# GABA, SUBSTANCE P AND THE EFFERENTS OF THE STRIATUM

by

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ABSTRACT

The efferent pathways from the striatum to the other nuclei of the basal ganglia were examined biochemically and histochemically. Acetylcholine, GABA, enkephalin and substance P have all been suggested to occur in striatal neurons, and markers for these possible transmitters were therefore measured in various nuclei of the basal ganglia following knife cuts of the striatal efferent fibres. These studies confirmed the existence of GABA projections from the head of the striatum to the globus pallidus (GP), entopeduncular nucleus (EP) and substantia nigra (SN). In addition the presence of substance P in the striatal projection to the EP was demonstrated and the substance P projection to the SN was confirmed using a radioimmunoassay. The first evidence suggesting the presence of both substance P and methionine-enkephalin in the striatopallidal fibres was also obtained. Also the important observation that methionine-enkephalin is not present in the projections from the head of the striatum to the EP and SN was noted.

In order to visualize substance P fibres in the brain a new method for immunohistochemical studies of the nervous system was developed based on the biotin-avidin system. Using this powerful technique substance P fibres and terminals were observed in the striatum, GP, EP and SN, as well as in various other areas including the amygdaloid complex, the habenula and the interpeduncular nucleus. This represents the first report of substance P fibres in the basal ganglia demonstrated using an immunoperoxidase procedure.

The enzyme GABA-transaminase (GABA-T) was examined as a potential marker for the GABA neurons of the basal ganglia. Using selective lesions and a biochemical assay procedure the enzyme was found to be present in the neurons of the striatum and in the striatonigral pathway. GABA-T was apparently not present in the glial elements of the striatum nor was it present in the nigrostriatal dopamine neurons. Histochemical experiments

demonstrated GABA-T to be present in the terminals of the striatal and pallidal efferents which are thought to use GABA as a transmitter. These experiments establish the usefulness of GABA-T histochemistry as a new method for the analysis of the topography of the GABA systems in the basal ganglia.

The response of the GABA and substance P cells in the basal ganglia to the selective removal of the dopamine cells of the SN was examined and compared with the pathological findings observed in Parkinson's disease. In contrast with the decrease reported in glutamate decarboxylase activity in the basal ganglia in Parkinson's disease, an increase in the activity of this enzyme was observed in the animal model. Also, a significant decrease in nigral and striatal substance P levels occurred following this lesion. The implications of these findings for the etiology and pharmacological therapy of Parkinsonism are discussed.

Finally, the nigrotectal pathway was examined ultrastructurally and biochemically since it represents a major output pathway of the basal ganglia. A selective decrease was found in the glutamate decarboxylase activity of the superior colliculus following lesions of the SN. This observation provides the first indication that the nigrotectal projection may use GABA as a transmitter. Electron microscopic examination of axon terminals of the nigrotectal pathway indicated the axons were probably myelinated and that the terminals form symmetric synapses with the major dendrites of neurons in the deep layers of the superior colliculus.

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| | |
|---|---|
| AOAA..... | amino-oxyacetic acid |
| BA..... | biotin-avidin |
| CAT..... | choline acetyltransferase |
| DOPAC..... | dihydroxyphenylacetic acid |
| EOS..... | ethanolamine-o-sulphate |
| EP..... | entopeduncular nucleus |
| GABA..... | γ-aminobutyric acid |
| GABA-T..... | GABA-transaminase |
| GAD..... | glutamate decarboxylase |
| GP..... | globus pallidus |
| HVA..... | homovanillic acid |
| KA..... | kainic acid |
| leu-enkephalin..... | leucine-enkephalin |
| met-enkephalin..... | methionine-enkephalin |
| 6-OHDA..... | 6-hydroxydopamine |
| PAP..... | peroxidase anti-peroxidase |
| PBS..... | phosphate buffered saline |
| SN..... | substantia nigra |
| SNC..... | substantia nigra pars compacta |
| SNR..... | substantia nigra pars reticulata |

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Edie McGeer for allowing me a free rein to explore any of the crazy ideas that popped into my head during the course of this work. The friendly interest, advice and encouragement of Dr. Chris Fibiger is also especially appreciated. I would like to acknowledge my debt to my two *sen sei* Drs. T. Hattori and H. Kimura, without whom none of the histological work would have been possible. Finally, it is a pleasure to remember the many members of the Kinsmen Laboratory and, in particular, Bill Staines and Jim Nagy, for making this work a pleasure.

# INTRODUCTION

The basal ganglia are a group of subcortical nuclei which includes the striatum, globus pallidus (CP), entopeduncular nucleus (EP), subthalamic nucleus and substantia nigra (SN). Together these structures comprise a major portion of what has been termed the extrapyramidal motor system and are involved in the control of posture and locomotion. Dysfunction of the basal ganglia result in a variety of clinical conditions involving motor behaviour. Parkinson's disease is the best understood of these disorders and is characterized by a loss of the dopamine-containing neurons of the SN and a corresponding reduction in striatal dopamine levels (Hornykiewicz, 1973).

Huntington's disease is another disorder involving the basal ganglia. It is characterized by neuropathological changes in the cortex and the striatum (Lange et al., 1976). Although choreiform movements are the hallmark of this disease, dementia is also a striking feature of Huntington's chorea (Garron, 1973).

The basal ganglia have also been implicated in the etiology of schizophrenia. Motor disturbances including chorea, akathisia, and oral dyskinesias occur frequently in psychotics, and this observation led Mettler (1955) to propose originally that schizophrenia is a disorder of the basal ganglia. With the introduction of the neuroleptic drugs for the symptomatic treatment of this disease attention has focused particularly on the role of the nigral dopamine neurons in schizophrenia.

These clinical conditions illustrate that disorders of the basal ganglia may be characterized not only by impairments of motor behaviour, but also by disruptions of cognitive function. Thus, study of the basal ganglia may provide insight into the neural mechanisms involved in both of these important processes. In particular, a study of the output pathways of the

basal ganglia may indicate the sites at which this system can interact with the rest of the nervous system to influence behaviour.

The striatum is the largest structure included in the basal ganglia and indeed, is the largest subcortical cell-mass in the mammalian brain. As such it probably represents the main integrative center of the basal ganglia. The striatum receives projections from all parts of the neocortex, including motor areas as well as areas implicated in perception, association and memory (Carmen et al., 1963; DeVito and Smith, 1964; Garcia-Rill et al., 1979; Glees, 1944; Goldman and Nauta, 1977; Jones et al., 1977; Kemp and Powell, 1970; Kunzle, 1975; 1977; Niimi et al., 1963; Webster, 1961; 1965; Yeterian and van Hoesen, 1978). This is the major input to the striatum and accounts for approximately one third of the afferent terminals to this nucleus (Kemp and Powell, 1971b). The cortico-striatal pathway is thought to be excitatory (Kitai et al., 1976a; 1976b; Kocsis et al., 1977) and to use glutamate as a transmitter (Divac et al., 1977; Kim et al., 1977b; McGeer et al., 1977; Reubi et al., 1979; Spencer, 1976). Electrophysiological (Kitai et al., 1976b) and anatomical (Endo et al., 1973) evidence indicates that the corticostriatal fibers are independent of the corticospinal or corticobulbar systems.

Although the parafasicular and centromedian nuclei provide the major thalamic inputs (Kuroda et al., 1975; Kalil, 1978; Royce, 1978) all of the intralaminar nuclei appear to project to the striatum (Jones and Leavitt, 1974). Together these inputs make up one quarter of the afferent terminals in the striatum (Kemp and Powell, 1971b). Although changes in choline acetyltransferase (CAT) and acetylcholine levels have been reported in the head of the striatum following parafasicular lesion (Saelens et al., 1979; Simke and Saelens, 1977) the anterior one-third of the striatum receives few if any parafasicular afferents (Jones and Leavitt, 1974). This and the lack of

staining of parafasicular cells for CAT (H. Kimura, personal communication) raises doubt about this being a cholinergic tract. Thus, at present the biochemical nature of this input remains a matter of speculation, although electrophysiological results suggest that it is an excitatory pathway (Kocsis et al., 1977; Malliani and Purpura, 1967; Purpura and Malliani, 1967).

The third major striatal afferent arises in the midbrain and supplies the striatum with approximately fifteen percent of its afferent terminals (Kemp and Powell, 1971b). This pathway contains dopamine and arises from both the SN and the adjacent ventral tegmental area (Andén et al., 1964; Beckstead et al., 1979; Fallon and Moore, 1978; Lindvall and Björklund, 1974). The nigro-striatal system was originally thought to be an inhibitory projection; but there is now considerable evidence that the dopamine terminals depolarize striatal neurons (Davies and Tongroach, 1978; Kitai et al., 1975; Richardson et al., 1977). A second non-dopaminergic nigrostriatal system has also been suggested on both physiological and biochemical grounds (Feltz and deChamplain, 1972; Fibiger et al., 1972; Ljundahl et al., 1975; Guyenet and Aghajanian, 1978).

In addition to these major striatal afferents, two aminergic afferents from the brainstem have also been identified. Serotonin is contained in the striatal afferents from the dorsal raphé nucleus (Azmitia and Segal, 1978; Jacobs et al., 1978; Miller et al., 1975). Biochemical studies suggest that this projection is most concentrated in the ventrocaudal region of the striatum (Ternaux et al., 1977). Also, the ubiquitous axons of the locus coeruleus have been shown to project to the striatum, providing a noradrenergic innervation to this area (Mason and Fibiger, 1979; Moore, 1978).

In contrast to the diversity of the afferents to the striatum, the output of this nucleus is quite restricted. The striatum is known to innervate directly only three structures, the GP, EP and SN. Thus, the entire output

of the striatum is confined to other nuclei within the basal ganglia. All parts of the striatum, including the head (Cowan and Powell, 1966; Graybiel et al., 1979, Nagy et al., 1978a, Tulloch et al., 1978) and tail (Tulloch et al., 1978) project to the pallidum. Although the EP receives fibers from the head of the striatum (Adinolfi, 1969; Nagy et al., 1978a) the extent to which it receives afferents from the tail of the striatum is uncertain. The SN receives a rich innervation from diverse areas of the striatum (Bunney and Aghajanian, 1976; Grofová, 1975; Kanazawa et al., 1976; Nagy et al., 1978a; Richardson et al., 1977; Tulloch et al., 1978) both to the pars compacta (SNC) and to the pars reticulata (SNR).

Which of the neuronal types of the striatum gives rise to these efferent pathways has been a matter of some debate. It has been estimated that over 95% of striatal neurons are medium size spiny cells (Kemp and Powell, 1971a). The striatum also contains a few small neurons as well as a population of giant aspiny cells (Kemp and Powell, 1971a). These giant cells, which account for only about one percent of the striatal neurons (Kemp and Powell, 1971a) were originally suggested on the basis of Golgi material to be the projection neurons of the striatum (Fox et al., 1971). However, with the introduction of retrograde transport as an anatomical tool, this conclusion has been questioned. Thus, these giant cells have not been labeled following the injection of horseradish peroxidase into the GP, EP or SN. Recently, however, some large neurons have been labeled following injections into the retrorubral area (Grofová, 1979), a dorsal extension of the SN (Beckstead et al., 1979; Nauta et al., 1978).

Although horseradish peroxidase injections into the GP, EP or SN proper have failed to label the giant aspiny neurons of the striatum, many medium size cells are labeled following these injections (Bunney and Aghajanian, 1976; Graybiel et al., 1979; Grofová, 1975; Kanazawa et al., 1976; Tulloch

et al., 1978). This indicates that these cells provide the major pathways for the striatal outflow.

Evidence indicates that the inhibitory neurotransmitter γ-aminobutyric acid (GABA) is contained in the striatal afferents to the GP (Fonnum et al., 1978; Hattori et al., 1973; Nagy et al., 1978a) EP (Fonnum et al., 1978a; Nagy et al., 1978a) and SN (Brownstein et al., 1977; Fonnum et al., 1978a; 1974; Gale et al., 1977b; Hattori et al., 1973b; Jessel et al., 1978; Kim et al., 1977a; Nagy et al., 1978a). This suggests that GABA is contained in at least some of the medium spiny cells of the striatum, and in fact some of these cells have been found to stain for the GABA synthetic enzyme glutamate decarboxylase (GAD) in immunohistochemical studies (Ribak et al., 1979).

Recently, evidence has been obtained that the neuropeptide substance P is also contained in the striatal projections to the nigra (Hong et al., 1977c; Kanazawa et al., 1977a; Mroz et al., 1977; Palkovits et al., 1978; Paxinos et al., 1978a) and EP (Paxinos et al., 1978b), suggesting that some of the medium spiny cells should contain this peptide. In fact, immunohistochemical studies have shown some small and medium size striatal cells do contain substance P immunoreactive material (Cuello and Kanazawa, 1978; Ljüngdahl et al., 1978a). Recent biochemical experiments have shown that the GABA and the substance P projections arise from different sites within the striatum (Brownstein et al., 1977; Gale et al., 1977; Jessel et al., 1978) indicating that these transmitters are probably contained in separate populations of striatal neurons.

The striatum has also been found to contain other peptides that may function as neurotransmitters. In particular some evidence suggests that the endogenous opioid peptides methionine- and leucine-enkephalin may be present in striatopallidal fibers (Cuello and Paxinos,1978b; Hong et al.,

1977a).

Although the direct output of the striatum is confined to the basal ganglia, pathways must exist to transmit this output beyond this system, ultimately to the motor neurons. Both the EP (Carpenter, 1973; Kim et al., 1976; Kuo and Carpenter, 1973; Larsen and McBride,1979; Nauta and Mehler, 1966) and SN (Beckstead et al., 1979) send projections to the pars compacta of the nucleus tegmenti pedunculopontis, a mesencephalic reticular formation nucleus lying within the brachium conjunctivum. The efferent connections of this nucleus are unknown, but preliminary reports suggest that its axons descend to the spinal cord (Ross et al., 1979) and ascend towards the forebrain, perhaps synapsing in the pallidum (Gonya-Magee and Anderson, 1979).

A population of dopamine cells in the medial SN and the ventral tegmental area (Fuxe et al., 1974; Lindvall et al., 1974; Thierry et al., 1973) and a population of cholinergic cells in and around the GP and EP (Kelley and Moore, 1978a; Lehmann et al., 1980) project to the cerebral cortex. However, these pathways have only recently been discovered and their connections with the striatum and their role in mediating basal ganglia output have not been examined.

The pathways most commonly thought to provide the main output for the basal ganglia arise from the EP and from the SNR. As discussed above these two nuclei both receive direct inputs from the striatum. In addition the GP projects directly to the SN (Bunney and Aghajanian, 1976; Grofová, 1975; Hattori et al., 1975; Kanazawa et al., 1976; Kim et al., 1976; Tulloch et al., 1978) and to the subthalamic nucleus (Hattori et al., 1975; Kim et al., 1976; Nauta and Mehler, 1966) which in turn projects to the EP (Carpenter and Strominger, 1967; Nauta and Cole, 1978; Whittier and Mettler, 1949) and the SN (Hammond et al., 1978; Kanazawa et al., 1976; Nauta and

Cole, 1978; Tulloch et al., 1978; Whittier and Mettler, 1949).

A major projection from the EP to the lateral habenula (Carter and Fibiger, 1978; Herkenham and Nauta, 1977; Kim et al., 1976; Larsen and McBride, 1979; Nauta, 1974) has been demonstrated and biochemical studies have suggested that GABA is a transmitter in this pathway (Gottesfeld et al., 1977; Nagy et al., 1978b). However, the lateral habenula is not thought to be critically involved in the control of motor behaviour. Rather, the projection from the EP to the thalamus has been suggested to serve such a function (Hassler, 1978). The principal sites of termination of pallidal fibers in the thalamus are the centromedian and parafasicular nuclei, and more importantly, the ventrolateral-ventroanterior complex and the ventromedial nucleus (Carter and Fibiger, 1978; Kim et al., 1976; Kuo and Carpenter, 1973; Larsen and McBride, 1979; Nauta and Mehler, 1966). Since the nucleus ventralis lateralis projects to the motor cortex areas four and six, the pallidal projection to this nucleus provides a pathway for the basal ganglia to affect directly the motor output of the cortex.

A substantial projection to the thalamus from the SNR has also been demonstrated. This is a non-dopaminergic projection and is distributed to the medial part of the nucleus ventralis lateralis, the magnocellular part of the nucleus ventralis anterior and the paralaminar part of the nucleus dorsomedialis (Beckstead et al., 1979; Bentiviglio et al., 1979; Carpenter et al., 1976; Carpenter and Peter, 1972; Cole et al., 1964; Faull and Mehler, 1978; Fibiger, et al., 1972; Mettler, 1970; Rinvik, 1975). A projection to the parafasicular nucleus has also been reported (Ahlenius, 1978; Beckstead et al., 1979; Clavier et al., 1976). Again, via the connection with the nucleus ventralis lateralis, this output pathway provides the basal ganglia with access to the motor cortex.

Finally, fibers from the SNR, arising at least in part as collaterals

of the nigrothalamic projection (Anderson and Yoshida, 1977; Bentivoglio et al., 1979), innervate the superior colliculus (Afifi and Kaelber, 1965; Beckstead et al., 1979; Faull and Mehler, 1978; Graybiel, 1978; Hopkins and Niessen, 1976; Jayaraman et al., 1977; Rinvik et al., 1976). The nigrotectal fibers terminate in the deeper layers of the superior colliculus (Anderson and Yoshida, 1977; Beckstead et al., 1979; Graybiel, 1978, Jayaraman et al., 1977; Rinvik et al., 1976), which are known to give rise to the tectoreticular and tectospinal pathways (Kuypers and Maisky, 1975). Indeed, electrophysiological experiments have indicated that the projection from the SNR to the superior colliculus interacts with the tectospinal system which regulates the neck muscles involved in head orientation (York and Faber, 1977). Thus, this system could well be involved in the expression of the motor asymmetries observed after unilateral manipulations of the basal ganglia.

## STATEMENT OF THE PROBLEMS TO BE EXAMINED

In the present study a biochemical and histological analysis of the output pathways of the basal ganglia is presented. We begin with an examination of the efferents of the caudate-putamen to the other nuclei of the basal ganglia. As mentioned in the Introduction, GABA, acetylcholine, enkephalin and substance P have all been suggested to be contained in striatal cells. In Experiment 1 the existence of these transmitters in the striatal projections to the GP, EP and SN was biochemically examined following lesions of the striatal output pathways.

The biochemical approach used in Experiment 1 can provide evidence that a chemical such as GABA or substance P is contained in a fibre tract. This technique will not however give information regarding the topography and synaptic relationships of the fibre systems. For this histological methods are essential. Therefore in Experiment 2 the distribution of the substance P fibres was examined using an immunohistochemical approach.

To study the GABA systems of the basal ganglia the localization of the enzyme GABA-transaminase (GABA-T), which catabolizes GABA, was examined. The biochemical studies in Experiment 3 suggest that GABA-T is localized in the GABA neurons of the striatum. Therefore, in Experiment 4, a histochemical method for this enzyme was adapted to examine the striatal and pallidal GABA efferents.

The striatal efferents are intimately associated with the nigrostriatal dopaminergic neurons. In order to examine the nature of this interaction the responses of the striatal GABA and substance P cells to the selective loss of the nigral dopaminergic neurons were biochemically examined in Experiment 5. As this lesion of the dopaminergic neurons has been used as a model of Parkinson's disease (Marsden, 1977), the results of this study have been compared with the biochemical changes reported to occur in

Parkinsonism.

Finally in Experiment 6 the biochemical nature of the nigrotectal pathway, which probably represents a major output of the basal ganglia, was examined by measuring various neurotransmitter markers in the superior colliculus following nigral lesions. The morphology of the nigrotectal projection was also examined using electron microscopy. The results provide the first indication that GABA may be a transmitter in the nigral output pathways. The implications of this observation for basal ganglia function are therefore discussed.

## EXPERIMENT 1: NEUROTRANSMITTERS CONTAINED IN THE EFFERENTS OF THE STRIATUM

Three neurotransmitter candidates have been found to be contained in the fibers emanating from the striatum. These are GABA, substance P and enkephalin.

GABA has been shown to be contained in striatopallidal (Fonnum et al., 1978a; Hattori et al., 1973b; Nagy et al., 1978a), striatoentopeduncular (Fonnum et al., 1978a; Nagy et al., 1978a) and striatonigral (Brownstein et al., 1977: Fonnum et al., 1978a; 1974; Hattori et al., 1973b; Jessel et al., 1978; Kim et al., 1971; Nagy et al., 1978a) fibers. The striato-entopeduncular and striatonigral projections have also been shown to contain substance P by immunofluorescent (Paxions et al., 1978a; 1978b) and radioimmunochemical (Brownstein et al., 1977; Gale et al., 1977; Hong et al., 1977c; Jessel et al., 1978; Kanazawa et al., 1977a) techniques, respectively. A striatopallidal leu-enkephalin-containing pathway has been demonstrated by immunohistofluorescent methods (Cuello and Paxinos, 1978) and pallidal met-enkephalin levels have been observed to decrease following kainic acid lesions of the striatum (Hong et al., 1977a). A comprehensive and systematic examination of each of these systems has not been conducted. The present study provides further information on the contribution of each of these substances to the striatal projections to the GP, EP and SN.

### METHODS

Male Wistar rats weighing about 300 g in the hemitransection group and 450 g in the GP island group were anesthetized with pentobarbital and placed in a Kopf stereotaxic instrument. For the semicircular cuts separating the GP from the striatum (GP islands) a Halasz knife four mm in radius and three mm in height was fashioned from tungsten wire and mounted in a 26 gauge cannula. After removal of a bone flap and deflection

of the dura the knife was lowered at a parasaggital orientation to an empirically determined set of coordinates. The axis of rotation of the knife was 3.5 mm posterior to bregma and 2.3 mm lateral to the midline. The tip of the knife was 8.1 mm ventral to the cranial surface. Once positioned the knife was rotated slowly 10-15 degrees towards the midline and then rotated 130 degrees laterocaudally. After returning to the entry orientation the knife was withdrawn and the wound treated with 0.1% Zephiran.

Hemitransections just anterior to the GP were produced with a straight tungsten wire lowered at the midline to the ventral surface of the brain and drawn to the lateral extreme in the plane of the anterior commissure.

Animals were sacrificed two to three weeks postoperatively by cervical fracture and the brains rapidly removed and placed on ice. All areas were dissected freehand from sections obtained on a freezing microtome. The head of the striatum was dissected only from those sections not visibly damaged by the knife. The GP was not assayed in animals in which the knife cut encroached on this area. The tail of the striatum was dissected from the same sections as the GP.

For enzyme activity measurements tissues were homogenized in 20 to 50 volumes of 50 mM Tris-acetate buffer, pH 6.4, containing 0.2% v/v Triton X-100. Protein was determined on these homogenates according to the method of Lowry et al. (1951).

A modification of the method of Albers and Brady (1959) was used for the assay of GAD activity (Chalmers et al., 1970). Tissue homogenate (20 µl) was added to 50 µl of incubation mixture containing (final concentrations) 2.0 mM [1-$^{14}$C]glutamate (specific activity 0.1 to 0.2 mCi/mmol, Amersham), 0.02% bovine serum albumin (Sigma) and 0.1 mM pyridoxal

phosphate (Calbiochem) in 20 mM potassium phosphate buffer, pH 7.4 in a small glass tube. Each tube was placed in a scintillation vial with half a gelatin capsule (Parke-Davis) containing a piece of filter paper to which 0.1 ml of hyamine hydroxide was added. The vials were capped with rubber septums and incubated at 37°C for 30 min. The reaction was stopped by the injection of 0.2 ml of 2M $H_2SO_4$ into the incubation tube through the rubber septum with a 19 gauge needle. After two hr the reaction tubes were removed from the vials and the trapped $^{14}CO_2$ counted in ACS (Amersham) with a liquid scintillation counter.

CAT was assayed according to the method of Fonnum (1975). The reaction was started by adding 20 µl of tissue homogenate to 50 µl of a mixture which contained (final concentrations) 300 mM NaCl, 8.0 mM choline chloride, 0.1 mM eserine, 20 mM EDTA, and 0.2 mM [1-$^{14}$C]acetyl coenzyme A (Amersham, specific activity 0.05 mCi/mmol) in 50 mM sodium phosphate buffer pH 7.4. The mixture was incubated at 37°C for 20 min in a scintillation vial. The reaction was then stopped by adding seven ml of a solution consisting of two parts acetonitrile and five parts ten mM sodium phosphate buffer pH 7.4 containing two mg/ml sodium tetraphenyl boron. Ten ml of toluene containing 0.4% 2,5-diphenyl-oxazole (PPO) and 0.015% p-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) were then added and the $^{14}$C-acetylcholine produced during the incubation was extracted into the organic phase with the tetraphenyl boron and counted in a liquid scintillation counter.

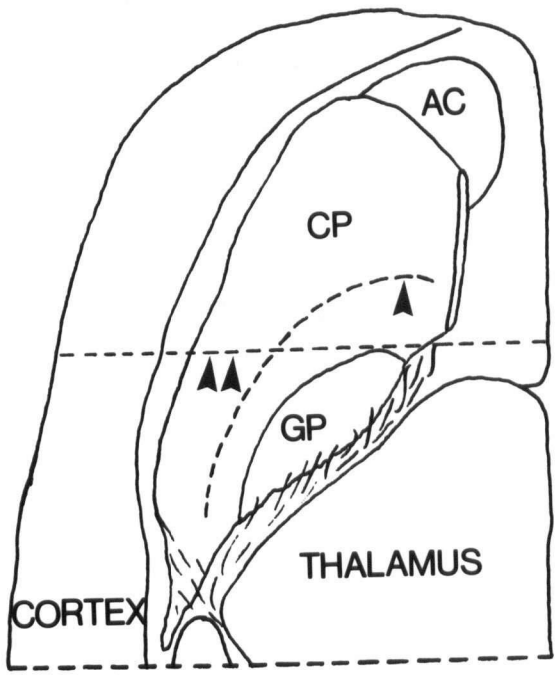
Substance P and met-enkephalin levels were determined by radioimmunoassay on lyophilized 1.0 N acetic acid extracts of weighed tissue (see Appendix).

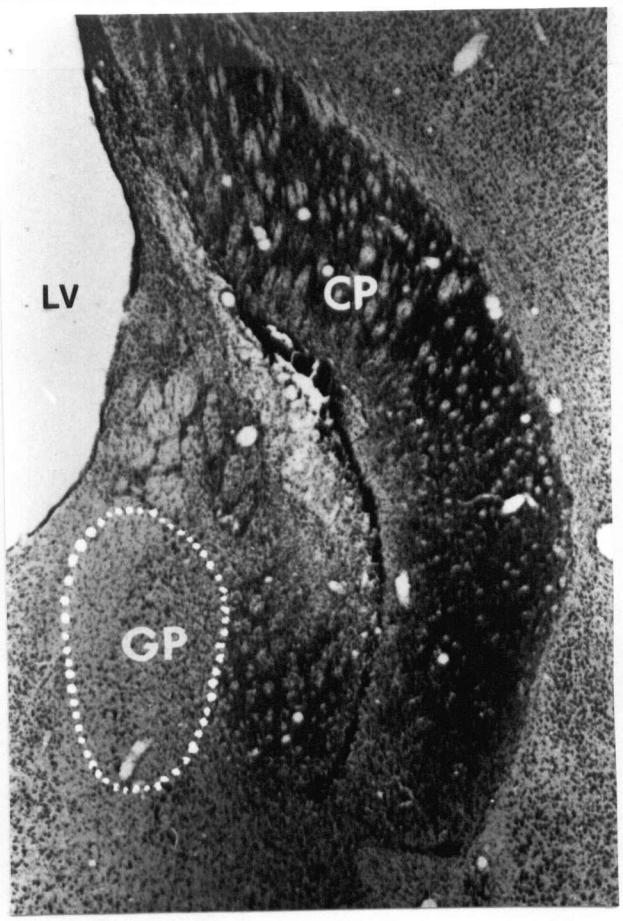
## RESULTS

The extent to which the knife cuts isolated the GP from the striatum (GP islands) is shown in Fig. 1. Rostrally, the lesions started just lateral

Figure 1. The knife cuts separating the globus pallidus from the striatum.

The diagram on the left represents a horizontal section through the forebrain showing the sites of both the circular cut forming the GP island (arrow) and the hemitransection (double arrow). The photograph on the right is a coronal section through the anterior globus pallidus showing the circular knife cut. The circular cut is seen lateral to the globus pallidus which is outlined in white dots. Shrinkage of striatal tissue is evidenced by the dilation of the lateral ventricle (LV). AC nucleus accumbens, GP globus pallidus, CP caudate-putamen. Magnification: 25X.


AC
CP
GP
THALAMUS
CORTEX


LV
CP
GP

to the septum and anterior to the decussation of the anterior commissure. From this point, the lesions proceeded laterally and then posteriorly to subscribe an arc surrounding the GP both dorsally and laterally. In order to avoid damage to the internal capsule the lesions were produced so as to isolate one-half to two-thirds of the GP from the tail of the striatum. Although direct damage to the pallidum was rarely observed, the lesions occasionally approached the lateral aspect of this structure. Some gliosis and shrinkage of the pallidum were evident probably resulting from the loss of striato-pallidal terminals and the retrograde loss of some pallidal projection neurons. Regions of the striatum anterior and lateral to the lesions exhibited some shrinkage although no gliosis was observed in these areas.

The effect of these knife cuts on GAD and CAT activity in the various brain areas examined are shown in Table 1. In the GP and EP GAD was reduced significantly by 44 and 22 percent, respectively. The activity of this enzyme was not significantly decreased in the SN by this lesion. The activity of CAT was not affected in the SN or EP but was reduced by 35 percent in the GP following this lesion. The production of the GP islands resulted in a 49 percent increase in GAD activity in the head of the striatum anterior to the lesion, without affecting CAT activity.

The effect of hemitransections just rostral to the GP on CAT and GAD is shown in Table 2. These lesions were more posterior and encroached upon the anterior pole of the GP. This probably explains the greater reductions of GAD observed in the GP and EP following this lesion compared with those observed after the circular cuts and could account for the significant reduction observed in nigral GAD. As in the case of the GP islands, CAT was significantly reduced in GP but unaltered in the EP or SN. Neither CAT not GAD was significantly altered in the tail of the striatum lateral to

Table 1. Glutamic acid decarboxylase and choline acetyltransferase activity in various areas after lesions isolating the globus pallidus from the striatum (GP islands).

| | Glutamic acid decarboxylase nmol/mg protein/hr | | Choline acetyltransferase nmol/mg protein/hr | |
|---|---|---|---|---|
| Area | Control | Lesion | Control | Lesion |
| Head of striatum | 235±14 | 350±16** | 168±9 | 173±10 |
| % of control | | 149 | | 103 |
| Globus pallidus | 498±30 | 279±26** | 55.8±7.2 | 36.4±3.3* |
| % of control | | 56 | | 65 |
| Entopeduncular nucleus | 290±27 | 225±14* | 20.2±1.5 | 16.9±1.3 |
| % of control | | 78 | | 84 |
| Substantia nigra | 683±78 | 545±50 | | |
| % of control | | 80 | | |

Values indicate means ± S.E.M. of eight determinations. *$p < .05$, **$p < .01$, Student's $t$ test.

the GP. This suggests the reduction in pallidal enzyme activities was not simply due to a posterior spread of tissue damage after the hemitransection lesions. As in the case of the GP islands, hemitransections significantly increased GAD activity by 54% in the head of the striatum while CAT activity remained unchanged.

Substance P levels in the head and projection areas of the striatum after lesions producing the GP islands are shown in Table 3. Expressed as percent of the unlesioned contralateral side, these lesions significantly reduced substance P levels by 48 percent in the GP, 58 percent in EP and 47 percent in the SN. The levels in the head of the striatum were unaffected. The levels of substance P in these same areas after hemitransections just anterior to the GP are shown in Table 4. The reductions of this peptide in the three projection areas of the striatum were comparable to those observed in the case of the GP islands. Similarly, substance P levels in the head of the striatum were unaltered.

The hemitransections significantly reduced met-enkephalin levels in the GP by 20 percent but did not alter levels in the EP or SN (Table 4). In addition, these lesions caused a 34 percent increase in met-enkephalin levels in the head of the striatum.

## DISCUSSION

Although it has been known for some time that the major outputs of the striatum are to the GP, EP and SN, there is still controversy over which neurotransmitters are involved in each of these systems. At present, the transmitters thought to be associated with striatal neurons include acetylcholine, GABA, substance P, and enkephalin. We have, therefore, examined the possible existence of each of these substances in the various projections of the striatum.

Table 2. Glutamic acid decarboxylase and choline acetyltransferase activity in various areas after hemitransections anterior to the globus pallidus.

| | Glutamic acid decarboxylase nmol/mg protein/hr | | Choline acetyltransferase nmol/mg protein/hr | |
|---|---|---|---|---|
| Area | Control | Lesion | Control | Lesion |
| Head of striatum | 285±23 | 440±33* | 224±15 | 199±18 |
| % of control | | 154 | | 89 |
| Tail of striatum | 326±19 | 417±44 | 167±12 | 139±18 |
| % of control | | 128 | | 83 |
| Globus pallidus | 506±22 | 215±24** | 62.8±6.5 | 41.4±2.9* |
| % of control | | 42 | | 66 |
| Entopeduncular nucleus | 338±25 | 196±21** | 28.5±5.4 | 25.4±5.5 |
| % of control | | 58 | | 89 |
| Substantia nigra | 632±39 | 369±37* | | |
| % of control | | 58 | | |

Values indicate means ± S.E.M. of seven determinations for SN and five for the remaining tissues. *$p < .025$, **$p < .005$, Student's $t$ test.

(a) The Globus Pallidus

The decrease in substance P levels in the GP observed following its separation from the striatum provides the first evidence that substance P may be a transmitter in the striatopallidal pathway. It would appear that half the substance P in the pallidum comes from the anterior striatum, while the remaining 50 percent could either arise from the tail of the striatum or could represent intrinsic substance P neurons of the GP. Substance P-like immunoreactive cells have been found in the GP (Cuello and Kanazawa, 1978). In this regard it is interesting to note that in Huntington's chorea a 50 percent decrease in substance P levels has also been observed in the GP (Gale et al., 1978).

Striatopallidal GAD-containing fibers have previously been demonstrated (Fonnum et al., 1978a; Hattori et al., 1973b; Nagy et al., 1978a). In the present study the reductions in pallidal GAD were only slightly less after GP island lesions than after hemitransections. This indicates that the GP receives the major portion of its GABA input from neurons originating rostral to both lesions in the striatum. This is supported by the observation that electrolytic lesions of the tail of the striatum do not reduce GAD activity in the GP (Nagy and Fibiger, 1980).

The GP has been reported to contain the highest levels of met-enkephalin in the brain (Gramsch et al., 1979; Hong et al., 1977b). Immunohistochemical evidence has been presented for a leu-enkephalin-containing striatopallidal projection (Cuello and Paxinos, 1978). The anti-serum employed in that study (Cuello and Paxinos, 1978) had little cross-reactivity with met-enkephalin. On the other hand, met-enkephalin levels have been observed to decrease in the GP following kainic acid (KA) injections into the striatum (Hong et al., 1977a). In that study, however, the KA lesion included not only the striatum but also the GP and part of

Table 3. Substance P levels in various areas after lesions isolating the globus pallidus from the striatum (GP islands).

| | Substance P pg/mg tissue | | | |
|---|---|---|---|---|
| Area | Control | Lesion | N | % of control |
| Head of striatum | 445±110 | 448±82 | 6 | 101 |
| Globus pallidus | 502± 90 | 206±45* | 5 | 41 |
| Entopeduncular nucleus | 993±114 | 416±90** | 6 | 42 |
| Substantia nigra | 2014±175 | 1067±215** | 6 | 53 |

Values are means ± S.E.M. of the number of determinations (N) indicated.

*$p < .025$, **$p < .01$, Student's $t$ test.

Table 4. The levels of substance P and met-enkephalin in various brain areas after hemitransections anterior to the globus pallidus

| | Substance P pg/mg tissue | | | Met-enkephalin pg/mg tissue | | |
|---|---|---|---|---|---|---|
| Area | Control | Lesion | N | Control | Lesion | N |
| Head of striatum | 322±16 | 301±24 | 15 | 1126±85 | 1531±110*** | 15 |
| % of control | | 94 | | | 134 | |
| Globus pallidus | 462±14 | 233±50*** | 16 | 3351±181 | 2675±249* | 16 |
| % of control | | 50 | | | 80 | |
| Entopeduncular nucleus | 614±99 | 300±56** | 9 | 728±80 | 749±96 | 8 |
| % of control | | 49 | | | 96 | |
| Substantia nigra | 1503±237 | 710±204*** | 5 | 152±28 | 187±28 | 8 |
| % of control | | 47 | | | 123 | |

Values represent the means ± S.E.M. of the number of determinations (N) indicated. *$p < .05$, **$p < .025$, ***$p < .01$, Student's $t$ test.

the thalamus. Thus, in view of the demonstration of distinct populations of leucine- and methionine-enkephalin-containing neurons in the caudate-putamen and GP (Larsson et al., 1979), the present results provide the first demonstration of the existence of a met-enkephalin containing striato-pallidal projection. The small but significant decrease in the levels of this peptide observed in the GP after a lesion which markedly depleted GAD and substance P, suggests an alternate source for the majority of the met-enkephalin in the GP than the head of the striatum. Since KA lesions of the striatum which include the GP lead to a 50% decrease in pallidal met-enkephalin levels (Hong et al., 1977a), it is probable that there are intrinsic pallidal met-enkephalin neurons and/or that the pallidum receives substantial met-enkephalin afferents from other sources.

Although CAT activity in the GP was decreased by both lesions in this study, these results are not sufficient to demonstrate the existence of a cholinergic striatopallidal projection. It has previously been observed that cortical lesions result in the retrograde degeneration of the intensely staining acetylcholinesterase neurons located in and around the GP which are thought to provide the cholinergic input to the cortex (Lehmann et al., 1980). It has also been observed (Wm. Staines and H.C. Fibiger, personal communication) that KA lesions confined wholly to the head of the striatum do not decrease pallidal CAT. The loss of pallidal CAT activity observed in the present study may therefore be attributable to the retrograde loss of the cholinergic pallidal neurons which project to areas rostral to the lesion sites such as the cerebral cortex (Johnston et al., 1979; Kelley and Moore, 1978a; Lehmann et al., 1980).

(b) The Entopeduncular Nucleus

The EP has previously been shown to contain substance P-like immunoreactivity by immunohistochemical methods (Cuello and Kanazawa, 1978; Ljüng-

dahl et al., 1978a; Paxinos e t al., 1978b). However, levels of substance P in the EP have not previously been quantified by biochemical methods. The levels measured in the striatum, GP and SN in the present investigation are similar to those reported in studies of the regional distribution of substance P (Brownstein et al., 1976; Kanazawa and Jessel, 1976) and from this it would appear that only the SN, and the trigeminal complex contain higher levels of substance P than the EP.

Paxinos et al. (1978b) have recently reported that substance P-like immunofluorescence in the EP disappeared following lesions similar to those used in the present study. The present data confirm that the majority of the substance P in the EP is probably present in afferents from the head of the striatum.

In addition to a GABA-containing striato-pallidal tract, GAD-containing striatoentopeduncular fibers have also been recently demonstrated (Fonnum et al., 1978a; Nagy et al., 1978a). The present results support the previous evidence for such a pathway. The hemitransections decreased EP GAD to the same extent as previously reported by Nagy et al. (1978a). In contrast, the lesions employed to produce the GP islands, which started more anterior than the hemitransections and swept wide of the GP laterally leaving some striatal tissue intact medial to the lesion, did not result in as large a decrease. This indicates that a major portion of the GABA input to the EP arises from striatal cells bordering the anterior pole of the GP. This is in agreement with the observations of Fonnum et al. (1978a) who found that successively more posterior lesions involving the head of the striatum led to progressively greater reductions in entopeduncular GAD activity.

The levels of met-enkephalin in the EP were found to be only slightly less than those in the striatum; however, they were not affected by either

type of lesion. This suggests that, in contrast to the striatopallidal projection, the afferents to the EP from the head of the striatum do not contain met-enkephalin.

Finally, since CAT activity in the EP was unaffected by any of the lesions it would appear that acetylcholine is not a transmitter in the striato-entopeduncular projection.

(c) The Substantia Nigra

The SN was found to contain very low levels of met-enkephalin. The lesion data indicate that the SN, like the EP, receives no cholinergic or met-enkephalin-containing input from the striatum. The absence of a cholinergic striatonigral projection is in agreement with previous reports (Fonnum et al., 1978a; McGeer et al., 1971).

Since the discovery of the existence of a GABA-containing projection from the striatopallidal complex to the SN, numerous studies have been conducted to determine the exact location of the neurons giving rise to this pathway (Brownstein et al., 1977; Fonnum et al., 1978a, Gale et al., 1977; Jessel et al., 1978). These recent studies and, in particular, that by Brownstein et al. (1977) have considerably clarified this issue. These workers suggested that there is a concentration of GAD-containing nigral projection neurons in the striatum just anterior to the GP. The present results are in agreement with this proposal. Hemitransections in the striatum near the rostral tip of the GP, reduced nigral GAD activity substantially. However, the GP island lesions, which began at a more rostral level, did not significantly decrease nigral GAD activity. These results together with recent observations (DiChiara et al., 1980; Nagy and Fibiger, 1980) that KA injections into the GP do not significantly alter nigral GAD are in full agreement with Brownstein et al. (1977) who have suggested that the origin of the majority of GAD in the SN is derived

from neurons located outside the pallidum but closely apposed to it. It is noteworthy that while GP island lesions did not decrease GAD in the SN and only produced a slight decrease in the EP GAD activity, the decrease in the GP was only slightly less than after the hemitransection. This indicates that the GABA-containing neurons innervating the SN and EP can at least partially be dissociated from those projecting to the GP.

Of all the brain areas that have been examined, the SN contains the highest levels of substance P (Brownstein et al., 1976; Kanazawa and Jessel, 1976). In Huntington's chorea substance P levels and GAD activity have both been found to decrease in the SN (Kanazawa et al., 1977b). A similar parallel decrease has been observed following hemitransections at the level of the lateral hypothalamus (Mroz et al., 1977). These results led to the suggestion that both GABA and substance P are transmitters in the striatonigral pathway (Hong et al., 1977c; Kanazawa et al., 1977b; Mroz et al., 1977; Paxinos et al., 1978b). More recent work has shown that the substance P and GAD-containing efferents appear to be dissociable. The substance P fibers appear to arise from the anterior striatum and not from the caudal and lateral areas (Brownstein et al., 1977; Gale et al., 1977; Jessel et al., 1978; Palkovits et al., 1978). In contrast and as discussed above, the GABA afferents to the SN arise from striatal neurons closely apposed to the GP (Brownstein et al., 1977). That the two lesions employed in the present study produced a similar depletion in nigral substance P, but had different effects on nigral GAD activity is in agreement with this segregation of these two nigral afferent systems.

(d) The Striatum

The increases in GAD activity and met-enkephalin levels in the striatum anterior to the hemitransections and GP island lesions could be due either to the shunting of enzyme and peptide to local collateral branches or

terminals after severing long axon efferent collaterals (Storm-Mathisen, 1975), or to an alteration in the activity of the neurons containing GAD and met-enkephalin. That this change does not merely reflect tissue shrinkage is indicated by the unchanged levels of substance P and the normal CAT activity in this same tissue. At least a portion of the GAD increase in the head of the striatum can be attributed to the severing of the dopaminergic input to this structure since we have found that 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway increase GAD activity in the striatum (Experiment 5). Hemitransections caudal to the GP do not appear to result in an increase in the met-enkephalin levels in the striatum (Pollard et al., 1978). This suggests that this increase is due to disruption of the striatopallidal met-enkephalin pathway. Further work is necessary to determine the significance of these changes in the functioning of the striatum and in its recovery from injury.

## EXPERIMENT 2: THE IMMUNOHISTOCHEMICAL DEMONSTRATION OF SUBSTANCE P IN THE BASAL GANGLIA

The undecapeptide substance P is contained in the efferents of the striatum, where it may function as a neurotransmitter. In Experiment 1, the existence of substance P-containing projections from the head of the striatum to the SN (Brownstein et al., 1977; Gale et al., 1977; Hong et al., 1977c; Jessel et al., 1978; Kanazawa et al., 1977a; Paxinos et al., 1978a) and EP (Paxinos et al., 1978b) was confirmed using a radioimmunoassay procedure. In addition, the presence of substance P in the striatal projection to the GP was demonstrated. In the present study an immunohistochemical approach was used to provide information on the distribution of substance P fibers in these and other brain nuclei.

Previous immunofluorescent studies have demonstrated dense plexi of substance P terminals in the SN (Cuello and Kanazawa, 1978; Cuello et al., 1979; Hökfelt et al., 1977; Jessel et al., 1978; Ljüngdahl et al., 1978a; 1978b; Paxinos et al., 1978a), EP (Cuello and Kanazawa, 1978; Ljüngdahl et al., 1978a; Paxinos et al., 1978b; Jessel et al., 1978), and GP (Ljüngdahl et al., 1978a). These fluorescent studies have been of great value in the mapping of the substance P systems of the brain. The fluorescent method does not however provide the resolution necessary to discern the precise synaptic relationships of the substance P terminals and cells. In particular the method is not compatible with electron microscopic studies. For this the immunoperoxidase method is available.

Immunoperoxidase staining for substance P has been performed at both the light and electron microscopic levels in the spinal cord (Barber et al., 1979; Hökfelt et al., 1977; Pickel et al., 1977; Vacca et al., 1980), medulla (Chan-Palay, 1978; Pickel et al., 1979) and amygdala (Pelletier et al., 1977) using the peroxidase-antiperoxidase (PAP) method developed by

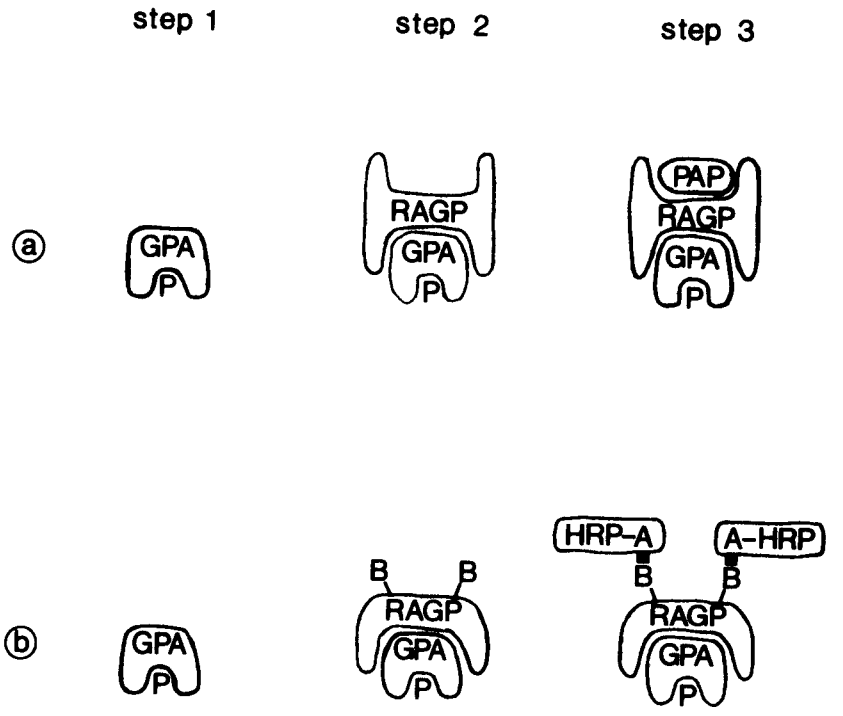
Sternberger et al. (1970). These studies have provided information on the localization of substance P in nerve terminals in synaptic relationships of various types. In the present study an immunoperoxidase procedure was used to demonstrate substance P in some terminal fields in the basal ganglia.

## METHODS

Initially the traditional PAP procedure of Sternberger et al. (1970) was attempted using the guinea pig antisera to substance P described in the Appendix. This method was abandoned when after a year of unsuccessful work a letter was received from Cappel Laboratories stating that their guinea pig PAP was being discontinued due to "technical problems".

As an alternative method, the biotin-avidin (BA) procedure has been utilized (Heitzmann et al., 1974; Guesdon et al., 1979). As this is the first time that this method has been used in immunohistochemical studies of the nervous system, the theoretical basis of this technique will be described in some detail and contrasted with the more familiar PAP method (Fig. 2). In both the BA technique and the PAP procedure, the initial incubation of the tissue in guinea pig anti-substance P sera is the same (Step 1). In the PAP procedure the second step involves incubation in rabbit anti-guinea pig sera. This must be used at high concentration in order to leave one binding site of the rabbit antisera free after reaction with the primary antibody. The use of high concentrations of this sera increases the amount of nonspecific binding of this sera to the tissue and this can increase the artifactual staining. In the BA procedure, the biotinylated rabbit anti-guinea pig sera is used in place of the rabbit antisera used in the PAP procedure. It has been found that extensive biotinylation of antibodies does not modify their antigen binding capacity (Guesdon et al., 1979). In the BA procedure the second step

Figure 2: A comparison of the biotin-avidin (BA) method (b) with the peroxidase-antiperoxidase (PAP) procedure (a) for immunohistochemistry. In Step 1 guinea pig-anti substance P sera (GPA) binds to substance P in the tissue. In Step 2 of the PAP procedure rabbit-anti guinea pig IgG (RAGP) binds the GPA and then the PAP in Step 3. In the BA procedure the Biotin residues attached to the RAGP bound in Step 2 are bound by avidin (A) which has been coupled to horseradish peroxidase (HRP).

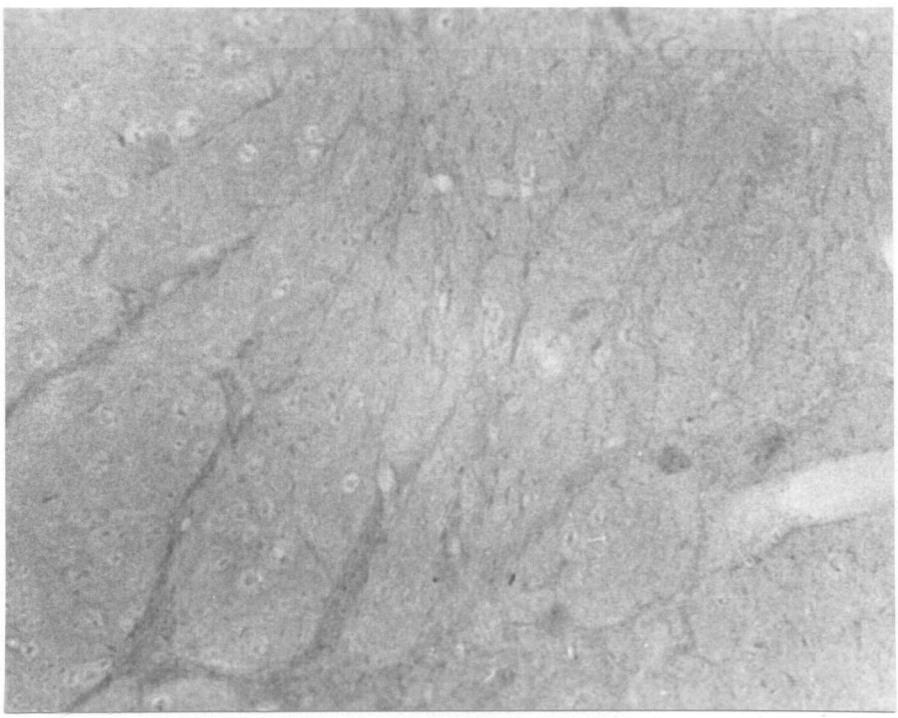

step 1
step 2
step 3
ⓐ
GPA
P
RAGP
GPA
P
PAP
RAGP
GPA
P
ⓑ
GPA
P
B
B
RAGP
GPA
P
HRP-A
A-HRP
B
B
RAGP
GPA
P

can be used at high dilution since it is not necessary to have a free antibody binding site available for Step 3.

It is in the third step where the BA system is most different from the PAP approach. In the PAP procedure, the free binding site left in the second step binds the PAP complex which acts as an antigen. In the BA method the biotin residues attached to the second antibody are bound by avidin to which horseradish peroxidase has been chemically coupled. The interaction between avidin and biotin is non-covalent, but extremely strong (Kd = $10^{-15}$M; Green, 1963). In fact the affinity is six to eight orders of magnitude higher than that of most antibodies for antigen. This means that the avidin-peroxidase complex can be used at very high dilution. This again greatly reduces the chances for non-specific binding. Also, extensive washing can be performed after the incubation without dissociation of the avidin-biotin complex since the half-time of dissociation requires months.

In the present study the distribution of substance P immunoreactivity was examined in the cat brain using the BA procedure. Adult male cats were deeply anesthetized with pentobarbital and perfused through the heart with phosphate buffered (0.1 M, pH 7.4) saline (PBS) followed by cold 4% paraformaldehyde and 0.3% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. The brain was removed, cut into 5 mm coronal sections and kept in 4% paraformaldehyde at 4°C for 6 hr. The tissue was then stored overnight in the buffer containing 15% sucrose and cut into 25 micron thick sections on a cryostat at -20°C. Sections for immunohistochemistry were washed in PBS containing 0.01% Triton X-100 for one hr at room temperature and then incubated overnight at 4°C in guinea pig-anti substance P sera (see Appendix) diluted 1:2,000 in PBS containing 0.05% Triton X-100. The sections were then washed in PBS and incubated for two

Figure 3. Control section of substantia nigra incubated with pre-absorbed anti-substance P sera. Only a light brown background exists, and no specific substance P staining occurs. 23X.



hr at room temperature with biotinylated rabbit anti-guinea pig IgG (E-Y Labs.) diluted 1:200 in PBS containing 0.01% Triton X-100. Again the sections were washed in PBS and finally incubated for one hr at room temperature in horseradish peroxidase-avidin (Vector Labs. Inc.) diluted 1:500 in PBS. The sections were finally washed in PBS for one hr and the peroxidase then reacted for 20 min with 0.02% (w/v) 3,3'-diaminobenzidine (Sigma) and 0.006% $H_2O_2$ in 50 mM Tris-Cl buffer, pH 7.6. The sections were then washed and mounted from PBS. The mounted sections were soaked in distilled water 30 min to remove salts and detergent and then treated with 0.01% (w/v) osmium tetroxide for three min to enhance contrast.

Control sections were processed identically except that the initial antisera was preabsorbed with 100 µg of synthetic substance P (Beckman) per ml at room temperature for four hr prior to use in immunohistochemistry. Sections incubated with this pre-absorbed sera did not exhibit any specific staining (Fig. 3).

## RESULTS

In sections from untreated adult cats which were stained for substance P immunoreactivity using the BA procedure immunoreactive cell bodies were not consistently detected. This is in agreement with previous immunofluorescent studies in the rat in which cell bodies could only be reproducibly demonstrated following colchicine pretreatment (Cuello and Kanazawa, 1978; Ljüngdahl et al., 1978a). This presumably indicates that the cell bodies normally contain very low levels of substance P.

Although positive cell bodies were not apparent, widespread networks of substance P-positive terminals and fibers were observed through the brain and spinal cord. In the present report we shall confine our discussion to substance P-positive structures in areas associated with the basal ganglia.

Both the putamen (Fig. 4a) and the caudate (Fig. 4b) contained a

delicate network of fine fibers which could be observed forming clusters of axo-somatic contacts with medium size striatal neurons. A similar network of fibers was also observed in the claustrum, particularly in the ventro-medial portion of this nucleus (Fig. 4c).

Within the GP (Fig. 4d) and the EP (Fig. 4f) the neuropil was rather densely stained. In both areas varicose fibers appeared to outline the dendritic processes of the pallidal neurons, presumably forming synapses along their length.

The ansa lenticularis, in which the striatonigral substance P fibers are thought to course (Palkovits et al., 1978), contained a dense network of varicose fibers (Fig. 4e).

Although not previously discussed in this report the amygdaloid complex is another telencephalic structure often included in the basal ganglia. Certain amygdaloid nuclei stain very strongly for substance P. The cortical nucleus contains an extremely dense network of positively stained punctate structures throughout its neuropil (Fig. 5b). A somewhat less dense network is present in the medial amygdala where long varicose fibers could often be seen (Fig. 5c).

A dense punctate network of substance P-positive fibers was also observed in the lateral habenula, particularly in the medial portion of this nucleus (Fig. 5a).

In the mesencephalon a diffuse network of varicose axons appears to form a shell over the dorsal and lateral borders of the interpeduncular nucleus (Fig. 6c). The staining is strongest in the ventro-lateral portion of this shell where it apparently is in continuum with the substance P immunoreactivity in the SN. Within the SN the substance P-positive structures form a massive, diffuse and punctiform pattern. The staining is most intense in the SNR (Fig. 6b). Bundles of intensely stained neuropil

Figure 4. Substance P immunoreactivity in coronal sections of the cat basal ganglia demonstrated with the biotin-avidin method.

In (a) a sparse network of positively stained puncta is apparent. These can often be seen forming terminals about the medium size cells (arrows).

Fig. (b) shows a similar pattern of substance P staining in the caudate nucleus. Again the punctate fibers can often be seen covering the unstained cell bodies (arrows).

Fig. (c) shows the same sparse pattern of punctate staining exists in the claustrum. The cell bodies show a slight non-specific background staining.

In (d) the neuropil of the globus pallidus can be seen to contain a dense network of substance P-positive fibers. These appear to cover the cell bodies and major dendrites (arrow) of the pallidum with many varicosities.

Fig. (e) demonstrates the substance P-like immunoreactivity in the fibers of the ansa lenticularis. These fibers appear to have many varicosities along their length.

The entopeduncular nucleus (f): the pattern of staining is very similar to that seen in the globus pallidus (d). Again the stained puncta cover the cell bodies (arrow) and appear to follow the major dendrites through the neuropil.

Calibration bars represent 20 microns in all figures.

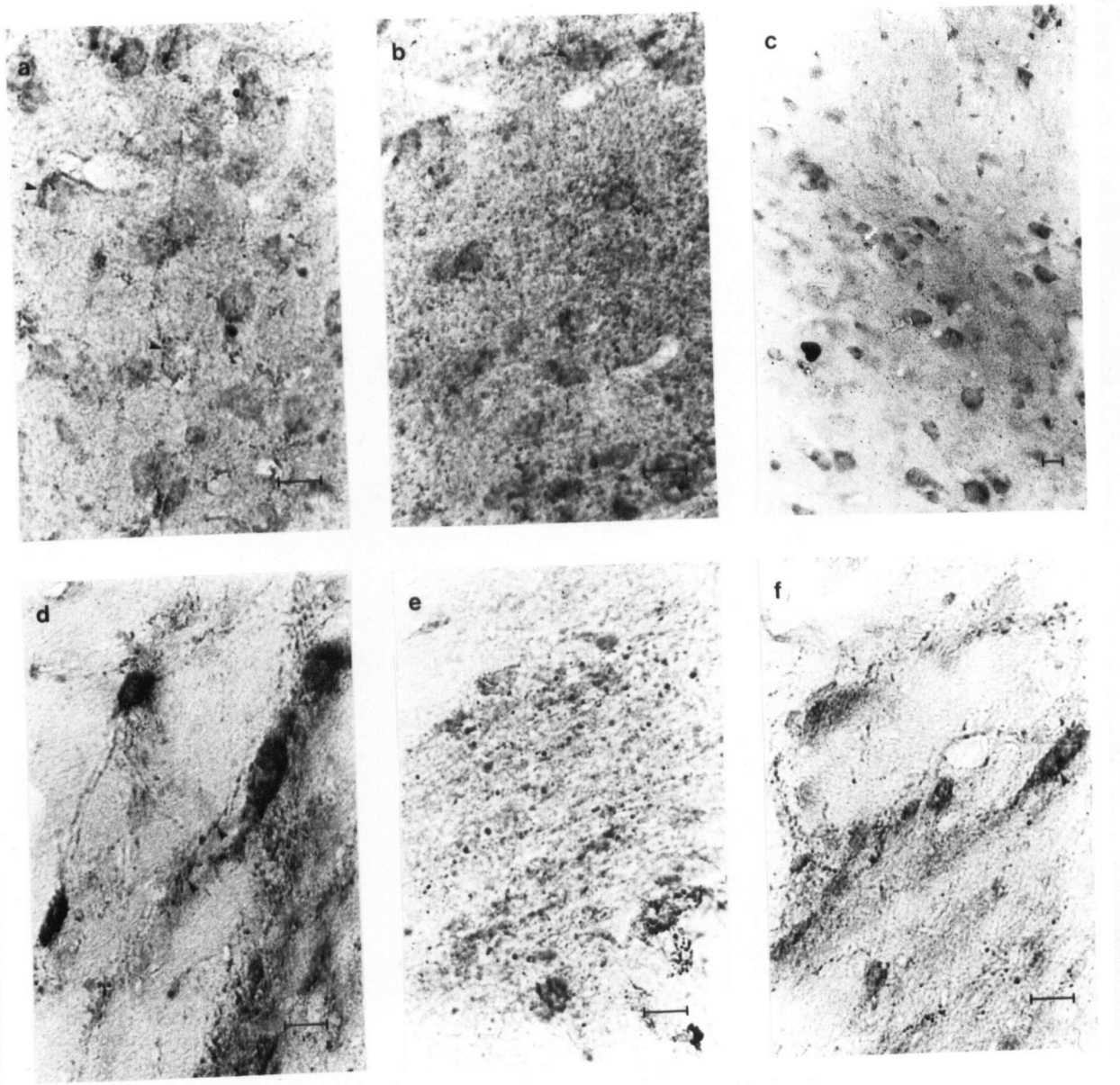

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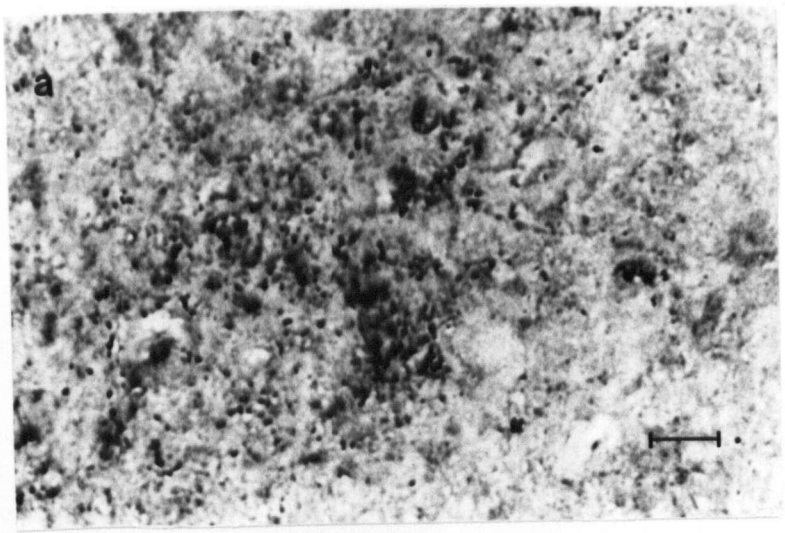
Figure 5. Substance P immunoreactive fibers demonstrated with the biotin-avidin immunoperoxidase procedure.

In (a) the dense network of positive puncta found in the medial portion of the lateral habenula nucleus is shown in a coronal section.

A similar dense network can also be seen in the neuropil of the corticomedial amygdala (b). In this coronal section the varicose fibers appear to branch throughout the neuropil.

Occasionaly, such as in this coronal section of the medial amygdala (c) substance P-containing varicose axons can be followed for some distance. The varicosities appear to be evenly spaced and about 2 microns apart.

Calibration bar represents 20 microns for all three photographs.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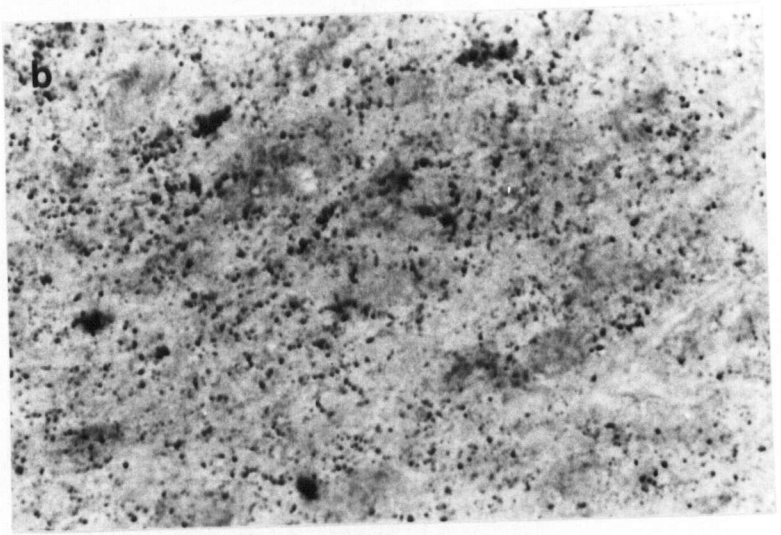
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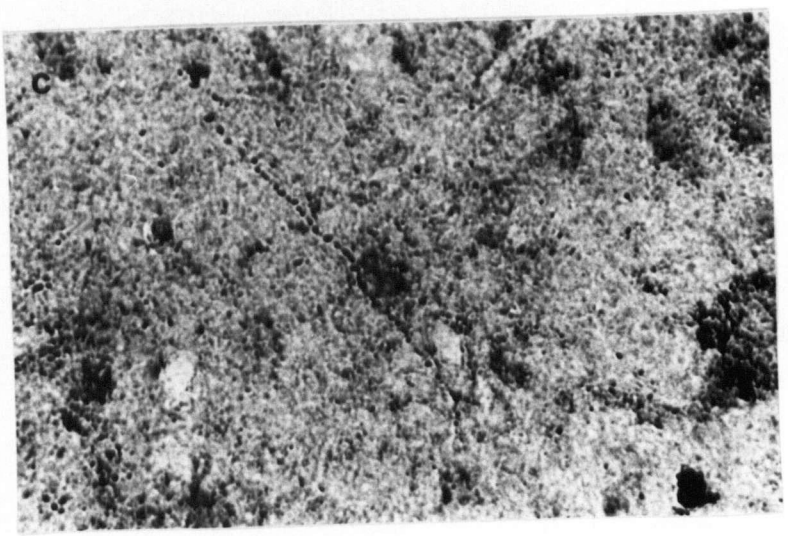

c

can be seen extending from the SNR into the subadjacent crus cerebri. In the SNC the substance P positive fibers appear to run in a horizontal orientation. The unstained cell bodies of the SNC are quite apparent and do not appear to receive many axo-somatic contacts.

## DISCUSSION

In the present study we have provided the first demonstration of substance P-immunoreactivity in the basal ganglia with an immunoperoxidase technique. The findings confirm and extend the previous reports of substance P-immunoreactivity in the rat brain using immunofluorescent techniques (Cuello and Kanazawa, 1978; Ljüngdahl et al., 1978a). In those studies a network of fine, weakly fluorescent structures were observed in the rat striatum. In our material a similar fine network of substance P-immunoreactivity was observed in both the caudate nucleus and the putamen of the cat. No differences in the staining between these two nuclei were apparent. This network probably arises from local collaterals of the substance P cell bodies which have been observed in the striatum (Cuello and Kanazawa, 1978; Jessel et al., 1978; Kanazawa et al., 1977).

In both the GP and the EP the dense plexus of fibers present likely represents the terminals of the striatopallidal substance P-containing pathways (see Experiment 1). These fibers often seemed to outline the major dendritic processes perhaps forming synapses en passant (Fox et al., 1974).

In previous immunofluorescent studies Cuello and Kanazawa (1978) reported a very compact network of fibers in the medial and the cortical amygdaloid nuclei. Ljüngdahl et al. (1978a) report very dense staining in the caudal portion of the medial nucleus, but only single fibers in the cortical amygdala. The medial nucleus in the rat has been found to contain very high levels of substance P by radioimmunoassay, while levels

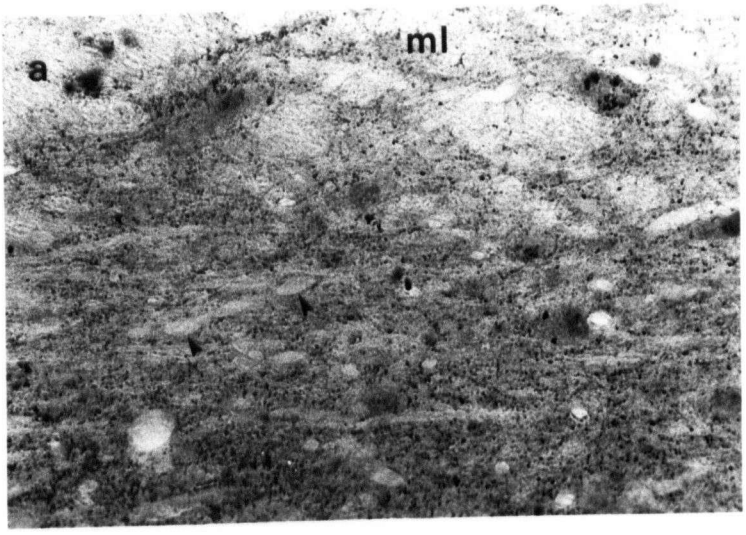
Figure 6. Substance P-like immunoreactivity in coronal sections of the ventral mesencephalon demonstrated with the biotin-avidin immunoperoxidase technique.

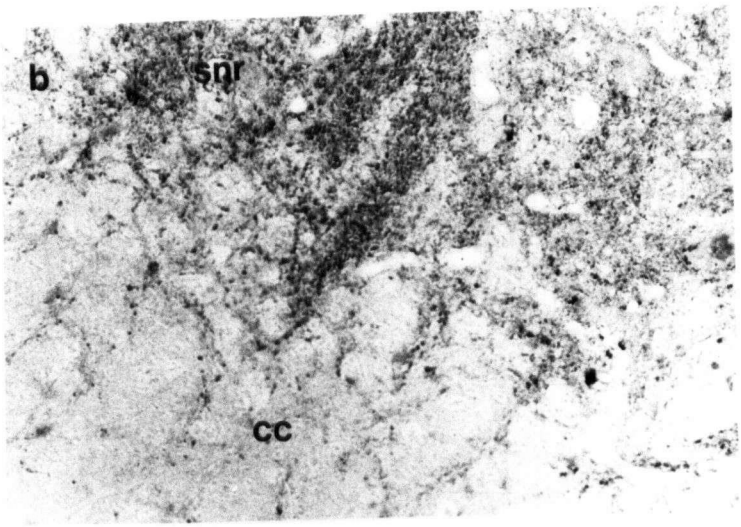
In (a) the dense fiber system occurs throughout the neuropil of the substantia nigra pars compacta. The cells of the pars compacta are unstained (arrows) and do not appear to receive many axosomatic substance P synapses. Some substance P-positive fibers extend dorsally from the nigra into the medial lemniscus (ml).

In (b) the intense staining which exists in the neuropil of the substantia nigra pars reticulata (snr) is demonstrated. Bundles of fibers can be seen extending ventrally from the nigra into the crus cerebri (cc).

In (c) a discrete network of substance P fibers can be seen at the ventro-lateral border of the interpeduncular nucleus (ip) adjacent to the ventral tegmental area (vta).

Calibration bar represents 50 microns for all three figures.


a
ml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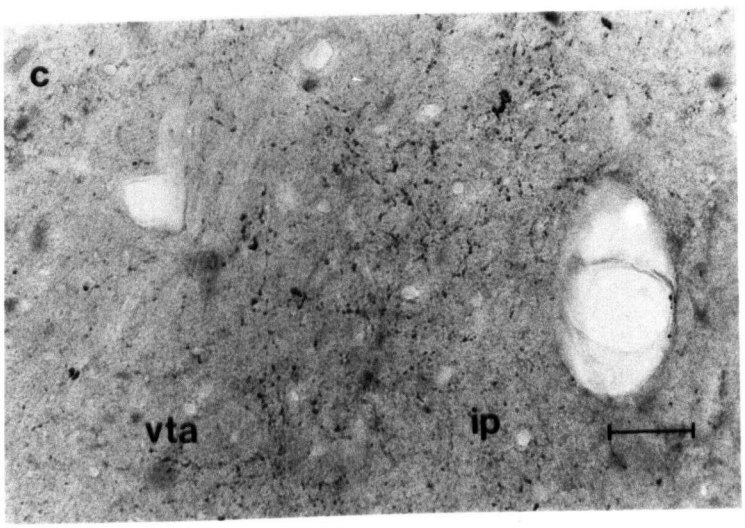
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in the cortical nucleus appear quite low (Ben-Ari et al., 1977). In the present study, the corticomedial nucleus of the cat was found to stain most intensely for substance P, with the fibers often forming basket-like structures about the cell bodies. Perhaps this indicates a homology between the nucleus amygdaloideus medialis of the rat and the cortico-medial nucleus of the cat. In both species it is apparent that most of the substance P is present in the more primitive components of the amygdaloid complex.

The intense staining observed in the lateral habenula is consistent with the previous immunofluorescent studies (Cuello and Kanazawa, 1978; Ljüngdahl et al., 1978a; Cuello et al., 1978). These studies have shown that the substance P terminals lie in the medial portion of the lateral habenula. A similar result was observed in the present study. In addition, a previously unknown band of dense terminals overlying the dorso-lateral surface of this nucleus was observed. The location of the substance P terminals in the lateral habenula appears to be quite distinct from that of the GABA terminals which appear to lie in the ventro-lateral portion of the lateral habenula (see Experiment 4). Although the substance P terminals have been suggested to arise from cells in the medial habenula (Cuello et al., 1978) anatomical studies have failed to demonstrate a connection between these nuclei (Herkenham and Nauta, 1979; Iwahori, 1977). Thus, the origin of the substance P in the lateral habenula remains in doubt.

The medial habenula has also been suggested to be the source of the substance P in the interpeduncular nucleus (Cuello et al., 1978; Emson et al., 1977; Hong et al., 1976; Mroz et al., 1976). In agreement with the immunofluorescent studies in the rat the substance P immunoreactivity found in the cat forms a shell about the dorsal and lateral borders of the

interpeduncular nucleus adjacent to the area of the dopaminergic A-10 cells (Dahlstrom and Fuxe, 1964). The central core of the interpeduncular nucleus is relatively barren of substance P terminals. As the medial habenula is known to provide the major innervation to this part of the interpeduncular nucleus (Herkenham and Nauta, 1979), it appears that the major transmitters in this system remain to be determined.

Within the SN the substance P immunoreactivity is most intense in the SNR, in agreement with radioimmunoassay data obtained in the cat SN (Gauchy et al., 1979). The staining in the SNC was less intense but many varicose fibers were still apparent. This may indicate that the dopamine cells of the A9 group and, in particular, their dendritic processes in the SNR are directly innervated by substance P terminals (Ljüngdahl et al., 1978b). This idea is supported by the observation that iontophoretically applied substance P excites the dopaminergic cells of the SN (Davies and Dray, 1976; Walker et al., 1976). Behavioural (James and Starr, 1977; 1979; Kelley and Iversen, 1978; 1979; Kelley et al., 1979; Olpe and Koella, 1977) and biochemical (Chéramy et al., 1977; 1978; Magnusson et al., 1976; Starr, 1978b) observations also support such an interaction. Hopefully the extension of the immunoperoxidase procedure developed in the present study to the electron microscopic level (Pickel, 1979) will allow the direct visualization of the synaptic relationships between the substance P terminals and the dopamine cells of the SN.

## EXPERIMENT 3: THE LOCALIZATION OF GABA-TRANSAMINASE IN THE STRIATO-NIGRAL SYSTEM.

In Experiment 1 the existence of GABA projections from the striatum to the GP, EP and SN was confirmed. Although these biochemical experiments provide good evidence for the existence of GABA pathways, they provide no information regarding the topographic distribution of the GABA innervations. For this a reliable morphological technique is required. A fluorescent method for GABA has been devised, but the results obtained with this technique have not been promising (Wolman, 1971). The immunohistochemical method for GAD has made possible the visualization of GAD-containing nerve terminals and cells (Roberts, 1979); however, this method has not been used to characterize the topography of GABA projections and the complexity of the technique precludes its general usage. Although a simple histochemical procedure for GABA-transaminase (GABA-T), the enzyme which catabolizes GABA, has long been known (Van Gelder, 1965) its use in examining GABA systems has not been explored.

High levels of GABA-T have been found in the basal ganglia (Salvador and Albers, 1959), and the striatum and SN have been found to exhibit intense histochemical staining for this enzyme (Robinson and Wells, 1973). However, the precise localization of GABA-T has not been clearly resolved. Histochemical studies have suggested that GABA-T activity could be present in nerve cells, terminals or glial elements in the basal ganglia, although which of these elements actually contains the enzyme has been difficult to discern (Robinson and Wells, 1973).

High affinity GABA uptake is thought to be primarily responsible for the removal of synaptically released GABA (Martin, 1976), but recent studies using specific GABA-T inhibitors have indicated that GABA-T may be directly involved in regulating the transmitter pool of GABA. Thus,

intracerebral or systemic injections of GABA-T inhibitors result in a marked elevation of brain GABA levels (Rando and Bangerter, 1977; Matsui and Kamioka, 1978). Also local injections of GABA-T inhibitors into the basal ganglia result in marked behavioural effects, involving both GABA systems and the nigrostriatal dopaminergic system (Pycock et al., 1976; Matsui and Kamioka, 1978). The localization of GABA-T in the basal ganglia has therefore been biochemically examined in order to evaluate the suitability of this enzyme for the histochemical demonstration of GABA pathways.

## METHODS

To destroy the striatal neurons, KA was injected into the caudate-putamen as previously described (McGeer and McGeer, 1976a). Nine rats received a unilateral injection of five nmoles of KA in 0.5 µl 50 mM $NaPO_4$, pH 7, at a rate of 1 µl/5 min. An additional group of rats received an injection of ten nmoles of KA in 1 µl. Two weeks later, the rats were sacrificed by cervical fracture, and the striatum was dissected on ice. The SN was obtained from sections cut on a freezing microtome.

Campochiara and Coyle (1978) and Lehmann and Fibiger (1979) have found that injections of KA into the striatum of neonatal rats preferentially deplete CAT as compared to GAD. In the present report we have examined the effect of such lesions on GABA-T activity. Ten day old rats were injected with 20 nmoles of KA in 1 µl of sodium phosphate buffered (pH 7.4) isosmolar Ringer solution. Fourteen days later the animals were sacrificed and the striatum dissected for enzyme analyses.

In an additional group of adult animals, 2.9 µg of 6-OHDA in 1 µl of 0.9% saline, 0.1% ascorbate was injected into the left nigrostriatal bundle to destroy selectively the nigrostriatal dopaminergic neurons (Clavier and Fibiger, 1977). The injections were made at A + 4.4; L + 1.8; DV - 2.4 mm,

according to the atlas of König and Klippel (1963). These animals received 25 mg/kg desipramine 30 min prior to the 6-OHDA to prevent damage to the noradrenergic neurons (Roberts et al., 1975). One month after the lesion the striatum and SN were dissected as described above.

For the biochemical analyses the tissues were homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.25% Triton X-100. Tyrosine hydroxylase was measured as previously described (McGeer et al., 1967). Forty µl of tissue homogenate were added to 80 µl of a mixture containing (final concentrations) 1.0 mM 2-amino-5-hydroxy-6,7-dimethyl tetrahydropteridine ($DMPH_4$, Sigma), 0.1 mM 1-[$^{14}$C(U)]tyrosine (specific activity 3 to 5 mCi/mmol, New England Nuclear), 0.3 mM ferric sulphate, 50 mM 2-mercapto-ethanol (Eastman) in 0.2 M sodium acetate buffer, pH 6.0. The reaction was incubated for 12 min at 37°C, then stopped by the addition of two ml of a solution containing 1.4% perchloric acid, 0.52% acetic acid and 0.5 µg/ml of dihydroxyphenylalanine. The reaction tubes were centrifuged at 1,000 g for five min and the supernatants transferred to 25 ml beakers. The pellets were rinsed with two ml of 0.35 M potassium phosphate buffer, pH 6.0, centrifuged as before and the supernatants added to those in the beakers. Twelve ml of 28 mM $Na_2EDTA$ were then added to each beaker and the samples were brought to pH 9 to 9.5 and then poured onto columns packed with about 0.3 g of alumina (Calbiochem, acid, $AG_4$, 100-200 mesh). The columns were washed with 35 ml of distilled water, eluted into scintillation vials with 2.0 ml of 0.5 M acetic acid and counted in 14 ml of ACS (Amersham).

The method of Sterri and Fonnum (1978) was used to assay GABA-T activity. Twenty µl of tissue homogenate was placed directly into scintillation vials in an ice bath. Fifty µl of reaction mixture containing (final concentrations) 5 mM γ-amino[U-$^{14}$C]butyric acid (specific activity 0.71 mCi/mmol, Amersham), 2 mM α-ketoglutaric acid (Sigma), 10 mM dithiothrietol

(Sigma), 3 mM nicotine adenine dinucleotide (NAD, Calbiochem), 1 mM succinate (Sigma), 0.3 mM pyridoxal phosphate (Calbiochem) in 50 mM Tris-Cl buffer pH 8.2, containing 0.2% Triton X-100 were then added and the vials incubated at 37°C for 20 min. The reaction was stopped by the addition of one ml of 0.1 M sodium phosphate buffer, pH 7.4. The labeled succinate formed in the reaction was extracted from the aqueous phase by the addition of one ml of isoamyl alcohol containing 0.2 M tri-n-octyl-ammonium phosphate, freshly prepared from tri-n-octylamine (Sigma) according to the method of Sterri and Fonnum (1978). Ten ml of the toluene-based fluor used in the CAT assay were then added and the radioactivity in the organic phase counted. Before use in the assay, the commercial sample of radioactive GABA was purified by extracting once with an equal volume of 20 mM tri-n-octylammonium phosphate in chloroform.

CAT and GAD were assayed according to the procedures outlined in Experiment 1, and protein was determined by the method of Lowry et al. (1951).

## RESULTS

One month after the injection of 6-OHDA, the activity of tyrosine hydroxylase in the striatum was reduced by half indicating a significant destruction of the nigrostriatal dopamine neurons (Table 5). The activities of CAT and GAD in the striatum were not significantly affected nor was GAD activity in the SN altered. This lesion had no effect on the GABA-T activity of either the striatum or the SN. This agrees with the report of Kim (1973) who found no change in striatal GABA-T activity four days after intraventricular 6-OHDA.

Following the injection of KA into the striatum, the activities of striatal CAT and GAD were reduced in a dose-dependent manner. A similar dose-dependent decrease was seen in striatal GABA-T activity (Table 6).

Table 5. THE ACTIVITIES OF NEUROTRANSMITTER-RELATED ENZYMES IN THE STRIATUM AND SUBSTANTIA NIGRA ONE MONTH AFTER INJECTION OF 6-OHDA INTO THE NIGROSTRIATAL BUNDLE.

| | Control (nmol/mg protein/hr | Lesion S.E.M.) | % of control |
|---|---|---|---|
| Striatum | | | |
| Tyrosine hydroxylase | 82.0±3.0 | 39.0±7.8 | 48* |
| Choline acetyltransferase | 140.4±6.7 | 142.5±2.7 | 101 |
| Glutamate decarboxylase | 48.2±1.4 | 54.3±3.3 | 113 |
| GABA-transaminase | 109.3±5.0 | 110.5±5.0 | 101 |
| Substantia nigra | | | |
| Glutamate decarboxylase | 255.6±14.4 | 218.8±17.8 | 86 |
| GABA-transaminase | 101.0±1.6 | 99.2±6.7 | 98 |

n = 12

*$p < .001$, Student's two-tailed $t$ test.

Table 6. The activities of neurotransmitter-related enzymes in the striatum and substantia nigra two weeks after the injection of five or ten nmoles of kainic acid into the striatum.

| | 5 nmol kainate (N=9) | | 10 nmol kainate (N=6) | |
|---|---|---|---|---|
| | Control | Lesion | Control | Lesion |
| | (nmol/mg protein/hr ± S.E.M.) | | | |
| **Striatum** | | | | |
| CAT | - | - | 105.9±3.2 | 18.9±1.7 (18% of control)*** |
| GAD | 72.6±5.2 | 46.5±5.0 (64% of control)*** | 91.2±4.5 | 22.6±2.6 (25% of control)*** |
| GABA-T | 107.7±3.2 | 66.8±4.7 (62% of control)*** | 107.3±3.1 | 35.9±2.4 (34% of control)*** |
| **Substantia nigra** | | | | |
| GAD | 254.6±6.1 | 188.6±8.9 (74% of control)** | 260.0±8.2 | 86.6±7.6 (33% of control)*** |
| GABA-T | 109.7±4.7 | 110.0±4.8 (100% of control) | 117.6±2.8 | 104.6±3.4 (89% of control)* |

*$p$ < .01; **$p$ < .005; ***$p$ < .001; Student's two-tailed $t$ test

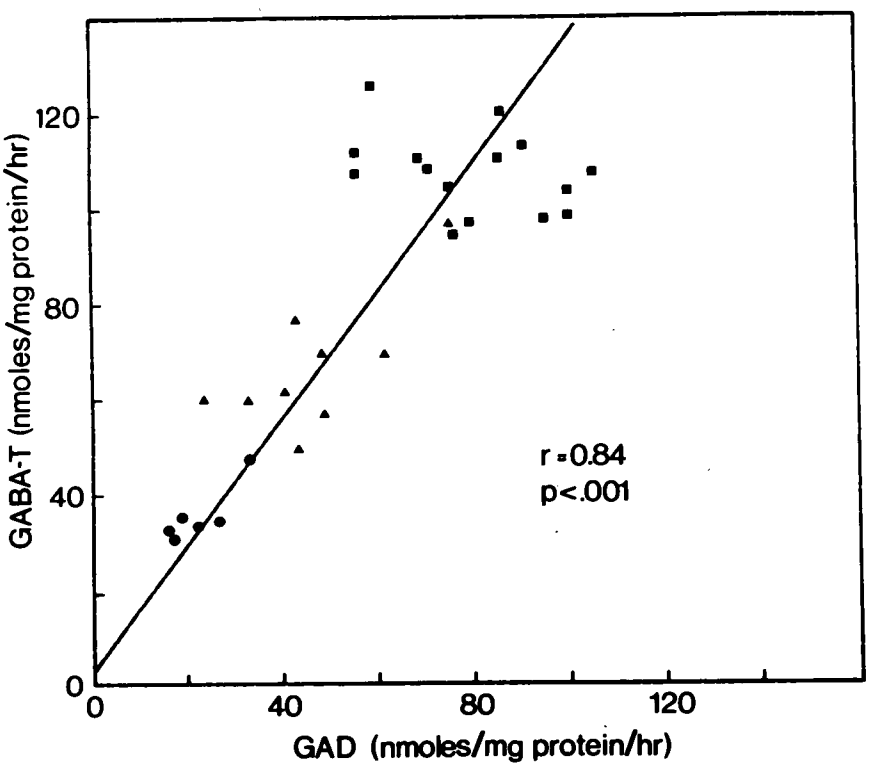
This decrease in striatal GABA-T activity correlated remarkably well with the decrease in striatal GAD activity (Fig. 7). The injection of five nmoles of KA into the striatum which resulted in a small but significant decrease in nigral GAD, did not significantly reduce the activity of GABA-T in the SN (Table 6). Following the injection of 10 nmoles of KA, however, nigral GAD was reduced by 75% and nigral GABA-T activity also showed a significant reduction (Table 6). As in the striatum, the decrease observed in GABA-T activity in the SN correlated significantly with the decrease in GAD (Fig. 8).

Following the injection of KA into the striatum of ten day old rats, the activity of CAT in the injected striatum was reduced by half (Table 7). This injection had no effect on the activities of GAD or GABA-T in the striatum.

## DISCUSSION

The dopamine neurons of the SN are thought to receive a major GABA innervation. Evidence suggests that a massive GABA projection to the SN arises in the striatum (Brownstein et al., 1977; Fonnum et al., 1978a; Nagy et al., 1978a). Immunocytochemical studies have reported that most of the boutons in contact with nigral dendrites stain for GAD (Ribak et al., 1976). Stimulation of the striatum can inhibit nigral cell firing (Yoshida and Precht, 1971), and this effect is blocked by picrotoxin (Precht and Yoshida, 1971) and mimicked by iontophoretic GABA (Feltz, 1971). A marked reduction in nigral $^{3}$H-GABA binding has been observed following 6-OHDA lesions of the nigrostriatal dopamine neurons (Guidotti et al., 1978). $^{3}$H-GABA binding has also been found to be reduced in the SN of Parkinsonian patients, in which the dopamine neurons have degenerated (Lloyd et al., 1977b; Rinne et al., 1978). A wealth of biochemical, anatomical and physiological evidence therefore indicates that the dopamine

Figure 7. The correlation between glutamate decarboxylase and GABA-transaminase activities in the striatum following kainic acid injections of the striatum. squares = control; triangle = 5 nmoles kainic acid; circles = 10 nmoles kainic acid. The line was drawn from the linear regression equation.


GABA-T (nmoles/mg protein/hr)
120
80
40
0
0
40
80
120
GAD (nmoles/mg protein/hr)
r=0.84
p<.001

neurons receive a major GABA innervation. Thus, the lack of change in GABA-T activity following 6-OHDA lesions of the dopamine neurons raises some interesting questions regarding GABA neurotransmission.

It has previously been suggested that GABA released at the synapse is taken up and catabolized in the postsynaptic neuron and in surrounding glial elements since these were the structures thought to contain most of the GABA-T (Baxter, 1976). However, the present results indicate that the dopamine neurons do not contain GABA-T, although they are thought to be postsynaptic to a major GABA system. This observation indicates that all neurons which receive GABA synapses do not necessarily contain GABA-T.

This situation is in sharp contrast with the situation for acetylcholinesterase, the enzyme which catabolizes acetylcholine. The dopaminergic neurons of the SN have been shown to contain appreciable levels of this enzyme (Lehmann and Fibiger, 1978). However, at present there is little evidence for the presence of cholinergic synapses onto the dopamine neurons. Thus, the function of the acetylcholinesterase in these neurons remains a mystery.

Schwarz et al., (1977) and Nicklas et al. (1979) have recently reported a decrease in GABA-T in the striatum following intrastriatal KA and have suggested a neuronal localization for this enzyme. The results of the present experiments with KA suggest that virtually all of the GABA-T activity in the striatum is neuronal. Thus, the dose-dependent decrease in GABA-T activity correlates with that of the neuronal marker GAD (Fig. 7). In fact, the regression line for this correlation approaches the origin, indicating that if GAD activity was completely abolished, the activity of GABA-T in the striatum would also approach zero.

Recent studies have shown that the injection of KA into the striatum of neonatal rats preferentially depletes CAT compared with GAD (Campochiara

Figure 8. The correlation between glutamate decarboxylase and GABA-transaminase in the substantia nigra following kainic acid injections of the striatum. squares = control; triangles = 5 nmoles kainic acid; circles = 10 nmoles kainic acid. The line was drawn from the linear regression equation.

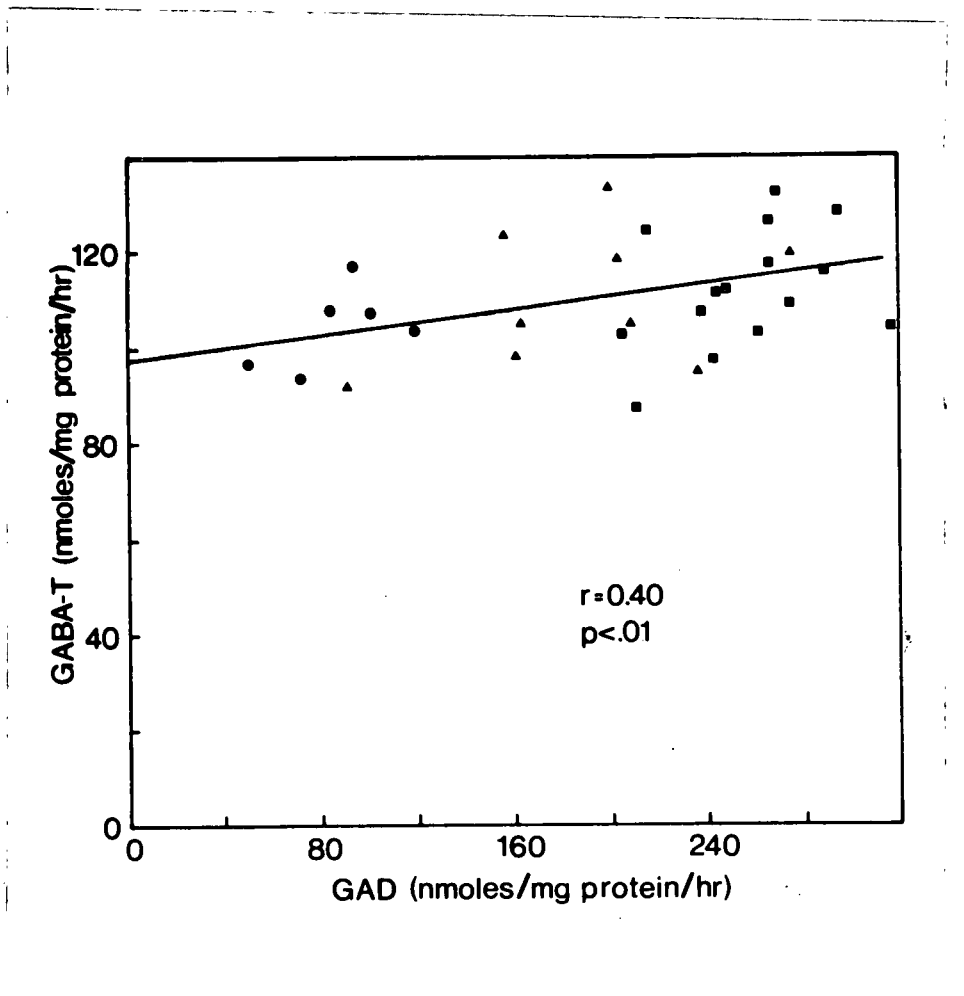

GABA-T (nmoles/mg protein/hr)
120
80
40
0
0
80
160
240
GAD (nmoles/mg protein/hr)
r=0.40
p<.01

Table 7. The activities of neurotransmitter-related enzymes in the striatum after the injection of twenty nmoles of kainic acid into the striatum of ten day rats.

| | Control (nmol/mg protein/hr | Lesion S.E.M.) | % of Control |
|---|---|---|---|
| Choline acetyltransferase | 286.3±5.4 | 124.1±24.9 | 43* |
| Glutamate decarboxylase | 102.1±6.8 | 95.0± 6.8 | 93 |
| GABA-Transaminase | 88.8±2.6 | 97.9± 3.3 | 110 |

n = 6; *$p$ < .001; Student's two tailed $t$ test

and Coyle, 1978; Lehmann and Fibiger, 1979). In fact, Lehmann and Fibiger (1979) have found that these injections selectively destroy the large aspiny neurons of the striatum, which are thought to be the cholinergic neurons (Lehmann and Fibiger, 1979; Kimura et al., 1980) and do not affect the density of the medium and small cells. In the present experiment this preferential decrease in CAT activity was again observed, while GAD and GABA-T activities were unaffected. This suggests that the large aspiny cholinergic neurons of the striatum do not contain GABA-T.

Both GABA and substance P neurons are known to project to the SN from the striatum (see Experiment 1). Therefore destruction of either of these systems could account for the decrease in nigral GABA-T seen after striatal KA injection. However, if GABA transmission within the striatum is similar in mechanism to that discussed above for the SN, then even if the striatonigral substance P neurons receive a GABA input in the striatum, this would not necessitate their containing GABA-T. On the other hand, just as acetylcholinesterase is found in very high concentrations in cholinergic neurons (Lehmann and Fibiger, 1979), perhaps it is the GABA neurons themselves which contain the GABA-T activity in this system. This hypothesis is supported by the significant correlation between GAD and GABA-T found in the striatum and SN after intrastriatal KA.

Striatal KA injections only reduced nigral GABA-T by ten percent. Kataoka et al. (1974) have reported a 25% decrease in GABA-T activity in the baboon SN after hemitransection between the striatum and SN which reduced nigral GAD by 70%. Kim et al. (1974) found a 30% reduction in GABA-T activity in the cat SN following removal of the caudate by suction, a procedure which reduced nigral GABA levels by 50%. The regression line for the correlation between nigral GAD and GABA-T following striatal KA

injections (Fig. 8) does not approach the origin. This contrasts with the situation in the striatum, where the regression line does approach the origin (Fig. 7). This may indicate that GAD and GABA-T are in different cellular compartments. In the striatum, KA injections destroy both the intrinsic GABA cell bodies and the terminals of these cells within the striatum. This results in a parallel decrease in striatal GAD and GABA-T. In the SN following the striatal lesion, the striatonigral terminals are destroyed and GAD is preferentially decreased compared with GABA-T. This suggests that a high GAD to GABA-T ratio may exist in GABA terminals, but not in GABA cell bodies.

Most of the GABA-T in the SN is in elements other than the striatonigral terminals. As discussed above, the dopamine neurons do not appear to contain this enzyme. GABA-T could be contained in other nigral afferents, in other nigral neurons, or in glial elements. It has been suggested that the SN contains a population of GABA neurons (Nagy et al., 1978d). Also, evidence is presented in Experiment 6 for a GABA projection from the SNR to the superior colliculus. In view of the arguments presented above, it would not be surprising if most of the GABA-T activity in the SN was contained in these GABA neurons.

## EXPERIMENT 4: THE HISTOCHEMICAL LOCALIZATION OF GABA-TRANSAMINASE IN THE BASAL GANGLIA.

As discussed previously, biochemical studies have suggested the existence of many GABA pathways in the basal ganglia. In Experiment 3 it was demonstrated that GABA-T is contained in the striatal projection to the SN and it was suggested that this enzyme may in fact be contained in the striatonigral GABA neurons. As a simple histochemical procedure for GABA-T has long been known (Van Gelder, 1965) the use of this technique for the histochemical demonstration of some GABA pathways in the basal ganglia was therefore examined.

### METHODS

Male Wistar rats weighing about 300 g were obtained from Woodlyn Laboratories, Guelph, Ontario and were used in all the experiments. Histochemical staining for GABA-T was performed by a modification of the procedure of Van Gelder (1965). Rats were perfused intracardially with 50 ml of ice-cold 0.1 M phosphate buffered saline, pH 7.4, followed by 200 ml of 2.0% paraformaldehyde and 2.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The free-floating sections were preincubated for 20 min at 37°C in a reaction mixture containing 5.0 mg/ml α-ketoglutarate 1.0 mg/ml malonate, and 0.05 mg/ml KCN in 50 mM Tris-Cl, pH 8.6. After the preincubation the substrate and dye were added to give final concentrations of 5.0 mg/ml GABA, 1.0 mg/ml nitro blue tetrazolium and 0.01 mg/ml phenazine methosulphate, and the incubation was continued for 30 min in the dark. The reaction was stopped by diluting in phosphate buffer, and the sections were then mounted, dehydrated and cover-slipped. Control sections incubated without GABA, or with the GABA-T inhibitor amino-oxyacetic acid (AOAA) showed no reaction product.

In order to destroy the striatal neurons, KA was injected into the

striatum as previously described (McGeer and McGeer, 1976a). Rats received a unilateral injection of 5 nmoles of KA in 0.5 µl of 50 mM $NaPO_4$, pH 7 at a rate of 1 µl/5 min. Two weeks later these animals were deeply anesthetized with pentobarbital and perfused and processed for GABA-T histochemistry at the injection site and in the projection areas of the striatal GABA efferents.

In a second study the GABA-T staining of the efferents of the pallidum was examined. One group of animals was given a unilateral stereotaxic injection of KA (2.0 nmol. in 0.25 µl) into the GP (AP + 8.0; ML + 2.8; DV + 3.6; with respect to stereotaxic zero) while another group received the same injection into the EP (AP + 6.3; ML + 3.0; DV + 2.2). One week later these animals were also processed for GABA-T histochemistry.

## RESULTS

### (a) GABA-T staining in control animals

All of the nuclei of the basal ganglia stain strongly for GABA-T in control animals. This is readily apparent in sagital sections through this region (Fig. 9). In fact, in many areas the staining is so intense as to obscure which cellular structures contain the enzyme. The striatum stains strongly for GABA-T. The reaction product is absent in the white matter coursing through the striatum but is diffusely present throughout the neuropil (Fig. 10). Stained neurons can not be clearly discerned and for the most part are hidden by the intense neuropil staining.

The neuropil of the GP also stains strongly for GABA-T activity and in addition some large, intensely stained neurons can be seen in this area (Fig. 11a). The white matter passing through the GP and the adjacent internal capsule are not stained. An even more intense staining pattern is observed in the neuropil of the EP, although stained neurons are not apparent here (Fig. 11c).

Figure 9. A sagital section through the rat brain stained histochemically for GABA-transaminase activity (magnification = 9.2. x)

Note the regional differences in the intensity of the staining. In particular areas associated with the basal ganglia show an intense reaction. ep entopeduncular nucleus; cp caudate-putamen; gp globus pallidus; lh lateral habenula; sc superior colliculus; sn substantia nigra; st subthalamic nucleus.

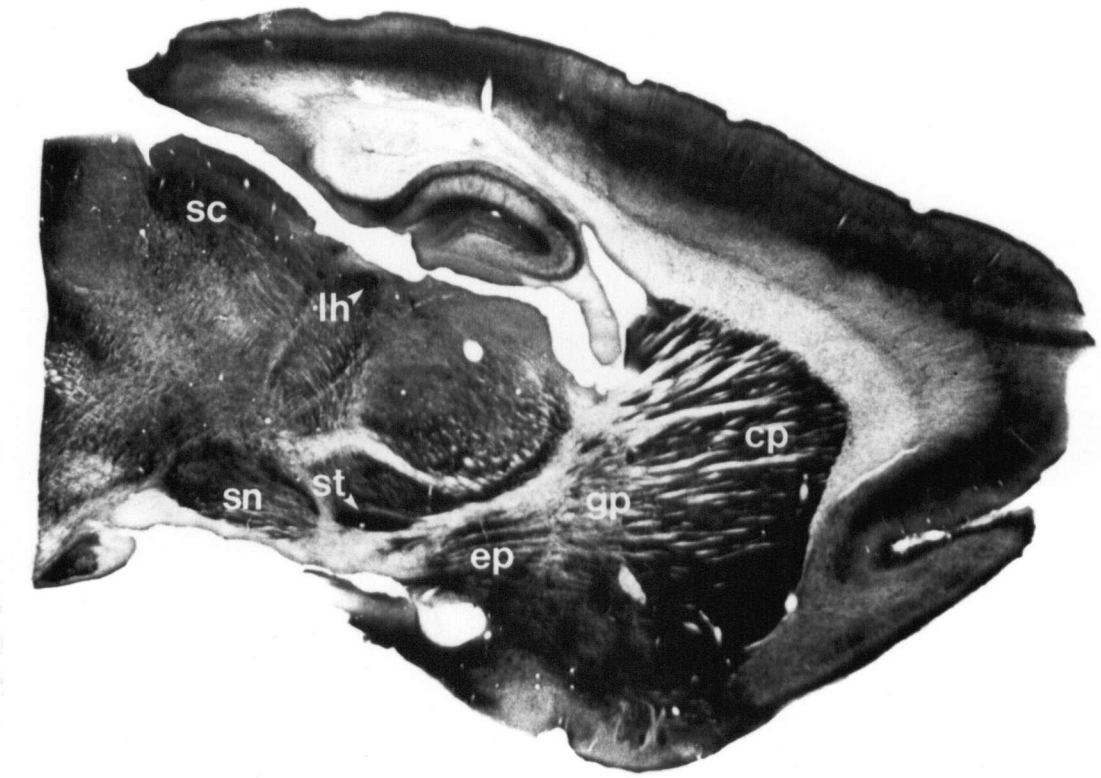

sc
lh
cp
sn
st
gp
ep

Figure 10. Fifty micron vibratome section through the rat forebrain stained histochemically for GABA-transaminase activity. The intense reaction product, which is present throughout the striatal neuropil of the control side (left), is almost completely absent on the contralateral side following the destruction of the striatal neurons by the injection of five nmoles of kainic acid. Some loss of staining can also be seen in the cortex on the lesioned side. This is most apparent along the needle tract (arrow). Magnification is 10 x.

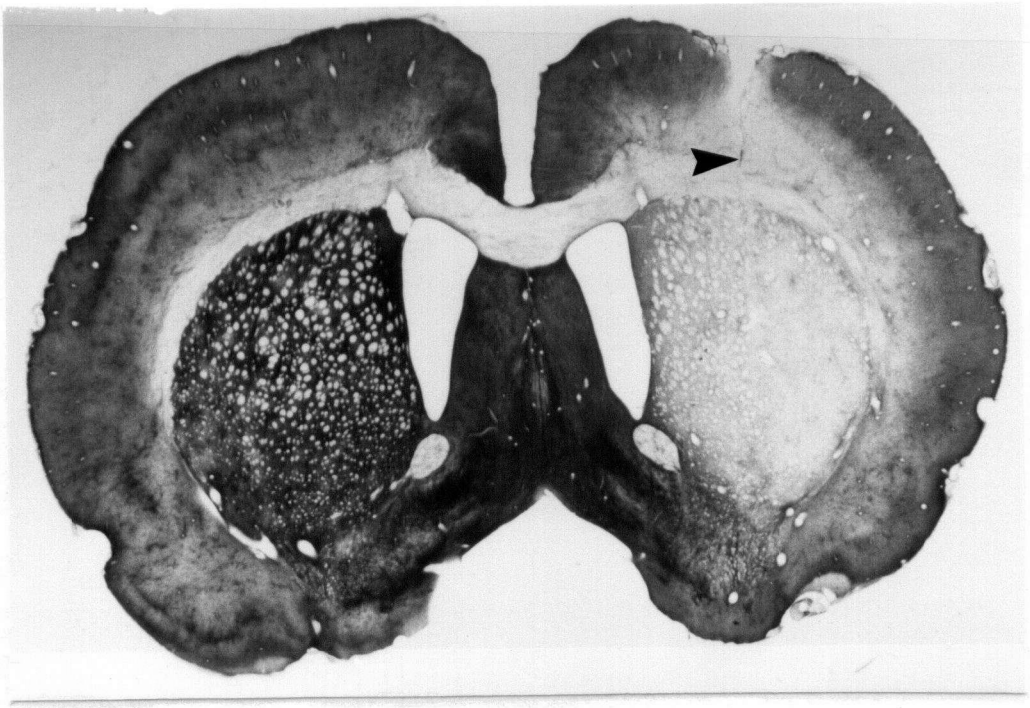


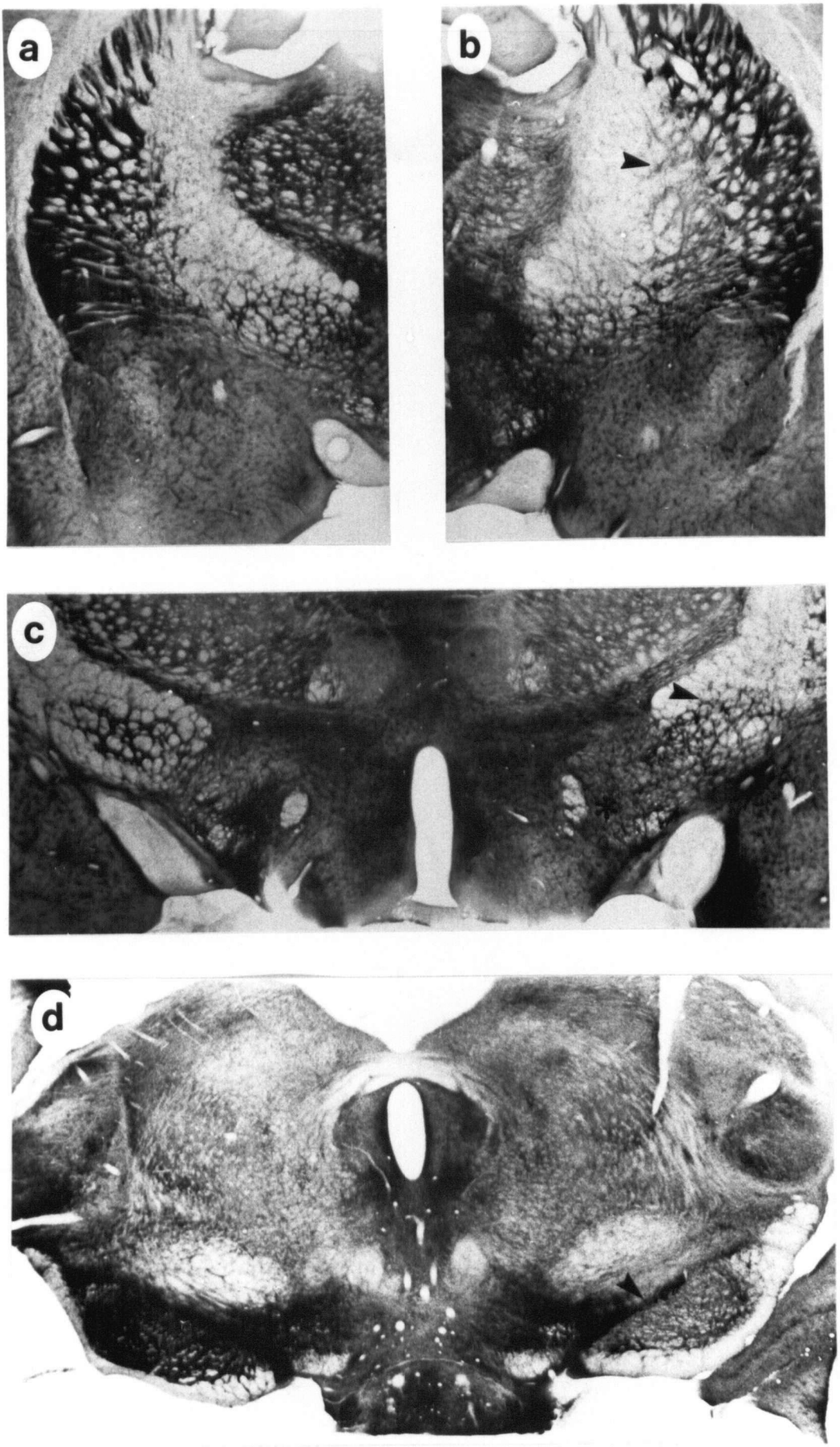
Figure 11. GABA-transaminase histochemistry following the injection of kainic acid into the head of the striatum.

(a) The control uninjected side of the brain can be seen in this coronal section through the tail of the caudate. Note the intense staining in the tail of the caudate and in the globus pallidus medial to it. Magnification is 16 x.

(b) On the injected side the staining in the globus pallidus is markedly reduced (arrow) but the GABA-T activity in the tail of the caudate lateral to the globus pallidus is unaffected by the kainate lesion of the head of the striatum. Magnification is 16 x.

(c) In this coronal section staining in the entopeduncular nucleus on the control side can be compared with that on the lesioned side. The GABA-T activity on the lesioned side (arrow) shows a marked reduction following the striatal kainic acid injection. A similar loss of staining can be seen in the lateral hypothalamic area (*) as well. Magnification is 15.1 x.

(d) The GABA-transaminase activity in the substantia nigra is also drastically reduced (arrow) following the striatal lesion when compared with the contralateral control nigra. This is true both in the pars compacta and the pars reticulata. Note the staining in the ventral tegmental area medial to the substantia nigra is unaffected by the striatal lesions. Magnification is 17 x.


a
b
c
d

Within the SN, the GABA-T staining is present as a dense band in the rostral SNC (Fig. 11d), while in the SNR the pattern of staining resembles that seen in the GP (Fig. 11d). A few large, stained neurons similar to those in the GP can also be found in the SNR.

The subthalamic nucleus shows the most intense GABA-T staining of any area yet examined. The reaction product is so strong in this nucleus that morphological features cannot be discerned (Fig. 9). Although the entire nucleus shows high GABA-T activity the medial half is the most intensely stained (Fig. 12). The crus cerebri passing ventral to the subthalamus is unstained except for some fine bands of reaction product extending from the subthalamic nucleus into the adjacent intrapeduncular area.

Finally, the lateral habenula stains quite strongly for GABA-T while minimal activity is present in the medial habenula (Fig. 13). The staining in the lateral habenula is most intense in the ventrolateral division and is noticeably weaker in the more medial portion of this nucleus. The dorsal aspect of the lateral habenula stains as weakly as the medial habenula for GABA-T.

(b) GABA-T staining after lesions of GABA pathways

Following the destruction of the neurons of the striatum by the injection of KA, the GABA-T staining is almost abolished in the striatal neuropil (Fig. 10). There is faint staining of macrophages or monocytes along the needle tract, and of astrocytes throughout the area of damage. Infiltration by macrophages which stain weakly for GABA-T was also observed in the corpus callosum dorsal to the lesioned striata. The lesion was confined to the striatum rostral to the decussation of the anterior commissure but extended ventrally to include the nucleus accumbens.

The injection of KA into the striatum also reduced the GABA-T staining

Figure 12. GABA-transaminase activity in the subthalamic nucleus of the rat is extremely strong on the control side (arrow), but is drastically reduced on the side in which the globus pallidus was injected with kainic acid (double arrow). Note that in the control subthalamus the staining is most intense in the medial portion of the nucleus. Also, bands of reaction product can be seen extending ventrally from the subthalamus into the adjacent intrapeduncular area. Magnification is 12.9 x.

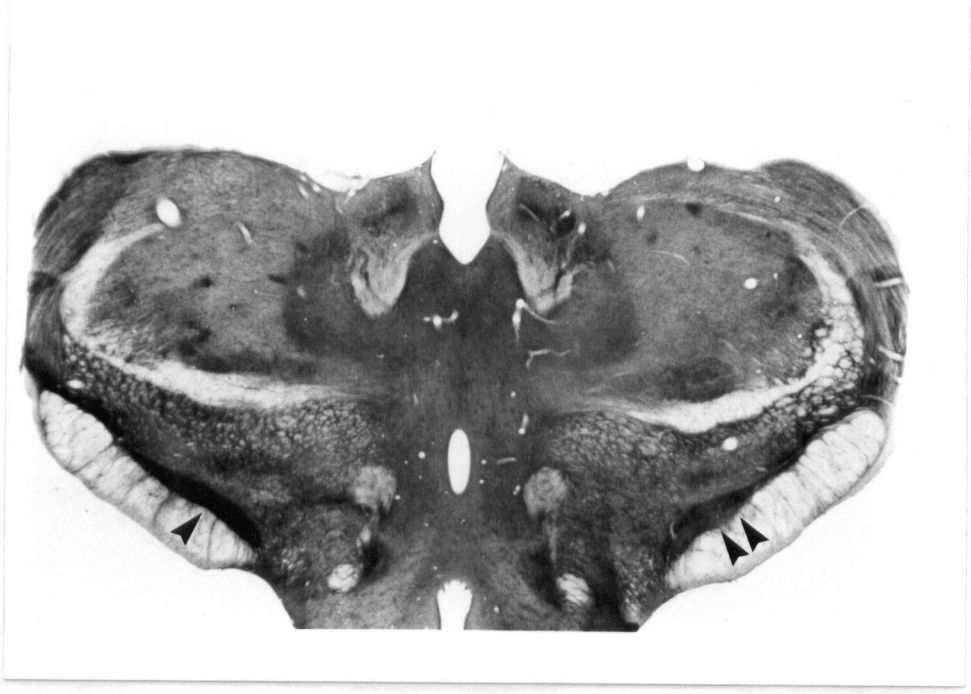
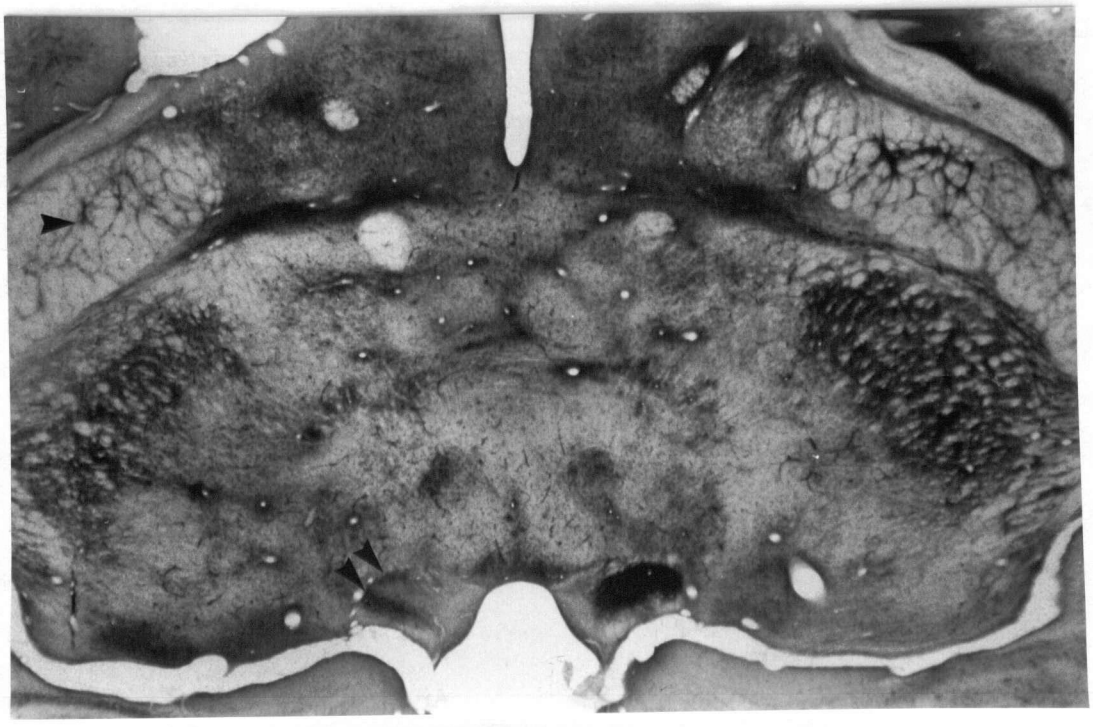
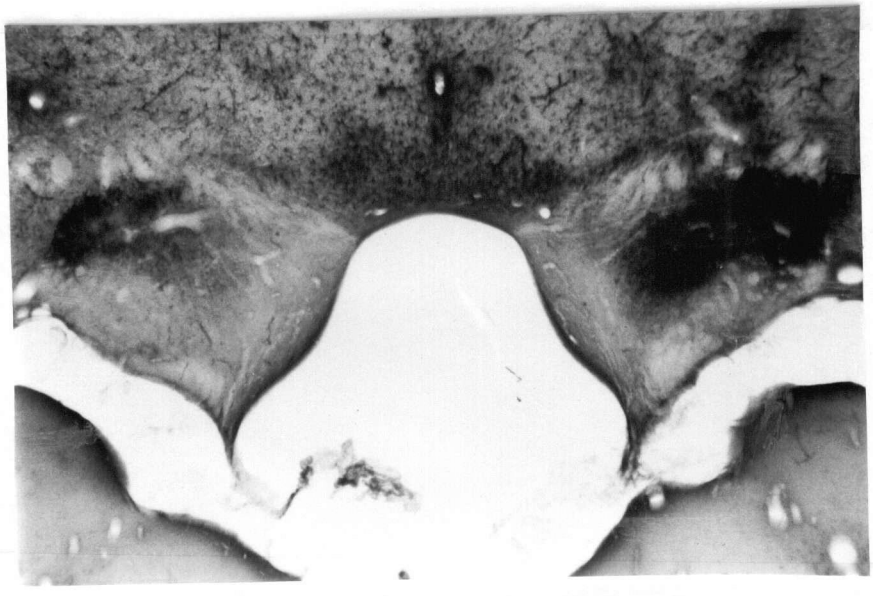


Figure 13. GABA-transaminase histochemistry in the habenula following kainic acid lesions of the entopeduncular nucleus. In the lower figure, which is a coronal section at the level of the injection site, the loss of staining in the entopeduncular nucleus following the kainic acid injections is readily apparent (arrow). This lesion dramatically reduced the GABA-T activity of the ipsilateral lateral habenula (double arrow) compared with the contralateral control. Magnification is 15.7 x.

In the upper figure the decrease in habenular GABA-T activity following the entopeduncular lesion can be seen in greater detail. Note that in the control habenula the GABA-T activity is most concentrated in the ventrolateral portion of the lateral habenula. Magnification is 36.6 x.





in the GP, EP and SN ipsilateral to the lesion (Fig. 11). The staining in the tail of the caudate was not affected by the lesion (Fig. 11b). This suggests that the reduced pallidal staining was not due to the diffusion of KA to this structure. Large GABA-T positive neurons were still visible in the GP and SN and a few small GABA-T positive cells could be seen in the EP. In the control side these cells are still concealed by the intense staining of the neuropil. Within the SN, both the intense staining of the SNC and the more diffuse SNR staining were markedly reduced by the striatal lesion (Fig. 11d).

Following the injection of KA into the GP a marked reduction in GABA-T activity was evident. In particular, the GABA-T positive neurons were no longer visible. The KA lesion of the GP also drastically reduced the GABA-T staining in the ipsilateral subthalamic nucleus. This is most evident in the medial portion of the nucleus (Fig. 12). The staining in the fields of Forel and the zona incerta around the subthalamic nucleus was not affected by the lesions.

Finally, the injection of KA into the EP resulted in a marked reduction in the GABA-T staining of this nucleus, although some diffuse reaction product was still present in the neuropil (Fig. 13). The GABA-T staining in the ipsilateral lateral habenula was also dramatically decreased by this lesion, while the staining of the contralateral habenula was not different from control (Fig. 13).

## DISCUSSION

In Experiment 3, biochemical evidence was presented suggesting that GABA-T activity in the striatum is contained in neurons. The present histochemical studies support such a conclusion. Thus, following the destruction of the striatal neurons with KA the GABA-T staining in this nucleus was almost completely abolished. The lesioned striata did

contain non-neuronal elements which stained weakly for GABA-T; however, invasion by these elements (monocytes, fibrous astrocytes) is probably related to the pathology of the lesion and they would not normally be present in the striatum.

Previous studies have found that lesions of the striatum result in a reduction in GAD activity in both the GP and the EP (see Experiment 1). The present experiments clearly illustrate that following the injection of KA into the head of the striatum a marked reduction in the GABA-T activity in these areas also occurs. This suggests that GABA-T is contained within the terminals of the striatopallidal GABA neurons. Some neurons in the GP and EP stain for GABA-T after the lesion, indicating the presence of this enzyme in some pallidal cell bodies as well as in afferent terminals. Both the GP (Fonnum et al., 1978b; Hattori et al., 1973b) and the EP (Nagy et al., 1978b) are thought to contain GABA neurons, suggesting that it is these cells which stain for GABA-T.

It is interesting to note that the injection of KA into the striatum reduces the activity of GABA-T in the lateral hypothalamic area ventromedial to the EP (Fig. 11c). This region stains intensely on the control side of the brain (Fig. 11c). The lateral hypothalamic area has been shown to possess the highest GABA levels (Kimura and Kuriyama, 1975a) and GAD activity (Kimura and Kuriyama, 1975b) in the hypothalamus. Anatomical studies have shown that the nucleus accumbens projects massively to this region (Nauta et al., 1978; Powell and Leman, 1976). Recently, biochemical studies have indicated that the accumbens projections to the substantia innominata, GP (Walaas and Fonnum, 1979), SN and ventral tegmental area (Waddington and Cross, 1978b; Walaas and Fonnum, 1980) may contain GAD. Together, these results suggest that the decrease in the GABA-T activity observed in the lateral hypothalamic area following KA

injections of the striatum may be due to the destruction of GABA neurons in the nucleus accumbens projecting to this area.

The existence of a striatonigral GABA pathway has been repeatedly demonstrated, although the precise origin of this pathway has been a matter of some debate (see Experiment 1). Recent evidence suggests that there is a concentration of striatonigral GAD-containing neurons in the striatum apposed to the anterior GP (Brownstein et al., 1977; Experiment 1). Anatomical studies suggest that these neurons project predominantly to the SNC, while the anterior striatal projection, which contains substance P (see Experiment 1) projects largely to the SNR (Hattori et al., 1975; Tulloch et al., 1978). Thus, the observation that the GABA-T staining although present throughout the SN, is most intense in the SNC correlates well with the proposed distribution of the terminals of the GABA projection from the striatum. It has been demonstrated biochemically that the dopaminergic neurons of the SNC do not contain GABA-T (Experiment 3). The loss of staining observed in the pars compacta following striatal lesions is consistent with this finding since the dopaminergic neurons are not damaged by this lesion. As in the pallidum, some large cells in the SNR stain for GABA-T both in control animals and after striatal lesions. These neurons may be the nigral cells which project to the tectum and the ventromedial thalamus and are thought to contain GABA (see Experiment 6).

Recent studies have shown that injections of GABA-T inhibitors into the GP or SN result in marked effects on motor behaviour (Matsui and Kamioka, 1978; Pycock et al., 1976). The present histochemical observations suggest that the GABA-T present in the terminals of the striatal GABA projections could be the site of action for these effects. This would be consistent with the hypothesis that GABA-T in GABA Nerve terminals is directly involved in the regulation of GABA transmission.

Lesions of the EP have previously been found to decrease GABA levels and GAD activity in the habenula indicating that this is a GABA pathway (Gottesfeld et al., 1977; Nagy et al., 1978b). In the present histochemical study, the activity of the enzyme GABA-T in the habenula was also found to decrease following EP lesions. This suggests that GABA-T in the habenula is contained in the terminals of the EP-habenula pathway. The GABA-T staining in the lateral habenula is most intense in the ventrolateral portion of this nucleus, and is weakest in the dorso-medial region. This may indicate that the GABA terminals in the lateral habenula are most concentrated in the ventrolateral area. The evidence from anatomical studies of the EP-habenula pathway is in full agreement with this hypothesis. In autoradiographic studies of the orthograde transport of radiolabeled protein from the EP the label has been found to distribute predominantly in the ventrolateral portion of the lateral habenula (Carter and Fibiger, 1978; Larsen and McBride, 1979; Nagy et al., 1978b) and not in the dorso-medial portion (Nauta, 1974). A similar topography has been obtained from retrograde studies with horseradish peroxidase (Herkenham and Nauta, 1977; Larsen and McBride, 1979).

The recent observation of Fonnum et al. (1978b) that GP lesions in the cat reduce subthalamic GAD activity indicates that this may also be a GABA pathway. Lesions of the GP in the rat reduce GABA-T activity in the subthalamic nucleus, suggesting that this enzyme is present in the terminals of the pallidal afferents to this nucleus. Fonnum et al. (1978b) have found that the concentration of GAD increases from the lateral to the medial part of the subthalamus. In the present experiments the intensity of GABA-T staining was found to follow a similar pattern. Again this agrees with the topography of the pallido-subthalamic pathway found in anatomical studies. Autoradiographic studies in the rat following GP injections of

tritiated amino acids have found the medial portion of the subthalamic nucleus to be most heavily labeled (Carter and Fibiger, 1978).

The present experiments indicate that GABA-T histochemistry may be a useful adjunct to biochemical studies for the analysis of GABA pathways. The localization of GABA-T staining in the efferents of the striatum corresponds to the distribution of the striatal GABA efferents. Also the distribution of GABA-T staining in the lateral habenula and subthalamic nucleus correlates well with the known topography of the pallidal GABA projections to these areas. Moreover, the combination of GABA-T histochemistry with selective lesions has provided a very graphic summary of what has been learned from several years of biochemical work. This approach provides a detailed picture of the topography of these GABA pathways previously only hinted at from correlations of lesion-biochemical work with anatomical studies. Thus, GABA-T histochemistry may provide a simple technique for the morphological analysis of known or suspected GABA pathways.

## EXPERIMENT 5: BIOCHEMICAL CHANGES FOLLOWING 6-HYDROXYDOPAMINE LESIONS OF THE NIGROSTRIATAL DOPAMINE NEURONS: AN ANIMAL MODEL OF PARKINSONISM?

Lesions of the nigrostriatal dopamine projection have been used as animal models of Parkinson's disease (see Marsden et al., 1975) since it was discovered that this system degenerates in this disorder (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1973). A detailed study of the effects of such lesions on the other transmitter systems of the basal ganglia has not been conducted. This is of great importance for, although the most striking aspect of Parkinson's disease is the marked decrease in nigral and striatal dopamine, changes in other transmitter systems have also been reported. A decrease in GAD activity in some nuclei of the basal ganglia has been consistently observed in Parkinson's disease (Lloyd and Hornykiewicz, 1973; McGeer et al., 1971; Rinne et al., 1974; 1979). This decrease has been reported to be reversed by chronic L-dopa therapy (Lloyd and Hornykiewicz, 1973; Rinne et al., 1979). Also, normal rats treated chronically with L-dopa have been reported to have an increased striatal GAD activity (Lloyd and Hornykiewicz, 1973). These results suggest an intimate interaction between the nigrostriatal dopamine system and the GABA neurons of the striatum.

Although the levels of substance P and met-enkephalin have not been measured in Parkinson's disease, experimental data indicate that the dopaminergic nigrostriatal system profoundly influences the neurons containing these peptides in the striatum. Acute amphetamine treatment has been found to decrease the substance P levels in the striatum (Pettibone et al., 1978a; 1978b). Chronic neuroleptic treatment decreases the levels of substance P in the SN (Hong and Costa, 1978) and increases the levels (Hong and Costa, 1978) and accelerates the synthesis of met-enkephalin

(Hong et al., 1978b) in the striatum, GP and nucleus accumbens.

The present series of experiments was designed to examine further the interactions between the dopaminergic neurons and the transmitters contained in the efferents of the striatum. Selective lesions of the nigrostriatal dopamine neurons were performed with 6-OHDA and the effects of such lesions on the GABA, substance P and met-enkephalin systems of the basal ganglia examined.

## METHODS

Male Wistar rats weighing about 300 g were placed under Nembutal anesthesia and given a unilateral stereotaxic injection of four µg 6-OHDA into the nigrostriatal pathway as described in Experiment 3. Control animals received an injection of vehicle only. All animals received desipramine (25 mg/kg) 30 min before the 6-OHDA injections.

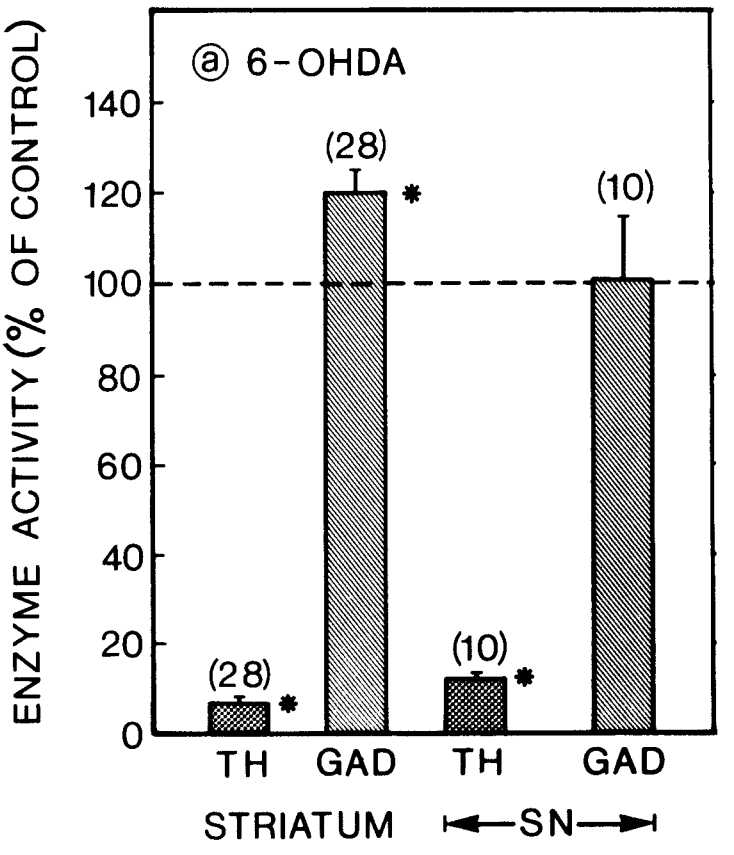
Following surgery the animals were singly housed in a 12 hr light-dark environment and given food and water ad libitum. The animals were sacrificed by cervical fracture at various times after the operation, the brains removed and dissected for biochemical analyses.

Tissues for the enzyme assays were homogenized in 20 to 30 volumes of 50 mM Tris-acetate buffer pH 6.4 containing 0.2% Triton X-100. GAD was assayed as described in Experiment 1, tyrosine hydroxylase as outlined in Experiment 3, and protein according to Lowry et al. (1951). The levels of substance P and met-enkephalin were measured by radioimmunoassay following extraction in one N acetic acid (Appendix).

## RESULTS

In the first experiment, animals were sacrificed 25 days after the injection of 6-OHDA into the left nigrostriatal pathway. The whole striatum, including the accumbens and GP was dissected freehand, while the SN was obtained from sections cut on a freezing microtome. This lesion

Figure 14. Enzyme activities in the striatum and substantia nigra (SN) 25 days after unilateral lesions of the nigrostriatal pathway, expressed as percent of control. Each column represents the mean of the number of rats indicated in brackets. Vertical bars represent the standard error of the mean. Absolute values for tyrosine hydroxylase (TH) and glutamate decarboxylase (GAD) in control striata were $4.2 \pm 0.2$ and $98.9 \pm 3.7$ nmoles/mg protein/h respectively. For control SN, the TH and GAD values were $2.3 \pm 0.1$ and $272 \pm 11$ nmoles/mg protein/h. * $p < .001$, paired $t$-test.


ⓐ 6-OHDA
ENZYME ACTIVITY (% OF CONTROL)
140
120
100
80
60
40
20
0
(28)
(28)
(10)
(10)
*
*
*
*
TH
GAD
TH
GAD
STRIATUM
SN

resulted in an decrease in striatal and nigral tyrosine hydroxylase activity of more than 90%, resulting from the orthograde and retrograde degeneration of the dopamine terminals and perikarya respectively. This lesion was associated with a highly significant increase in the activity of GAD in the striatum on the injected side. In contrast, GAD activity in the SN was not affected by the lesion (Fig. 14). These results have recently been published (Vincent et al., 1978b).

Since the striatal sample in which the GAD increase was observed in the preliminary experiment included the caudate-putamen, the accumbens and the GP, the experiment was replicated and GAD was assayed in discrete nuclei of the basal ganglia in an attempt to localize the site of this change in activity. One month after the 6-OHDA lesion animals were killed by cervical fracture and the nucleus accumbens, the head and the tail of the striatum, the GP and the SN were dissected from coronal sections obtained on a freezing microtome and assayed for GAD activity. These lesions were found to reduce tyrosine hydroxylase more than 92% in the head of the striatum indicating that the lesion of the dopamine neurons was essentially complete. As seen in Table 8, these lesions increased GAD activity in the nucleus accumbens, the head and the tail of the striatum, and the GP by about the same extent. There was a tendency for GAD to increase in the SN but this did not reach statistical significance ($.05 < p < .1$).

To determine if the observed increase in striatal GAD activity was a transient or a permanent change, the activity of GAD was measured in the striatum and SN three months after the unilateral injection of 6-OHDA into the nigrostriatal bundle. The levels of the neuropeptides substance P and met-enkephalin were also measured at this survival time, and in addition a group receiving a unilateral injection of vehicle only was

Table 8. Glutamic acid decarboxylase activity in various brain areas after unilateral lesions of the nigrostriatal pathway with 6-OHDA.

| Area | Enzyme (nmol/mg protein/hr) Control | Lesioned | % of control |
|---|---|---|---|
| Accumbens | 158±5.3 | 198±8.3 | 125** |
| Body of caudate-putamen | 109±7.6 | 135±7.9 | 124*** |
| Tail of caudate-putamen | 99.4±6.7 | 127±4.2 | 128** |
| Globus pallidus | 221±7.8 | 266±5.7 | 120*** |
| Substantia nigra | 293±8.9 | 321±13 | 110 |

Enzyme activities are the mean ± S.E.M. of the lesioned and contralateral side of 12 rats. Statistical evaluation was conducted using the paired *t* test. **p < .01, ***p < .001.

included in the analysis.

As shown in Table 9, the decrease in striatal tyrosine hydroxylase in this study was again virtually complete. This indicates that the lesion was total and no regeneration of the dopamine neurons had occurred. The vehicle injected control group showed a small reduction in mean tyrosine hydroxylase activity in the striatum, however, this did not reach statistical significance at the five percent level. It is worth noting in this regard that previous reports have found ascorbate-containing vehicle to be slightly toxic to the nigral dopamine cells (Wolfarth et al., 1977).

The increase in GAD activity observed at three months following 6-OHDA was identical to that seen at 28 days. The GAD activity in the denervated striatum was significantly higher than that in either the contralateral uninjected striata or in the saline injected striata of the control group.

The lesion of the nigrostriatal dopamine system with 6-OHDA dramatically reduced the levels of substance P in both the head of the striatum and in the SN. Saline injected animals showed no change in substance P levels. The levels of met-enkephalin were unaffected in either the striatum or the SN following the 6-OHDA lesions (Table 9).

## DISCUSSION

Although 6-OHDA has been found to be a selective neurotoxin for catecholamine neurons (Hökfelt and Ungerstedt, 1973; Maler et al., 1973) the changes observed in GAD activity and substance P levels in the present study make it evident that secondary and probably indirect effects on other neuronal systems can also occur following 6-OHDA injections. Thus, the selective lesion of the nigrostriatal dopamine system resulted in an enhanced striatal GAD activity. As GAD activity was measured at substrate

Table 9. Enzyme activities and neuropeptide levels in the striatum and substantia nigra three months after the injection of saline or 6-hydroxydopamine into the left nigrostriatal pathway.

| | Striatum | | Substantia nigra | |
|---|---|---|---|---|
| | R | L | R | L |
| Saline injections: | | | | |
| Tyrosine hydroxylase (nmol/mg protein/hr) | 1.62± .33 | 0.98±.17 | | |
| Glutamate decarboxylase (nmol/mg protein/hr) | 60.9± 4.3 | 56.2±4.3 | | |
| Substance P (pg/mg tissue) | 369± 26 | 353±25 | 1918±155 | 1854±189 |
| 6-OHDA injections: | | | | |
| Tyrosine hydroxylase (nmol/mg protein/hr) | 1.18±.12 | 0.01±.01* (.85% of control) | | |
| Glutamate decarboxylase (nmol/mg protein/hr) | 56.1 ±3.7 | 66.7±4.0** (119% of control) | | |
| Substance P (pg/mg tissue) | 393±21 | 232±12* (59% of control) | 1997±170 | 1013±67* (51% of control) |
| Met-enkephalin (pg/mg tissue) | 533±84 | 696±128 | 153±18 | 135±8 |

*$p < .001$ compared to contralateral side or ipsilateral saline injection (2-tail $t$ test)

**$p < .05$ compared to contralateral side (1 tailed $t$ test) or ipsilateral saline injection (2-tail $t$ test)

concentrations well above saturation, it appears that the increased activity measured indicates an increased enzyme velocity, indicating an actual increase in the amount of enzyme rather than an activation of existing enzyme. This has in fact been found in a recent kinetic analysis of the GAD increase following 6-OHDA lesions (Fibiger et al., 1980).

It is evident from the regional examination that these lesions increase GAD activity in the caudate-putamen as well as in the nucleus accumbens and the GP. A non-significant tendency toward increased GAD activity was also observed in the SN. Inasmuch as it is known that the distribution of GAD within the SN is not homogeneous (Fonnum et al., 1978a; Fonnum et al., 1974) it is possible that there occurred significant regional increases in nigral GAD which may have been obscured when the whole SN was assayed. Saavadra et al. (1978) have recently provided evidence that such regional increases in nigral GAD do indeed occur after 6-OHDA lesions of the dopamine neurons.

These changes in GAD activity in the basal ganglia stand in marked contrast to those observed in Parkinson's disease which the 6-OHDA lesion is thought to mimic. In Parkinsonism, a consistent decrease in basal ganglia GAD activity has been observed (Lloyd and Hornykiewicz, 1973; McGeer and McGeer, 1976b, McGeer, et al., 1971; Rinne et al., 1974). This decrease has led to the proposal that in Parkinson's disease the decreases in striatal and nigral GAD activity are compensatory changes and represent an attempt to maintain nigrostriatal dopamine transmission (Lloyd and Davidson, 1979). The present observations would seem to make this hypothesis untenable. Thus, complete destruction of the nigrostriatal dopamine system with 6-OHDA results not in a compensatory decrease in GAD activity, but rather, in a significant increase.

The present findings thus have important implications for the etiology

of Parkinson's disease. They strongly suggest that in addition to the demonstrated pathology of the dopaminergic systems in this disease a pathology of the striatal GABA neurons may also be present. The decrease observed in pallidal and nigral GAD in Parkinsonism (Lloyd and Hornykiewicz, 1973; McGeer and McGeer, 1976b; McGeer et al., 1971) is entirely consistent with this hypothesis and suggests that the striatopallidal and striatonigral GABA tracts atrophy in this disease.

In this regard, it has been found that the cerebrospinal fluid levels of GABA are markedly reduced in Parkinsonian patients (Lakke and Teelken, 1976) and in fact are even lower than those found in patients with Huntington's disease where a degeneration of striatal GABA neurons is considered to be a main pathological finding (Chase and Tammiga, 1979). Also, Rinne et al. (1979) report a significant reduction in GABA levels in the cerebral and cerebellar cortices in Parkinsonism. Rinne et al. (1979) have also observed significant correlations between GAD activity and the symptoms of Parkinson's disease.

These deficits in the GABA system may have some importance in designing a pharmacological therapy for this disease. We have seen that in the animal experiments striatal GAD increases in response to the loss of the dopamine neurons. In Parkinson's disease this apparently is not possible because of the concomitant atrophy of the GABA neurons. Thus, just as L-dopa has been used to replace the lost dopamine innervation in the striatum, perhaps a GABA agonist could be of use in replacing the GABA deficit, particularly in the SN where both GAD and GABA receptors are reduced (Lloyd et al., 1977b; Rinne et al., 1978; 1979). In this regard Bartholini et al. (1979) have found in preliminary studies that the GABA agonist and prodrug SL 76003, when combined with L-dopa therapy, prevents L-dopa-induced involuntary movements and increases the dosage of L-dopa that can be given, resulting

in greatly ameliorated Parkinsonian symptoms with the absence of involuntary movements.

Pharmacological manipulations of the nigrostriatal dopamine system have been found to affect the levels of substance P in the striatum and SN (Hong and Costa, 1978; Hong et al., 1978a; Pettibone et al., 1978a; 1978b). The present experiments have shown that the destruction of the nigrostriatal dopamine system with intracerebral 6-OHDA is associated with a marked reduction in substance P levels in the striatum and the SN. This could represent a change in substance P turnover in these areas, or it could be due to the actual destruction of striatonigral substance P neurons by 6-OHDA. Several observations argue against the latter interpretation. In Experiment 1, complete hemitransections at the anterior pole of the GP reduced substance P levels in the EP, GP and SN, but did not affect the levels of substance P in the head of the striatum. This indicates that destruction of substance P axons projecting to the pallidum and SN does not result in the death of the striatal substance P neurons, presumably because they possess many intrinsic collaterals (Experiment 2). Also, the 6-OHDA injection was made into the nigrostriatal pathway at a level at which the descending striatonigral system is separated from the ascending dopamine fibers (Tulloch et al., 1978). In addition, it has been reported that intracisternal injections of 250 µg of 6-OHDA does not affect spinal cord substance P levels (Singer et al., 1979) indicating that 6-OHDA is not in general toxic to substance P neurons. Incidentally, these lesions, had no effect on the met-enkephalin levels in the striatum.

The observations that pharmacological manipulations (Hong and Costa, 1978; Hong et al., 1978a; Pettibone et al., 1978a; 1978b) or lesions of the nigrostriatal dopamine neurons can reduce substance P levels in the striatum and SN suggest that great caution should be exercised in interpreting the

results of lesion studies where a decrease in peptide levels is observed. Although such decreases could indicate the interruption of a peptide pathway, in the absence of other evidence, a secondary change in peptide turnover due to disruption of some unknown system would seem an equally likely explanation.

The decreased levels of substance P in the SN probably represent an increase in the turnover of substance P. A similar decrease has been observed after chronic haloperidol treatment (Hong and Costa, 1978; Hong et al., 1978a). Intranigral substance P increases the firing of the nigrostriatal dopamine neurons (Davies and Dray, 1976; Walker et al., 1976) thereby increasing the release of dopamine in the striatum (Chéramy et al., 1977). These observations suggest that substance P turnover increases in the nigra in response to a decrease in dopamine transmission in the striatum. The decrease in striatal substance P levels seen in the present study may be a manifestation of the prolonged activation of these substance P neurons and may not have been apparent at the shorter survival times used in the chronic haloperidol studies (Hong and Costa, 1978; Hong et al., 1978a). This hypothesis suggests that drugs which increase the effectiveness of substance P transmission, particularly in the SN, may be of value in the therapy for Parkinson's disease.

## EXPERIMENT 6: THE NIGROTECTAL PROJECTION: A BIOCHEMICAL AND ULTRASTRUCTURAL STUDY

As confirmed in Experiment 1, the SN receives both a GABA and a substance P projection from the striatum. In return, the SN provides the striatum with a dense dopaminergic innervation arising from the SNC. The non-dopaminergic SNR cells have also been suggested to send a sparse projection to the striatum (Fibiger et al., 1972) and in addition this area projects massively to the superior colliculus (Faull and Mehler, 1978; Graybiel, 1978; Hopkins and Niessen, 1976; Rinvik et al., 1976) and the thalamus (Carpenter and Peters, 1972; Carpenter et al., 1976; Clavier et al., 1976; Faull and Mehler, 1978; Rinvik, 1975). Lesion studies have shown that the striatonigral pathway is essential for the expression of some types of striatal-mediated behaviour (Lee et al., 1980; Marshall and Ungerstedt, 1971). For example, the rotatory behaviour induced by dopamine agonists in rats with unilateral lesions of the nigrostriatal dopamine cells is dependent upon the integrity of this pathway (Marshall and Ungerstedt, 1971).

It has been suggested that the projection from the SNR to the superior colliculus interacts with the tectospinal system which regulates the neck muscles involved in head orientation (York and Faber, 1977). Thus, this pathway may be the output of the basal ganglia involved in rotational behaviour. In this study, the effects of lesions of the SN on neurochemical parameters in the superior colliculus was examined in an attempt to determine the transmitters in this system. The fine structure of the terminal boutons of this projection was also examined to understand further the synaptic relationships of this projection with the collicular cells.

### METHODS

Male Wistar rats weighing about 300 g were used for all experiments. For electron microscopic analysis six rats received a stereotaxic injection

of 10 μCi of [$^3$H]leucine (New England Nuclear, 80 Ci/mmol) in 0.5 μl saline into the left SNR. The coordinates of the injection were AP + 2.9; ML + 2.1; DV + 3.0, with respect to stereotaxic zero, with the incisor bar 5 mm above the horizontal plane. With these coordinates the needle tract completely avoids the superior colliculus. The injection was 20 min, and the cannula was left in place an additional five min after the injection. The rats were perfused through the heart 24 hr later with 500 ml of 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.6% dextrose in 0.1 M sodium phosphate buffer, pH 7.4. One mm cubes were cut from the deeper layers of the ipsilateral superior colliculus and processed for electron microscopic autoradiography according to the method of Hattori et al. (1973a). The blocks were postfixed in the above solution overnight and then in 1% buffered osmium tetroxide (pH 7.4) for two hours. The tissue was then embeded in epon-araldite mixture and gold sections cut on an LKB microtome. Sections were picked up on formvar-coated copper grids, and Ilford L4 emulsion was applied by the standard loop technique. After one month exposure at 4°C the sections were developed in Microdol X and fixed in Kodak Rapid Fix. Sections were counterstained with uranyl acetate and lead citrate. Grain distribution and area determinations were performed on electron micrographs taken with a Philips 201 electron microscope, and the relative grain density over various cell structures was calculated according to Salpeter and McHenry (1973).

Thirty micron sections through the injection site were cut on a freezing microtome and examined with a light microscopic autoradiographic technique. Slides were dipped in emulsion (Kodak NTB 3), dried and then stored in light-tight boxes containing silica at 4°C for two weeks. The slides were developed in D-19, fixed and counterstained with cresyl violet.

In the biochemical studies, the left SN was injected with five nmoles

of KA in one µl of buffered saline. Three weeks later, the animals were killed by cervical fracture. The superior colliculi were dissected fresh from coronal slices and homogenized in 30 volumes of 0.32 M sucrose. A 75 µl aliquot was removed and added to 75 µl of 50 mM Tris-acetate buffer (pH 7) containing 0.2% Triton X-100. Aliquots of this were used to assay GAD and CAT as described in Experimental 1. The $P_2$ fraction was obtained from the original homogenate by the method of Simon et al. (1976) and was resuspended in 50 volumes of 0.32 M sucrose. Aspartate and glycine uptake were measured in 5 min incubations of 60 µl of this suspension in Krebs-Ringer phosphate buffer with $10^{-6}$M of [U-$^{14}$C]aspartate (New England Nuclear 30 Ci/mol), or [U-$^{14}$C]glycine (New England Nuclear; 68 Ci/mol). After incubation, the samples were rinsed onto Millipore filters and washed with 0.9% saline. The filters were counted in a mixture of one ml water and 9 ml of a solution of 10% naphthalene and 0.4% PPO in dioxane. In control incubations sodium ion was replaced by choline or potassium in the buffer. Protein was determined on both the sucrose homogenates used for uptake and on the samples used for the enzyme assays by the method of Lowry et al. (1951).

## RESULTS

The results of this study have recently been published (Vincent et al., 1978a). In the autoradiographic experiments to be discussed the injection site was confined to SNR (Fig. 15). The distribution of silver grains in the superior colliculus one day after the injection of [$^{3}$H]leucine into the SNR is summarized in Table 10. Myelinated axons were preferentially labeled compared to unmyelinated axons. Boutons forming symmetrical synapses with major dendrites were labeled with a high relative grain density of 2.4, indicating that label transported from the SN was localized in a preferential manner in these structures (Salpeter and McHenry, 1973). The labeled

Figure 15. Schematic representation of the site and extent of the [$^3$H]leucine injections in the substantia nigra pars reticulata (SNR). CC crus cerebri; IP interpeduncular nucleus; LM medial leminiscus; SNC substantia nigra pars compacta.

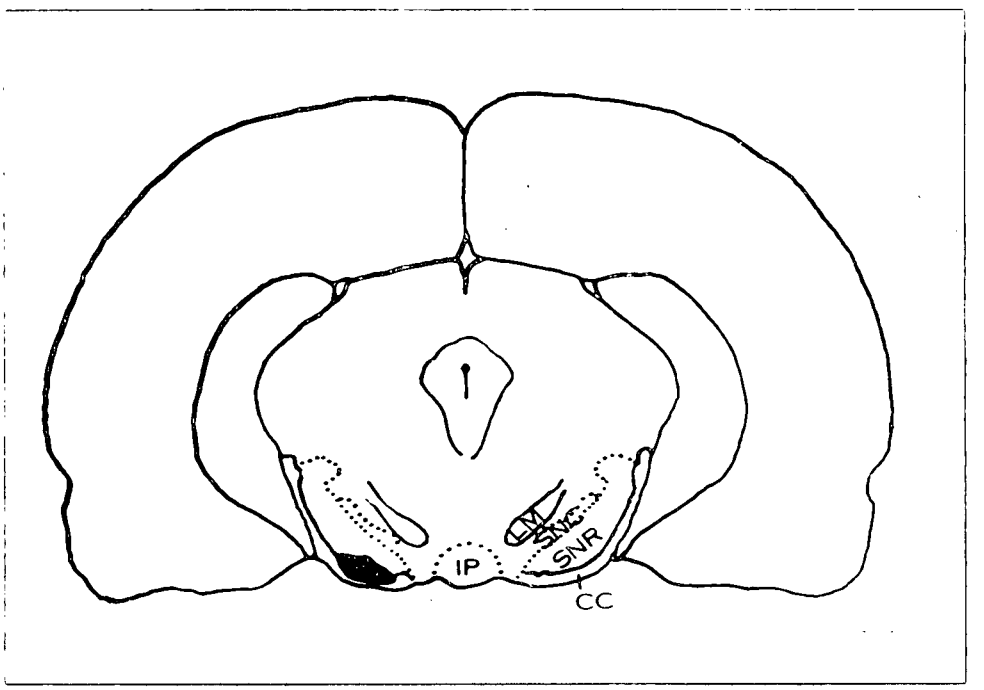

LM
SNC
SNR
IP
CC

Table 10. Distribution of silver grains in the superior colliculus 24 hr after the injection of [$^3$H]leucine into the substantia nigra.

| Structure | Number of grains | Percent grains | Percent area | Relative grain density |
|---|---|---|---|---|
| Bouton | | | | |
| Symmetric | 51 | 15.9 | 6.5 | 2.4* |
| Asymmetric | 5 | 1.4 | 4.4 | 0.32 |
| Preterminal | 8 | 2.5 | 2.6 | 0.96 |
| Axon | | | | |
| Myelinated | 98 | 30.5 | 21.5 | 1.4* |
| Unmyelinated | 40 | 12.4 | 15.3 | 0.81 |
| Soma | 23 | 7.2 | 10.8 | 0.67 |
| Dendrite | 66 | 20.7 | 24.3 | 0.85 |
| Glia | 27 | 8.2 | 12.8 | 0.64 |
| Blood vessel | 4 | 1.2 | 1.8 | 0.67 |
| Total | 322 | 100 | 100 | |

*RGD > 1.

boutons were one to two microns in diameter and contained moderately packed slightly pleomorphic vesicles (Fig. 16).

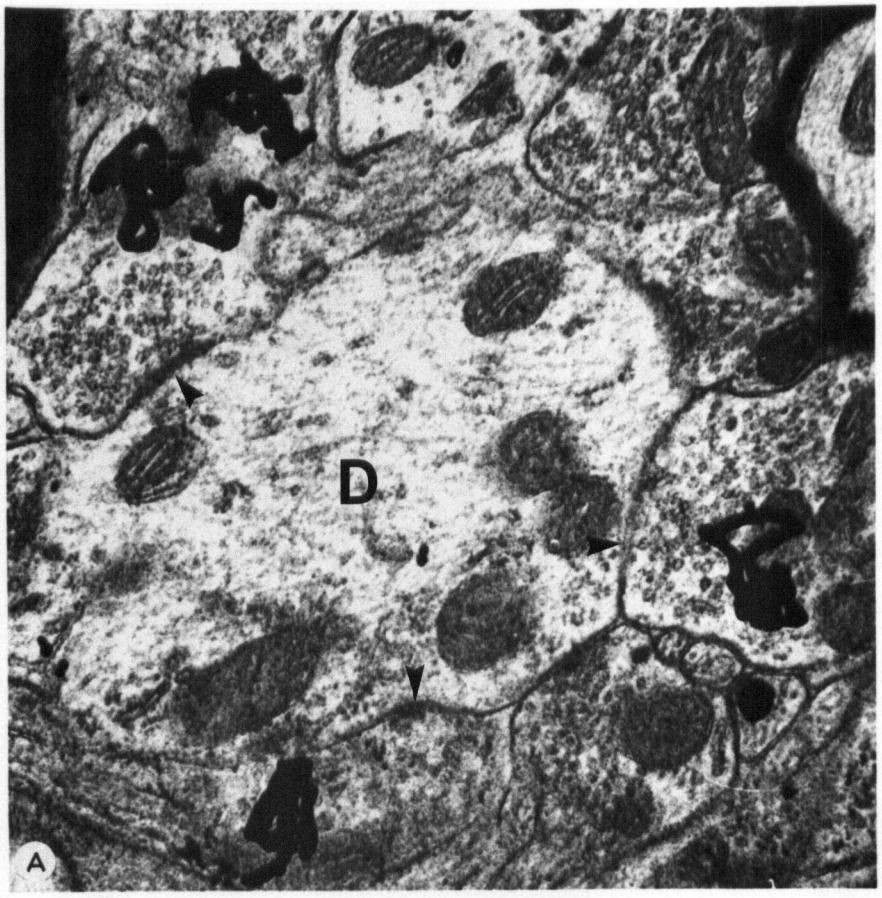
The results of the biochemical studies of the superior colliculus after unilateral KA lesions of the SN are summarized in Table 11. No significant changes were observed in aspartate or glycine uptake or in the activity of CAT in the superior colliculus after lesioning the SN. However, GAD activity showed a significant decreases in the superior colliculus ipsilateral to the lesion when compared to the contralateral control side.

## DISCUSSION

Glutamic acid decarboxylase is the rate limiting enzyme in the synthesis of GABA and is a useful marker for GABA nerve endings. The distribution of GAD correlates well with that of GABA (Fahn, 1975) and GAD activity is concentrated in synaptosomes in areas known to receive GABA afferents (Fonnum and Walberg, 1973). Thus, the significant drop in GAD activity observed in the superior colliculus following the SN lesion suggests that a GABA projection to the tectum was lesioned. It has been shown that intranigral injections of KA similar to those used in the present study result in a substantial reduction in nigral GAD activity (Nagy et al., 1978d). Injections of KA have been suggested to damage only neurons with cell bodies at the site of injection, sparing fibers of passage (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976a). Thus, the decrease in GAD activity observed in the colliculus appears to be due to the loss of nigral GABA neurons which project to the tectum.

The morphological data obtained in the present study confirm earlier reports of a projection from the SNR to the superior colliculus. Furthermore, ultrastructural examination revealed that the terminals labeled after SN injections form symmetrical synapses with the major dendrites of tectal neurons and contain pleomorphic vesicles. This morphology is identical

Figure 16. Examples of labeled boutons in the superior colliculus following injection of [$^3$H]leucine into the substantia nigra pars reticulata. The labeled terminals form symmetrical synaptic contacts (arrow heads) with major dendrites (D) and contain slightly pleomorphic vesicles. Magnification A: X 46,309; B: X 62,842.


D
A

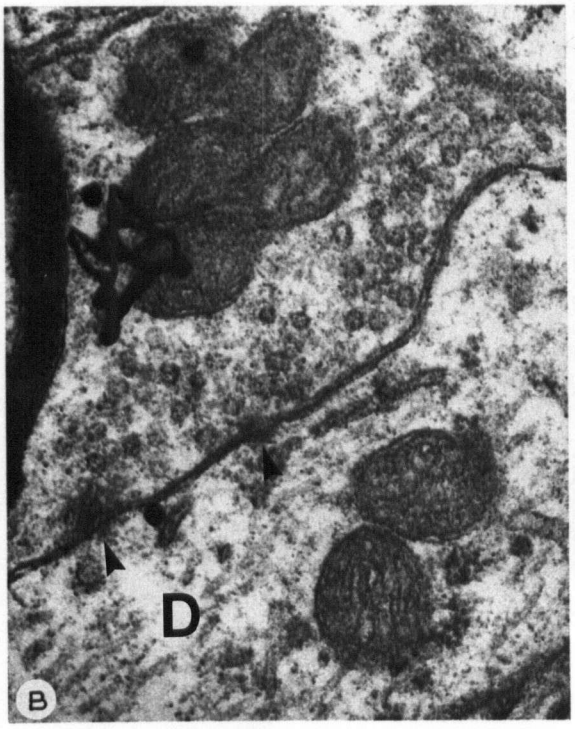

D
B

Table 11. Biochemical changes in the superior colliculus after lesions of the substantia nigra. Uptake expressed as pmol/mg protein/5 min. Enzyme activity as nmol/mg protein/hr.

| | n | Control | Lesion | % of control |
|---|---|---|---|---|
| Glycine uptake | 6 | 209±27 | 223±29 | 107 |
| Aspartate uptake | 6 | 352±40 | 302±30 | 86 |
| Choline acetyltransferase | 12 | 18.2±1.3 | 18.5±1.2 | 102 |
| Glutamate decarboxylase | 8 | 221±5.7 | 132±14 | 60* |

*$p < 0.001$; Student's $t$ test

to that of Purkinje cell terminals in the deep cerebellar nuclei labeled by radioactive GABA transport (McGeer et al., 1975) and with presumed GABA boutons in the SN labeled by uptake of radioactive GABA (Hattori et al., 1973b). They are also similar to those synapses of the cerebellar cortex or SN which stain immunocytochemically for GAD (McLaughlin et al., 1974; Ribak et al., 1976).

Anderson and Yoshida (1977) have recently suggested that some nigrotectal neurons send collaterals to the ventromedial thalamus, and have shown that SN stimulation results in monosynaptic inhibition in the thalamus. Anatomical studies have also found that many nigral neurons project to both the tectum and the ventromedial thalamus (Bentivoglio et al., 1979), and the morphology of the nigrothalamic boutons is identical to that of the nigrotectal terminals (Kultas-Ilinsky et al., 1978). This suggests that the nigrotectal projection may also be inhibitory. Although York and Faber (1977) originally reported that a few tectal units were activated by stimulation in the SN, more recent studies have demonstrated that tectal units are monosynaptically inhibited by SN stimulation (Deniau et al., 1978). This is consistent with the present evidence for a GABA-containing projection from the SNR to the superior colliculus and suggests that the nigrothalamic projection may utilize GABA as well. In fact, evidence has very recently been published supporting this hypothesis (DiChiara et al., 1979; Felter et al., 1979).

## GENERAL DISCUSSION

The striatum has been implicated in both motor behaviour and in more cognitive functions. In the present study the pathways by which the striatum can affect these processes have been examined using biochemical and histological techniques. Evidence has been provided indicating that a parallel pair of striatal efferent systems exists projecting to the GP, EP and SN. One of these systems contains GABA, the other substance P. A met-enkephalin-containing striatopallidal pathway has also been proposed (Experiment 1). Further experiments have suggested a role for GABA in the output pathways of the basal ganglia. The histochemical results obtained with GABA-T support the hypothesis of a pallidohabenular GABA pathway, while the results of Experiment 6 provided the first evidence for a nigrotectal GABA projection. These pathways are summarized in Fig. 17. In the following discussion some speculations on the possible role of these pathways in mediating the functions of the basal ganglia will be offered. As little is known concerning the functions of the striatal projections to the GP and EP, the discussion will concentrate on the striatonigral system and the nigral output pathways.

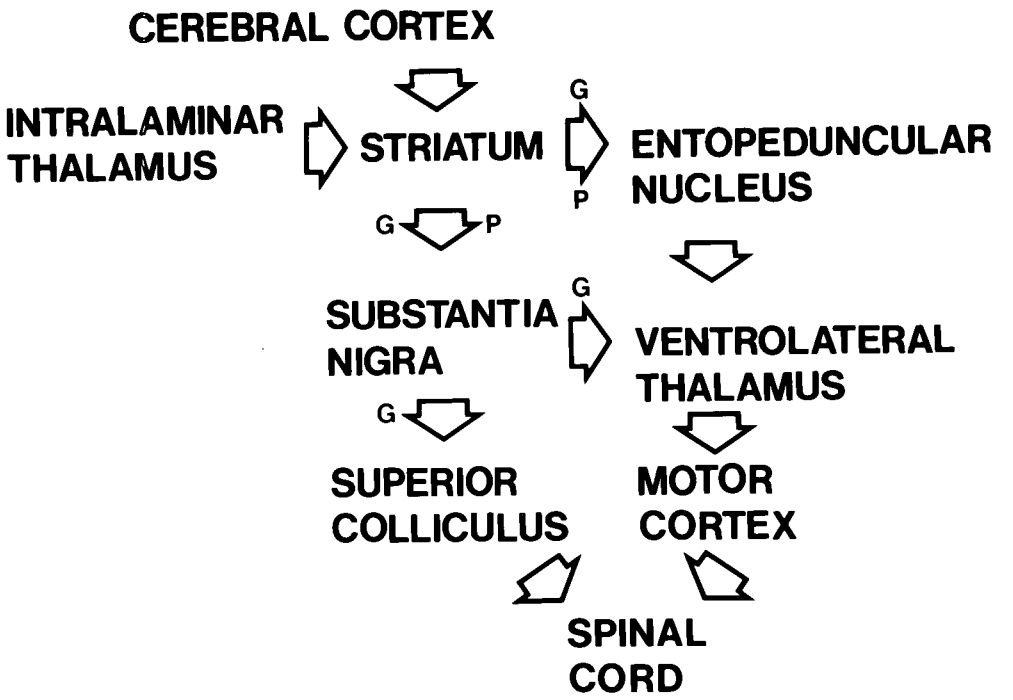
## NIGROSTRIATAL REGULATION

The striatonigral pathway appears to serve two major functions: it provides an output pathway from the striatum and, in addition, it serves as a feedback system regulating the nigrostriatal dopamine system. The regulation of the nigrostriatal dopamine neurons has been a subject of intense debate for almost twenty years. In 1963 Carlsson and Linqvist noted that neuroleptic drugs increased the turnover of dopamine in the striatum and suggested that this was due to a compensatory activation of the monoamine neurons subsequent to dopamine receptor blockade. The observations of Kehr et al. (1972) that the dopamine agonist apomorphine

Figure 17: Summary diagram of the inputs and outputs of the basal ganglia.

G = GABA pathway

P = Substance P pathway


CEREBRAL CORTEX
INTRALAMINAR THALAMUS
STRIATUM
G
P
ENTOPEDUNCULAR NUCLEUS
G
P
SUBSTANTIA NIGRA
G
VENTROLATERAL THALAMUS
G
SUPERIOR COLLICULUS
MOTOR CORTEX
SPINAL CORD

and antagonist haloperidol could influence dopamine metabolism in the striatum even after hemitransections separating the dopamine terminals from their cell bocies led Carlsson (1975) to postulate the existence of "autoreceptors" or dopamine receptors on the dopamine terminals.

Autoreceptors for dopamine have also been postulated to exist in the SN (Aghajanian and Bunney, 1973; Groves et al., 1975). Iontophoretically applied dopamine or apomorphine inhibits the dopamine cells in the nigra in a haloperidol-reversible manner (Aghajanian and Bunney, 1973). In fact the dopamine cells have been found to be much more sensitive than striatal neurons to the action of dopamine agonists (Skirboll et al., 1979). Electrophysiological studies have found that systemic apomorphine inhibits dopamine cell firing even after destruction of the striatal neurons with KA (Baring et al., 1980), suggesting that the SN is the site of action for this drug. This is supported by the observation that intranigral and systemic apomorphine have similar effects on striatal dopamine turnover (Maggi et al., 1977).

The concept of nigral dopamine receptors has also received support from anatomical and biochemical demonstrations of the mechanisms necessary for dopamine transmission in the nigra. Thus, nigral dendrites have been shown to contain dopamine (Björklund and Lindvall, 1975; Felton, 1977) as well as the synthetic enzyme tyrosine hydroxylase (Pickel et al., 1976). Also specialized organelles which accumulate the dopamine analog 5-hydroxydopamine have been observed (Hattori et al., 1979; Mercer et al., 1979; Wilson et al., 1977) and both dendro-dendritic (Hajdu et al., 1973; Wilson et al., 1977) and dendro-axonic (Hattori et al., 1979; Reubi and Sandri, 1979) connections have been reported. In addition, a calcium-dependent dopamine release (Hefti and Lichtensteiger, 1978; Geffen et al., 1976; Korf et al., 1976; Nieoullon et al., 1977; Paden et al., 1976),

dopamine binding sites (Nagy et al., 1978c; Quick et al., 1979) and a dopamine-sensitive adenylate cyclase (Kebabian and Saavadra, 1976; Phillipson and Horn, 1976; Spano et al., 1976; Traficante et al., 1976) have been shown to exist in the SN.

Although the existence of dopamine autoreceptors in the striatum and SN appears to explain the actions of systemic apomorphine on dopamine neuronal activity, some difficulties arise when the actions of other dopaminergic drugs are considered. Amphetamine is thought to release dopamine and to block the neuronal uptake of this amine in both terminal regions (Carlsson et al., 1965; Glowinski et al., 1966) and at the dopamine cell bodies (Groves et al., 1975; Nieoullon et al., 1977; Paden et al., 1976). This is accompanied by a depression of neuronal firing in both the striatum and SN (Bunney and Aghajanian, 1973; Rebec and Groves, 1975). The inhibition of dopamine cell firing is markedly attenuated by lesions of the striatonigral pathway providing support for the hypothesis of Corrodi et al. (1967) that amphetamine acts via a neuronal feedback loop to modulate dopamine cell activity. However, amphetamine also appears to act in the SN to inhibit dopamine cells since at high doses its depressant effect on nigral cells still occurs in the absence of the striatonigral system (Bunney and Aghajanian, 1978). The effect of amphetamine on dopamine turnover in the striatum is also not abolished by the destruction of the striatonigral pathway (Argiolas et al., 1978).

Systemic dopamine antagonists such as haloperidol increase the firing rate of the dopamine neurons of the SN. In contrast to the inhibition of dopamine cell firing by apomorphine and amphetamine which are respectively unaffected or partially attenuated, the actions of haloperidol on dopamine cell firing are completely abolished by interruption of the striatonigral

pathway (Kondo and Iwatsubo, 1980). This indicates that the striatonigral feedback system mediates the actions of neuroleptics on dopamine neuron activity.

Strong support for this hypothesis comes from observations on the kinetic activation of striatal tyrosine hydroxylase. This effect is dependent upon an increase in impulse flow in the nigrostriatal neurons (Roth et al., 1975) and occurs following systemic neuroleptic treatment (Zivkovic et al., 1974). Neuroleptic-induced activation, like the increase in dopamine cell firing is abolished by lesions of the striatonigral pathway (Gale et al., 1978). Also direct application of neuroleptics to the striatum results in activation (Gale et al., 1978) while nigral application does not (Gale and Guidotti, 1976; Gale et al., 1978). Thus, it appears that systemic dopamine antagonists affect the dopamine neurons predominantly via the striatonigral feedback loop. However, it must be kept in mind that haloperidol can block the actions of apomorphine and amphetamine which seem to be independent of this feedback system.

## GABA REGULATION OF THE NIGROSTRIATAL DOPAMINE SYSTEM

The presence of a striatonigral pathway has been known for quite some time, and much evidence indicates that GABA is a transmitter in this system (see Experiment 1). Stimulation of the striatum results in a picrotoxin-reversible inhibition of nigral neurons (Feltz, 1971; Precht and Yoshida, 1971). This suggests that GABA could function in the striatonigral feedback system controlling dopamine cell activity. Support for this comes from the observation that intravenous injections of picrotoxin reverse the amphetamine-induced inhibition of nigral cells, and this effect is dependent upon the striatonigral neurons (Bunney and Aghajanian, 1978). This suggests that amphetamine acts in the striatum to increase the firing rate of striatonigral GABA neurons which in turn inhibit the

dopamine cells of the SN.

Intracerebroventricular (Biswas and Carlsson, 1977a) and intraperitoneal (Biswas and Carlsson, 1977b) injections of GABA have been reported to increase striatal dopamine levels suggesting an inhibition of dopamine cell firing. This has also been observed with the GABA agonist and prodrug SL 76002 (Lloyd et al., 1979) or with muscimol, another GABA agonist (Andén et al., 1979; Lloyd et al., 1979). Elevation of endogenous GABA levels by systemic injection of GABA-T inhibitors such as AOAA also increases striatal dopamine levels (Andén, 1974; Biswas and Carlsson, 1977b) and AOAA reverses the increase in dopamine turnover induced by neuroleptics (Andén, 1974; Lahti and Losey, 1974), while isoniazid or picrotoxin enhance haloperidol-induced activation of striatal tyrosine hydroxylase (Gale et al., 1978). Finally, chronic elevation of brain GABA levels results in dopamine receptor supersensitivity in the striatum, suggesting that the nigrostriatal dopamine cells have been chronically inhibited (Ferkany et al., 1980). These results indicate that measures which increase central GABA function inhibit the firing of nigral dopamine cells.

Although GABA may have some action on dopamine release directly in the striatum (Doble et al., 1980; Giorgieff-Chesselet et al., 1979; Starr, 1978a; Stoof and Mulder, 1977; Stoof et al., 1979) its main influence clearly occurs in the SN. $^{3}$H-GABA receptors appear to occur on the dopamine cells of the SN, since their density decreases following 6-OHDA lesion (Guidotti et al., 1978). Similar decreases in $^{3}$H-GABA binding have been observed in the SN of Parkinsonian patients (Lloyd et al., 1977b; Rinne et al., 1978; 1979) in which the nigrostriatal dopamine cells are known to degenerate. Lesions of the striatonigral pathway increase the density of $^{3}$H-GABA or $^{3}$H-muscimol binding sites (Gale and Iadorola, 1980; Guidotti et al., 1979; Waddington and Cross, 1978a) in

a way suggestive of denervation supersensitivity. The observation of Bunney and Aghajanian (1978) that KA lesions of the striatonigral system greatly increase the responses of SNC cells to iontophoretic GABA further supports the idea that these receptors can display supersensitivity.

Anden and Stock (1973) and Kelly and Moore (1978b) have found that the local application of GABA onto nigral dopamine neurons causes an increase in striatal dopamine levels similar to that seen after systemic GABA agonists. This does not occur after intrastriatal GABA application (Anden and Stock, 1973). These results suggest that nigral GABA receptors are involved in the regulation of dopamine cells. Gale and Guidotti (1976) also point to the role of GABA in the SN with their observation that intranigral muscimol blocks the neuroleptic-induced activation of striatal tyrosine hydroxylase. Bicuculline injections of the SN block this effect indicating that specific GABA receptors are involved.

Walters et al. (1979) have found that muscimol or GABA-T inhibitors also inhibit the haloperidol-induced increase observed when tyrosine hydroxylase activity is measured in vivo. Their observation that n-dipropylacetate (Valproate) is more effective than AOAA is consistent with the observation of Iadoraola and Gale (1979) that Valproate selectively increases GABA levels in the striatonigral terminals while AOAA appears to increase primarily other nigral GABA pools. These observations support the hypothesis put forward in Experiment 3 that the GABA-T present in the striatonigral terminals may be directly involved in controlling nigral GABA transmission.

In summary, the results described above indicate that GABA released from the striatonigral terminals acts to inhibit the firing of the nigrostriatal dopamine neurons. In times of increased dopaminergic

stimulation in the striatum (i.e. after amphetamine or L-dopa) the striatonigral GABA neurons would show an increase in their activity, thereby inhibiting the nigral dopamine cells. Conversely, during a time of decreased dopaminergic activity in the striatum (i.e. after neuroleptics) the activity of the striatonigral GABA neurons decreases, allowing the dopamine cells of the SN to increase their firing rate.

SUBSTANCE P REGULATION OF THE NIGROSTRIATAL DOPAMINE SYSTEM

Substance P is also present in the striatonigral system (Experiment 1) and thus could be invoked as a participant in the feedback control of nigral dopamine cells. Systemic substance P has been reported to increase striatal dopamine turnover (Starr et al., 1978) as have injections of substance P into the lateral ventricle (Magnusson et al., 1976). Intranigral application of substance P has been found to produce a similar increase in striatal dopamine turnover, as evidenced by increased striatal levels of the dopamine metabolites DOPAC and HVA (Waldemier et al., 1978). James and Starr (1979) report that this increase in striatal dopamine turnover is associated with substance P injections of the SNC. When the injection is made into the SNR a decrease in striatal HVA results.

In vivo studies have also shown that intranigral application of substance P increases dopamine release in the ipsilateral caudate nucleus (Chéramy et al., 1977). In contrast, nigral application of antibodies against substance P, which function as substance P antagonists, produces a decrease in striatal dopamine release (Chéramy et al., 1978).

Behavioural experiments also suggest a role for the striatonigral substance P system in the regulation of dopamine neuronal activity. Olpe and Koella (1977) report that unilateral intranigral substance P injections induce contralateral rotation. James and Starr (1979) have found that this rotation following SNC injections is associated with an

increased turnover of dopamine in the ipsilateral striatum, and were able to block this response with haloperidol (James and Starr, 1977). These observations agree with the hypothesis that animals rotate away from the side with increased striatal dopamine function (Ungerstedt et al., 1969).

Injection of substance P either into the lateral ventricle (Katz, 1979) or the SN (Kelley et al., 1979) produces a grooming response which can be blocked by lesions of the dopamine terminals in the striatum with 6-OHDA (Kelley and Iversen, 1978; 1979). Bilateral nigral application results in a strong stereotyped rearing and sniffing with no concurrent enhancement of locomotion (Kelley and Iversen, 1979). This response is also blocked by striatal 6-OHDA lesions (Kelly and Iversen, 1979). These results are consistent with the hypothesis that stereotypy results from an activation of the nigrostriatal dopamine neurons (Kelly et al., 1975) and that such an activation can be produced by substance P.

Intranigral substance P has also been found to produce a drastic retrograde amnesia for a passive avoidance task (Huston and Staubli, 1978). This effect too can be explained by a stimulation of the nigrostriatal dopamine neurons (Fibiger and Phillips, 1976).

Finally, injections of substance P into the mesolimbic A-10 dopamine cell region produce an increased locomotor response which can be blocked by the infusion of neuroleptics into the nucleus accumbens or by 6-OHDA lesions of these neurons (Kelley et al., 1979). Also the locomotor response to systemic amphetamine is potentiated by these substance P injections (Stinus et al., 1978). These results are consistent with the hypothesis that enhanced locomotor activity results from activation of the mesolimbic dopamine neurons (Kelly et al., 1975) and indicate that substance P could play a role in this response.

In summary it appears that the efferents of the striatum can exert

opposite effects on the nigral dopamine neurons. The GABA system provides a negative feedback system inhibiting dopaminergic activity, while the substance P system acts to increase the activity of these neurons. Perhaps through these two systems the firing rate of the dopamine neurons can be controlled with great precision.

## GABA, SUBSTANCE P AND THE OUTPUT OF THE STRIATUM

In addition to their role in the regulation of the nigral dopamine cells, the efferents of the striatum must provide output pathways for the expression of the many functions in which dopamine and the striatum have been implicated. Dyskinetic disorders have been attributed to a hyperdopaminergic activity or to a cholinergic-dopaminergic imbalance in the striatum. GABA involvement in dyskinesic syndromes has also been suggested due to the significant reduction in striatonigral GABA and GAD observed in Huntington's disease (Bird and Iversen, 1974; Enna et al., 1976; McGeer and McGeer, 1976; Perry et al., 1973; Urquhart et al., 1975). A reduction in cerebrospinal fluid levels of GABA has also been found in tardive dyskinesia patients (Neophytides et al., 1978). In animal studies it has been found that decreasing striatal GABA function by the intrastriatal injection of GABA antagonists results in a GABA-reversible dyskinesia (Robin et al., 1979). These results suggest that treatment with drugs which increase GABA function in the striatum could be of benefit in treating dyskinesias in man.

Both muscimol (Chase and Tamminga, 1979) and Valproate (Linnoila el al., 1976) have been found to be of some benefit in tardive dyskinesia. Also, the direct GABA agonist SL 76002 has been found to be of benefit in treating the dyskinesias resulting from L-dopa therapy in Parkinson's disease (Bartholini et al., 1979). It has also been found to be of some value in the early stages of Huntington's disease. However, Valproate has not been found beneficial in Huntington's disease (Lenman et al., 1976;

Shoulson et al., 1976). The poor results obtained with GABA drugs in Huntington's disease, especially in its later stages, could be due to the loss of striatal GABA receptors in this disease (Iversen et al., 1979; Lloyd et al., 1977a). Also, as the usual treatment for Huntington's disease involves neuroleptic drugs, the decrease in GABA receptors produced by these drugs (Trabbuchi et al., 1978) could also tend to render GABA agonists ineffective in this disease.

Akinesia and catalepsy are thought to result from an inhibition of the action of dopamine in the striatum. Thus, the motor deficits produced by haloperidol or reserpine are reversed by L-dopa or apomorphine. It has been reported that AOAA, muscimol and SL 76002 injected systemically potentiate neuroleptic-induced catalepsy (Biggio et al., 1977; Kaariainen, 1976; Keller et al., 1976; Lloyd and Davidson, 1979; Worms and Lloyd, 1978). It was suggested that this was due to a facilitation of the GABA inhibition of the dopamine neurons in the SN (Keller et al., 1976; Lloyd and Davidson, 1979; Matsui and Deguchi, 1977). However, the observation (Costall and Olley, 1971) that lesions of the GP antagonize haloperidol catalepsy indicates that the striatopallidal pathway may be involved in this response. Indeed, it has been found that direct elevation of pallidal GABA levels or the injection of muscimol into the GP potentiates the cataleptic action of haloperidol (Matsui and Kamioka, 1978). Also ethanolamine-o-sulphate (EOS, a GABA-T inhibitor) injections of the GP result in an akinesic state which is not blocked by amphetamine (Pycock et al., 1976). The EOS injection of the GP also blocks the hyperactive response induced in the rat by direct stimulation of the dopamine receptors in the accumbens (Pycock and Horton, 1976). Perhaps this indicates that inhibition of the recently discovered GABA pathway from the accumbens to the GP (Walaas and Fonnum, 1979) mediates the increased locomotor response to amphetamine.

Neuroleptic drugs have been found to increase the turnover of GABA in the GP and the nucleus accumbens (Marco et al., 1976). The increased turnover in the accumbens persists after chronic haloperidol (Marco et al., 1976) suggesting that this change could be a biochemical marker for the mechanisms involved in the symptomatic relief of schizophrenia elicited by these drugs (Costa et al., 1978). In contrast, tolerance develops to the increase in the turnover of GABA in the GP (Marco et al., 1976). This may be a biochemical indicator of the tolerance that occurs to the cataleptogenic properties of neuroleptics (Ezrin-Waters and Seeman, 1977). Also, Moroni et al. (1979) have observed an increased GABA turnover in the GP following either systemic or intrastriatal morphine and suggest that this may also be indicative of opiate catalepsy.

Evidence also exists implying that the elevation of nigral GABA levels does not potentiate neuroleptic catalepsy. Elevation of nigral GABA levels by the local injection of EOS (Dray et al., 1975) or gabaculline, another GABA-T inhibitor induces hyperactivity which is blocked by intranigral picrotoxin (Matsui and Kamioka, 1978). Furthermore, intranigral injection of muscimol produces behavioural stimulation and antagonizes haloperidol-induced catalepsy (Scheel-Krüger et al., 1977). Elevation of nigral GABA levels by local injections of gabaculline also antagonizes haloperidol catalepsy (Matsui and Kamioka, 1978). As discussed below, these behavioural effects probably depend upon the inhibition of efferents from the SNR.

In summary, these results indicate that the systemic elevation of brain GABA function potentiates neuroleptic-induced catalepsy and antagonizes the hyperactive response to dopamine agonists via an action in the GP and not the SN. This suggest that some of the behavioural functions of the nigrostriatal dopamine system might be mediated through an action on the striatopallidal GABA pathway.

Rats with unilateral 6-OHDA lesions of the nigrostriatal dopamine system rotate away from the side with the lesion when challenged with apomorphine (Ungerstedt et al., 1969). This has been attributed to denervation supersensitivity of the dopamine receptors following the lesion. This response has been found to be reduced by electrocoagulation of the caudate nucleus ipsilateral to the 6-OHDA lesion (Marshall and Ungerstedt, 1977) indicating that striatal neurons are involved. As crus cerebri lesions or hemitransections just rostral to the SN also reduce this rotational response, it has been suggested that the striatonigral fibers mediate this behaviour (Marshall and Ungerstedt, 1977). Garcia-Munoz et al. (1977) have found that lesions of the striatonigral pathway results in a marked turning response towards the lesioned side with both apomorphine and amphetamine, in spite of intact dopamine neurons. Similar effects have been found after extensive unilateral ablations of the striatum (Andén et al., 1966) or more recently following KA induced degeneration of striatal cell bodies (Schwarcz et al., 1979). Also, electrical stimulation of the striatum has been found to result in contralateral head turning, and this behaviour is abolished by lesions of the GP or SN on the stimulated side (Lee et al., 1980). These results indicate that some of the motor asymmetries observed after manipulations of striatal dopaminergic function are mediated through the striatonigral pathway.

## OUTPUTS OF THE SUBSTANTIA NIGRA

It is known that the efferents which arise in the head of the striatum synapse preferentially in the SNR on non-dopaminergic neurons (Hattori et al., 1973b; Tulloch et al., 1978). The SNR contains neurons projecting predominantly to the VM-VL thalamus (Beckstead et al., 1979; Carpenter et al., 1976; Carpenter and Peter, 1972; Cole et al., 1964; Faull and Mehler, 1978; Fibiger et al., 1972; Mettler, 1970; Rinvik, 1975) the

the superior colliculus (Afifi and Kaelber, 1965; Beckstead et al., 1979; Graybiel, 1978; Hopkins and Niessen, 1976; Jayaraman et al., 1977; Rinvik et al., 1976) and the nucleus tegmentus pedunculopontis, par compacta (Beckstead et al., 1979). Electrophysiological and anatomical evidence suggests that many SNR neurons project to both the ventrolateral thalamus and the superior colliculus (Anderson and Yoshida, 1977; Bentivoglio et al., 1979). It is therefore of interest that Deniau et al. (1976) have shown that those neurons in the SNR which are inhibited after stimulation of the striatum are also antidromically driven from the ventrolateral thalamus. This indicates that the nigral efferents to the thalamus and tectum may represent major output systems for the striatum.

The behavioural effects seen following the injection of KA into the SN strongly support this suggestion. Thus, unilateral, intranigral administration of KA results in chronic turning away from the lesioned side (DiChiara et al., 1977; Olianas et al., 1978a), while bilateral nigral injections produce chronic stereotyped behaviours (Olianas et al., 1978a). These effects mimic a striatal dopamine receptor stimulation, although they are independent of the nigrostriatal dopamine pathway (Olianas et al., 1978a). Thus, the stereotypy is not antagonized by haloperidol, which also fails to produce catalepsy in these animals (Olianas et al., 1978a). These results suggest that dopamine receptor stimulation in the striatum results via the striatonigral pathway in the inhibition of the SNR output cells. This could be accomplished by either an increase in the firing of the striatonigral GABA cells or by a decrease in the activity of the striatonigral substance P cells.

As discussed above, the behavioural responses to intranigral substance P appear to be dependent upon the ascending dopamine systems. In contrast, intranigral administration of GABA agonists produces an acute syndrome

similar to that seen after KA lesions of the SN. That is, unilateral nigral injections of GABA, muscimol or GABA-T inhibitors results in contralateral turning (Dray et al., 1975; Olianas et al., 1978b; Oberlander et al., 1977; Scheel-Krüger et al., 1977), while that of GABA antagonists produces ipsilateral rotation (Olianas et al., 1978b; Scheel-Kruger et al., 1977). Bilateral injections of picrotoxin results in catalepsy which is resistant to apomorphine administration ((linanas et al., 1978b), while bilateral injections of muscimol produce intense stereotypy that is unaffected by haloperidol (Olianas et al., 1978b; Scheel-Krüger et al., 1977). It can, therefore, be suggested that striatal dopamine receptor stimulation results in increased activity in the striatonigral GABA neurons increasing nigral GABA release and thereby inhibiting the non-dopaminergic SNR output cells. This leads to stereotypy. Conversely, catalepsy in response to striatal dopamine receptor blockade depends upon a reduction in GABA release within the SN, with the consequent activation of the nigral non-dopaminergic efferents.

This hypothesis suggests that neuroleptics should decrease nigral GABA release during catalepsy. However, we have already seen that neuroleptics increase GABA action in the GP (Marco et al., 1976). This implies that the striatopallidal and striatonigral GABA systems are functionally dissociable. This is in agreement with the results found in Experiment 1 that these two systems are anatomically distinct.

What neurotransmitters are contained in the nigral neurons which provide these output pathways from the basal ganglia? Recently, a decrease in nigral GAD has been observed following the destruction of nigral neurons with KA (Nagy et al., 1978d). This has led to the suggestion that the nigra, in addition to receiving a GABA innervation from the striatum, contains a population of GABA neurons (Nagy et al., 1978d). Could GABA be

contained in the nigral output pathways? This possibility was examined in Experiment 6 where a significant decrease in GAD activity was observed in the superior colliculus following KA lesions of the SN. This suggests that GABA is a transmitter in the nigral efferents to the tectum.

Electrophysiological (Anderson and Yoshida, 1977) and anatomical (Bentivoglio et al., 1979) evidence indicates that the nigrotectal neurons send collaterals to the VM-VL thalamus. This implies that the nigrothalamic neurons may also utilize GABA as a transmitter. In fact, Yoshida and Omata (1978) have reported that the monosynaptic inhibition of the ventromedial thalamic neurons in response to nigral stimulation is blocked by picrotoxin. Also, a decrease in GAD activity in the thalamus has recently been found following nigral KA or electrolytic lesions (DiChiara et al., 1979a; Felter et al., 1979).

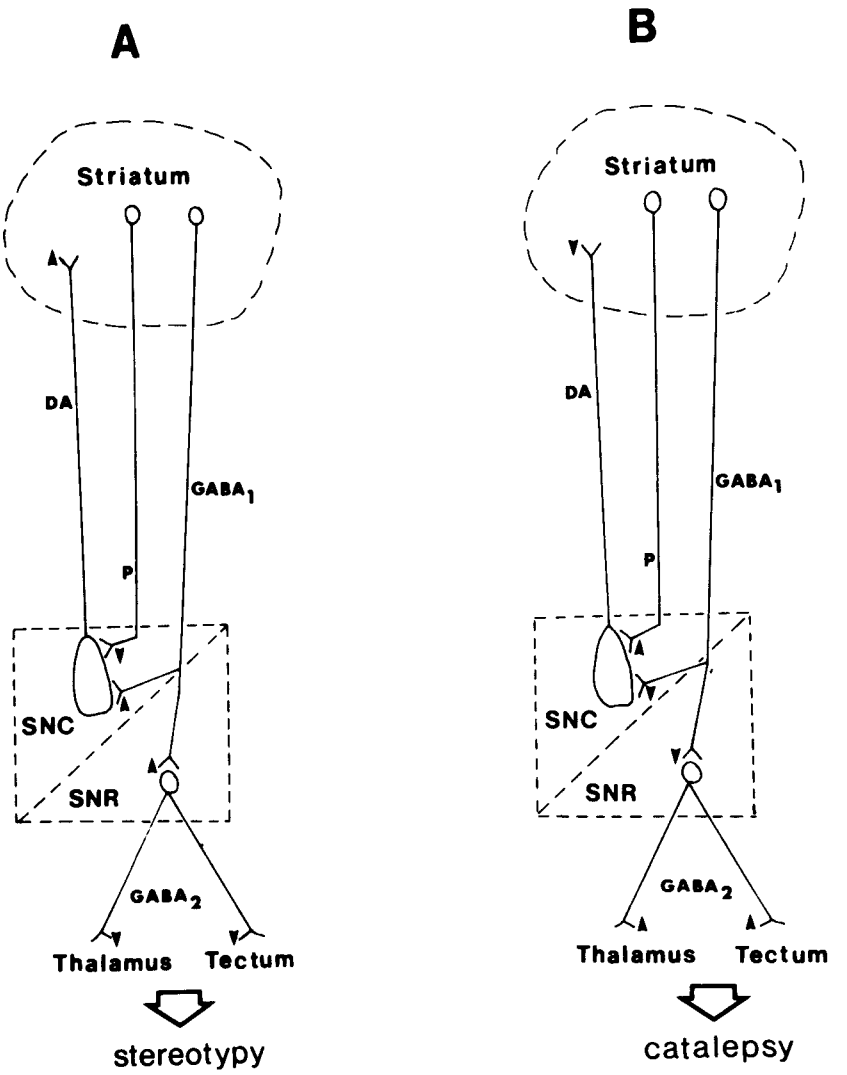
The GABA nigrotectal and nigrothalamic projections are thus likely candidates for the mediation of striatal efferent information. In this scheme, dopamine agonists, by increasing the activity of the striatonigral GABA system and thus inhibiting the nigral GABA efferents in the SNR, would act to release certain thalamic and tectal units from inhibition. Conversely, neuroleptics, by decreasing the activity of the striatonigral GABA cells would inhibit certain tectal and thalamic units via these pathways. These two conditions are summarized in Fig. 18.

DiChiara et al. (1979b) have examined the possibility that the GABA pathway to the ventromedial thalamus may mediate basal ganglia output. These workers have found that the injection of muscimol into the ventromedial thalamus produces a catalepsy which is not blocked by apomorphine. Also, the intrathalamic injection of muscimol had no effect on the stereotypy induced by apomorphine. Thus, these animals display both catalepsy and stereotypy simultaneously. Picrotoxin injections of the

Figure 18. Hypothetical mechanisms of action for haloperidol and amphetamine.

In condition A amphetamine causes an increased release of dopamine (DA) within the striatum. This results in a decrease in the firing of the striatonigral substance P cells (P) and an increase in the activity of the striatonigral GABA fibers ($GABA_1$). Together these systems act to decrease the effect of amphetamine on striatal dopamine release by inhibiting the nigral dopamine neurons. In addition, the increased activity of the striatonigral GABA fibers inhibits the GABA cells of the SNR which project to the thalamus and tectum ($GABA_2$). This inhibition is behaviourally expressed in the form of stereotypy.

In condition B haloperidol acts to inhibit dopamine activity in the striatum. This results in an increase in striatonigral substance P activity and a decrease in striatonigral GABA activity, effects which act to increase the activity of the nigrostriatal dopamine neurons. The decrease in activity of the striatonigral GABA neurons also results in increased activity in the GABA projection neurons of the SNR. This leads to the development of catalepsy.


A
Striatum
DA
GABA1
P
SNC
SNR
GABA2
Thalamus
Tectum
stereotypy
B
Striatum
DA
GABA1
P
SNC
SNR
GABA2
Thalamus
Tectum
catalepsy

ventromedial thalamus produce hyperactivity but no stereotypy, and in addition reverse haloperidol catalepsy. Thus, the catalepsy produced by the blockade of striatal dopamine receptors appears to result from the activation of the nigrothalamic neurons.

It is noteworthy that manipulation of GABA in the ventromedial thalamus affects cataleptic behaviour but not stereotypy. As discussed above, increasing GABA function in the SN results in profound stereotyped behaviours that are independent of the dopamine system (Olianas et al., 1978b; Scheel-Krüger et al., 1977). Thus, we would expect that some nigral efferents would be involved in this process. A clue to which efferents are involved may come from the work of Cools (1979) who has found that the blockade of GABA action in the parafasicular-centromedian complex produces stereotypy which is not blocked by haloperidol. As SNR neurons are known to project to this region (Ahlenius, 1978; Beckstead et al., 1979; Clavier et al., 1976) this observation raises the possibility that inhibition of a GABA projection from SNR to the parafasicular-centromedian complex could be the basis for the stereotypic response to dopamine agonists.

The electrophysiological evidence that the projection from the SNR to the superior colliculus interacts with the tectospinal system which regulates the neck muscles involved in head orientation (York and Faber, 1977) suggests that this pathway may mediate the rotational behaviours associated with unilateral manipulations of the nigrostriatal dopamine system. Support for this concept comes from the observation that lesions of the tectospinal pathway or the superior colliculus markedly attenuate apomorphine-induced rotation in rats with unilateral 6-OHDA lesions (Wirtshafter et al., 1978). However, other workers have questioned this result (Crossman and Sanbrook, 1978; Lee et al., 1980; Reavill et al.,

1979) leaving the role of the nigrotectal system in these responses in doubt.

The deep layers of the superior colliculus appear to be involved in optokinetic nystagmus and in visual tracking (Sprague et al., 1973). Thus, the termination of the nigrotectal pathway within this zone (i.e. the upper part of the intermediate grey layer) could provide the basal ganglia with access to the occulomotor mechanisms. In this regard, lesions of the striatum have been found to result in eye deviations (Chandler and Crosby, 1975) and to impair visual tracking (Bowen, 1969). Mohler and Wurtz (1976) have found that units in the upper part of the intermediate grey layer of the superior colliculus discharge with the shortest time-lead before saccades. These workers have also recorded neural responses in this layer interpreted as signalling "readiness" of the visuomotor system (Mohler and Wurtz, 1976). Thus, in view of the theory that the basal ganglia act in the initiation of movement (Denny-Brown and Yanagisawa, 1976) the role of the nigrotectal pathway in the generation of these "readiness" potentials may be considered.

Finally, the basal ganglia have been suggested to function as an internal feedback system for visually and somatically guided movements (Anderson et al., 1979). The spinal projections arising in the tegmentum and superior colliculus are involved in controlling the integrated movements of the head and body (Kuypers, 1973). Thus, the nigrotectal pathway may provide a route by which the basal ganglia can act in such a feedback regulation of motor behaviour.

## A MODEL OF THE BASAL GANGLIA

It is often useful to formulate models to explain the behaviour of complex systems, even if those models appear premature and oversimplified. Roberts (1976) has employed the concept of disinhibition in discussing the functions of the basal ganglia. In view of the many recently discovered GABA pathways in this system such a view may be appropriate, and it may be useful to expand upon this model. As shown in Fig. 17, the striatum receives input from the entire cerebral cortex and from the intralaminar thalamus, which receives afferents from the reticular formation. Thus, the striatum is in a position to receive information of a general nature regarding the emotional and physical state, the level of consciousness and the degree of alertness of the animal.

The striatum also receives an input from the dopamine cells of the SNC. In a highly simplified scheme this input might be thought of as providing "requests" to the striatum for certain movements or behaviours to occur. The striatum could then compare these requests with the general state of the animal and make a "decision" as to whether to allow the response or not.

This response would then be sent out via the striatal efferents. The striato-nigral pathways could provide a feedback via the GABA and substance P neurons to the dopamine cells informing them that the response either has or has not been made. If the response has been made the striatonigral GABA fibers would increase their firing rate, while the activity of the substance P cells would be decreased. This would prevent further activation of the appropriate dopamine cells until the need for the response again arose. Certain SNR cells would also be inhibited as well. These GABA projection neurons, which may be tonically active, would then decrease their firing rate and thereby disinhibit certain tectal and thalamic units allowing the requested response to occur. This may be thought of as the

disinhibition of a pre-programmed neuronal circuit for patterned postural control, normally held in a tonic inhibition by these GABA neurons.

If other striatal activities are incompatible with the response requested and it is not complied with a different result would ensue. In this case the striatonigral substance P cells would increase their rate and the GABA cells decrease their rate of firing, thereby increasing the activity of the nigral dopamine cells. This would also result in the disinhibition of the nigral projection neurons. They would therefore fire more and act to inhibit the appropriate tectal and thalamic cells thus blocking the response.

Can this scheme be related to what is known regarding the activity of the basal ganglia? In Parkinson's disease, or in an animal following the injection of neuroleptics, the dopaminergic input to the striatum is reduced. In this condition there is difficulty in initiating movements. In the present model the dopaminergic signals necessary for such initiation would be very weak and this would be associated with a decrease in the activity of the striatonigral GABA fibers and an increase in the activity of the substance P system. As a feedback system this response would act to increase the activity of the dopamine neurons. It would also increase the activity of the nigrotectal and nigrothalamic GABA neurons and thereby prevent the appropriate response patterns from being initiated.

In contrast, during times of dopaminergic hyperactivity, i.e. in Huntington's disease or following amphetamine administration, an inappropriate release of action patterns results. The model would suggest that this is due to a relatively greater activity of the striatonigral GABA neurons as compared to the substance P cells. This response would tend to decrease the activity of the nigral dopamine cells in an

attempt to return the striatal dopaminergic activity to normal. It would also result in the inhibition of the nigrotectal and nigrothalamic GABA neurons. This would result in the release of the thalamic and tectal units controlling posture, resulting in inappropriate movements.

Although models such as this may appear overly simplistic in view of the complexity of the basal ganglia, it is useful to attempt to gain a perspective on the function of the basal ganglia as a whole. Such an overview often yields new predictions and insights which may result in further testable hypotheses. Hopefully, as further knowledge of the basic anatomy, physiology and chemistry of the basal ganglia becomes available a better understanding of the functions of this system will be possible.

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# APPENDIX

## THE SUBSTANCE P RADIOIMMUNOASSAY

### (a) Preparation of the Antigen

Substance P is a relatively small molecule and must therefore be couple to a larger carrier molecule to give an antigen large enough for immunization. In the first report of a radioimmunoassay for substance P, Powell et al. (1973) coupled synthetic substance P to bovine γ-globulin with a carbodiimide. In the present study synthetic substance P (Beckman) was coupled to bovine serum albumin following the method developed by Goodfriend et al. (1964). Albumin (3.72 g., bovine RIA grade, Sigma), 1.90 mg synthetic substance P (Beckman) and 100 mg 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluene-sulphonate (Aldrich) were dissolved in this order in 0.5 ml distilled water and agitated gently at room temperature for one hr. The gelatinous mixture was then dialysed against distilled water for 24 hr at 4°C and lyophilized.

### (b) Immunization

The immunization procedure of Vaitukaitis et al. (1971) was used to produce specific antisera to substance P. Our initial attempts to raise antisera to substance P in rabbits met with limited success and so guinea pigs were used in the present studies. For the initial immunization one mg of antigen was dissolved in three ml water and emulsified with three ml Freund's complete adjuvant (Cappel Labs). One ml of this emulsion was injected per guinea pig intradermally in 20-30 wheals on the back. Animals received a booster injection of 100 µg antigen in 200 µl water and 200 µl Freund's incomplete adjuvant (Cappel Labs) every four weeks.

### (c) Screening of Antisera

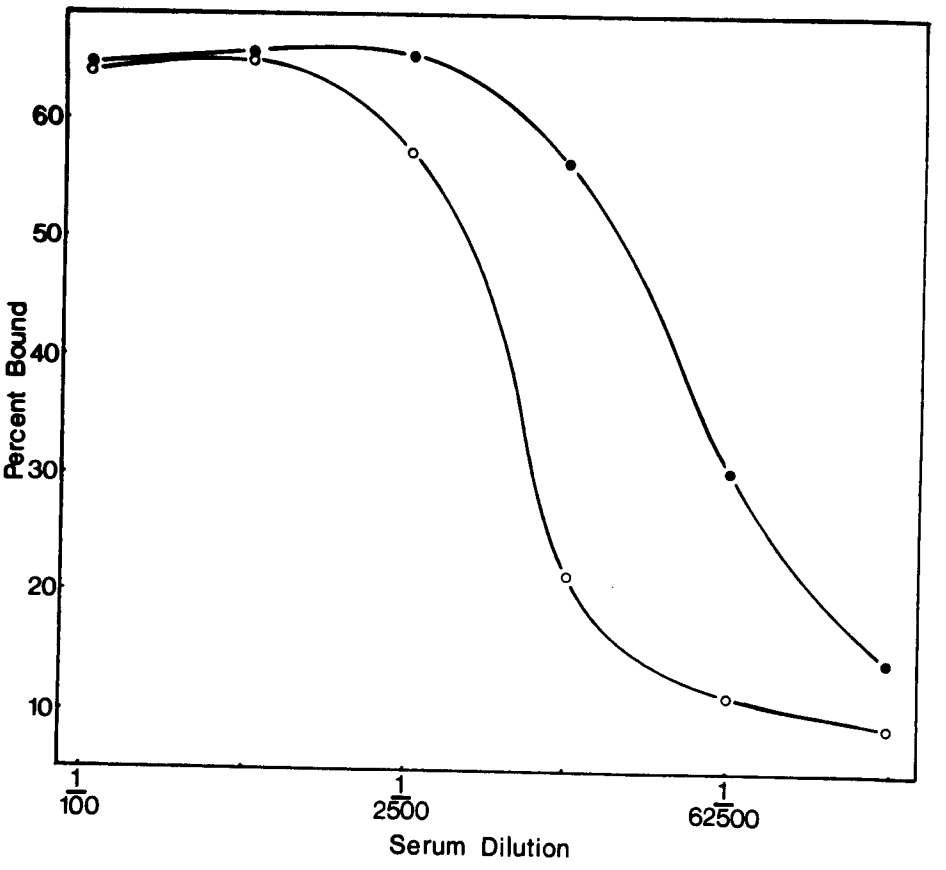
Sera were examined for their ability to bind substance P two weeks after each booster. The guinea pigs were bled by cardiac puncture while

under halothane anesthesia. The blood was allowed to stand at room temperature for one hr, then overnight at 4°C to facilitate clotting. Following centrifugation at 3,000 g for 15 min to remove cells, the sera were kept frozen at -20°C. Each antisera was checked for its ability to bind tracer, and for the ability of a small amount of unlabeled substance P to displace the tracer. A range of antisera dilutions (1:100 to 1:312,500) was incubated with about 5,000 cpm of tracer both with and without an additional one ng unlabeled substance P. With the antisera used in all the experiments reported here, about 30% of the tracer was bound at a sera dilution of 1:62,500 and 65% of this could be displaced by one ng of unlabeled substance P (Fig. I). The cross-reactivity of this antisera with physalamen and eledoisin, two nonmammalian peptides structurally similar to substance P and with the peptides somatostatin, bacitracin, and leu- or met-enkephalin was less than one percent. Although only one immunoreactive fraction was obtained from tissue extracts chromatographed on Sephadex G-25, peptides other than substance P would contribute to this immunoreactivity. Therefore the substance P levels reported in these experiments and the immunohistochemical staining should properly be termed substance P-like immunoreactivity.

(d) Preparation of $^{125}$I-substance P

The chloramine T procedure of Greenwood et al. (1963) can be used to iodinate peptides or proteins possessing a tyrosine residue. Substance P does not contain a tyrosine group, however, one may be substituted for the phenylalanine residue in position eight without seriously affecting the biological or radioimmunological activity of the peptide. Ten μg of (Tyr$^8$)-substance P (Beckman) in 100 μl of 500 mM sodium phosphate buffer pH 7.4 was added to one mCi of Iodine 125 (Amersham IMS-30) in a pyrex tube (#9820). The reaction was started by the addition of 52 μg chloramine T

Figure I. The effect of sera dilution on the amount of displaceable $^{125}$I-substance P bound. closed circles = total bound; open circles = binding in the presence of one ng unlabeled substance P.


Percent Bound
60
50
40
30
20
10
1/100
1/2500
1/62500
Serum Dilution

(Sigma) in 20 µl $H_2O$. After 12 sec the reaction was stopped by the addition of 185 µg sodium metabisulphite in 50 µl $H_2O$.

The iodinated substance P was then purified according to the method of Yalow and Berson (1966). One ml of water was added to the reaction mixture followed by 10 mg of microfine silica (QUSO G32, Philadelphia Quartz Co.). After standing for 10 min at room temperature the suspension was centrifuged at 1,000 g for five min and the supernatant discarded. The pellet was washed five times with one ml of distilled water and the labeled substance P then extracted into one ml of 20% acetone-1% acetic acid, or one ml of the buffer. Both extraction procedures yielded about 0.2 mCi of tracer, and both showed equal apparent binding in the absence of antibody (damaged label) of 5%. Upon storage at 4°C the "damage label" of the tracer stored in buffer increased more than that in acid-acetone but either tracer was usable for at least 10 weeks. The tracer used in the present experiments was always prepared within one month of use.

(e) Sample Preparation

Tissue samples were boiled in about 20 volumes of 1.0 N acetic acid for five min, placed on ice and homogenized, boiled again for five min and centrifuged at 1,000 g for 10 min. The supernatant was then lyophilized. The sample was resuspended in in an appropriate volume of assay buffer immediately before assay and, if necessary, spun to remove sediment When $^{125}$I-substance P was added to the original homogenate in this procedure the recovery was greater than 90%.

(f) The Assay

The assay buffer was 50 mM sodium barbital titrated with acetic acid to pH 8.6. It contained an antibacterial agent (0.001% merthiolate), a protease inhibitor (500 KIU/ml aprotinin, Sigma) and 0.2% bovine serum albumin (RIA grade, Sigma) to minimize the loss of substance P onto the

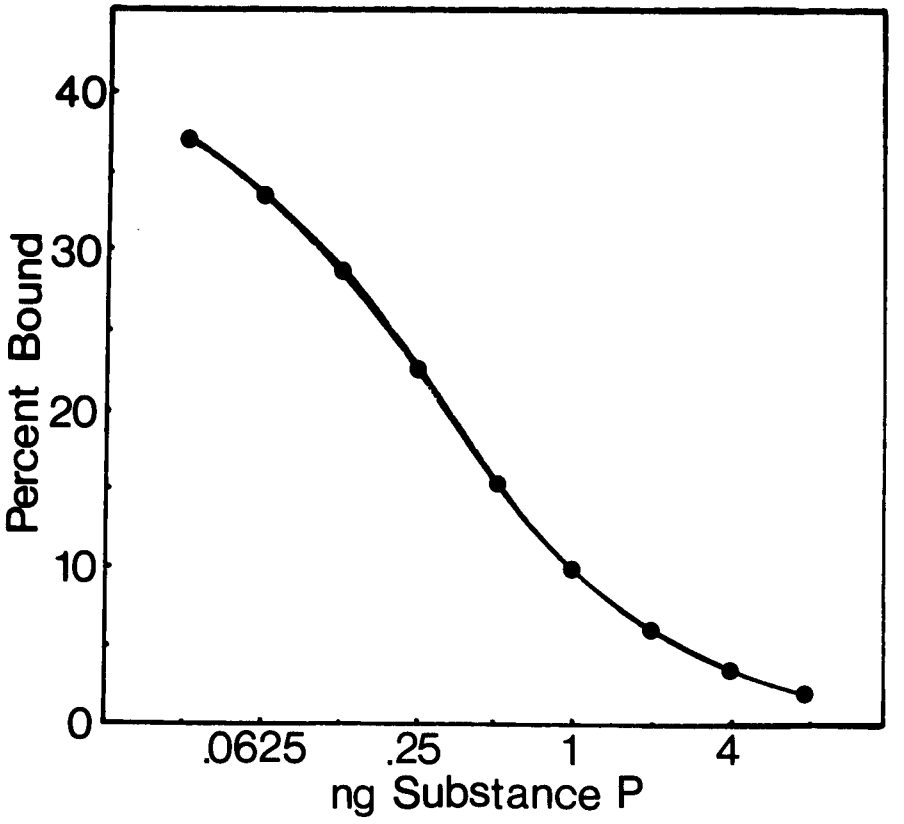
surface of the incubation tubes. The assay was set up on ice in disposable borosilicate tubes (10 x 75 mm). A standard curve was set up in triplicate with concentrations of unlabeled substance P ranging from 31.25 pg to eight ng. Determination of the concentration of substance P in preliminary extractions allowed the samples to be assayed to be taken up in an appropriate volume of buffer such that the concentration of substance P in each lay approximately at the midpoint of the standard curve. Assay of tissues diluted over a three-fold range gave a dose-response curve parallel to the standard curve.

The final volume of each incubation was 0.5 ml. Antisera (diluted in buffer to give a final concentration of 1:60,000) was mixed with buffer to give 300 µl, and sample or standard in 100 µl was then added. After a five hr preincubation at 4°C the labeled $^{125}$I-substance P was added (about 5,000 cpm/tube) in 100 µl and the tubes left at 4°C for 48 hr.

To separate the tracer bound to antisera from free tracer, the coated charcoal technique was used (Herbert et al., 1965). A suspension of 10 g charcoal (Norit A neutral decolorising carbon) and 0.1 g Dextran T-40 (Pharmacia) was prepared in 10 ml of the assay buffer without the aprotinin. After the incubation of the assay, 200 µl of the charcoal-dextran suspension was added to each incubation tube, and, after standing at 4°C for five min the tubes were spun for 10 min at 1,000 g. A 500 µl aliquot of each supernatant was sampled and counted for four min in 10 ml of ACS (Amersham) in a liquid scintillation counter at a relative efficiency of 80% as determined by the channels ratio method using chloroform quenched standards of $^{125}$I-substance P. The concentration of substance P in each sample was the determined from the standard curve (Fig. II).

To practical sensitivity of the assay was defined as 10% displacement of tracer (equivalent to 50 fmoles of substance P per sample). A substance

Figure II. Inhibition of $^{125}$I-substance P binding by unlabeled substance P; the substance P standard curve.


40
30
20
10
0
Percent Bound
.0625
.25
1
4
ng Substance P

P concentration of 186 fmol/assay gave a 50% displacement of bound tracer. In the absence of antisera about five percent of the tracer remained in the supernatant and this blank (damaged label) was subtracted from all samples.

It is interesting to compare the present assay with the original substance P radioimmunoassay reported by Powell et al. (1973). The sera dilution in that report was 1:8,000 compared with 1:60,000 in the present assay. The "damage label" or blank in both assays was five percent, however, the sensitivity of the present assay is an order of magnitude greater than that reported by Powell et al. (1973).

THE METHIONINE-ENKEPHALIN RADIOIMMUNOASSAY

Rabbit antisera to methionine-enkephalin was obtained from immuno Nuclear Corporation, Stillwater, Minnesota. The antisera could be used at a final dilution of 1:1,000 in the assay (Fig. III). Methionine-enkephalin labeled with tritium on the tyrosine residue to a specific activity of 18.77 Ci/mmol (New England Nuclear) was used as tracer at about 5,000 cpm per assay. Unlabeled methionine-enkephalin (Sigma) was used at concentrations from 78 pg to 10 ng for a standard (Fig. IV). In this assay two pmoles of synthetic met-enkephalin caused displacement of 50% of the bound tracer, while the practical limit of sensitivity was 0.5 pmoles, which gave a 10% displacement. This is similar to the sensitivity reported by others (Sullivan et al., 1977; Wesche et al., 1977; Yang et al., 1977).

The tissue preparation and assay conditions were identical to those described above for the substance P assay, except that the incubation was in polypropylene tubes as recommended by Yang et al. (1977). The bound tracer was counted in a liquid scintillation counter at an efficiency of 35% for tritium. The cross-reactivity of the assay with leucine-enkephalin

Figure III. The effect of sera dilution on the amount of displaceable $^{3}H$-methionine enkephalin bound. closed circles = total bound; open circles = binding in the presence of one ng unlabeled methionine enkephalin.

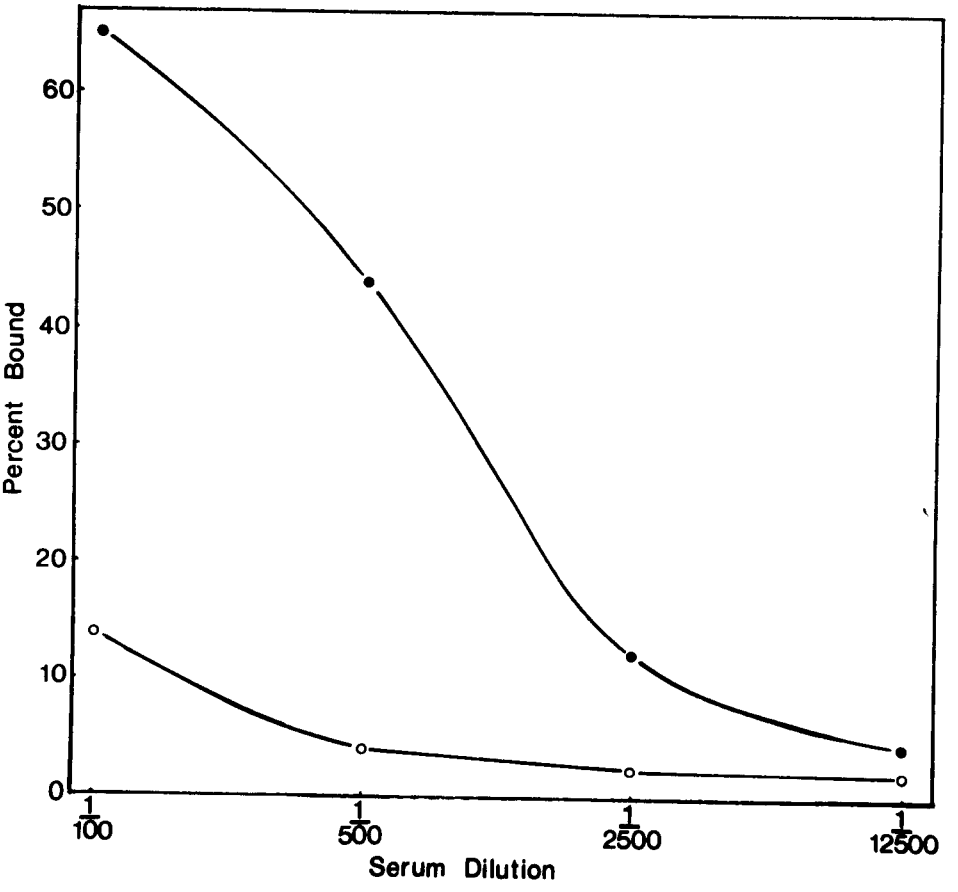
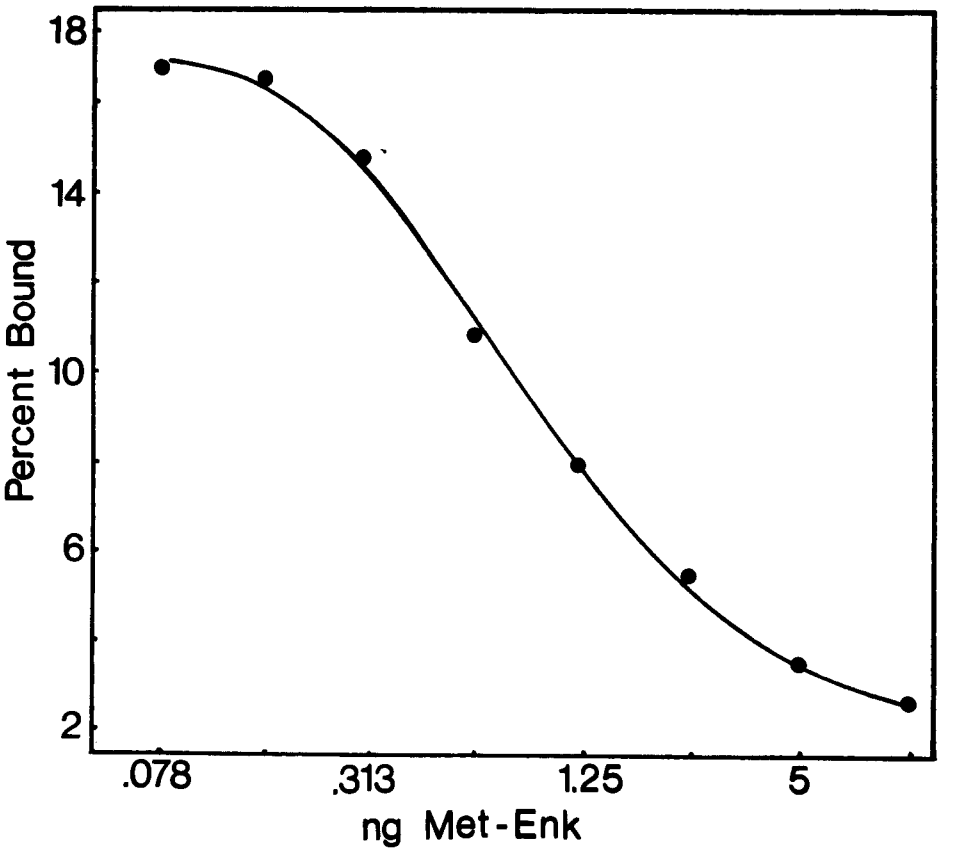

Percent Bound
60
50
40
30
20
10
0
1/100
1/500
1/2500
1/12500
Serum Dilution

Figure IV. Inhibition of $^{3}H$-methionine enkephalin binding by unlabeled methionine enkephalin; the methionine-enkephalin standard curve.


18
14
10
6
2
Percent Bound
.078
.313
1.25
5
ng Met-Enk

(Sigma) was found to be less than two percent.

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