbit anti-WGA and visualized with goat-anti-rabbit-rhodamine.

Figure 54 A 4 um glycol methacrylate section was incubated with rabbit anti-GAD-fluorecene. Arrowhead shows a positive GAD neuron which does not react in fig. 55 for anti-WGA. Bar = 20 μm.

Figure 55 Same section as fig. 54 but was re-incubated with 1:100 rabbit anti-leucine enkephalin and visualized with goat-anti-rabbit-rhodamine.

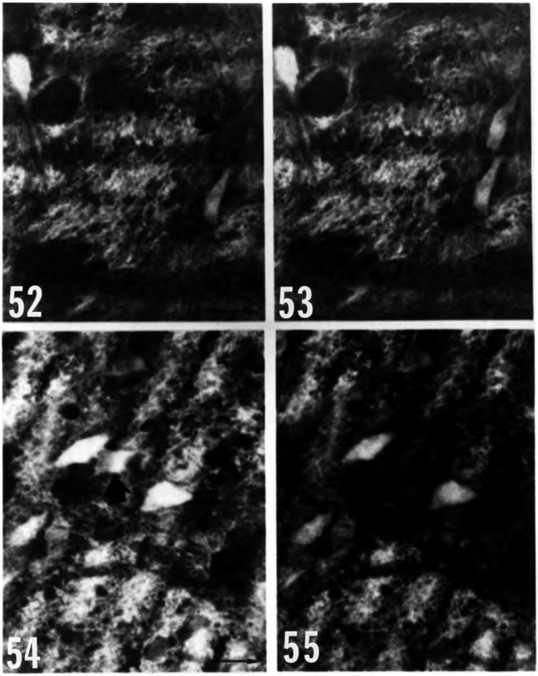
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Figure 56 Diagramatic representation of immunocytochemical findings of the topography of neurotransmitters as seen in saggittal sections of the rat brain.

▲ = methionine enkephalin

△ = GAD

○ = substance P

● = double label neurotransmitters (GAD & LE, ME)

Cortex = Cx

Subthalamus = ST

Substantia nigra = SN

Caudate-putamen = Cd

Globus pallidus = gp

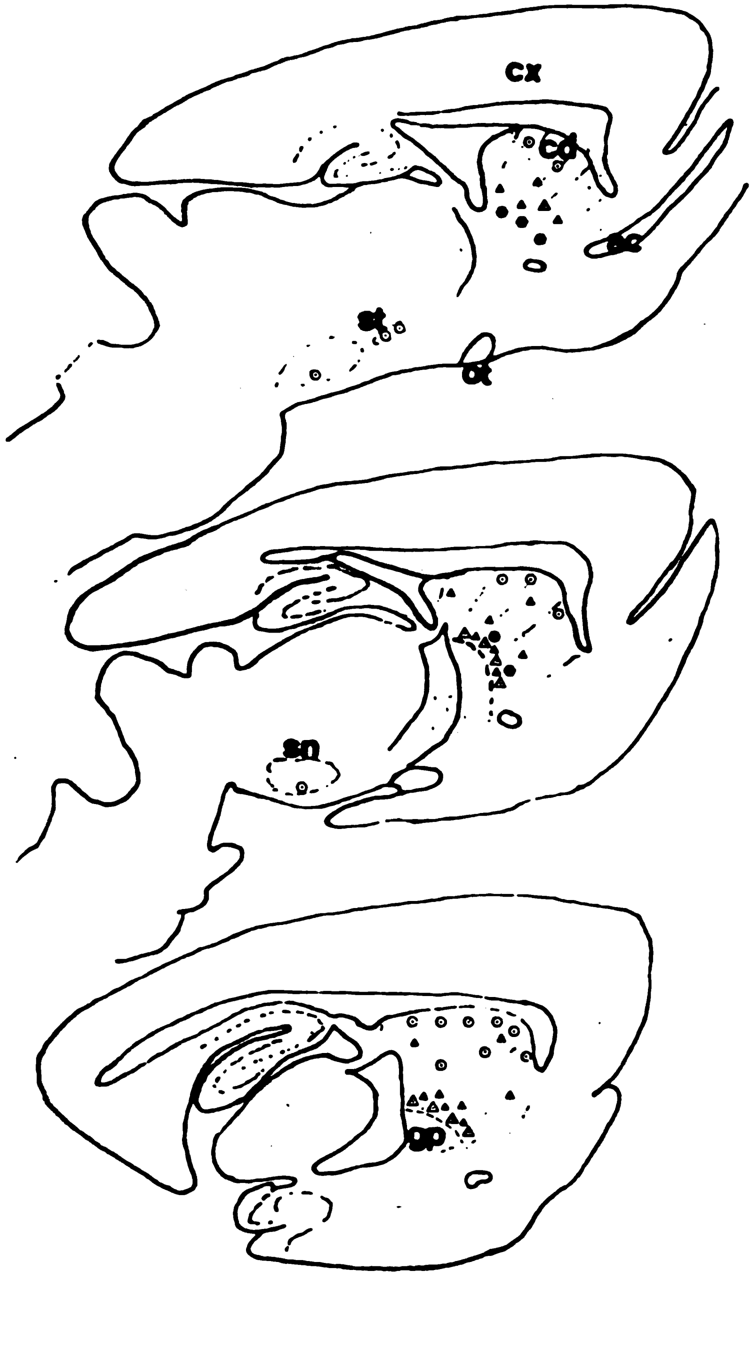

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Figure 57 Frontal sectional of the topographical distribution of immunocytochemically localized neurotransmitters which was seen within striatum.

▲ = methionine enkephalin

△ = GAD

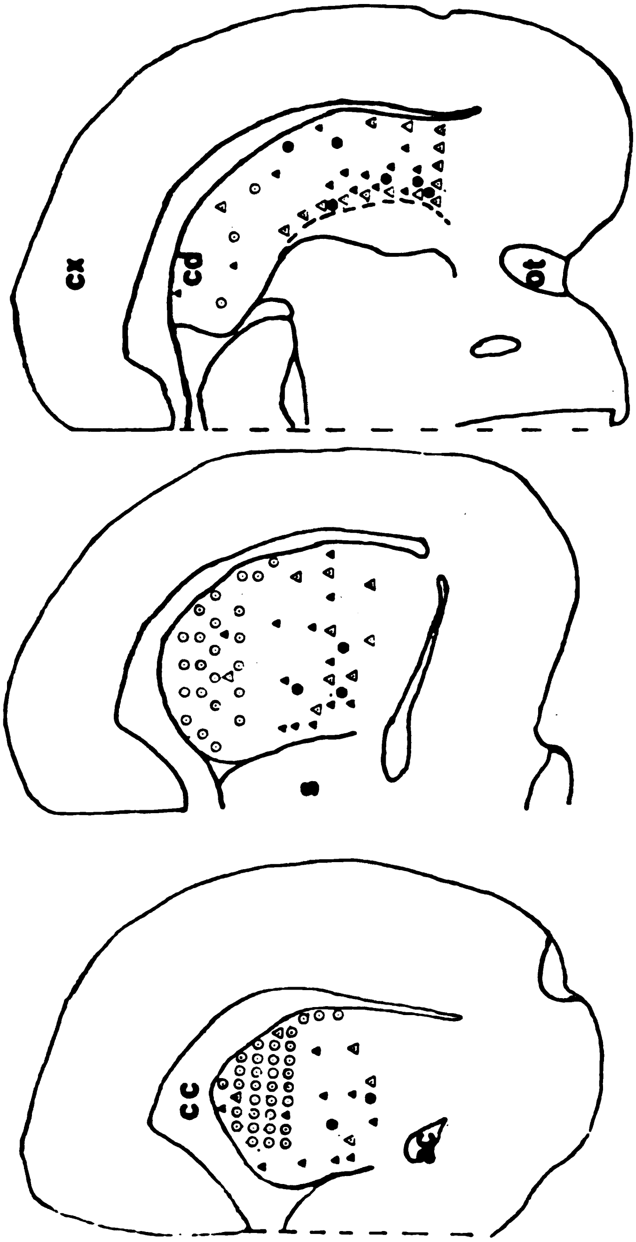
⊙ = substance P

● = double label for neurotransmitter (GAD & ME)

Cortex = Cx

Anterior commissure = Ac

Caudate-putamen = Cd



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F. Electron Microscopy

Ultrastructural observations on neurotransmitter localization within neostriatum were carried out on rats that received bilateral colchicine injections with a survival time of 48 hours. Vibratome sections (60 μm thick) were incubated with the appropriate neurotransmitter antiserum and these neurotransmitters were visualized with the modified avidin:biotin complex (ABC) method as described in the Material and Methods.

Vibratome sections incubated with a control sera (substance P plus rabbit anti-substance P) were found to exhibit no identifiable immunoreactivity at the light level (Fig. 59) when compared to sections incubated with rabbit anti-substance P (Fig. 58). When these control sections were observed at the electron microscope level no immunoreactivity was seen (Fig. 60). It was mentioned earlier that the swelling in the neuropil was due to the colchicine injections and not due to the processing of the tissue for immunocytochemical localizations.

Substance P immunoreactive medium-sized neurons (10-15 um in diameter) in rostral striatum possessed a round nucleus with a thin rim of immunoreative cytoplasm (Fig. 61). Immunoreactive dendrites of varying sizes were observed throughout the cytoplasm (Figs. 61, 62, 63). Numerous substance P immunoreactive spine laden dendrites were seen receiving unlabeled symmetrical synapses (Figs. 62, 63, 64). It was a common feature to find many labeled dendrites receiving unlabeled synapses from axons. There were no observations of labeled dendrodendritic synapses or labeled axoaxonic synapses within the neuropil.

Substance P immunoreactive presynaptic boutons made asymmetrical type synapses (Figs. 65, 66). The vesicles within these labeled presynaptic terminals were small and round in configuration. The average diameter of the labeled vesicle was 45 nm (n=75) with a range of 35 - 53 nm. Observations of labeled or unlabeled axoaxonic terminals were not seen.

Methionine enkephalin immunoreactive medium-sized neurons were seen scattered throughout the striatal neuropil. The heaviest concentration of these medium-sized neostriatal neurons was seen in ventral striatum. These methionine enkephalin immunoreactive medium-sized neurons had somatic diameters of 10-14 um which contained a round, smooth, unindented and pale nucleus. Juxtaposition to the nucleus could be seen a thin rim of immunoreactive cytoplasm (Fig. 67, 68, 69). Numerous other medium-sized neurons could be seen unlabeled within the neuropil (Fig. 67). Labeled dendritic profiles (large and small) were seen adjacent to labeled neurons (Fig.67, 68, 69). The localization of the ABC immunoperoxidase in somata was consistently found on rough endoplasmic reticulum (Fig. 69), within microtubules (Figs. 70, 71) and on the surface of mitochondria (Figs. 69, 70, 71). This type of immunoperoxidase labeling was seen in all the methionine enkephalin labeled profiles. Numerous dendritic spines were seen labeled throughout the neuropil (Figs. 70, 72). A majority of the time these dendritic spines were seen receiving asymmetrical synpases (Fig. 71). Numerous presynaptic terminals were seen to contain methionine enkephalin immunoreactivity throughout the neuropil. An example of one of these presynaptic terminals was seen synapsing onto a labeled dendrite making a symmetrical synapse (Fig. 70). Such labeling of axodendritic structures was rarely seen. The more typical presynaptic labeling was that exhibited by Figure 72, with the labeled presynaptic terminal being small in configuration and synapsing onto a dendritic spine. The average diameter of the labeled vesicles contained within the presynaptic bouton was 55 nm (n = 76) with a range of 54 - 61 nm. There was no labeled axoaxonic terminals observed.

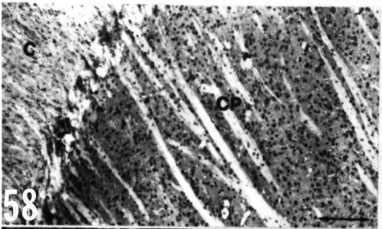
Vibratome sections incubated with rabbit anti-GAD revealed immunoreactive medium-sized neostriatal neurons concentrated mainly in the caudolateral region of striatum. Ultrastructural observations of these GAD immunopositive medium-sized neurons with a somatic size of 10-13 μm possessing a smooth, round and unindented nucleus (Figs. 73, 74, 75). These GAD immunoreactive neurons were seen to have a thin rim of cytoplasm with immunoreactivity on different cytoplasmic organelles. A typical medium-sized neostriatal neuron exhibiting immunoreactivity is seen to receive an unlabeled asymmetrical synapse (Fig. 74). The immunoreactivity within this medium-sized neuron was observed to be localized on coated vesicles, mitochondria and polyribosomes (Fig. 75). Numerous dendritic profiles are seen labeled which are adjacent to the labeled somata (Fig. 76). Numerous dendritic spines were seen labeled throughout the neuropil. The majority of the dendritic spines that were labeled for GAD were seen to receive asymmetrical synapses that contained no GAD labeling (Fig. 76). Another common occurrence was to see a labeled dendrite of small diameter receiving a symmetrical synapse which was not GAD labeled (Fig. 77). The observations of GAD labeled symmetrical synapses were rarely seen in the neuropil. Labeled presynaptic vesicles were found to have an average diameter of 33 nm (n - 69) with a range of 32 - 36 nm. GAD positive labeled axoaxonic terminals were not observed.

Labeling of GABA-T immunoreactive neurons was homogenous throughout striatum as well as other basal ganglia structures. As mentioned in the light microscopic observations of GABA-T immunoreactive regions of the rat brain included not only the basal ganglia but almost every other region as well. GABA-T immunoreactive neostriatal neurons possessed ultrastructural characteristics similar to those of a type II medium neuron, namely a medium aspiny neuron (Fig. 79). This GABA-T immunoreactive neurons possessed a deeply invaginated nucleus with dark heterochromatin. This type of characteristics were seen in a vast majority of the neostriatal neurons stained for GABA-T. On occasion a medium-sized GABA-T immunoreactive neuron was seen to possess a round and smooth unindented nucleus (Fig.80). These GABA-T immunoreactive neurons were seen to possess a thin rim of immunoreactive cytoplasm (Fig. 81). The immunoperoxidase localization was seen in the cytoplasm on rough endoplasmic reticulum, mitochondria and polyribosomes (Figs. 80, 81). Immunoreactive dendrites (Fig 82) with large and small diameters were routinely found throughout the neostriatum. Smaller labeled dendritic spines were occasionally observed (Fig. 83) receiving unlabeled presynaptic boutons. Labeling of presynaptic and axoaxonic synapses were not observed.

Figure 58 A vibratome section of dorsorostral striatum was incubated with 1:250 rabbit anti-substance P Corpus callosum (C); Caudate-putamen (CP). Bar = 180 μm.

Figure 59 A control section from rostral striatum. Substance P (10 ug/ml) was added to 1:250 rabbit anti-substance P and spun down. The supernate was then applied and the rest of the avidin:biotin technique followed. This vibratome section was then osmicated to identify any reactive cells. Bar = 180 μm.

Figure 60 Ultrastructural analysis of the control section from striatum is seen to lack any reaction product within the neuropil or cytoplasm of a neuron (N). Stacks of rough endoplasmic reticulum (rer) are seen within the cytoplasm. Bar = 2 μm.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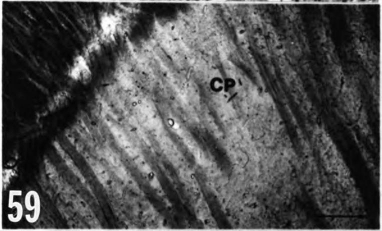
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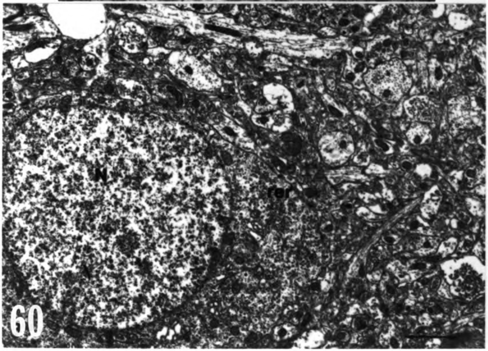
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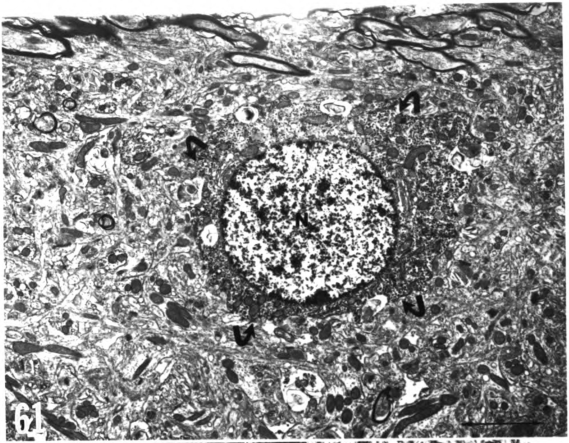
Figure 61 An ultrathin section of neostriatum was incubated in a 1:1000 rabbit anti-substance P and visualized with the avidin:biotin technique. Curved arrows outline a immunopositive neuron (N). This section was cut and viewed unstained (all ultrastructural analysis were performed in this fashion). Bar = 3 um.

Figure 62 Intermediate magnification micrograph of substance P immunoreactivity within a spine of a dendrite located within the neostriatal neuropil. Curved arrows outline the dendritic spine and small arrows show a synapse onto the dendrite. Arrowheads show a labeled dendrite. Bar = 2 μm.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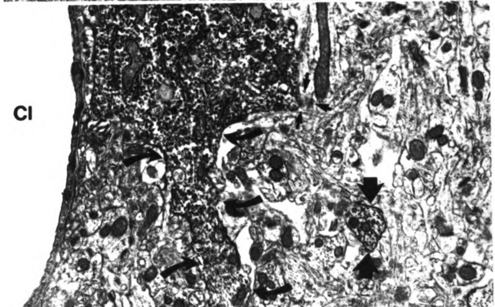
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Figure 63 A substance P labeled proximal dendrite is from rostral neostriatum. Arrowheads depict a symmetrical synapse onto the labeled dendrite. Open arrowheads show there is labeling located on the rough endoplasmic reticulum. Mitochrondria (M) are seen to appear to have little or no immunoreactivity. An unlabeled asymmetrical synapse (curved arrow) is seen adjacent within the neuropil. Bar = 2 μm.

Figure 64 A substance P labeled dendritic spine within neostriatum was seen to recieve a asymmetrical synapse. Labeling is seen within the cytoplasm and on mitochrondria. Bar = 0.5 μm.

Figure 65 Neostriatal neuropil is labeled for substance P. Arrows outline a labeled pre-synaptic terminal making a asymmetrical synapse. Bar = 0.5 um.

Figure 66 A substance P labeled pre-synaptic terminal is observed making a asymmetrical synapse with a dendritic spine head (small arrows). Bar = 0.5 um.

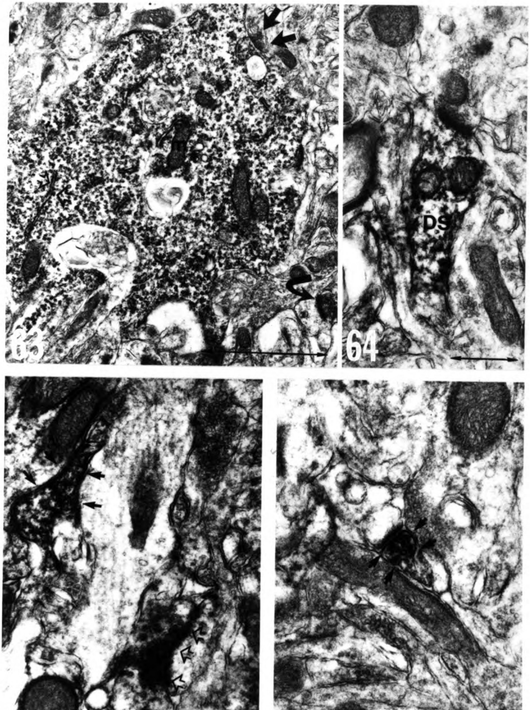
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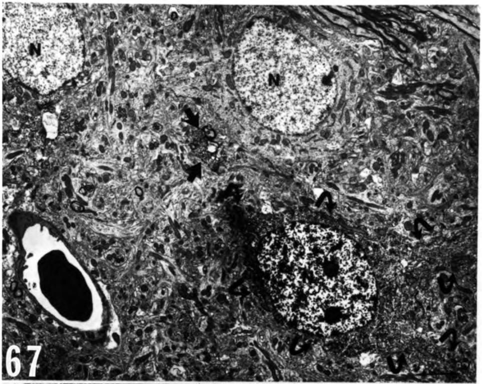
Figure 67 The ventral region of striatum was incubated 1:1000 rabbit anti-methionine enkephalin and visualized with the avidin:biotin technique. Two unlabeled neurons are seen on the top of the micrograph. Curved arrows outline a labeled neostriatal neuron and its primary dendrites. Solid arrowheads show one labeled dendrite in the neuropil. Bar = 2 μm.

Figure 68 An intermediate magnification micrograph of ventral striatum which was labeled for methionine enkephalin. This labeled neostriatal neuron (N) contains labeled rough endoplasmic reticulum (arrows) and other cytoplasmic organelles. Numerous dendritic profiles are labeled throughout the neuropil (arrowheads). Bar = 2 μm.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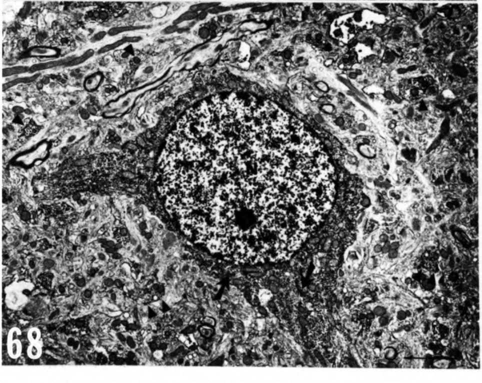
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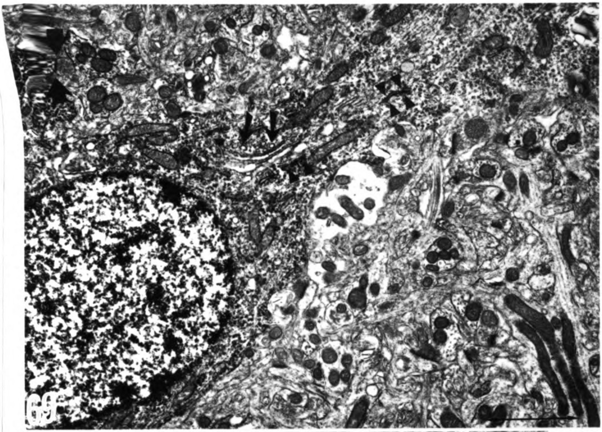
Figure 69 An intermediate magnification micrograph of ventral striatum which was incubated with rabbit anti-methionine enkephalin. Labeling is seen within a medium-sized (spiny) neuron and within its proximal dendrite. Labeling is seen localized on rough endoplasmic reticulum (arrowheads) and vesicles (arrows) forming off the forming face of the Golgi complex. Bar = 1 μm.

Figure 70 Labeling for methonine is seen within the neuropil in dendrites (open arrowheads) and a pre-synaptic terminal (solid arrows) making a symmetrical synapse. Bar = 1 μm.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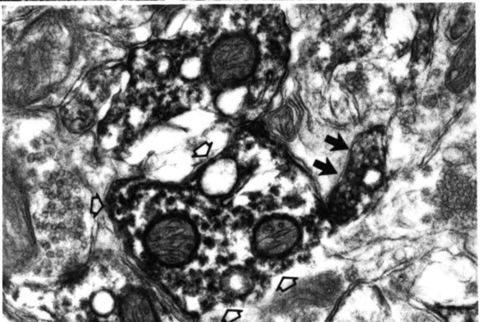
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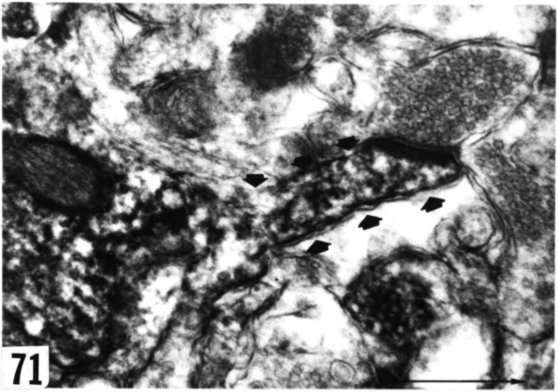
Figure 71 Methionine enkephalin is seen localized within a dendritic spine (solid arrowheads) which is recieving a asymmetrical synapse. Bar = 0.5μm.

Figure 72 A methionine enkephalin labeled terminal (open arrowhead) is observed making an asymmetrical synapse onto a spine head. Bar = 0.5μm.



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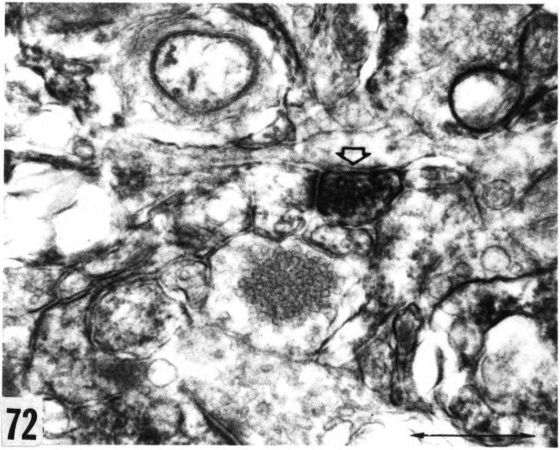

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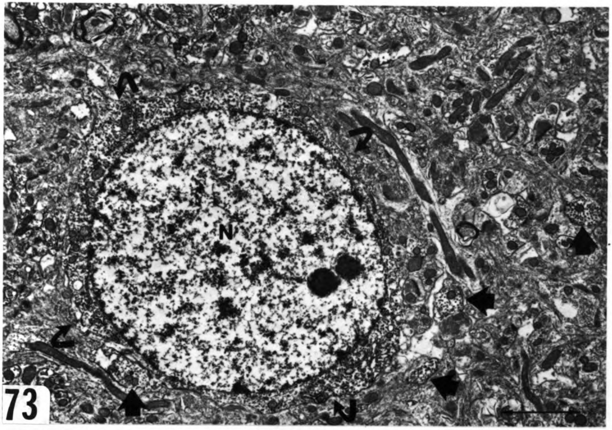
Figure 73 A ventrocaudal region of striatum was incubated with 1:1000 rabbit anti-GAD and visualized with avidin:biotin technique. A labeled medium-sized neuron (spiny) is outlined (N) with curved arrows. Several dendritic profiles are labeled within the neuropil (solid arrowheads). Bar = 2 μm.

Figure 74 A GAD labeled neuron (N) is seen to have a somatic synapse (solid arrowheads). Seen within the somatic cytoplasm is labeling localized onto ribosomes, rough endoplasmic reticulum, and occasionally on mitochondria. Bar = 2 μm.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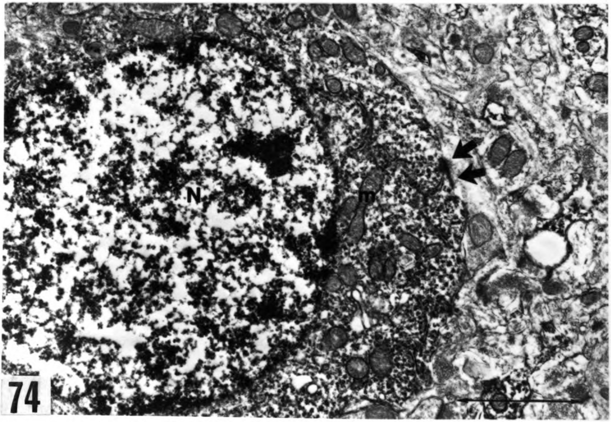
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Figure 75 A GAD labeled neuron (N) from neostriatum is observed to possess a somatic synapse (curved arrows). The synaptic vesicles are pleomorphic in size with a dense core vesicle within the terminal (arrow). Contained within the cytoplasm of the labeled neuron is observed labeling on coated-vesicles (solid arrowheads). Mitochondria (m) are occasionally seen to possess labeling on their surface. Bar = 0.5μm.

Figure 76 GAD labeling is seen within the neuropil in dendrites (solid arrowheads) and in dendritic spines (arrows). The dendritic spine has labeling throughout the cytoplasm. Bar = 0.5μm.

Figure 77 A GAD positive dendrite which has labeling within microtubules (arrow) and on the surface of mitochondria (m). A labeled dendrite is seen recieving a symmetrical synapse. Bar = 0.5μm.

Figure 78 A single terminal is seen making two symmetrical synapses and the terminal is labeled for GAD (curved arrows). Numerous small round vesicles are contained within the terminal. Bar = 0.5μm.

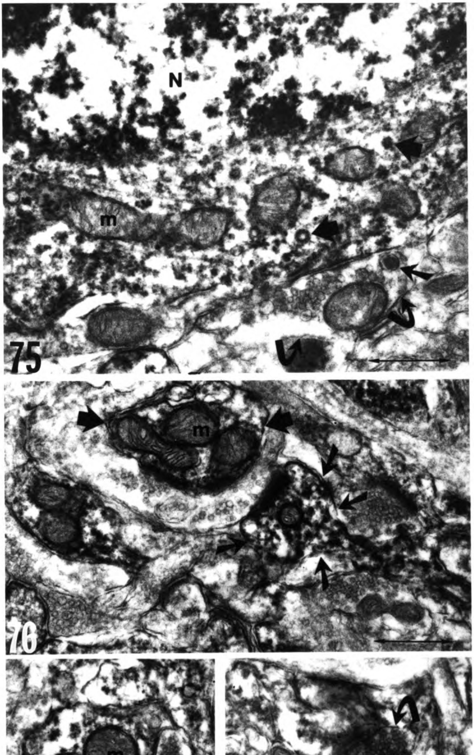
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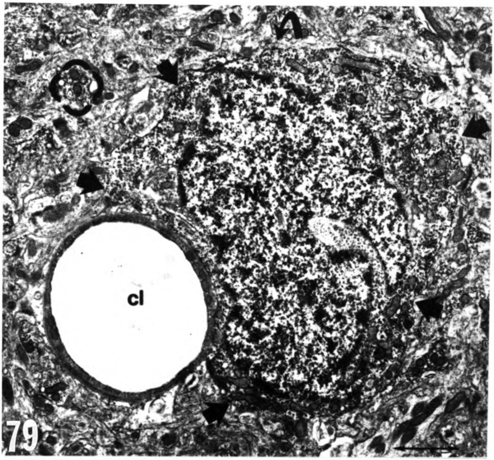
Figure 79 A section from the ventral region of striatum which was incubated with 1:500 rabbit anti-GABA-transaminase. This micrograph is exhibiting a labeled neuron (arrowheads) with a deeply indented nucleus (N) and labeling is seen throughout the cytoplasm. A somatic synapse is observed on the labeled neuron (curved arrows). A labeled dendrite is also observed within the neuropil (brackets). Bar = 2 μm.

Figure 80 A GABA-transaminase labeled neostriatal neuron (N) is seen to possess a somatic synapse (curved arrows) and labeling on mitochondria, rough endoplasmic reticulum and ribosomes. A lack of labeling is noted on the multivesicular body (arrowhead). Bar = 2 μm.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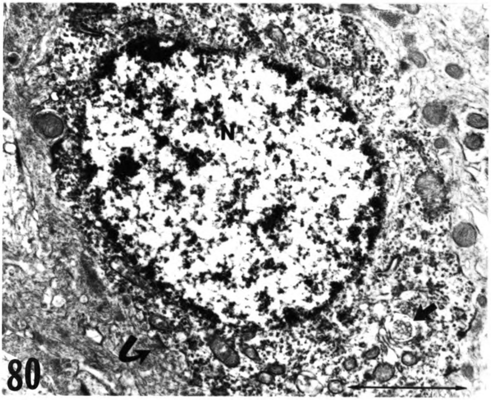
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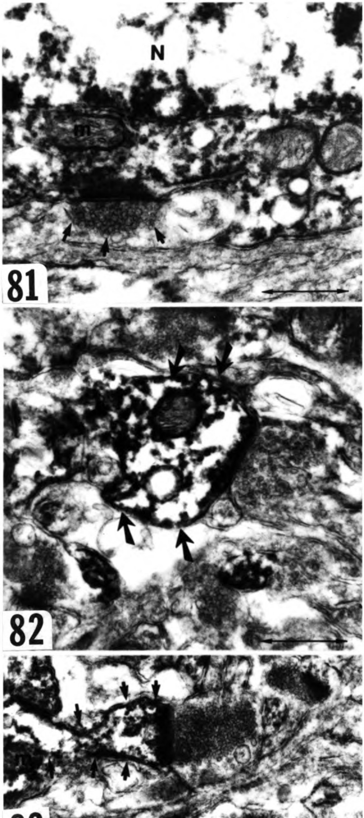
Figure 81 A GABA-T labeled soma is seen within neostriatum exhibiting a somatic synapse (arrows) and labeling is on mitochondria (m). Nucleus (N). Bar = 0.5 um.

Figure 82 A GABA-T labeled dendrite (arrows) is observed to possess heavy labeling on the mitochrondria as well as throughout the cytoplasm. Bar = 0.5 um.

Figure 83 A dendritic spine (arrows) is seen labeled for GABA-T. Labeling is observed throughout the cytoplasm and on the mitochondrial membrane (m). Bar = 0.5 μm.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Chapter 4: Discussion

A. Neostriatal Efferents and Utilization of Wheat Germ Agglutinin

The major target of neostriatal projection neurons have been identified as globus pallidus and substantia nigra. The neurotransmitters localized within these striatopallidal and striatonigral neurons have not been directly analyzed. Various anatomical and electrophysiological techniques have been used to demonstrate the striatopallidal (Bishop et al., 1978; Cuello and Paxinos, 1978; Fonnum et al., 1978; Fox and Rafols, 1976; Grofova, 1979; Jessell et al., 1978; Kemp, 1970; Nagy et al., 1978; Obata and Yoshida, 1973; Pasik et al., 1976; Preston et al., 1980; Szabo, 1962; Szabo, 1970; Szabo, 1981 and Wilson and Phelan, 1982) and striatonigral projections (Bak et al., 1975; Bak et al., 1978; Bunny and Aghajanian, 1976; Carpenter, 1976; Domesick, 1977; Fonnum et al., 1974; Fonnum et al., 1978; Fox and Rafols, 1976; Gale et al., 1977; Grofova, 1975; Grofova, 1979; Grofova and Rinvik, 1970; Hattori et al., 1973; Hong et al., 1977; Garcia et al., 1977; Jessell et al., 1978; Kanazawa et al., 1977; Kemp, 1970; Kim et al., 1971; Kitai et al., 1975; Kocsis and Kitai, 1977; Mroz et al., 1977; Nagy et al., 1978; Nauta et al., 1975; Preston et al., 1980; Ribak et al., 1980; Rosegay, 1944; Somogyi et al., 1981; Somogyi and Smith, 1979; Spano et al., 1977; Szabo, 1962; Szabo, 1970; Szabo, 1981).

In order to label projection neurons and their neurotransmitter, WGA, N-[acetyl-^{3}H] was stereotaxically injected into substantia nigra or globus pallidus. Visualization of retrogradely transported WGA antigens were accomplished with rabbit anti-WGA and neurotransmitter candidates were visualized within the identified projection neuron with rabbit anti-neurotransmitters (methionine enkephalin, substance P and GAD). Striatal neurons that were retrogradely labeled with WGA have a similar distribution as seen by previous workers. Several other considerations of WGA are detailed below.

In recent studies utilizing WGA, N-[acetyl-^{3}H] for bidirectional labeling, it was found that this axonal tracing technique was extremely effective and sensitive (Steindler and Bradley, 1983 for a review; Margolis et al., 1981; Ruda and Coulter, 1980; Steindler and Deniau, 1980). WGA was used in the current study due to the high concentration found in striatal cells when retrogradely labeled from globus pallidus or substantia nigra injections. Injections of WGA into substantia nigra were found to cover both compacta and reticulata which resulted in a homogenous labeling within striatum. These results are in agreement with previous reports (see Grofova, 1979, for a review). Injections into globus pallidus were seen to be confined to a small area. Injections of globus pallidus were not seen to spread into striatum and were small in comparison to other HRP labeling studies. We were able to utilize WGA because of its antigenic qualities and our ability to localize WGA by autoradiography if the need arose.

Column IgG-purified rabbit anti-WGA sera was made available to us from Dr. Myron Lyon for immunocytochemical identification of neostriatal projection neurons. Commercially available rabbit anti-WGA sera were analyzed as well and it was found to identify the WGA antigen. We were unable to utilize HRP retrograde labeling due to the fact that peroxidase is employed in peroxidase-anti-peroxidase and avidin:biotin complex. It is additiona-ly known that utilization of WGA has a specific uptake mechanism (Gros et al., 1977; Lechan et al., 1981 and Nagata and Burger) and probably does not involve fibers of passage at the injection site (Steindler, 1982; Steindler and Bradley, 1983; Lechan et al., 1981). WGA, N-[acetyl-^{3}H] utilized as an axonal tracing technique has proven to be compatible with our neuroimmunocytochemical procedures and its antigenic nature has not been significantly altered by colchicine injections.

B. Neurotransmitter Candidates

In the late nineteenth century it was realized that the nervous system consisted of independent cells which possessed a nucleus, membrane, axon and dendrites to communicate with each other (Cajal, 1894). Cajal proposed sites where neurons make contact with otherneurons whereby allowing communication between these cells. The term synapse was introduced by Sherrington (1904) and Elliot (1904) suggested that adrenalin was released by these synapses in the peripheral nervous system. This idea of chemical transmission was proven later by Loewi (1921). Dale (1935) proposed that this principle of chemical transmission was also utilized by the central nervous system. Eccles (1954) was originally opposed to these ideas, but it was his experiments on spinal cord which finally proved chemical transmission within the central nervous system.

Our basic concepts regarding chemical neurotransmission were derived from studies on cholinergic and noadrenergic transmission in a small population of peripheral tissues or specialized structures. The structure most studied is the neuromuscular junction. The possible neurotransmitter role of a substance in the CNS presupposed that there is a great deal of analogy between central neurotransmission and peripheral transmission-cholinergic and noradenergic mechanisms (Krnjevic , 1974 and Orrego, 1979). The major presynaptic criteria that have been utilized for the CNS are as follows: 1) the neuron has the necessary enzyme(s) to synthesize the transmitter candidate; 2) the transmitter is stored and localized within synaptic vesicles; and 3) that the neurotransmitter candidate is released in a calcium dependent manner thereby causing upon its release depolarization of the nerve terminals (Mulder, 1982 and Erulker, 1979).

Discussion of the neurotransmitter candidates within the basal ganglia will be restricted herein to methionine enkephalin, substance P and GABA. This is not to say that there are not other neurotransmitter candidates. A brief review of other investigator's efforts to identify these neurotransmitter candidates within the basal ganglia are as follows: acetylcholine (Butcher and Bilezikjian, 1975; Butcher and Butcher, 1974; Chubb et al., 1980; Fonnum et al., 1978; Fonnum and Walaas, 1979; Hattori and McGeer, 1974; Kimura et al., 1980; Kitai et al., 1978; Kravitz, 1967; Mao et al., 1977; Mulder, 1982; Dalkovits, 1978 and Swaab, 1982) cholecystokinin (Fonnum and Walaas, 1979; Robberecht et al., 1978), dopamine (Buijis, 1982; Fallon and Moore, 1978; Fibiger et al., 1972; Fonnum et al., 1978; Fonnum and Walaas, 1979; Fuxe, 1965; Gelfen et al., 1976; Giorguieff et al., 1978; Hattori et al., 1973; Korf et al., 1976; McGeer et al., 1971; Mulder, 1982; Oertel et al., 1982; Pollard et al., 1977; Ross ane Reis, 1974; Sokoloff et al., 1980; Seeman, 1981; Ungerstedt, 1971; Ungerstedt and Arbuthnott, 1970; Ungerstedt et al., 1982 and Versterg et al., 1976), glutamate (Cotman et al., 1981; Divac et al., 1977; Fagg and Lane, 1979; Fonnum et al., 1978; Fonnum et al., 1979; Frotscher et al., 1981; Kravitz, 1967; McGeer et al., 1977; Nistri and Constanti, 1979; Storm-Mathisen, 1977; Storm-Mathisen et al., 1983; Walaas, 1981 and Zieglgansberger, 1982), norepinephrine (Aghajanian and Bloom, 1967; Buijs, 1982; Fonnum and Walaas, 1979; Fuxe, 1965; McGeer et al., 1971; Mulder, 1982 and Versteeg et al., 1976), neurotensin (Leeman and Carraway, 1982; Kobayashi et al., 1977; Fonnum and Walaas, 1979 and Uhl and Snyder, 1976), serotonin (Amin et al., 1954; Bobillier et al., 1975; Bobillier et al., 1976; Carlsson et al., 1958; Fonnum and Walaas, 1979; Fuxe and Jonsson, 1974; Mulder, 1982; Park et al., 1982; Pasik et al., 1981; Ternaux et al., 1977 and Van der Kooy, 1979), somatostatin (DiFiglia and Aronin, 1982; Fonnum and Walaas, 1979 and Kobayashi et al., 1977) and vasoactive intestinal peptide-VIP (Fonnum and Walaas, 1979; Fuxe et al., 1977; Giachetti et al., 1977 and Palkovits, 1978).

1. Enkephalins

The enkephalins have received widespread acclaim due to their binding to opiate receptors within the central nervous system (Atweh and Kuhar, 1977; Buijs, 1982; Herkenham and Pert, 1980; Herkenham and Pert, 1981; Osborne et al., 1978 and Palkovits, 1978) and their possible role in pain pathways. Immunohistochemical investigations have shown striatum to contain enkephalin (methionine or leucine) immunoreactive terminals and neurons (Cuello and Paxinos, 1978; DiFiglia et al., 1982; Elde et al., 1976; Emson et al., 1980; Graybiel et al., 1981; Hokfelt et al., 1977; Hong et al., 1977; Osborne et al., 1978; Pickel et al., 1980; Sar et al., 1978; Simantov et al., 1976; Simantov et al., 1977; Somogyi et al., 1982; Wamsley et al., 1980; Watson et al., 1977 and

Yong et al., 1977) as well as its release from striatal slice preparation upon appropriate stimulation (Henderson et al., 1978 and Iversen et al., 1978). The possible action of the enkephalins on sensitive neurons are to decrease excitability (Fry and Zieglgansberger, 1979 and Zieglgansberger, 1982). Recent evidence has shown the enkephalins to decrease substance P release from rat trigeminal nucleus (Jessell and Iversen, 1977) and an apparent inhibitory effect on substance P release as well as decreased action potential of cultured sensory neurons (Mudge et al., 1979). Finally, an enzyme involved in neuronal synthesis of enkephalin (enkephalinase) has recently been identified (De la Baume et al., 1981; Schwartz et al., 1980). Experiments to date have identified only cells that contain enkephalin immunoreactivity and correlated the somatic architecture to separate anatomical or electrophysiological HRP studies.

In the present study, WGA containing projection neurons (striatopallidal and striatonigral) and their putative neurotransmitter methionine enkephalin were localized within the same neuron. Light microscopic analysis of the size of neurons were entirely made up of a medium-sized neostriatal population. The distribution of these enkephalin containing neurons correlates to previous studies (DiFiglia et al., 1982; Khacheturion et al., 1983; Watson et al., 1977; Sar et al., 1978 and Pickel et al., 1980). There were no observations of a patch-like distribution of enkephalin immunoreactivity as was seen by Graybiel (1981) in the cat caudate nucleus.

The present study utilizes a 48 hour survival time after colchicine injections which has the effect to increase the amount of immunoreactive cell bodies. Similar results have been found by other investigators utilizing colchicine to increase somatal concentrations of putative neurotransmitter (DiFiglia et al., 1982; Hokfelt et al., 1977 and Ribak et al., 1979).

Ultrastructural observations of methionine enkephalin immunoreactive (MEI) neurons were seen to possess medium-spiny neuronal characteristics. These MEI neurons had a smooth round unindented nucleus and thin rim of MEI cytoplasm. These MEI neurons also have been identified by other investigators (DiFiglia et al., 1982; Pickel et al., 1980 and Somogyi et al., 1982). Labeling was also seen in dendrites, spines of dendrites and presynaptic boutons. The type of synapses made by MEI presynaptic terminals in this study were of symmetrical configuration (Gray type II) and to equal extent were asymmetrical (Gray type I). The labeled vesicle size was an average of 55 nm and the range was narrow (55-61). The sampling size was not as extensive as the study by Somogyi (1982) and coworkers. However, great care was taken when viewing synaptic configurations in the electron microscope. If a synapse appeared fuzzy or not distinct, the section was tilted in the microscope to aid in photographing and eventual analysis. Somogyi (1982) found an equal number of symmetrical and asymmetrical synapses, whereas DiFiglia (1982) and Pickel (1980) found more labeled asymmetrical synapses than labeled symmetrical synapses. These discrepancies are easily attributed to the small sampling size utilized at the electron microscopic level. A more fastidious study is needed before any conclusions can be derived such immunocytochemical experiments.

The findings of two types of MEI boutons raises an interesting question as to their origin. The first and easiest response would be to explain all immunocytochemical investigations as not specific or artifactual. This possibility can be only answered by each investigator.

In the present study, a modified avidin:biotin complex technique was utilized for the identification of neurotransmitters. It is the belief that the immunoperoxidase technique is entirely specific and sensitive enough to detect picograms of antigen (Parson and Erlandsen, 1974; Sternberger, 1974).

A second theory of the two types of labeled boutons (symmetrical and asymmetrical) origin are that they may originate from two separate populations of cells. Recent evidence by Somogyi (1982) has indicated such a theory. Symmetrical synapses are known to be derived from neostriatal projection neurons from their axon collaterals (Wilson and Groves, 1980). However, it is known from degenerative studies that asymmetrical synapses are derived from afferent inputs and these terminate onto spines (Hassler et al., 1977 and Somogyi et al., 1981). Somogyi (1982) stated he was unable to observe labeled presynaptic terminals forming a symmetrical synaptic appearance. In this study, I have observed several labeled presynaptic terminasl that were labeled onto dendritic spines forming a symmetrical synapse were observed (Fig. 72).

2. Substance P

Substance P was the first neuropeptide to be pharmacologically characterized. It displays a wide distribution in both the peripheral and central nervous system whereby it displays some type of neuronal function (Zieglgansberger, 1982). However, little is known about its cellular mechanism of synthesis or breakdown. Gamse (1979) and coworkers have shown substance P release from slice after addition of capsaicin and it is known that capsaicin inhibits uptake of substance P (Mudler, 1982). Immunohistochemical studies have shown substance P to be localized within the basal ganglia (Amin et al., 1954; Brownstein et al., 1976; Brownstein et al., 1977; Buijs, 1982; Cuello et al., 1977; Cuello and Kanzawa, 1978; DiFiglia et al., 1981; Duffy et al., 1975; Emson et al., 1980; Fonnum and Walaas, 1979; Gale et al., 1977; Hokfelt et al., 1977; Hong et al., 1977; Jessell et al., 1978; Kanzawa et al., 1976; Kanzawa and Jessell, 1976; Mulder, 1982; Palkovits, 1978; Pelletier et al., 1977; Pickel et al., 1979; Scully et al., 1978 and Walker et al., 1976). However, the highest concentrations of substance P were found within the substantia nigra (Brownstein et al., 1976) and it is known that the major afferent inputs to substantia nigra arise from neostriatum (Grofova, 1979 for a review). To date there has been no direct observations of substance P within neostriatal neurons.

In the present study, substance P was found to be localized within neostriatal neurons. The light microscopic characteristics of these neurons were of medium-size possessing a round nucleus and a thin rim of substance P immunoreactive (SPI) cytoplasm. SPI neurons were seen in both striatopallidal and striatonigral neurons. These substance P projection neurons were mianly located in clusters (4-6) within the rostral regions of striatum.

Ultrastructural characteristics of these substance P immunoreactive SPI neurons were consistently similar to that of a medium-spiny neuron (Wilson and Groves, 1980; Chang, 1981). The SPI neurons were seen to possess an unindented, smooth, round nucleus and a thin rim of SPI cytoplasm. The cytoplasmic elements labeled were restricted to rough endoplasmic reticulum, polyribosomes and microtubules. All of these known cytoplasmic constituents are presumed to be involved with synthesis and transport of proteins. Labeling of SPI could also be seen in dendrites, spines of dendrites and presynaptic terminals.

The types of synapse that were observed consisted of a preponderance of asymmetrical and to a lesser extent symmetrical. The labeled terminals were found to contact round and clear vesicles with an average diameter of 44 nm (n = 75) possessing a range from 35-52 nm. DiFiglia (1981) and coworkers have recently found SP labeled vesicles within substantia nigra to range from 35 nm to 50 nm in size and these investigators have shown asymmetrical labeled boutons within substantia nigra. In the present study, I was unable to find labeled large granular vesicles within striatum as compared to what DiFiglia (1981) observed within substantia nigra. Labeled post-synaptic structures (i.e. somata, dendrites, spines of dendrites) were observed to receive symmetrical and asymmetrical synapses. The origin of the labeled symmetrical synapses are probably due to axon local collaterals from neostriatal projection neurons (Wilson and Groves, 1980). The origin of asymmetrical synapses are probably from afferent inputs from other nuclei.

3. GABA [GAD]

It has long been known that amino acids are primarily concerned with general metabolic functions of the cell. It was the work of Eccles (1962) who postulated that GABA was the most promising neurotransmitter candidate in the spinal cord for mediating primary afferent depolarization. The general mechanism of GABA is believed to be that the pre- and post-synaptic effects dealing with increases of Cl permeability (Ten Bruggencate and Enberg, 1979). This increase of Cl permeability was shown to result in hyperpolarization rendering the cell less excitable to chemical or electrical synaptic stimulation (see Zieglgansberger, 1982 for a review). Recent evidence has suggested that there are at least two GABA receptors with spatially different conformations (Nistri and Constanti, 1979). The neurons of sympathetic ganglia, interneurons and motor neurons are found to possess one configuration, whereas GABAergic actions on cortical neurons, dorsal root ganglia and primary afferent terminals might be mediated through another type of receptor (Zieglgansberger, 1982). The binding sites for the benzodiazepines are found to be GABAergic in nature. Thus, the effects of the benzodiazepines to relieve anxiety, insomnia, convulsions and muscle spasms could be functionally linked to GABAergic transmission (Haefely et al., 1978). Phenobarbital which is used clinically for its ability to induce a general anesthetic state and its actions of phenobarbital are apparently GABAmimetic whereby it activates Cl channels coupled to a GABA-receptor (Barker and Mathers, 1981). Additionally, Perry (1973) and coworkers were able to find a decrease in GABA in striatum from Huntington Chorea patients. Lloyd (1979) and coworkers were able to find a decrease in GABA in Parkinson's patients. GABA has been shown to excitatory (Obata, 1976) in the CNS, involved as a transmitter in the peripheral nervous system, a vasodilator of cerebral blood vessels and the more accepted function is an inhibitory neurotransmitter (Wu et al., 1982). It has also been shown that GABA can activate release of dopamine from rat striatal slice (Giorguieff et al., 1978).

Immunohistochemical and biochemical-lesion studies into the location of GABA within the basal ganglia have been extensive (Bolam et al., 1983; Brownstein et al., 1977; Fonnum et al., 1978; Fonnum et al., 1974;

Fonnum and Walaas, 1979; Fry and Zieglgansberger, 1979; Gale et al., 1977; Giorguieff et al., 1978; Kim et al., 1971; Kravitz, 1967; Mao et al., 1977; Hattori et al., 1973; McGeer and McGeer, 1975; McLaughlin et al., 1974; Nagy et al., 1978; Nistri and Constanti, 1979; Oertel et al., 1982; Ribak et al., 1976; Ribak et al., 1977; Ribak et al., 1978; Ribak et al., 1979; Ribak et al., 1980; Storm-Mathisen, 1977; Storm-Mathisen et al., 1983; Vincent et al., 1982a, b and Wu et al., 1982). Additional studies on GABA have been performed on its location within cerebellum (Chan-Palay, 1982; Chan-Palay, 1978 a, b and Chan-Palay, 1979).

In the present study, antisera to glutamate decarboxylase (GAD) were used to identify GABA containing neurons within striatum. Comparison of rabbit anti-GAD to rabbit anti-GABA-T were made in order to determine the best marker for GABA neurons. At the light microscopic level, we were able to identify GAD immunoreactive neurons (GAD-I) throughout striatum with the heaviest concentration in dorsolateral distribution. Heavy GAD fiber staining was observed in globus pallidus and substantia nigra. Neuronal staining was observed in subthalamus and substantia nigra, but there were very few neurons labeled in globus pallidus. The morphological characteristics of GAD-I neostriatal neurons were that of a medium-sized neuronal population, consisting of a round nucleus and a thin rim of GAD-I cytoplasm. However, GABA-T immunostaining was shown to be in medium-sized and large-sized neuronal populations. McGeer (1975) and Vincent (1982) have consistently used a histochemical technique for GABA-T and hypothesized that these histochemically stained neurons were the GABAergic cells of striatum. In the present fluorescence studies of neurotransmitter-projection neuron, evidence was presented that only medium-sized projection neurons contained GAD-I. Additionally, Vincent (1982) and co-workers utilizing this GABA-T histochemical technique have concluded the major GABAergic pathway to substantia nigra arises from the globus pallidus. However, the present study shows little or no neuronal staining was obtained within globus pallidus. These results are consistent with other biochemical-lesion studies (Fonnum et al., 1978; Gale et al., 1977 and Fonnum et al., 1979). The striatonigral neurons and striatopallidal were found in this study to contain GABA and I believe that anti-GAD is a better marker of GABA neurons than is GABA-T. It is not surprising that other investigators have found different results, since GABA-T is known to break down GABA into succinic semialdehyde and the same enzyme converts α-ketoglutarate into fumurate. Therefore, the specificity of GABA-T as a marker for GABA is questionable.

Bolam (1983) and co-workers have recently utilized injections of ^{3}H-GABA to identify neostriatal GABA containing cells. Their studies concluded that the GABA concentrating neurons were of a medium-aspiny type. In the present study, only medium-spiny GAD immunoreactive labeled neurons were identified at the ultrastructural level. These neurons possessed a smooth, round and unindented nucleus with a thin rim of GAD-I cytoplasm. These characteristics are identical to those medium-spiny neuron characteristics that have been described by Chang (1981) and Wilson and Groves (1980). The GABA-T immunoreactive cells were seen to be either medium-aspiny neurons, medium-spiny neurons or large neurons. GABA-T immunoreactivity was seen in only post-synaptic structures including labeled somata, labeled dendrites and spines of dendrites. Therefore, GABA-T is probably a good marker for cells receiving GABA synapses.

The GAD-I labeled synapses were found to consist mainly of symmetrical synapses and to a lesser degree asymmetrical synapses. These labeled synapses were only occasionally found. Th- sample size here is limited due to the poor labeling of GAD terminals within striatum. The symmetrical GAD-I synapses are probably due to axon collaterals of medium spiny neurons which project out of striatum (Wilson and Groves, 1980 and Chang, 1981). The labeled synaptic vesicles were found to be small and clear within shape with an average of 33 nm (n = 69) and a range of 32 nm to 36 nm in size. The extrinsic source of GAD labeled asymmetrical synapses could possibly originate from substantia nigra and there has been a recent report which suggests that nigrothalamic neurons send collaterals to striatum (Steindler and Deniau, 1980). It is assumed that the nigrothalamic neurons could utilize GABA as their neurotransmitter and that the inhibitory effects from the nigrostriatal axons could in part be due to GABA (Ferger et al., 1979). However, this does not exclude the possibility that these GAD-I asymmetrical synapses are derived either from cortex or thalamus (Grofova, 1979).

Ribak (1979) and co-workers postulated that these GAD-I labeled asymmetrical synapses could be derived from interneurons within striatum. To date, there has been a lack of documentation supporting this possibility.

In the present study GAD immunoreactivity was found to coexists within the same neuron labeled for methionine enkephalin or leucine enkephalin. Coexistence of two neurotransmitter candidates is not a new concept (Brownstein et al., 1974; Burnstock, 1976 and Osborne, 1977). This idea of more than one neurotransmitter contradicts Dale's (1935) original hypothesis that one transmitter is utilized by a neuron. However, this hypothesis still may be true because the exact mechanism of action of the enkephalins and GABA are not yet resolved.

Summary

It has been shown in this study that striatopallidal and striatonigral neurons possess methionine enkephalin, substance P and GAD immunoreactivity. It has been demonstrated that at least some neostriatal cells possess enkephalin immunoreactivity and GAD-immunoreactivity within the same neuron.

Ultrastructural characteristics of these labeled neurons were that of a medium-spiny neuron possessing a smooth, round and unindented nucleus. The immunoreactivity (ME, SP, GAD) was localized within the cytoplasm is on rough endoplasmic reticulum, microtubules and mitochondria. The labeled synaptic characteristics were both symmetrical and asymmetrical. The origin of the labeled symmetrical are probably from axon collaterals of neostriatal projection neurons. The synaptic vesicles with the labeled boutons varied in size according to the transmitter labeling. GAD-I labeled vesicles were found to range from 32-36 nm; substance P vesicle size was found to range from 35-61 nm in size. What must be emphasized here is that these sizes are totally dependent upon fixatives utilized and buffers.

Therefore, it is concluded, from the present study, that by using the microtubule stabilizing buffer (360 nm Osm) and low aldehyde concentrations, the vesicles were observed consistently small and round. The labeled vesicles were found to exhibit different sizes when they were labeled for different neurotransmitters. This may vary from investigator to investigator and therefore may be of little consequence.

Possible clinical implications can be drawn from the presence of GABA containing striatonigral and their possible interaction in degenerative diseases states such Huntington Chorea and Parkinson's disease. It is of particular interest to note the interactions of benzodiazepines with GABA receptors within the central nervous system and especially these interactions within the basal ganglia. Clinical implications in regards to the other neurotransmitter candidates, methionine enkephalin and substance P, has to be further investigated.

In conclusion, I have shown that some striatopallidal and striatonigral neurons utilize GABA and methionine enkephalin as a possible neurotransmitter. The topographical arrangement between these two neurotransmitter candidates also share similar arrangements. Substance P is located in a different population of topographically arranged striatopallidal and striatonigral neurons. Additionally, existence of leucine enkephalin and GAD immunoreactivity were exhibited to occur within the same neostriatal neuron.

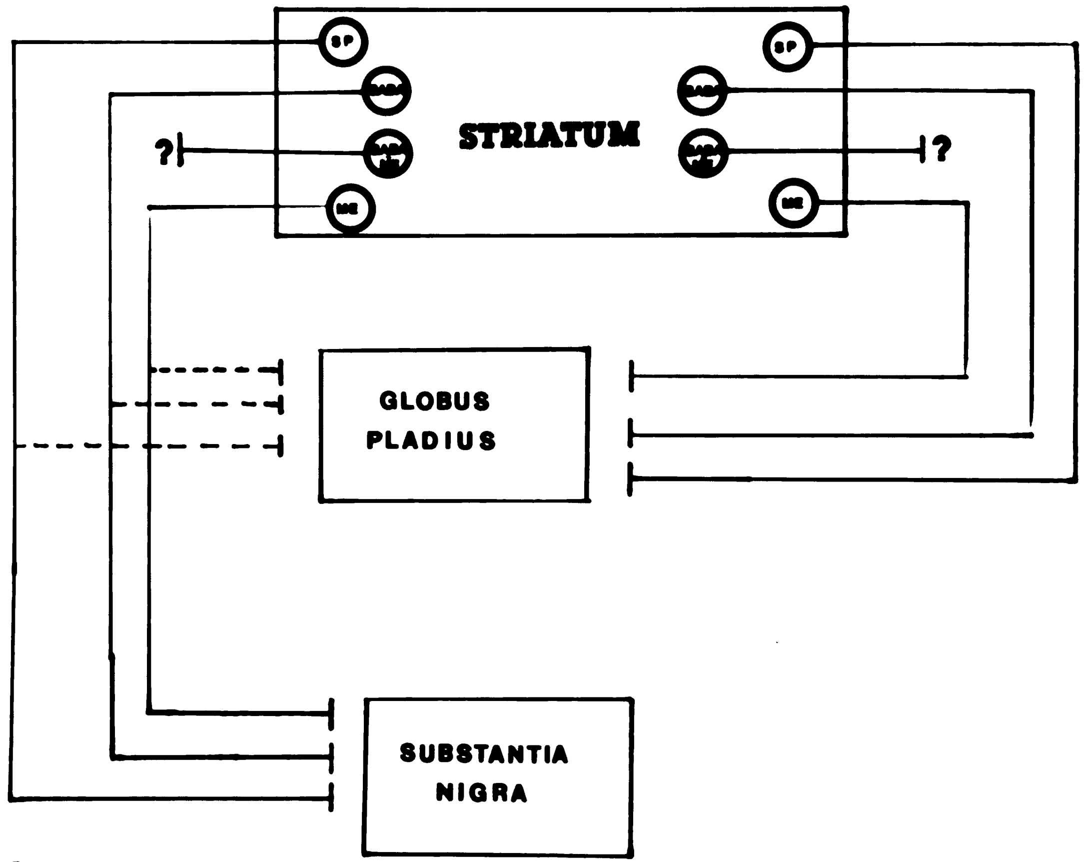
Figure 84 A diagramatic representation of the projections (striatopaliidal and striatonigral) neurons.

substance P = SP

methionine enkephalin = ME

gamma aminobutyric acid = GABA

gamma aminobutyric acid plus methionine enkephalin = GABA + ME



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