NEUROCHEMICAL CHANGES IN CENTRAL CATECHOLAMINE SYSTEMS FOLLOWING FRONTAL CORTICAL ABLATION IN RATS

by

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Abstract

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Lesions of the frontal cortex have been found to result in a gradually increasing hypersensitivity to the locomotor effects of the catecholamine-releasing drug amphetamine. It was therefore decided to study the effects of frontal lesions on catecholamine systems in the brain.

The frontal cortex of rats was removed by aspiration, and striatal and hypothalamic catecholamines were measured at different times after the lesion. NE levels in the hypothalamus of frontal animals were 24% lower than in sham animals at one week after surgery; this difference was absent two weeks and four weeks after surgery. A significant difference in striatal DA levels was first detected two weeks after surgery, when frontal animals had 26% more striatal DA than sham animals. By four weeks after the lesion, frontal animals had 36% more striatal DA.

DA metabolism was studied by measuring striatal HVA and DOPAC before and after the administration of probenecid. In spite of their higher DA levels, frontal animals were found to have less striatal DOPAC, a metabolite which has been found to correlate with dopaminergic activity. The rate of striatal DA turnover was estimated by measuring HVA and DOPAC levels after the administration of pargyline, a drug which inhibits the formation of these

metabolites. Frontal animals were found to have a lower k_{DA} or fractional rate constant for DA turnover, 0.50 hr^{-1} as compared to 0.81 hr^{-1} for sham animals.

It was hypothesized that the increase in striatal DA levels in frontal animals was due to the collateral sprouting of the intact dopaminergic nigro-striatal system to reinnervate the postsynaptic sites that were denervated by the frontal lesion. It was therefore decided to measure the <u>in vitro</u> uptake of H^3-DA by striatal synaptosomes, hoping that the maximal velocity of uptake would correlate with the number of dopaminergic terminals present. Kinetic analysis of synaptosomal H^3-DA uptake revealed no significant differences in either Vmax or K_T between frontal and sham animals.

It was concluded that the increased levels of striatal DA in frontal animals may contribute to the hypersensitivity that these animals show to the locomotor effects of amphetamine.

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ACh	acetylcholine
AMPT	α-methyl-para-tyrosine
ATP	adenosine triphosphate
CA	catecholamine
COMT	catechol-O-methyl transferase
DA	dopamine
DBH	dopamine-β-hydroxylase
DOPA	dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DOPPA	3,4-dihydroxyphenylpropionic acid
GABA	γ-aminobutyric acid
5-HIAA	5-hydroxyindoleacetic acid
HMPPA	4-hydroxy-3-methoxyphenylpropionic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
HVA	homovanillic acid
MAO	monoamine oxidase
MD	dorsomedial nucleus of the thalamus
MFB	medial forebrain bundle
3-MT	3-methoxytyramine
NE	norepinephrine
6-OH-DA	6-hydroxydopamine

INTRODUCTION

The frontal cortex has been the object of intense investigation with regard to behavioral recovery of function. For example, Glick, Nakamura and Jarvik (1971) have shown that mice with frontal cortical lesions initially show a deficit in passive avoidance learning, but recover over a two to three week period of time, independent of experience. Harrell and Isaac (1969) made deep frontal lesions in rats and found large increases in activity during the first two weeks after surgery; however, three weeks after the operation, activity level in the light had returned to control levels. Kolb and Nonneman (1975) reported that orbital frontal lesions produce aphagia and adipsia in rats, but that eating and drinking behavior resume by the second week after surgery. These investigations demonstrate that experimental brain damage can produce behavioral deficits which change as a function of time after surgery. This suggests that, following brain damage, there is some reorganization of function in the brain secondary to, or in response to the original damage.

Structural changes have been reported to take place in the nervous system secondary to an experimental lesion. For example, transsynaptic atrophy may be noted in cells that were not originally damaged. In this case, the changes that occur following the primary lesion result in increased neuronal damage and could conceivably be responsible for further functional impairment.

Transsynaptic atrophy has been studied extensively in the visual system. After section of the optic tract, cells in the lateral geniculate nucleus show signs of shrinkage and atrophy (Garey et al., 1973; Matthews

et al., 1960; Glees et al., 1966). Cragg (1971) found evidence of fewer terminals in the visual cortex as well as atrophic changes in the lateral geniculate nucleus after optic tract section. Atrophic changes can take place in the opposite direction, that is, in the neuron preceding the injured cell, as well as in the neuron receiving information from the injured cell. Thus, after ablation of the occipital cortex, optic nerve terminals show degenerative changes (Horoupian et al., 1973) as well as retinal ganglion cells (Van Buren, 1963).

There are reports in the literature of regeneration by lesioned central nervous system neurons. Fiber systems containing the biogenic amines can be examined by fluorescent histochemical techniques and have been extensively studied for signs of regeneration. After making electrolytic lesions in rat mesencephalon, Katzman et al. (1971) found that numerous fine, fluorescent catecholamine (CA) fibers developed around the area of the lesion between 7 and 19 days after surgery. These fibers remained after removal of the superior cervical ganglia, indicating that they were of central, not peripheral origin. They were believed to represent regenerative sprouting of CA axons that had been cut by the lesion. This abnormal fluorescence was no longer present 7 weeks after surgery, except for those fibers invading blood vessels. Katzman and his colleagues offer two possible explanations. Either the attempt at regeneration was abortive, or the terminals established synaptic contacts away from the site of the lesion. The histochemical method is not sensitive enough to detect the low concentration of amines in the preterminal part of axons without special treatment. Thus the axons of the regenerated cells would no longer be visible after their growth was completed and contacts had been established.

Björklund and Stenevi (1971) made lesions in the mesencephalon of rats and studied the growth of the cut axons into transplanted smooth muscle. CA fibers were seen to vigorously invade transplants of iris and mitral valve tissue. Fibers containing either norepinephrine (NE) or dopamine (DA) grew into the iris transplants and were found up to six months after surgery. When tissues which normally have little CA innervation were transplanted, such as diaphragm, uterus, and striated muscle, they were rarely invaded by the abundantly sprouting axons around them. The study of Björklund and Stenevi extends the findings of plasticity of central CA neurons and suggests that variables such as type of fiber and target tissue are important.

Björklund et al. (1973) made discrete lesions in rat brain by injecting 5,6-dihydroxytryptamine into the lateral ventricle. This drug causes degeneration of terminals containing 5-hydroxytryptamine (5-HT). This method of lesioning would cause less morphological damage with less necrosis and scar tissue formation than would electrolytic or mechanical lesions. Signs of sprouting of 5-HT axons were detected 10 to 17 days after surgery. Three months after surgery, abundant 5-HT fibers were seen in the diencephalon and medulla resembling normal 5-HT terminals in their morphological characteristics. New fibers were seen a) in areas that weredenervated by the lesion, b) in areas that were not denervated but which normally contain 5-HT fibers and were therefore hyperinnervated, and c) in areas that normally do not contain 5-HT fibers. Apparently the discrete chemical lesion created conditions favorable for reinnervation of areas normally containing 5-HT terminals. However, abnormal growth was also detected. Thus, lesioned axons may be capable of re-establishing appropriate connections under special circumstances, but may also show what appears to

be random and abnormal growth.

Collateral sprouting is a phenomenon similar to regenerative sprouting. This takes place when a system which is not injured sprouts to reinnervate synapses left bare by a system which has been lesioned. Goodman and Horel (1966) found histological evidence of sprouting of optic tract fibers after removing the occipital cortex of rats. Sprouting was found only in specific loci of various optic nuclei and various reasons were discussed to account for the specificity. Convergence of the two fiber systems at the specific locus appears to be necessary but not sufficient. There may be other afferents present that, if they are more numerous, successfully compete for the denervated sites. Furthermore, there may be topographic differences in the synaptic contacts made by different afferents. Cortical and optic afferents may terminate on different neurons or on different parts of the same neuron. Another possibility mentioned was that specificity is determined by the chemical coding of synaptic terminal areas.

Raisman (1969) and Raisman and Field (1973) studied collateral sprouting on the electron microscopic level. They lesioned the fimbria, thus removing the hippocampal input to the septal area. These fibers normally terminate on the dendrites of septal neurons. When animals were killed one to six months after the initial lesion, it was discovered that these denervated sites had been reinnvervated. The number of terminals making multiple contacts was increased, suggesting that the intact terminals in the area had grown to reinnvervate empty synaptic areas. A subsequent lesion of the medial forebrain bundle (MFB) input to the septal area caused many of these multiple contact profiles to disappear, indicating

that the MFB was one source of the new terminals. Many of the new multiple contract profiles remained after MFB lesion, so other fiber systems must have sprouted as well. In a complementary experiment, lesions were made in the MFB. These fibers normally terminate on both the dendrites and somata of septal cells. It was found that fimbrial fibers sprouted to take up the denervated sites, even though they do not normally terminate on cellbodies. The reinnervation appeared to reach a peak approximately one month after the initial lesion.

Lynch et al. (1972) have found evidence of collateral sprouting in another area of the brain. The hippocampus receives projections from the entorhinal cortex and the septal area. The septal projection is thought to be cholinergic. After the entorhinal cortex is lesioned, histochemical examination of the hippocampus reveals the development of an acetylcholinesterase band in the area normally innervated by entorhinal afferents. This band develops over a period of 30 to 40 days and is eliminated by septal lesions. The authors interpret these results as indicating that the cholinergic septal terminals sprout in response to the partial denervation of the hippocampus.

Similarly, Lynch et al. (1973) studied the commissural projection to the hippocampus after entorhinal lesions. Using histological techniques, they found that the commissural projection increased following removal of the entorhinal input. Interneurons containing γ-aminobutyric acid (GABA) may also sprout following entorhinal lesions, since there is a time-dependent increase in glutamate decarboxylase activity in the hippocampus of lesioned animals (Nadler et al., 1974). This series of experiments suggests that there is a lack of specificity regarding the type of terminal that

can reinnervate a structure. Many of the systems that project to a partially deafferented structure can sprout to innervate new sites.

The functional significance of collateral sprouting is not known. Some investigators think it may contribute to recovery of function after brain damage. In most experiments, it is not known whether or not the new connections are functional. Steward, Cotman and Lynch (1974) reported electrophysiological evidence of functional new contacts. After removing the entorhinal input to the dentate gyrus in rats, they found that fibers from the contralateral entorhinal cortex sprouted to innervate the deafferented dentate gyrus. By stimulating the contralateral entorhinal cortex, they were able to record a short latency evoked potential in the previously deafferented dentate gyrus. This indicates that functional synapses were established. In this case the reinnervation may have some significance for recovery of function since the reinnervating fibers are of the same type as the lesioned fibers.

On the other hand, collateral sprouting may increase the disability brought about by the initial lesion by establishing improper or abnormal connections. Thus, when the hippocampal input to the septal nuclei is removed, this area becomes hyperinnervated by fibers from the MFB. In this case, sprouting may be increasing the imbalance in synaptic connections brought about by the lesion. Some investigators suggest that collateral sprouting may interfere with the successful re-establishment of normal contacts by occupying deafferented sites before regeneration is completed (Raisman, 1969).

A third possibility is that new abnormal connections are non-functional and are essentially neutral in their effects.

Denervation supersensitivity is an example of a functional change which takes place in response to nervous system damage and which takes a number of weeks to reach its full extent. This phenomenon is well known in the peripheral nervous system, where its characteristics and time course have been studied.

After denervation of the nictitating membrane, the ensuing supersensitivity of the membrane to NE can be divided into two components (Trendelenburg, 1966). The first component is due to loss of the re-uptake mechanism. Since re-uptake is the major mechanism of CA inactivation, its loss would lead to more transmitter remaining in the synapse and thus a greater effect of a given amount of transmitter. This component is supposed to develop rapidly, being complete by the second postoperative day. However, in a system where re-uptake is not the major means of transmitter inactivation, this component of supersensitivity does not exist. For example, after denervation of skeletal muscle, the first component does not develop since a cholinergic system is involved.

The second component in the development of supersensitivity is equivalent to the decentralization effect. Decentralization refers to the destruction of preganglionic fibers, as opposed to postganglionic fibers. Postganglionic fibers remain intact so that inactivation by re-uptake can still take place. However, there is still a loss of input to the nictitating membrane or other target organ, and a compensatory supersensitivity develops. This component, which is thought to be postsynaptic, develops gradually at least up to four weeks after decentralization (Langer, Draskoczy, and Trendelenburg, 1967). This gradual component is the only one seen after cholinergic denervation since re-uptake is not the major means of ACh inactivation.

Evidence consistent with the occurrence of denervation supersensitivity in the central nervous system has also been reported. For example, Ungerstedt (1971a) injected 6-hydroxydopamine (6-OH-DA) unilaterally into the substantia nigra of rats. This resulted in the unilateral degeneration of the nigro-striatal DA tract and the denervation of the striatum. When apomorphine, a dopaminergic agonist was administered to these animals, they rotated toward the intact side. The direction of the rotation indicated that there was greater activation of the striatum on the lesioned side. This was taken as evidence of the increased sensitivity of the denervated side to agonist agents. Sensitivity continued to increase for a period of at least one month after the operation. Ungerstedt stated that since apomorphine is not thought to be inactivated by re-uptake, the increased response to this drug was probably due to postsynaptic supersensitivity.

These various structural and functional changes that have been found after experimental brain damage must be correlated with neurochemical changes as well. The frontal cortex has been extensively studied with regard to the changes that occur after it is lesioned. Changes in behavior and in drug sensitivity that take place give clues to some aspects of the time-dependent reorganization that the brain undergoes in response to damage.

Behavioral Changes that Occur After Frontal Lesions in Rats

The possible functions of the frontal cortex in rats have been studied using the method of ablation. Examination of the behavioral changes that occur after frontal lesions might suggest which neurochemical systems are affected.

Divac (1971) made frontal cortical lesions in rats and studied the effect on spatial reversal retention. The animals had to choose which compartment of a two-chamber testing apparatus to enter. The correct response consisted of alternating sides from one trial to the next. He found that rats with medial frontal ablations were temporarily impaired on the retention of this task. Another group of animals underwent caudate lesions and were even more impaired. The observation that frontal and caudate lesions can produce similar deficits is not unusual. Since the frontal cortex projects to the caudate nucleus (Knook, 1966), it is easy to understand how their functions might be interrelated. In confirmation of these results, Wikmark et al.(1973) reported that rats with lesions in the anteromedial frontal cortex or the caudate nucleus were impaired on the retention of a spatial delayed alternation task. Again, the animals with lesions in the caudate nucleus were more severely impaired than the frontal animals.

Both the frontal and striatal systems have been implicated in feeding behavior. Lesions of the nigro-striatal system produce severe aphagia and adipsia (Ungerstedt, 1971c;Oltmans and Harvey, 1972). The animals will die if not tube fed, and they remain aphagic and adipsic for varying periods of time depending on the extent of the striatal damage (Ungerstedt, 1971c). Kolb and Nonneman (1975) reported that orbital frontal lesions in rats produced deficits in eating and drinking behavior. The animals, if sustained by forced feeding, gradually resume eating behavior.

The effects of frontal and caudate lesions are not always the same, nor could they be expected to be identical. The two structures differ in their afferent and efferent connections, and in their histology. However, since a projection system connects the two areas, it is logical to suppose

that the activity of one will affect the activity of the other.

Animals with frontal cortical lesions show a difference in sensitivity to amphetamine, a drug which is known to affect catecholamine systems in the brain. Adler (1961) removed the frontal cortex of rats and studied their locomotor response to amphetamine at different times after surgery. He found that they were hypersensitive to the drug. This hypersensitivity increased gradually, asymptoting approximately 4 weeks postoperatively, and was evident at least up to 26 weeks after surgery. Because of this gradual time course, Adler proposed that the hypersensitivity was due to denervation supersensitivity, a phenomenon which is known to develop gradually in response to deafferentation.

Adler's results were confirmed by Glick (1970), and by Iversen, Wilkinson and Simpson (1971). These latter authors reported that frontal rats showed increased stereotyped behavior as well as increased locomotor behavior in response to amphetamine. Stereotyped behavior is usually characterized by repetitive biting, gnawing, licking, sniffing and head movements. It usually emerges at the higher dose levels of amphetamine.

Paradoxically, animals with frontal lesions display hyposensitivity to the effects of amphetamine under some circumstances. Glick (1971) tested frontal rats on a spatial discrimination reversal task two weeks after surgery. The animals were confronted with two levers; the correct one to press for a water reward alternated on succeeding days. Both amphetamine and scopolamine interfere with the performance of this task, but frontal rats were less sensitive to the disrupting effect of amphetamine than sham-operated animals. The two groups did not differ in their sensitivity to scopolamine. In another experiment (Glick, Nakamura and Jarvik, 1971), frontal mice were tested on one-trial passive avoidance learning. Animals were

shocked upon stepping from one compartment to the other of the testing apparatus. When tested the following day, they could show their retention of the procedure by not stepping into the next chamber, or by doing so only after a long latency. If mice with frontal lesions were tested soon after surgery, they showed a deficit in learning. If they were tested three weeks postoperatively, their performance was equal to that of sham animals, even though they had received no previous training. Amphetamine disrupts this task in normal animals, but frontal mice became increasingly hyposensitive to the disrupting effects of the drug as their recovery progressed. When their locomotor activity was studied in response to the drug, frontal mice displayed the typical hypersensitivity. Thus the change in sensitivity depends on the nature of the task. Glick and Marsanico (1974) attribute the contradictory results to the complex dose-response curve obtained with amphetamine, with facilitory effects at low doses and depressant effects at high doses. They believe that frontal animals show increased sensitivity to all actions of amphetamine but that this is more so for the facilitory effects than for the depressant effects.

Dependency of Amphetamine Action on Catecholamine Systems

The finding that frontal animals are hypersensitive to the stimulation of locomotor and stereotyped behavior by amphetamine is not unexpected. There has been a great deal of research on the neurochemical basis for these responses to amphetamine. Randrup and Munkvad (1966) inhibited the synthesis of both NE and DA in rat brain by administering α-methyl-p-tyrosine (AMPT). The subsequent administration of amphetamine failed to

produce the usual hyperactivity and stereotyped behavior. However, if 3,4-dihydroxyphenylalanine (DOPA) was administered so that CA synthesis could be resumed, the usual responses to amphetamine were restored. This study shows that the catecholamines are important for amphetamine-induced behavior, but it does not differentiate between NE and DA. Other investigators have tried to inhibit the synthesis of NE selectively by administering a DA-β-hydroxylase (DBH) inhibitor. Randrup and Scheel-Kruger (1966) gave rats diethyldithiocarbamate to inhibit NE synthesis. They found that the locomotor response to amphetamine was blocked whereas the stereotypic response was not. They concluded that DA mediates the latter effect while NE mediates the former. Svensson (1970) reported that FLA-63, another DBH inhibitor, partially inhibited amphetamine hyperactivity, while H44/68, a tyrosine hydroxylase inhibitor, completely inhibited such activity. The conclusion was that both NE and DA are important for the effect. These results were challenged by Thornburg and Moore (1973) who, unlike the previous investigators, administered the synthesis inhibitors in the diet of mice rather than by intraperitoneal injection. These drugs, when given intraperitoneally, result in irritation and thus are stressful. Under these conditions they reduce spontaneous motor activity as well as amphetamine-induced activity. When Thornburg and Moore administered the DBH inhibitors in the diet, there was no depression of spontaneous motor activity, although NE levels were as low as those found after AMPT. Furthermore, the locomotor stimulant action of amphetamine was unaltered after DBH inhibition. However, when the synthesis of both NE and DA was blocked by AMPT, amphetamine hyperactivity was successfully inhibited. The authors concluded that DA is most important for the locomotor effect. Rolinski and Scheel-Kruger (1973)

reported that very low doses of DA antagonists blocked the locomotor effects of amphetamine, while very high doses of NE antagonists produced only a partial blockade. This also indicates that dopaminergic mechanisms mediate the locomotor effect.

Most authors agree that stereotyped behavior is mediated by a dopaminergic system. When DOPA is administered to rats pretreated with a monoamine oxidase (MAO) inhibitor, they display stereotyped behavior. Scheel-Krüger and Randrup (1967) found that blocking the synthesis of NE but not DA with a DBH inhibitor does not interfere with the production of stereotyped behavior by DOPA.

There is some controversy concerning which area of the brain is the primary site of action for the locomotor and stereotypic actions of amphetamine. Simpson and Iversen (1971) destroyed the dopaminergic substantia nigra of rats electrolytically and found that the normal locomotor response to amphetamine was prevented, while stereotyped behavior was unaltered. Since spontaneous motor activity was unimpaired in lesioned animals, they concluded that motor pathways had not been damaged by the lesion. The results indicate that the locomotor effects of amphetamine are mediated by the nigro-striatal system, whereas the stereotypic effects are not.

In a later experiment, Creese and Iversen (1972) obtained opposite results. Lesions were made in the substantia nigra by the injection of 6-OH-DA. This presumably would result in a more discrete or selective lesion than electrolytic methods. In this experiment, lesioned animals displayed an increased locomotor response to amphetamine, while stereotyped responses were abolished. These results suggest that the nigro-striatal system mediates the stereotypic effects of amphetamine, but not the locomotor effects. Biochemical studies showed that striatal DA had been

reduced by 85-90 per cent. Denervation supersensitivity might account for the increased locomotor response to amphetamine, but this would not explain the lack of stereotyped behavior, unless there is a differential localization of the two functions in the striatum. The authors concluded that the loss of the locomotor response to amphetamine in the previous paper (Simpson and Iversen, 1971) was due to the accidental electrolytic destruction of components other than the DA system. However, this does not explain why the stereotypic response was left intact in one experiment but not in the other. Naylor and Olley (1972) made lesions in the striatum of rats and found that amphetamine-induced stereotypy was either abolished or diminished. Locomotor effects appeared unaltered.

In a later paper, Creese and Iversen (1975) concluded that the nigro-striatal DA system mediates both the locomotor and stereotypic responses to amphetamine. Bilateral injections of 6-OH-DA into the substantia nigra of rats resulted in the reduction of more than 99 per cent of striatal tyrosine hydroxylase activity. Furthermore, both the locomotor and sterotypic responses to amphetamine were abolished. To control for the effects of the NE depletions which also occurred, the investigators made lesions of the dorsal or ventral NE pathways, which had no effect on striatal tyrosine hydroxylase activity. In these cases, the locomotor and stereotypic responses to amphetamine were not blocked. DA depletion must be virtually complete for the locomotor response to amphetamine to be blocked. This may be the reason for the conflicting results in the literature. Electrolytic lesions are neither complete nor discrete. If even 10 to 15 per cent of the striatal DA terminals remain intact, an increased locomotor response to amphetamine occurs (Creese and Iversen, 1972), most likely due to denervation supersensitivity. Thus, the authors conclude that stereo-

typed behavior requires a high degree of striatal DA activity, while locomotor activity requires only a low degree of DA activity.

Chemical implantation studies add support to the data implicating striatal DA in the development of stereotyped behavior. Ernst and Smelick (1966) implanted crystals of DOPA or apomorphine into the striatum of rats. Both of these drugs produced stereotyped behavior. Fog and Pakkenberg (1971) injected DA into the striatum, resulting in stereotyped behavior and hyperactivity. Striatal NE injections had no effect, nor did DA injections into the thalamus or hippocampus. Furthermore, bilateral electrical stimulation of the caudate nucleus produces stereotyped behavior in rats (Zimmerberg and Glick, 1974).

Thus, in spite of some degree of controversy, striatal DA has been strongly implicated in the motor effects produced by amphetamine. Since animals with frontal lesions are hypersensitive to these effects, it seemed reasonable to propose that frontal lesions cause a change in striatal function. The frontal cortex has been found to project to the striatum (Knook, 1966; Leonard, 1969), so that its removal would produce a partial denervation of this area. This denervation might be at least partly responsible for the hypothesized change in striatal activity. It was decided to remove the frontal projection system to the striatum by making frontal cortical lesions and to study the effects of this on the dopaminergic nigro-striatal system.

Since amphetamine releases NE as well as DA, it was decided to examine an area of the brain which has high NE levels, the hypothalamus. The hypothalamus also receives a moderate input from the frontal cortex (Knook, 1966; Leonard, 1969).

Neuroanatomy of the Frontal Cortex

Knook (1966) undertook a detailed study of the connections of the rat forebrain. He found that the rat striatum receives fibers from all areas of the cerebral cortex. These fibers appear to be collaterals of the cortical projection system that runs in the internal capsule before distributing to other brain areas. No evidence could be found for a reverse projection, i.e. fibers running from the striatum to the cortex.

Cortical fibers, especially from the frontal cortex, were found to terminate in the lateral preoptic and lateral hypothalamic areas. A smaller number of fibers were found to terminate in other hypothalamic areas, such as the mammillary bodies, the posterior hypothalamic area, and the supramammillary region.

A large number of cortical fibers were found to leave the internal capsule to project to practically all thalamic nuclei. Still others terminate in the interpeduncular nucleus, the zona incerta, and the corpus Luysi.

Some mesencephalic nuclei receive cortical projections via the pedunculo-mesencephalic tract which is largely of frontal cortical origin, with a considerable parietal component. Areas innervated by this tract include the central grey, the oculomotor nucleus, the red nucleus, the reticular formation, and the substantia nigra. The projection to the substantia nigra appears to be very small.

No cortical projection fibers could be found in the nucleus accumbens or the amygdaloid complex, two areas of the brain which contain dopaminergic terminals.

The thalamic connections of the frontal cortex are considerable. The prefrontal cortex (that is, the cortex of the frontal lobes not including motor cortex) is frequently defined as the projection area of the dorsomedial (MD) nucleus of the thalamus. Leonard (1969) undertook a detailed study of the cortical projections of this nucleus in the rat. By making lesions in the MD nucleus and studying the resulting anterograde degeneration, she found evidence of terminal degeneration in sulcal cortex and medial cortex. Sulcal cortex covers the dorsal lip of the rhinal sulcus and seems to receive input from the medial part of the MD nucleus. This area was not ablated in the present study. Medial cortex would include the medial wall of the frontal lobe; it receives a projection from the lateral part of the MD nucleus. This area, in addition to the dorsal surface of the frontal lobe, was lesioned in the present experiment. No evidence was found of a projection from MD to the dorsal surface of the frontal lobe.

When Leonard lesioned the medial cortex and studied the resulting degeneration pattern, she found that it projected to the striatum, subthalamus, pretectal area, superior colliculus, midbrain, and midline structures such as thalamus and hypothalamus. The medial frontal cortex sends fibers to the lateral MD nucleus of the thalamus as well as receiving fibers from it. The projection from the MD nucleus to the frontal cortex gives off collateral fibers that innervate the striatum and reticular nucleus. Lesions of the frontal cortex did not produce retrograde degeneration in the MD nucleus, indicating that the collateral innervation remains intact.

Lesions of the ventromedial nucleus of the thalamus produced evidence of a diffuse projection to the entire frontal cortex. This nucleus is the homologue of the ventral lateral and ventral anterior nuclei in the primate,

thalamic nuclei which are involved in the control of motor behavior. In addition, diffuse projections exist between ventral areas of the medial cortex and the anterior thalamic nuclei.

The prefrontal cortex of the rat lacks the granular layer that characterizes this area in primates. It also lacks the considerable number of intracortical association fibers characteristic of higher animals. Furthermore, the frontal projections to the temporal and limbic cortical areas found in higher species have not been found in the rat.

Striatal Connections

The striatum receives three main afferent systems: from the cerebral cortex (Knook, 1966), the substantia nigra (Ungerstedt, 1971b), and certain thalamic nuclei (Nauta and Mehler, 1969). The cortical projection is topographical, with the prefrontal cortex projecting to the anteroventral region of the caudate nucleus (Webster, 1965; Leonard, 1969). The dopaminergic innervation of the striatum originates in the zona compacta of the substantia nigra. There is also some evidence of a non-dopaminergic pathway running from the nigra to the striatum (Fibiger et al., 1972). In the thalamus, the intralaminar nuclei and the dorsomedial nucleus project to the striatum (Nauta and Mehler, 1969). The MD nucleus has reciprocal connections with the prefrontal cortex (Leonard, 1969). Collaterals of the same neuron in the MD nucleus may innervate the prefrontal cortex and anterior striatum.

The striatum sends its fibers to the substantia nigra (Bedard and Larochelle, 1973) and to the globus pallidus (Szabo, 1962). Some of the fibers terminating in the globus pallidus may be axon collaterals of fibers innervating the substantia nigra. The pathway running from the striatum to

the substantia nigra may serve as a feedback system modulating nigral activity. Stimulation of caudate cells or the fibers of the striato-nigral pathway results in inhibition of nigral cells.(Yoshida and Precht, 1971; McNair et al., 1972). The globus pallidus is the output part of the basal ganglia. It projects to nuclei of the thalamus which are involved in motor behavior (Nauta, 1966). These nuclei, ventral lateral and ventral anterior, project in turn to premotor cortex. In addition to the thalamus, the globus pallidus projects to the nucleus tegmentalis pedunculopontinus in the caudal mesencephalic tegmentum.

Most of the neurons in the striatum are interneurons, that is, their axons do not leave the striatum (Mensah and Deadwyler, 1974). Less than 5 per cent of striatal neurons are output cells. Many of the striatal interneurons are thought to be cholinergic (McGeer et al., 1971; Butcher and Butcher, 1974).

Catecholamine Pathways in the Central Nervous System

The CA pathways in the brain have been traced using the fluorescence histochemical method developed by Falck and Hillarp (Falck, 1962; Falck et al., 1962). When tissues are treated with paraformaldehyde gas under the proper conditions, the catecholamines present react to form intensely fluorescent isoquinoline derivatives. CA pathways can then be traced under the fluorescence microscope.

Special methods must be used to distinguish between the NA and DA fluorophores. After treatment with HCl, the excitation spectra of the two fluorescent products will differ and they can be identified using microspectrofluorometry.

I. DA Pathway

1. The Nigro-Striatal DA System: This pathway originates in the zona compacta of the substantia nigra. The fibers ascend in the crus cerebri and internal capsule, spread out into the globus pallidus, and innervate the caudate nucleus and putamen. Fibers of this system are also believed to terminate in the central nucleus of the amygdala.

2. The Mesolimbic DA System: The cell bodies of these DA cells lie dorsal to the interpeduncular nucleus. Their fibers ascend just dorsal to the medial forebrain bundle and terminate in the nucleus accumbens, the nucleus interstitialis striae terminalis, and the tuberculum olfactorium.

3. The Tubero-Infundibular DA System: There are DA cell bodies peresent in the hypothalmus. The cells in the nucleus arcuatus innervate the median eminence. Cells that may be entirely intrahypothalamic lie lateral to the periventricular nucleus. A third group which lies dorsolateral to the dorsomedial nucleus may give rise to ascending fibers.

4. Cortical DA Terminals: Hökfelt et al. (1974) reported DA terminals in the frontal, cingular, entorhinal, amygdaloid, and hippocampal areas.

II. NE Pathways

There are two main ascending NE pathways.

1. The Dorsal NE Pathway: This originates in the locus coeruleus, a nucleus made up entirely of NE cell bodies. Fibers originating in this nucleus innervate the cerebral, hippocampal, and cerebellar cortices, as well as lower brain stem nuclei. Collateral fibers from the dorsal pathway innervate the inferior and superior colliculi, the geniculate bodies and, the thalamic nuclei.

2. The Ventral NE Pathway: This pathway receives fibers from many nuclei in the pons and medulla. These fibers innervate the medulla, pons, mesencephalon, diencephalon (including the hypothalamus), and septal area.

There is some degree of overlap in the termination areas of the two pathways. The dorsal as well as the ventral system contributes terminals to the medulla and pons (Ungerstedt, 1971b). Loizou (1969) found evidence of dorsal termination in many hypothalamic nuclei as well. Furthermore, some NE terminals in the cerebral and cerebellar cortices were found to originate from one of the brain stem nuclei which contributes to the ventral system (Andén et al., 1967).

Catecholamine Synthesis and Metabolism

The initial step in CA synthesis, the hydroxylation of tyrosine to DOPA is the rate-limiting step (Levitt et al., 1965). The enzyme catalyzing this reaction, tyrosine hydroxylase, requires tetrahydropteridine, Fe^{++}, and O_2 for maximal activity (Nagatsu, Levitt, and Undenfriend, 1964). The enzyme is specific for L-tyrosine. Similar compounds such as D-tyrosine, DL-m-tyrosine, tyramine, and L-tryptophan will not serve as satisfactory substrates (Nagatsu, Levitt, and Undenfriend, 1964) but phenylalanine will be hydroxylated. Alpha-methyl-p-tyrosine, a competitive inhibitor, is converted to some extent to α-methyl DOPA (Spector, Sjoerdsma, and Undenfriend, 1965; Nagatsu, Levitt, and Undenfriend, 1964). A number of aromatic compounds related to tyrosine and DOPA can inhibit the enzyme. Many tyrosine analogues inhibit by competing with the substrate (Undenfriend, Zaltzman-Nirenberg, and Nagatsu, 1965). Catechol compounds inhibit by

competing for the reduced pteridine cofactor; such inhibition is not reversed by substrate, but by the addition of cofactor (Udenfriend, Zaltzman-Nirenberg, and Nagatsu, 1965). Thus, end-product inhibition may serve to help regulate CA biosynthesis *in vivo*. Indeed, when tissue levels of CA are increased by the administration of a monoamine oxidase inhibitor, the synthesis rate declines (Javoy, Agid, Bouvet, and Glowinski, 1972).

DOPA is decarboxylated to DA by the action of L-aromatic amino acid decarboxylase. As the name implies, it is rather non-specific, catalyzing the decarboxylation of other aromatic L-amino acids, such as phenylalanine, tryptophan, 5-hydroxytryptophan, and histidine in addition to DOPA (Lovenberg, Weissbach, and Udenfriend, 1962). It is specific for the L-isomer. Pyridoxal phosphate is required as a cofactor and is also present as a prosthetic group bound as a Schiff's base (Awapara, Sandman, and Hanly, 1962; Christenson, Dairman, and Udenfriend, 1970).

The conversion of DA to NE by DA-β-hydroxylase may be a regulatory step in the synthesis of this catecholamine (Thierry, Blanc, and Glowinski, 1971). DBH is not very specific; it can hydroxylate other phenylethylamines (Levin and Kaufman, 1961). It requires ascorbic acid and O_2 (Levin, Levenberg, and Kaufman, 1960). The enzyme contains Cu^{++} which is reduced by an electron donor such as ascorbic acid; the reduced form acts in the presence of oxygen to hydroxylate the substrate and is again reoxidized (Friedman and Kaufman, 1965). Many inhibitors of DBH contain thiol groups which bind the Cu^{++}.

There are two major enzymes active in CA catabolism. Catecholamines can be deaminated by the action of monoamine oxidase or O-methylated by catechol-O-methyltransferase (COMT) or both. Monoamine oxidase is associated with mitochondria (Schnaitman, Erwin, and Greenawalt, 1967). The product of

this reaction is an aldehyde which is then either reduced to an alcohol or oxidized to an acid. In the case of DA, the acidic metabolites (dihydroxyphenylacetic acid or DOPAC and homovanillic acid or HVA) predominate in brain, where little of the alcohol is found (Wilk and Zimmerberg, 1973). The acid is formed by the action of aldehyde dehydrogenase (Duncan and Sourkes, 1974). The situation is reversed in NE metabolism in brain, with the glycols (3-methoxy-4-hydroxyphenylglycol and 3,4 dehydroxyphenylglycol) predominating in brain (Rutledge and Jonason, 1967; Mannarino, Kirshner, and Nashold, 1963). Using a sensitive mass fragmentographic procedure, Sjöquist (1975) was able to detect the low levels of 3-methoxy-4-hydroxymandelic acid (VMA) present in brain and cerebrospinal fluid, and confirmed that cerebral NE is metabolized preferentially to the glycol rather than to the acid.

Monoamine oxidase is rather nonspecific. Its inhibition can result in increased tissue levels of NE, DA, and 5-HT (Neff and Costa, 1968; Tozer, Neff, and Brodie, 1966).

Catecholamines which have been released into the synapse can be metabolized by COMT. NE is converted to normetanephrine, and DA is converted to 3-methoxytyramine (3-MT). The methyl donor in this reaction is S-adenosylmethionine; divalent cations are required by the enzyme and sulfhydryl group blocking agents and tropolones will inhibit it (Axelrod and Tomchick, 1958). A wide variety of catechol compounds can be methylated by this enzyme, which transfers the methyl group to the hydroxy group in the 3 position.

ASSESSMENT OF CATECHOLAMINE LEVELS IN STRIATUM AND HYPOTHALAMUS

Methods

Surgery

Male and female Sprague-Dawley rats, approximately 3 months old and weighing approximately 250 g were subjected to frontal cortical ablation by aspiration of tissue. After induction of anesthesia with methohexital (40 mg/kg ip), an incision was made along the midline of the head, and subcutaneous tissue was deflected. Bilateral burr holes about 5mm in diameter were drilled through the skull approximately 0.5mm from the midline and 0.5 mm in front of the coronal suture. The frontal cortex was removed by suction through a 20 gauge needle, and the incision was closed. Burr holes were drilled through the skulls of sham animals, but no tissue was removed.

An additional control group was prepared to see if results were specific to the frontal cortex. In this group, burr holes were placed 0.5 mm anterior and lateral to lambda, and the posterior cortex was removed by suction.

Histology

Histological examination of the brains of a separate group of animals revealed the extent of the lesions. Animals were perfused with 10% formalin. Brains were removed and kept in formalin for a week. Forty μ sections were cut and stained with Luxol blue and cresyl violet.

Frontal lesions extended from the frontal pole to the genu of the corpus callosum. Posterior lesions extended caudally to the occipital pole and rostrally 5 mm anterior to the pole. Photographs of similar lesions

can be seen in the paper of Glick and Greenstein (1973a)

Dissections and Tissure Preparation for Assay

At 3 days, 1 week, 2 weeks, and 4 weeks after surgery, groups of animals were killed, their brains quickly removed, and the striatum and hypothalamus dissected according to the method of Glowinski and Iversen (1966a). A transverse cut was made caudal to the mammillary bodies. Another cut was made at the level of the optic chiasm, marking the anterior limit of the hypothalamus. The hypothalamus was dissected by making a horizontal cut at the level of the anterior commissure. The striatum, having been divided by the cutting of the optic chiasm, lay partially in the most anterior part of the brain, and partially in the section posterior to the chiasm. It was removed from both portions with the lateral ventricle as the medial limit and the corpus callosum as the lateral limit; tissue with a non-striated appearance was discarded.

Each sample was homogenized in 10 ml 0.4N perchloric acid and centrifuged at 27,000 g in the cold for 15 minutes. The supernatants were subjected to analysis of DA in striatal samples and NE in hypothalamic samples by a modification of the method of Weil-Malherbe (1968).

Assays

Reagents for the dopamine assay:

1. 1M phosphate buffer, pH 7.2: Dissolve 13.8g $NaH_2PO_4 \cdot H_2O$ in water. Adjust pH to 7.2 with 5N NaOH and bring volume to 100 ml with water.

2. 2% sodium metaperiodate ($NaIO_4$): Should be freshly prepared.

3. 25% sodium sulfite (Na_2SO_3): Should be freshly prepared.

4. 5N NaOH.

5. NaOH-sodium sulfite solution: Mix 5N NaOH and 25% sodium sulfite in a ratio of 4 volumes to 1 volume immediately before use.

6. 7.2M phosphoric acid: Bring 125 ml of 85% H_3PO_4 to 250 ml with water.

7. Stock DA solution: 100 µg/ml in 0.1N HCL. Stored at 4°C; prepared fresh every 4 weeks.

Reagents for the norepinephrine assay:

1. 0.25% potassium ferricyanide ($K_3Fe(CN)_6$): Prepared fresh every week.

2. mercaptoethanol-sodium sulfite solution: Add 0.25 ml mercaptoethanol to 25 ml of a 20% sodium sulfite solution; should be freshly prepared.

3. NaOH-mercaptoethanol-sodium sulfite solution: Mix equal volumes of the mercaptoethanol-sodium sulfite solution and 5N NaOH immediately before use.

4. 5N NaOH.

5. 10M acetic acid (CH_3COOH)

6. Stock NE solution: 100 µg/ml in 0.1 N HCl. Stored at 4°C, prepared fresh every 4 weeks.

General Principles of the Hydroxyindole Method of CA Analysis: Tissue samples are homogenized in acid and generally kept at an acid pH since catecholamines are unstable at alkaline pH. The catecholamines are then isolated by adsorption onto alumina and elution with acetic acid. In general, alumina (aluminum oxide, Al_2O_3) will adsorb catechol compounds maximally at pH 8 to 8.5 and these compounds can be eluted with acid.

The catecholamines are converted to highly fluorescent hydroxyindoles by a reaction which proceeds in two stages, oxidation and intramolecular rearrangement. They are first oxidized to an adrenochrome-like structure. This molecule undergoes an intramolecular rearrangement in the presence of concentrated alkali to which has been added a reducing agent. The reducing agent is necessary since the fluorophore is unstable and must be protected from further oxidation. In this way, the fluorescent hydroxyindole

derivative is formed.

A faded tissue blank is run by adding the reducing agent after the alkali instead of together with it, thus allowing the unstable fluorophore to be destroyed.

Calculations are based on the fluorescence produced by the oxidation of a known amount of amine which has been added to an aliquot of sample.

Modification of the Fluorometric Method: Initially, brain samples from two animals had to be pooled for this assay. The procedure had to be scaled down so that pooling would not be required. It was necessary to elute the catecholamines from the alumina column in a smaller volume, thus making the eluate more concentrated. The elution pattern was followed with H^3-DA. The purity of the labeled catecholamine was assessed by thin layer chromatography using a butanol: acetic acid: H_2O (25:4:10) system.

Eluting with molarities of acetic acid higher than 0.2M did not result in a more concentrated eluate. Decreasing the amount of alumina in each column from 0.7 to 0.5 g allowed the elution volume to be reduced from 10 ml to 5 ml.

It was next necessary to scale down the oxidation reaction so that it could be performed on smaller volumes. Special microcuvettes were used to accommodate the smaller volumes.

Final Procedure: Alumina (Woelm) was first purified of fines, and fluorescent, alkaline, and metal contaminants by heating in HCl. Two hundred grams of alumina were stirred into 1 liter of 2N HCl. The HCl was then heated to the boiling point for 20 minutes with constant and vigorous stirring. After filtering on a sintered glass funnel, the alumina was washed with 1 liter of hot 2N HCl, and then rinsed with large

amounts of distilled water. The alumina was then transferred to a large beaker and gently mixed with 1 liter of distilled water. The water was poured off after the larger particles had settled, thus removing the fine particles which might interfere with sample flow. The process was repeated until the water was at neutral pH. The alumina was filtered under suction and dried in an oven at 300°C for 2 hours. It was stored in a desiccator in a tightly closed container.

Brain samples were homogenized in 10 ml 0.4N perchloric acid containing 0.5 ml 10% EDTA and centrifuged in the cold at 27,000 g for 15 minutes. The supernatant was stored in the freezer and assayed within 3 days.

For each sample to be run, 0.5 g of the purified alumina was weighed out in a large test tube and suspended in 10 ml of 0.1M ammonium acetate solution which had been adjusted to pH 8.0 by the addition of 1M ammonium hydroxide. To this suspension was added enough IN NaOH to bring the final pH to 8.3 $\pm$ 0.1; the amount of NaOH needed differed for each batch of alumina. The alumina was poured into glass columns (Kontes, Chromaflex, 7 mm ID) in which glass wool was used to plug the constriction. Distilled water was used to wash the alumina remaining in the test tube and to wash down the alumina in the columns. The columns were never allowed to run dry.

The brain samples in perchloric acid were thawed and adjusted to pH 8.4 using 5N, IN, and finally 0.1N NaOH with constant stirring. The pH had to remain constant at 8.4 for at least 3 minutes.

Samples were then poured over the alumina columns. As the meniscus approached the top of the alumina, the columns were washed with 10 ml ice-cold water. Before the columns could run dry, the catecholamines

were eluted with 5 ml 0.2M acetic acid.

The eluates were adjusted to pH 5.9 ± 0.1 with 1N NaOH and brought to a constant volume, usually 7 ml, with water. Three 1 ml aliquots were removed from the eluate. One aliquot was the unknown, one (the standard) had a known amount of amine (50 ng DA or 25 ng NE) added to it, and one served as the tissue blank. The known amount of amine was added in a volume of 0.1 ml; therefore 0.1 ml of water was added to the unknown and the tissue blank. The subsequent reaction differed for DA and NE.

DA reaction: 0.15 ml 1M phosphate buffer, pH 7.2 was added to all tubes. The unknown and standard were oxidized with 0.05 ml 2% sodium metaperiodate. After 1 minute, the oxidation was interrupted by the addition of 0.15 ml of the NaOH-sodium sulfite solution. Four minutes later, 0.15 ml 7.2 M phosphoric acid was added. The sequence of additions was changed for the tissue blank. The blank was oxidized with 0.05 ml 2% sodium metaperiodate. After 1 minute, 0.12 ml 5N NaOH was added. After another 10 minutes the procedure was completed by adding 0.15 ml 7.2M phosphoric acid and 0.03 ml 25% sodium sulfite.

The tubes were then covered with parafilm and put in a 50°C water bath for 45 minutes. After cooling to room temperature, fluorescence spectra were recorded using a Hitachi spectrofluorometer (MPF-2A) at the following settings: activation wavelength 340 nm; activation slit 12 nm; emission wavelength 382 nm; emission slit 12 nm.

NE reaction: The unknown and standard were oxidized with 0.05 ml 0.25% potassium ferricyanide. Five minutes later, 0.025 ml of the NaOH-mercaptoethanol-sodium sulfite solution was used to interrupt the oxidation. After another 4 minutes 0.15 ml 10M acetic acid was added. Again, the sequence of additions differed for the tissue blank. Five minutes after the

blank had been oxidized with 0.05 ml 0.25% potassium ferricyanide, 0.125 ml 5N NaOH was added. After waiting 10 minutes, the reaction was completed by the addition of 0.125 ml of the mercaptoethanol-sodium sulfite solution and 0.15 ml 10M acetic acid. Fluorescence spectra were recorded at the following settings: activation wavelength 400 nm; activation slit 12 nm; emission wavelength 472 nm; emission slit 10 nm.

Calculations: The fluorescence of the unknown is subtracted from that of the internal standard to arrive at a figure for a known amount of amine. Similarly, the blank reading is subtracted from that of the unknown. A proportion is set up using these figures.

$$\frac{\text{internal standard (ng)}}{\text{internal standard — unknown (fluorescence units)}} = \frac{X\ \text{(ng)}}{\text{unknown — blank (fluorescence units)}}$$

The figure arrived at for the unknown is multiplied by a correction factor to account for the dilutions that were made after the sample was applied on the alumina column. The final value is expressed in terms of μg DA/g striatum or μg NE/g hypothalamus.

Many identical samples were run with added NE or DA to test recovery and reproducibility. The NE assay gave 77% recovery with a variability of ±6% (SEM). The recovery of DA was 75% with a variability of ± 5% (SEM).

RESULTS

Changes in Hypothalamic NE Following Lesions

Table 1 shows the hypothalamic NE levels of frontal, posterior, and sham animals at various times after surgery. Sham animals were compared to frontal and posterior animals by analysis of variance. If this test indicated significance, the differences were explored further using students' t-test. Analysis of variance comparing frontal and sham animals over time revealed significant differences between the two groups ($p < 0.05$) and among the various time periods ($p < 0.01$). T-tests revealed that NE levels in the hypothalamus of frontal animals, while not different from sham values at 3 days after surgery, showed a 24% decrease at 1 week ($p < 0.05$). This difference was absent at 2 weeks and 4 weeks after surgery, that is, the NE levels returned to control values. Changes in NE levels in posterior animals seemed to parallel those in frontal animals, but did not attain statistical significance at any time point.

Interestingly, the operation itself appeared to have some nonspecific effect on NE levels since sham values were found to be significantly lowered at 3 days as compared to 4 weeks after surgery ($p < 0.025$).

TABLE 1

HYPOTHALAMIC NE LEVELS AFTER SURGERY

Time After Surgery	Sham	Frontal	Posterior
3 days	1.9 ± 0.1 (N=13)	1.8 ± 0.1 (N=9)	1.8 ± 0.2 (N=5)
1 week	2.1 ± 0.2 (N=9)	1.6 ± 0.1 (N=9)*	1.7 ± 0.3 (N=8)
2 weeks	2.2 ± 0.2 (N=6)	2.2 ± 0.3 (N=5)	2.5 ± 0.5 (N=4)
4 weeks	2.2 ± 0.1 (N=16)**	2.1 ± 0.1 (N=16)	2.3 ± 0.1 (N=9)

*$p < 0.05$ as compared to 1 week sham animals.
**$p < 0.025$ as compared to 3 day sham animals.
Values are in μg/g ± SEM

Changes in Striatal DA Following Lesions

Striatal DA levels after surgery are shown in Table 2. When frontal and sham animals were compared using analysis of variance, significant differences were revealed between groups and over time. No such differences were found between posterior and sham animals using this method of analysis.

T-tests revealed that at 3 days and 1 week following surgery, DA levels in the striatum of frontal animals were not significantly different from sham values. However, at 2 weeks after surgery, frontal animals were found to have 26% more striatal DA than sham animals ($p < 0.025$). By 4 weeks after the lesion, there was 36% more DA in the striatum of frontal animals ($p < 0.001$).

Again, results after posterior lesions seemed to parallel those after frontal lesions. They were much more variable and were not statistically different from sham animals.

In order to judge the permanence of the effects of the frontal lesion, striatal DA levels were assayed 3 months after lesioning. Frontal animals still had significantly higher DA levels ($p < 0.025$). However control or sham levels of DA were higher than previously found ($p < 0.005$). Although this could represent a true developmental increase, it is most likely due to unknown and uncontrolable factors since the 3 month group was assessed 2 years after the original series of experiments.

TABLE 2

STRIATAL DA LEVELS AFTER SURGERY

Time After Surgery	Sham	Frontal	Posterior
3 days	5.9 ± 0.4 (N=11)	6.6 ± 0.3 (N=14)	5.8 ± 0.7 (N=4)
1 week	5.4 ± 0.6 (N=7)	5.0 ± 0.5 (N=7)	4.9 ± 0.4 (N=10)
2 weeks	5.3 ± 0.3 (N=10)	6.7 ± 0.5 (N=10)*	6.3 ± 0.1 (N=6)
4 weeks	5.6 ± 0.2 (N=5)	7.6 ± 0.3 (N=9)**	6.4 ± 0.6 (N=6)
3 months	6.9 ± 0.4 (N=7)	8.4 ± 0.4 (N=6)*	

* $p < 0.025$

** $p < 0.001$

Values are in ug/g ± SEM

DISCUSSION

Frontal lesions were found to cause a temporary lowering of hypothalamic NE levels. However, there was some evidence that the sham operation had an effect on NE levels, although a lesser one than frontal cortical ablation. NE levels were lower in the sham group at three days than at later times after surgery.

Thus, the stress of the operation may have had some nonspecific effect resulting in a temporary lowering of NE levels, an effect which was increased by frontal lesions. An alternate explanation would be that the sham operation, in which burr holes were drilled in the skull and bone flaps removed, caused some minimal frontal cortical damage. This is probably so, since, when the brains of sham animals were examined three days after surgery, some degree of swelling was almost always observed.

In removing the frontal cortex, there was loss of a moderate projection to the hypothalamus. This projection is not thought to be noradrenergic. However, frontal lesions do remove the terminals of noradrenergic fibers originating in the locus coeruleus (Ungerstedt, 1971b). Instead of a lowering of NE levels in lower brain regions, we might expect a temporary increase due to the amine accumulation that takes place proximal to the lesion after an axon is transected (Dahlström and Fuxe, 1965). Similarly, if there are axon collaterals of the system which terminate at lower levels, these might be expected to show an increase due to the re-channeling of the flow of NE storage granules from the transected fibers to the intact collaterals (Olson and Fuxe, 1971). However, such increases were not found after frontal lesions.

A related experiment was conducted by Robinson et al. (1975). They occluded the middle cerebral artery of rats, producing a lesion of the lateral part of the fronto-parietal cortex. They observed decreases in CA levels in both the cortex and the brain stem. This could be attributed according to the authors, to the release of catecholamines locally after the interruption of pathways originating in the brain stem. Alternatively, the injury to cortical terminals could cause a shift in synthesis from the production of catecholamines to the production of materials for axonal sprouting. This could account for temporary decreases in CA levels. However, most of the decreases that were found were permanent. Only in the cortex, where NE levels were decreased at 5 days after the operation, did these levels return to control values by 20 and 40 days after surgery. The hypotheses presented could help to explain the temporary decrease in hypothalamic NE levels found in the present experiment.

The changes found in the striatal DA system after frontal lesions were much more interesting. The results confirm a report by Nielson (1966) who ablated the frontal pole of rats and found an increase in DA, NE, and 5-HT in the caudate nucleus two to three weeks after surgery. The increase in CA levels was disputed by Iversen (1971). She made frontal cortical ablations in rats and found no increase in forebrain DA or NE three weeks postoperatively. It is quite likely that her results are negative because the tissue assayed was not limited to the striatum. This would tend to dilute the concentration of DA and make it much more difficult to detect significant differences. The control level of DA found by Iversen (0.69 ug/g) is far lower than that commonly reported in the literature for rat striatum. Nielson (1966) reported a value of 4.66 μg/g while Valzelli and Garattini (1968) quote a figure of 5.25 ug/g, much closer to the levels

found in the present experiment.

Results after posterior lesions appeared to be similar to those after frontal lesions but more variable and not as great; analysis of variance revealed no significant differences between sham and posterior animals. Both the frontal cortex and the posterior cortex project to the striatum. It would thus be logical to expect posterior lesions to affect the striatum as well as frontal lesions. However, the terminations of the frontal and posterior projections within the striatum are not the same. The cortex projects to the striatum topographically (Webster, 1961). Anterior cortical areas project to the anterior part of the striatum, while posterior cortex projects to caudal striatum. There is a similar topographical organization in the mediolateral plane.

Although the difference in DA levels between frontal and sham animals is not significant at three days after the operation, there is some suggestion that there is a change brought about by the operation at this time point. As in the results of the NE experiment, the sham operation may have had some slight effect on DA levels, and for this reason, the difference between frontals and shams did not reach statistical significance. Indeed, the three-day DA levels in the sham group is slightly higher than those found at other time points. In the frontal group, the three-day DA levels are higher than all sham values and there is a significant difference between the three-day value and the one week value, which is closer to sham levels. There may be a short-term increase in DA levels after frontal ablation, and the mechanism for this increase would be expected to be different from that underlying the long-term increase. The permanent increase appears only after two weeks.

It is not unusual for the changes that take place after brain damage to have a complex time-course, reflecting multiple processes. This is evident in Ungerstedt's data (1971a) on the effects of unilateral nigro-striatal lesions. An imbalance in nigro-striatal dopaminergic function between the two hemispheres results in postural asymmetry and rotation away from the side of increased activity. If the nigro-striatal DA system on one side of the brain is lesioned by the unilateral injection of 6-OH-DA, the animal assumes a postural asymmetry toward the side of the lesion immediately upon waking up from the operation. This might be expected, since it reflects greater activity of the intact DA system. However, between 24 and 34 hours after the lesion, the animal has a tendency to rotate towards the intact side. This tendency most likely reflects the release of DA from degenerating terminals and can be intensified by administering an MAO inhibitor. After the amine has disappeared from the degenerating axons, the animal may show a chronic deviation toward the lesioned side, since dopaminergic activity is obviously greater in the intact side. However, the development of denervation supersensitivity on the lesioned side serves to decrease the imbalance between the sides. At this point, the administration of a DA receptor stimulator, such as apomorphine, will force the animal to rotate toward the intact side, demonstrating that the denervated striatum is more sensitive to the drug.

Thus, a system that is degenerating may actually show increased activity due to release of amine stores. This phenomenon is well-known in the periphery, where the response of the nictitating membrane of the cat after denervation is also complex (Langer, 1966). Immediately after denervation, the membrane relaxes. However, a "denervation contraction" begins about 18

to 30 hours after the operation and lasts for approximately 10 to 14 hours, reflecting the release of NE as the postganglionic fibers degenerate. Thus, the changes after lesioning may not be unidirectional, but may reflect several different mechanisms, resulting in swings between decreased and increased activity until a stable end state is reached.

The time-course of amine depletion and the resulting functional manifestations of amine release will differ in different systems and will be affected by the length of the degenerating axon and where along its length the lesion is made. In the system examined by Langer (1966), the NE level of the nictitating membrane is normal 24 hours after denervation, but is depleted 36 hours after lesion (Kirpekar et al., 1962). The interval of amine release coincides very well with the time-course of the degeneration contraction. Ungerstedt (1971a) found that DA depletion was complete 48 hours after a lesion of the nigro-striatal system with 6-OH-DA. This fits fairly well with the time course of the behavioral results. However, Faull and Laverty (1969) found a more extended time-course for DA depletion in the striatum after placing electrolytic lesions in the substantia nigra of rats. They found decreased levels by three days after surgery, with levels continuing to fall up to ten days after surgery. Thus the time-course of changes found after brain damage will differ according to the experimental conditions. If lesions are made in the cell bodies of neurons and changes are noted in their terminals, the time-course will be longer than that following a more proximal lesion. The time-course of changes in the present experiment would be expected to be long since the cell bodies of neurons of the fronto-striatal pathway were removed and the effects of this were studied in a structure innervated by the terminals of these neurons.

Some of the changes noted soon after brain damage may be nonspecific and temporary in nature. Decreased activity can result from the nonspecific shock that accompanies brain damage (Glassman, 1971) and decreases in activity are frequently associated with the build-up of amine levels (Roth et al., 1973). Glassman (1971) reported that a cortical lesion causes surrounding non-lesioned tissue to show temporary deficits in function. He destroyed part of the somatosensory cortex of cats and recorded evoked potentials from other parts of this area. He found an initial loss of responsiveness in these unlesioned areas followed by slow recovery over about a month. He postulates that the recovery of excitability might be due to such factors as the decrease in edema and in toxic substances, glial proliferation, or the re-establishment of blood supply to the area.

Irwin, Criswell and Kakolewski (1973) recorded nonlocalized prolonged slow potential brain waves and episodes of cortical spreading depression during a five day recovery period from brain surgery in rats. There was also a decrease in multiple nerve cell activity and in motor behavior. Thus, early changes detected after brain surgery may be nonspecific and temporary.

There are not many reports in the literature of increases in amine levels in response to nervous system lesions. Examining these reports may help us to formulate hypotheses concerning the mechanism responsible for the increase in the present study.

After an axon is transected, histochemical examination of the tissue reveals an accumulation of transmitter proximal to the lesion (Dahlström and Fuxe, 1965; Katzman et al., 1971). This phenomenon has been used to help trace aminergic pathways in the central nervous system, since axonal amine levels are normally too low to be detected by histofluorescent techniques. The transected fibers appear swollen and distorted and show intense fluorescence due to amine accumulations. The build-up takes place very rapidly, reaching

a peak 24 hours after the lesion. The levels may remain high for about 12 days, and then gradually decline (Dahlström and Fuxe, 1965). This process cannot be responsible for the increases in DA seen in the present study, since the nigro-striatal system itself was not transected.

A related phenomenon is that of collateral accumulation. If the axon of a neuron has more than one branch, transecting one branch may result in increased amine levels in collateral fibers. For example, neurons in the locus coeruleus innervate both the cerebral and cerebellar cortices. When the rostral projection to the cerebral cortex is cut, the NE nerve terminals of the cerebellar cortex show an increase in fluorescence seven days after the lesion (Olson and Fuxe, 1971). The authors suggest that branches of the same cell terminate in the cortex and cerebellum. When the flow of amine-containing storage granules is blocked rostrally by transection, the flow of the granules is directed into axon collaterals to the cerebellum.

Moore et al. (1971) reported a personal communication from Ungerstedt on the time-course of increased amine levels due to collateral accumulation. According to Ungerstedt, the increase reaches a maximum within a few days after surgery and gradually subsides over a period of several weeks. Similarly, Pickel et al. (1973) reported that the CA accumulations present in the intact collaterals of severed axons at five days after surgery had disappeared by 15 days after surgery. Since it appears too rapidly and is not permanent, the phenomenon observed by Pickel et al (1973) does not explain the permanent increase in DA levels found in the present experiment.

Pickel et al. (1974) found evidence for the sprouting of intact collaterals of lesioned axons. She made partial lesions of the superior cerebellar peduncle, thus injuring NE axons originating in the locus coeruleus

and terminating in the cerebellum. Thirty days later, using radioautographic methods, she found an increase in the noradrenergic innervation of the hippocampus, an innervation which also originates in the locus coeruleus. Presumably the hippocampal afferents sprouted in response to injury of collaterals innervating the cerebellum. Sprouting is not detectable until around two weeks after surgery (Pickel et al., 1973; Moore et al., 1971). It will be considered in more detail later on.

There is some possibility that collaterals of the nigro-striatal system were injured when the frontal cortex was ablated. DA-containing nerve terminals have been found in the cerebral cortex (Hökfelt et al., 1974; Thierry and Glowinski, 1973). Hökfelt et al. (1974) found histochemical evidence of DA terminals in the frontal, cingular, entorhinal and amygdaloid areas, as well as parts of the hippocampal formation. They saw no evidence of DA cell bodies, concluding that the DA terminals have an extracortical origin.

Lindvall et al. (1974) tried to discover the origin of the cortical DA terminals by lesioning DA-containing cell groups in the mesencephalon and determining whether this removed the DA fluorescence in the cortex. Their data indicate that the DA nucleus which surrounds the anterior part of the interpeduncular nucleus (A10 in the terminology of Dahlström and Fuxe, 1964) projects to the frontal cortex, while the DA innervation of the anterior cingulate gyrus has its origin in the lateral part of the substantia nigra. The A10 cell group also projects to the nucleus accumbens and the olfactory tubercle; the DA system arising in the substantia nigra also projects to the striatum. It is quite possible that collaterals from the same neuron innervate more than one structure. Furthermore, lesions of the medial frontal cortex as performed in the present study infringed upon the anterior part of

the cingulum. The lesion extended posteriorly to the anterior part of the corpus callosum, and the cingulate gyrus curves around the callosum. If collaterals of dopaminergic nigral neurons were injured in the operation, it could be expected to affect other areas innervated by the same system by the processes already discussed.

The concentration of DA in the cortex is low (approximately 0.1 to 0.2 µg/g, Thierry et al., 1973). The question arises as to whether such a low concentration could account for the magnitude of the changes seen in the striatum. However, collateral damage could affect the activity of the entire neuron. Injury to a collateral can result in decreased turnover of CA throughout the neuron as a result of a "functional lesion." This phenomenon was described by Ungerstedt (1974). After cortical lesions destroying noradrenergic fibers arising from the locus coeruleus, increased amine levels are noted in the collateral fibers innervating the cerebellum. In spite of increased NE levels, the cerebellar Purkinje cells surrounded by these terminals increase their activity. They are thus responding as though there were a decrease in noradrenergic transmission, or as though they were denervated. Thus, the increase in amine levels may be due to decreased turnover and metabolism. The authors suggest that lesioning one collateral of a neuron results in a "functional lesion" of the entire collateral network of that neuron. The duration of this decreased activity was not determined.

Increases in amine levels following lesions can be due to increased synthesis as well as decreased metabolism. The increase in striatal DA levels following lesions of the nigro-striatal system has been studied in depth by Roth, Walters, and Aghajanian (1973). If the impulse flow in

dopaminergic neurons is blocked either by lesioning the nigro-striatal pathway or by injecting into this pathway a local anesthetic, an increase in striatal DA levels results (Walters et al., 1973; Stock et al., 1973; Agid et al., 1974). This can be partially explained by the decreased metabolism of DA since DOPAC levels fall as well as the rate of DA disappearance after the administration of α-methyl-p-tyrosine (Walters et al., 1973; Roth et al., 1973). However, although the activity of the dopaminergic system is sharply inhibited, the rate of DA synthesis is increased. This has been shown by demonstrating an increased incorporation of labeled tyrosine into DA, and an increase in DOPA levels after DOPA decarboxylase inhibition (Agid et al., 1974; Kehr et al., 1972). There is much data to indicate that tyrosine hydroxylase activity is modulated by the degree of DA receptor activity in the synaptic area. DA receptor stimulating and blocking agents have produced effects which support this theory. The administration of apomorphine, a DA receptor stimulant, will result in a decrease in DA synthesis (Kehr et al., 1972). A DA receptor blocking agent such as haloperidol or chlorpromazine will cause an increase in DA synthesis (Kehr et al., 1972; Nybäck and Sedvall, 1971). To show that these effects are receptor-mediated and not dependent upon firing rate, the nigro-striatal pathway was lesioned (Kehr et al., 1972). The increase in tyrosine hydroxylation usually found after such a lesion was antagonized by the administration of a receptor stimulating agent. When impulse flow is interrupted, there will be less DA released into the synaptic area and less DA receptor activation. This condition serves to activate tyrosine hydroxylase and to increase DA synthesis. Thus there is a compensatory mechanism to counteract decreased dopaminergic activity.

Once the new steady-state level of DA has been established after a lesion, the rate of DA synthesis and the levels of DOPAC return to normal,

while DA levels remain high (Roth et al., 1973; Walters et al., 1973). The authors propose that either the feedback mechanism of end-product inhibition is relatively insensitive under these conditions, or that somehow the accumulated DA is not able to influence the rate-limiting step. They believe that the newly synthesized DA is particulate-bound and therefore does not have access to tyrosine hydroxylase. Furthermore, the administration of α-methyl-p-tyrosine after the new DA levels have been reached has no effect on the increased levels (Walters et al., 1973), suggesting that the increased levels of amine are in some way protected from release and metabolism.

Stimulation of the nigro-striatal system, both electically and by the administration of drugs such as haloperidol and chlorpromazine which cause increased firing rate, also results in increased DA synthesis. However, in this case, there is an increase in the levels of DOPAC (Bunney et al., 1973). This is in contrast to the increase in synthesis and decrease in metabolism that take place after the interruption of impulse flow by lesioning. Thus, the nigro-striatal system appears unique since it responds to both an increase and decrease in activity with an increase in synthesis. After nigro-striatal lesions the DA levels must eventually decline as the system degenerates, so the ultimate time-course of this phenomenon is not yet known. If frontal lesions result in injury or shock to the nigro-striatal system as discussed above, this process could contribute to changes in striatal DA levels that occur soon after surgery. However this process would not account for an increase that did not become evident until two weeks after surgery.

Denervation supersensitivity is a phenomenon that takes an extended period of time to fully develop. Although loss of the re-uptake inactivation mechanism and loss of metabolizing enzymes may play a role, supersensitivity is thought to involve a post-synaptic adaptation similar to that following denervation of skeletal muscle (Axelsson and Thesleff, 1959). When muscle is denervated, there gradually develops an enlargement of the chemosensitive area, until the entire surface of the muscle fiber is as responsive to ACh as the end plate region. This is thought to be due to the development of ACh receptors along the surface of the muscle fiber, whereas the receptors are normally confined to the end plate region (Berg et al., 1972; Kimura and Kimura, 1973).

Ungerstedt (1971a) studied the supersensitivity that develops following nigro-striatal lesions. When the DA innervation of the striatum is removed, it becomes more sensitive to dopaminergic agents. A frontal lesion also results in a partial denervation of the striatum and this might result in the development of a denervation supersensitivity. This may be why animals with frontal lesions display a hypersensitivity to catecholaminergic drugs. However, although the transmitter involved in the fronto-striatal projection is unknown, it is not thought to be a catecholamine. This nonspecificity could be accounted for by proposing that the frontal and striatal systems synapse close to each other on the same cells in the striatum. Specificity may depend on the juxtaposition of synaptic terminations.

Denervation supersensitivity which develops peripherally can show some degree of nonspecificity (Sharpless, 1964). When the adrenergic innervation of the nictitating membrane is removed, the membrane becomes more sensitive to the actions of ACh and 5-HT as well as NE. Section of the sympathetic fibers to the salivary gland results in increased sensitivity to both adrenergic

and cholinergic agents; section of the parasympathetic innervation brings about the same result.

Glick and Greenstein (1973b) have published some data suggesting that a unilateral frontal ablation results in a denervation supersensitivity of the ipsilateral striatum to the effects of amphetamine. Amphetamine will cause animals with a unilateral frontal ablation to rotate towards the intact side, indicating that there is greater dopaminergic activity in the denervated striatum. Furthermore, it takes two weeks for this rotational behavior to emerge. This can be explained by postulating that the partially denervated striatum becomes increasingly more sensitive to dopaminergic agents, even though the system that was removed is not dopaminergic. If frontal lesions made the partially denervated striatum hypersensitive to its dopaminergic innervation, it could result in decreased turnover of the DA system by feedback mechanisms. This in turn might bring about an increase in DA levels as supersensitivity progressed.

The time-course of the development of denervation supersensitivity must be considered and related to the results in the present experiment. Although supersensitivity takes several weeks to reach its maximum, it becomes evident shortly after surgery. When the nictitating membrane of the cat is decentralized, the re-uptake mechanism and metabolizing enzymes are left intact. Under these conditions, postsynaptic supersensitivity develops gradually for at least four weeks (Langer, Draskóczy, and Trendelenburg, 1967). Ungerstedt (1971a) first noted supersensitivity to apomorphine between one and two days after lesioning the nigro-striatal system with 6-OH-DA. It increased gradually for up to 121 days after surgery. If a slowly increasing supersensitivity to DA resulted in a decrease in DA turnover with a consequent use in DA levels, it might become evident before a two week delay.

Another possibility that must be considered is that the synapses denervated by the frontal lesion are reinnervated by collateral sprouts from the nigro-striatal system. This would result in a gradual increase in striatal DA levels. Collateral sprouting of an adrenergic system was demonstrated by Moore, Björklund, and Stenevi (1971). These authors lesioned the hippocampus in rats, thus removing the hippocampal projection to the septal nuclei. The septal area also receives a noradrenergic input via the medial forebrain bundle. Histochemical examination revealed little or no change in the noradrenergic innervation three or eight days postoperatively. However, by 15 days after lesioning, there was a clear increase in the number of catecholamine terminals in the denervated septal area. The increase was even more marked in the brains of animals examined 30, 60 and 100 days after surgery. Thus, the increase appears to be permanent, at least up to 100 days. Biochemical studies also showed that NE levels increased with time after the lesion. The time-course of this phenomenon agrees very well with the increase in DA levels found in the present experiment.

A dopaminergic system was examined for collateral sprouting by Moore, Björklund, and Stenevi (1974). By ablating the olfactory bulb, they removed a primary projection to the olfactory tubercle. The olfactory tubercle is known to receive a considerable DA innervation. Histochemical examination suggested an increase in the DA innervation, but this was very difficult to distinguish because of the density of the normal innervation. Biochemical studies indicated that there was a 45% increase in DA levels at 30 days after the operation. It was suggested that the dopaminergic system sprouted to reinnervate the synapses left bare by the olfactory bulb ablation.

Some investigators think that changes that take a long time to appear

may be due to transsynaptic mechanisms. For example, Moore and Heller (1967) made lesions in the medial forebrain bundle of rats and found that it took 12 days for the levels of brain 5-HT and NE to stabilize at 60% of control levels. Because of this prolonged time-course, they attributed the change to transsynaptic effects, although other interpretations are possible.

Transsynaptic mechanisms have been implicated in the time-dependent changes in brain NE levels which occur following olfactory bulb ablation (Pohorecky and Chalmers, 1971; Pohorecky, Laren, and Wurtman, 1969; Pohorecky, Zigmond, Heimer, and Wurtman, 1969). The authors found a fall in telencephalic NE which they considered to be due to the degeneration of NE neurons originating in the olfactory bulb. Brain stem NE was unchanged 1, 7 and 14 days after bulb ablation, but was increased by 15% 21 and 45 days after the lesion. Because of the prolonged time-course, and because no direct connections are known to exist between the olfactory bulb and the brain stem, the authors concluded that the changes observed were due to transsynaptic effects.

One could postulate many different neuronal circuits whereby frontal lesions could affect the nigro-striatal system transsynaptically. The frontal cortex projects to many lower brain areas. Some of these areas in turn may normally modulate the activity of the nigro-striatal system. Loss of frontal input would disrupt such modulation.

Some investigators have found evidence of a small cortical projection to the nigra itself (Knook, 1966), although there is some controversy about this (Goswell and Sedgwick, 1973). The slow rise in DA levels may reflect some kind of response to the loss of frontal input to the nigra.

As another example, the frontal cortex is known to send projections to the dorsomedial and intralaminar nuclei of the thalamus (Knook, 1966;

Leonard, 1969). These projections would degenerate after frontal lesions. The dorsomedial and intralaminar nuclei in turn send fibers into the striatum, thus modulating its activity (Nauta and Mehler, 1969). It has been found that electrical stimulation of an intralaminar nucleus results in the release of DA from the striatum (McLennan, 1964). With the loss of frontal input, the thalamic effect on the nigro-striatal system would be disturbed and might result in a change in amine levels. Many other such transsynaptic pathways from the frontal cortex to the nigro-striatal system can be postulated.

Examples have been given of increased amine levels associated with decreased amine metabolism. However, instances of increased amine levels and increased turnover have also been described. For example, Salama and Goldberg (1973) found a 12% increase in NE levels in the hindbrain of rats seven days following lesioning of the septal area. No other postoperative time point was examined, so that time-course of this increase is unknown. The authors estimated NE turnover in the hindbrain by administering α-methyl-p-tyrosine and calculating the rate constants for NE decline. They estimated that turnover was increased by 760% in lesioned animals. The authors offered as a possible explanation that a lesion of the septal area resulted in the release of inhibitory influences on hindbrain structures and this increased neural activity.

Some authors have a similar conception of the effect of frontal lesions on the striatum (Bolme, Fuxe, and Lidbrink, 1972; Iversen, Wilkinson and Simpson, 1971). Behavioral and electrophysiological studies suggest that the frontal and nigral inputs to the striatum have antagonistic effects, with frontal stimulation exciting, and nigral stimulation inhibiting striatal cells (Buchwald et al., 1973; Connor, 1970). Removal of the frontal projection leaves the striatum under predominantly dopaminergic control. As already discussed,

activation of the nigro-striatal dopaminergic system results in an increase in motor behavior. When the opposing frontal system is no longer present, behavioral activation and a hypersensitivity to drugs affecting the nigro-striatal system might be expected. Furthermore, DA turnover might increase since the system opposing its activity has been removed.

On the other hand, this imbalance between frontal and nigral input to the striatum might initiate a compensatory decrease in DA metabolism and this decrease in metabolism could contribute to the build-up of DA levels.

Obviously, steady-state amine levels tell little about the activity of the system. Increased levels can be associated with either an increase or decrease in activity. It seemed most important to determine whether the increase in striatal DA levels in frontal animals was associated with a change in the activity of the dopaminergic system.

DOPAMINE METABOLITE STUDIES

Different Approaches to the Estimation of Turnover

Turnover is the rate at which a biochemical is renewed. In a neural system, it is thought to be a reflection of the activity of the system. Turnover is the result of a balance between the rate of amine synthesis and the rate of metabolism. At steady-state conditions, it is assumed that the rate of synthesis is equal to the rate of metabolism. Similarly, the rate of metabolite formation must be equal to the rate of metabolite elimination if steady-state conditions prevail. Most of the different methods that are used to estimate turnover make use of these relationships.

1. Intracerebral injection of labeled DA: Labeled DA is injected into the ventricular cavities of the animal or directly into a desired brain structure using stereotaxic coordinates. The disappearance of the label is followed by killing the animal at different times after injection and measuring the decline in the specific activity of DA. It is assumed that the rate of disappearance of injected amine will reflect the rate of utilization of endogenous stores.

DA will not cross the blood brain barrier if administered systemically, and so must be injected intracerebrally. This means that the animal must be subjected to a stressful operation. It is hoped that when injected in low concentrations, the DA will be taken up specifically by DA neurons. Unfortunately, as Snyder and Coyle (1969) have shown, the NE uptake system appears to have a greater affinity for DA than for NE. Fuxe and Ungerstedt (1966) found that NE and α-methyl-NE injected intraventricularly can be taken up by DA neurons and that 5-HT neurons can take up α-methyl-NE. In addition to diffuse accumulation in brain tissue, there was extraneuronal

binding of NE in pericytes and endothelial cells. They also observed that uptake was limited to those parts of the neuron that were near the ventricle. Uptake appears to be greatest at the site of injection and decreases with increasing distance from the site, rather than reflecting endogenous levels. Furthermore, even very small amounts of amine will change steady state levels when injected locally, and subsequent metabolism may not reflect turnover under steady-state conditions. Also, injected amine may be metabolized before being taken up by neurons so that its metabolic fate would not be a good indicationof what occurs physiologically. Finally, when exogenous amine is taken up by a neuron, its intracellular distribution may not reflect that of endogenous stores (Glowinski and Iversen, 1966b).

2. Injection of labeled precusor: If a labeled precursor is administered and the amine is synthesized endogenously, its distribution should better reflect that of the naturally occurring substance. Furthermore, the precursor, unlike the amine itself, can cross the blood brain barrier and can therefore be administered systemically. In the case of DA, two different precursors are available:

(a) Tyrosine: The conversion of tyrosine to DOPA is rate-limiting in CA biosynthesis. Therefore, the rate of DA synthesis as well as the rate of DA loss can be studied after the administration of this precursor. However, the amount of label incorporated into DA will be very small. This would make accurate measurements very difficult since the decline in specific activity over time is exponential. Because tyrosine is involved in many different metabolic pathways, it is difficult to successfully separate the metabolites one is interested in from the others. Furthermore, the labeled tyrosine remains in the brain for a long period after injection.so that synthesis continues and turnover is underestimated (Udenfriend and Zaltzman-Nirenberg,

1963). The DA specific activity will be in part determined by the specific activity of the precursor. Therefore it is important to correct for the changing specific activity of the precursor in making calculations. However, since tyrosine acts as a precursor in many metabolic pathways, its total specific activity may not accurately reflect the specific activity of the pool involved in CA synthesis.

(b) DOPA: By injecting labeled DOPA, one can by-pass the rate-limiting step in CA biosynthesis and obtain a greater degree of incorporation of label than with tyrosine. However, the rate of incorporation cannot be used as an index of the rate of synthesis since the rate-limiting step is not involved in the conversion of DOPA to DA. Furthermore, since the enzyme which decarboxylates DOPA to DA (L-aromatic amino acid decarboxylase) is relatively non-specific, DOPA can be decarboxylated in non-dopaminergic neurons.

3. Synthesis inhibition: The rate-limiting step in CA biosynthesis can be inhibited by injection of α-methyl-p-tyrosine. It is assumed that after synthesis inhibition, the rate of amine disappearance will be correlated with the rate of amine utilization. AMPT is a reversible and competitive inhibitor of the conversion of tyrosine to DOPA, and adequate concentrations of the drug must be maintained in order to ensure continued maximal inhibition.

A drug which inhibits CA synthesis would be expected to have central nervous system as well as autonomic effects. Spector et al. (1965) found a reduction in motor activity and muscle tone as well as miosis after the administration of AMPT. These effects could be reversed by the administration of DOPA, but not 5-HTP.

4. Inhibition of metabolism: A monoamine oxidase inhibitor is administered to inhibit the metabolism of the amine. The resulting decrease in metabolite levels or increase in amine levels is measured over time. It is hoped that the rate at which these changes take place reflects the rate of amine metabolism under normal conditions.

Tozer, Neff, and Brodie (1966) used this method to estimate 5-HT turnover in rat brain. They administered an MAO inhibitor and measured both the increase in 5-HT levels and the decrease in metabolite (5-hydroxy-indoleacetic acid, 5-HIAA) over time. The levels of 5-HIAA declined exponentially after the administration of an MAO inhibtor. Plotting the log of 5-HIAA concentration as a function of time produced a straight line and the slope of this line enabled a rate constant for 5-HIAA formation to be calculated. Multiplying the rate constant by the steady-state level of 5-HIAA gave a figure for the turnover of 5-HIAA. This figure should be equal to the rate of 5-HT turnover under steady-state conditions. They also measured changes in 5-HT levels after MAO inhibition, and found linear increases up to 90 minutes. Calculations of 5-HT synthesis based on 5-HT levels after MAO inhibition agreed very well with the calculations based on 5-HIAA levels.

Methods for turnover estimation which depend on metabolite formation and elimination are most suitable for substances such as 5-HT for which there is thought to be one main metabolite. When the metabolic pathway is more complex, as it is for the catecholamines, these methods may be less accurate. It is always possible that an important metabolite is not being measured or that metabolism is being shifted to an alternate route. Similarly, if metabolism is blocked, some amine may be lost by diffusion.

5. Inhibition of metabolite transport: Probenecid is a drug which has been found to block the active transport of some acidic metabolites out of the brain (Neff, Tozer, and Brodie, 1967; Werdinius, 1967). It appears to work by competitive inhibition of the organic acid transport system (Fishman, 1966).

Neff, Tozer, and Brodie (1967) measured the rate of 5-HIAA accumulation in the brains of rats after the administration of probenecid. They found that the levels of 5-HIAA increased linearly for 90 minutes. The rate of accumulation was assumed to be equal to the rate of 5-HIAA formation. The turnover figure arrived at agreed very well with the one calculated after inhibiting the formation of 5-HIAA by administering an MAO inhibitor.

Probenecid also inhibits the transport of HVA out of the brain. Werdinius (1967) found a 2 to 3 fold increase in striatal HVA levels 3.5 hours after the administration of 200 mg/kg probenecid to rats.

When drugs are used to measure turnover, there are certain considerations that should be kept in mind. First of all, when an experimental and control group are being compared there is the possibility that the two groups might show differential sensitivity to the drug or that there might be an interaction betweeen the effect of the drug and the experimental conditions. Also, most drugs have more than one effect, even though the investigator may not be aware of all of them. Drugs that have central nervous system effects change the behavior of the animal and may have secondary effects because of this. Furthermore, one is attempting to gain information about the normal state of the system by interfering with steady-state conditions, that is, by inhibiting synthesis or metabolism. Synthesis inhibition may interfere with amine utilization and may give results which do not accurately reflect

steady-state conditions. Similarly, inhibition of CA metabolism with an MAO inhibitor is known to decrease the rate of amine synthesis by a process of end-product inhibition (Javoy et al., 1972).

Present Approach

It was decided to adopt an approach toward turnover that would involve measuring metabolites. This would avoid the dangers of labeled DA being taken up nonspecifically and changing steady-state conditions of DA, or labeled DOPA being metabolized to DA in non-dopaminergic neurons. Furthermore it was felt that measuring metabolites would result in the most detailed analysis of the system since present concepts concerning CA metabolism describe two routes of inactivation. The amine may be deaminated intracellularly by mitochondrial MAO, or it may be inactivated extraneuronally by COMT.

Metabolite levels were measured under the following conditions:

a) baseline levels

b) levels following probenecid

c) levels following pargyline

Methods

Flurometric Assay of DA Metabolites

Fluorometric methods of measuring HVA have been described in the literature. Initial attempts to set up the method of Gerbode and Bowers (1968) were unsuccessful. Brain samples were homogenized in 0.4 N perchloric acid and centrifuged. The pH was adjusted to 5.5 with K_2CO_3 and the samples were centrifuged again. The supernatant was acidified, saturated with NaCl, and the acids were extracted into ethyl acetate. The acids were then back extracted into phosphate buffer, pH 8.5. After adjusting to an alkaline pH, HVA was oxidized with $K_3Fe(CN)_6$ and the oxidation interrupted with cysteine. Fluorescence spectra were recorded.

This method, originally intended for cerebrospinal fluid, gave very high blanks which would obscure the HVA peaks. The source of these elevated blanks was found to be the ethyl acetate, which would mix to a certain extent with the phosphate buffer. Efforts to reduce the blanks by using different organic solvents, different buffers, different concentrations, etc. were all unsuccessful.

Better results were obtained using the fluorometric method of Andén et al. (1963). After homogenization in 0.4N perchloric acid and centrifugation, brain samples were adjusted to pH 6.5 with K_2CO_3 and re-centrifuged. The supernatant was washed with chloroform. The aqueous phase was acidified, saturated with NaCl, and the acids were extracted into ether. Any salt water which might have remained in the organic phase was removed by placing the ether in a dry ice-acetone bath for 1 hour, then immediately filtering. The acids were back extracted into Tris-acetate

buffer, pH 8.5. After adding NH_4OH, HVA was oxidized with $K_3Fe(CN)_6$. The oxidation was stopped by the addition of cysteine and the fluorescence spectrum recorded.

Results were good, with satisfactory blanks and 88% recovery. However, the sensitivity of the method would not allow determinations on samples from single animals. Several brain samples would have to be pooled, as routinely reported in the literature. Furthermore, Kirschberg (1972) found that other acid metabolites present in biological samples interfere with the formation of the HVA fluorophore (a dimer of HVA), and this gives falsely lowered values.

Initially, DOPAC was also measured flurometrically. The extraction procedure was identical to the one used in the HVA assay. After the DOPAC was extracted into Tris-acetate buffer, pH 8.5, it was reacted with a fresh mixture of redistilled ethylenediamine and 4M NH_4Cl (1:1.3 v/v). The samples were heated in a water bath at 50°C for 20 minutes with shaking and the exclusion of light. After cooling to room temperature, samples were read in the fluorometer.

The most severe limitation of this assay was establishing a satisfactory blank. The use of H_2O_2 to destroy fluorescence was unsatisfactory. An unheated blank gave falsely lowered fluorescence. Running a brain area which should have low levels or no DOPAC (such as the cerebellum) was decided upon as the best solution, but this was far from an ideal blank.

Since the fluorometric methods available for the determination of HVA and DOPAC were less than satisfactory, an attempt was made to devise a sensitive and specific gas chromatographic method.

Gas Chromatographic Assay of DA Metabolites

An initial attempt to measure HVA involved extracting from an acidified sample into ethyl acetate and back extracting the acids into K_2CO_3. The

aqueous phase was acidified and saturated with NaCl, and HVA was extracted into ethyl acetate. The acids were esterfied using pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFP). In using this method, the salts used in the extraction procedure were carried over and, being hydroscopic, led to hydrolysis of the derivative.

A simplified procedure involved washing the acidified samples with toluene, then extracting the acids into ethyl acetate. HVA was reacted with PFPA and PFP.

Results from the last procedure were very variable, frequently yielding no peaks. Eventually it was discovered that a contaminant in the ethyl acetate was oxidizing the acids. Different brands and batches of ethyl acetate were tested, giving variable results. Substitution of ether for ethyl acetate resulted in lower background, as the ether extracted less interfering material from the sample. The addition of sodium diethyldithiocarbamate to the ether (1 mg/lb ether) resulted in improved recovery and reproducibility of the method. Moreover, it was found that DOPAC could be extracted together with HVA and both metabolites measured simultaneously.

The propionic acid homologs of HVA and DOPAC were used as internal standards.

Final Procedure

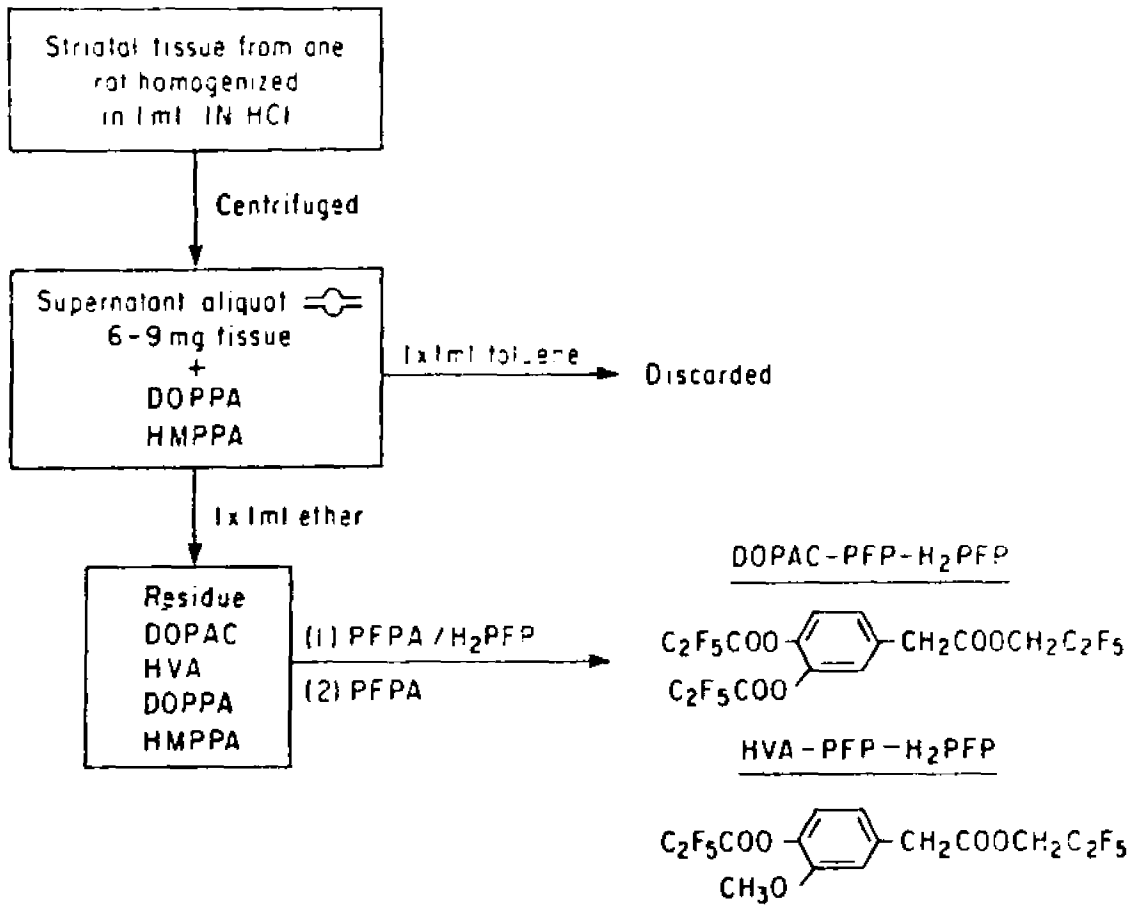
Materials and reagents:

1. Pentafluoropropionic anhydride (PFPA): Pierce Chemical Co., Rockford, Ill.
2. 2,2,3,3,3-pentafluoro-1-propanol (PFP): Peninsula Chemical Research, Gainesville, Fla.
3. Homovanillic acid (HVA): Sigma Chemical Co., St. Louis, Mo.
4. 3,4-dihydroxyphenylacetic acid (DOPAC): Sigma Chemical Co.
5. 4-hydroxy-3-methoxyphenylpropionic acid (HMPPA): Prepared from 4-hydroxy-3-methoxycinnamic acid (Aldrich Chemical Co., Milwaukee, Wis.) by catalytic hydrogenation (Sjöquist and Änggård, 1972).

6. 3,4-dihydroxyphenylpropionic acid (DOPPA): Aldrich Chemical Co.
7. Ether: Electronicgrade E-138, Fisher Chemical Co., Pittsburgh, Pa. 1 mg sodium diethyldithiocarbamate was added to each 1 lb can of ether to retard peroxide formation and increase recovery. The ether was stored in the refrigerator.
8. IN HCl
9. Toluene: Nanograde, Malinkcrodt.

Rats were killed by decapitation and the striatum rapidly dissected out and homogenized in 1 ml IN HCl. The homogenate was centrifuged in the cold at 12,000 g for 10 minutes. Typically 100 μl of the supernatant was removed for analysis. This was usually equivalent to 6-9 mg of tissue. HMPPA and DOPPA, the propionic acid homologs of HVA and DOPAC, were added as internal standards. The samples were extracted with 1 ml toluene, which was then discarded. They were then extracted with 1 ml cold ether. The ether was transferred to a small silanized glass tube and evaporated to dryness under nitrogen. The residue was derivatized by reacting with 40 μl of PFPA and 10 μl of PFP for 10 minutes at 75°C. The tubes were cooled under tap water, and the excess reagents blown off under nitrogen. An additional 25 μl of PFPA was added and allowed to react for 5 minutes at 75°C. Again the tubes were cooled under tap water and the excess reagent removed under nitrogen. The residue was dissolved in 100 μl ethyl acetate and, typically, 1 μl was injected onto the gas chromatograph for analysis. The flow-diagram for the procedure can be seen in Figure 1.

Figure 1. Extraction and derivatization procedure for the assessment of DOPAC and HVA.


Striatal tissue from one rat homogenized in 1 ml 1N HCl
Centrifuged
Supernatant aliquot
6-9 mg tissue
+
DOPPA
HMPPA
1 x 1 ml toluene
Discarded
1 x 1 ml ether
Residue
DOPAC
HVA
DOPPA
HMPPA
(1) PFPA / H2PFP
(2) PFPA
DOPAC-PFP-H2PFP
C2F5COO
C2F5COO
CH2COOCH2C2F5
HVA-PFP-H2PFP
C2F5COO
CH3O
CH2COOCH2C2F5

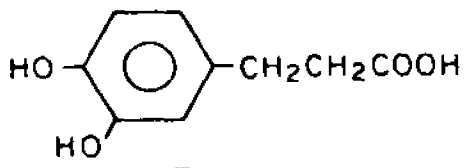
A Packard 7400 series gas chromatograph was used. Injections were made onto a coiled glass column measuring 6 ft by 4 mm ID and packed with 3% JXR coated on gas chrom Q 100/120 mesh (Applied Science Labs., State College, Pa.) The tritium source of the electron capture detector was a 150 mCi tritium foil.

Nitrogen was used as the carrier gas at a flow rate of 60 ml/min. The inlet temperature was 175°C, the column 135°C, and the detector 180°C.

Calculations were based on the peak height obtained with known amounts of HVA and DOPAC as compared to their respective internal standards. These were 4-hydroxy-3-methoxyphenylpropionic acid (HMPPA) and 3,4-dihydroxyphenylpropionic acid (DOPPA), the propionic acid homologs of HVA and DOPAC. These contain the same functional groups as HVA and DOPAC, differing only by the presence of one additional methylene group on the side chain (Fig. 2). The increased length of the side chain increased the retention time of these compounds, enabling them to be separated from HVA and DOPAC. No peaks were found in striatal tissue coinciding with those of the internal standards. The HMPPA: HVA and DOPPA: DOPAC response factors were estimated and used to determine the unknown amounts of HVA and DOPAC in the samples.

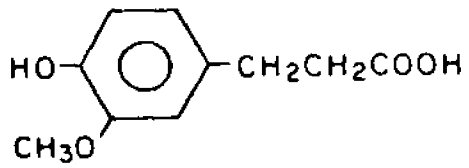
Figure 2. Internal standards for DOPAC and HVA assay.

INTERNAL STANDARDS FOR DOPAC AND HVA ASSAY



3,4-Dihydroxyphenylpropionic acid

(DOPPA)



4-hydroxy-3-methoxyphenylpropionic acic

(HMPPA)

Results

The derivatization reactions for DOPAC and for HVA are shown in Figures 3 and 4 respectively. The structures of the derivatives wefe confirmed by mass spectrometry (Figures 5 and 6).

Figure 7 shows a typical chromatogram assessing metabolite levels in 7 mg rat striatal tissue. The sensitivity of the procedure allowed for the quantitation of both metabolites in as little as 1 mg striatal tissue (Figure 8). Control rat striatal tissue was found to contain 0.90 μg/g $\pm$ 0.21 S.D. (N=12) DOPAC and 0.66 μg/gm $\pm$ 0.16 S.D. (N=12) HVA.

Specificity was determined on several column packings. The retention time of the sample HVA derivative was compared to that of the standard HVA derivative on columns of JXR, OV-1. OV-17, Silar 5-CP, and OV-225. The sample and standard DOPAC derivatives were compared on columns of JXR, OV-1, and SE-54. Best results were obtained on a 3% JXR column at 115^{o}C.

Further evidence for specificity was the absence of DOPAC and HVA peaks in samples obtained from rat hypothalamus (Figure 9) an area in which DA levels are low (Laverty and Sharman, 1965).

Drug studies were carried out to obtain further evidence for specificity. Rats were injected with probenecid (200 mg/kg, ip) and their striatal metabolite levels measured two hours later. The HVA concentration doubled but there was no significant change in DOPAC levels. Another group of rats was injected with pargyline (75 mg/kg, ip). An hour later the levels of both DOPAC and HVA had fallen to undetectable amounts.

Metabolite recovery and the reproducibility of the method were assessed by adding known amounts of HVA and DOPAC to distilled water and running multiple samples through the procedure. The recovery of HVA was 95% with a standard deviation of $\pm$ 4%. DOPAC recovery was 84% with a standard deviation of $\pm$ 5%.

Figure 3. Derivatization reaction for DOPAC.

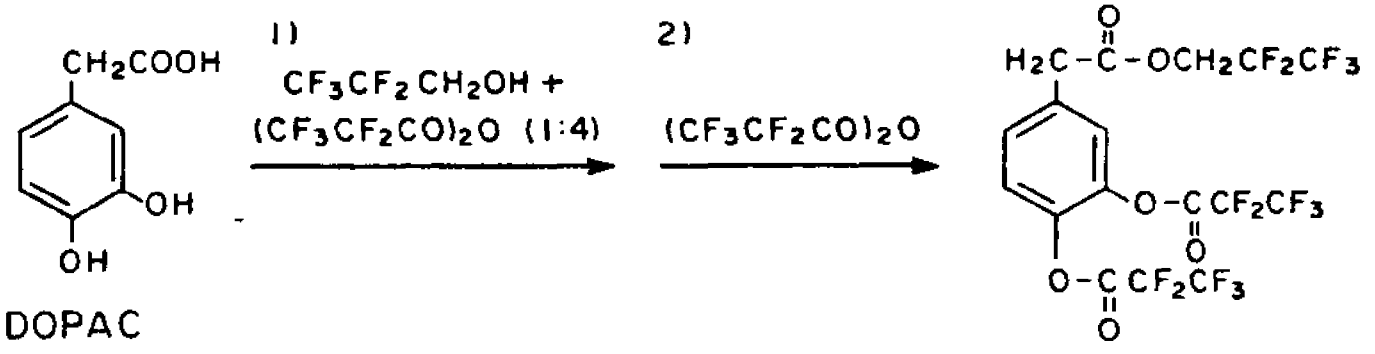

CH2COOH
OH
OH
DOPAC
1)
CF3CF2CH2OH +
(CF3CF2CO)2O (1:4)
2)
(CF3CF2CO)2O
H2C-C-OCH2CF2CF3
O-C CF2CF3
O-C CF2CF3

Figure 4. Derivatization reaction for HVA.

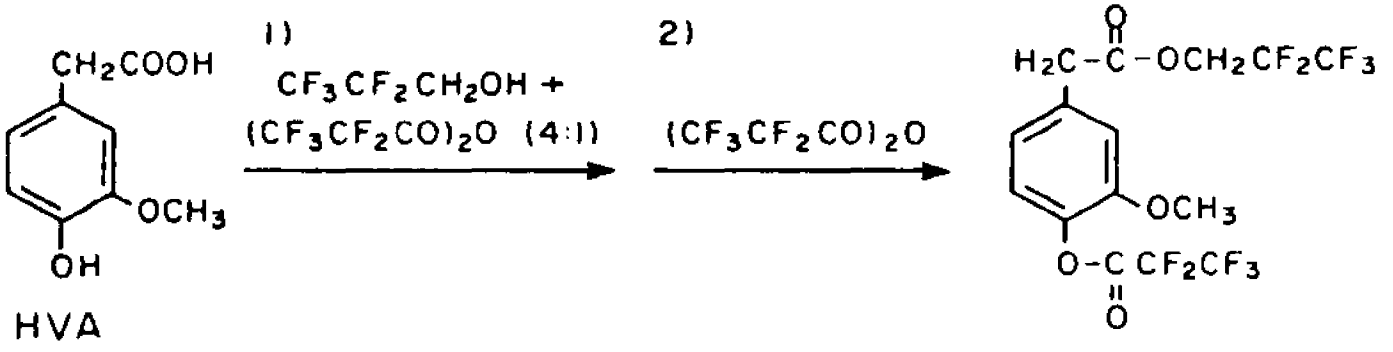

CH2COOH
OCH3
OH
HVA
1)
CF3CF2CH2OH +
(CF3CF2CO)2O (4:1)
2)
(CF3CF2CO)2O
H2C-C-OCH2CF2CF3
OCH3
O-C CF2CF3

Figure 5. Mass spectrum of DOPAC derivative.

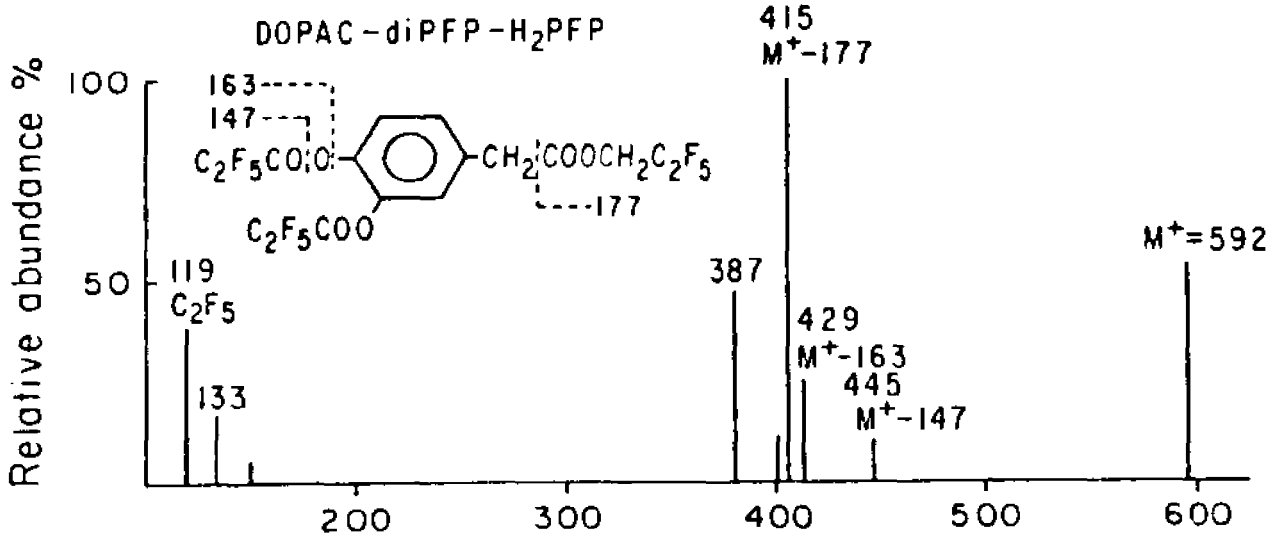

DOPAC-diPFP-H2PFP
Relative abundance %
100
50
163
147
C2F5CO
CH2COOCH2C2F5
177
C2F5COO
119
C2F5
133
387
415
M+-177
429
M+-163
445
M+-147
M+=592
200
300
400
500
600

Figure 6. Mass spectrum of HVA derivative.

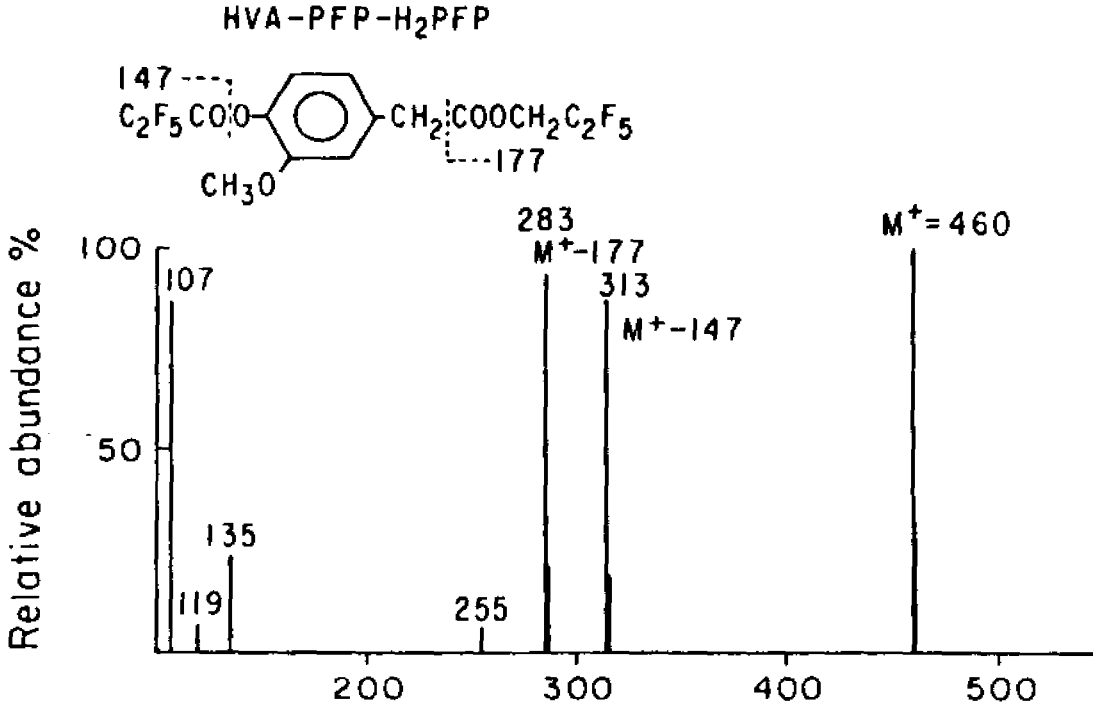

HVA-PFP-H2PFP
147
C2F5COO
CH2COOCH2C2F5
177
CH3O
Relative abundance %
100
50
107
135
119
255
283
M+-177
313
M+-147
M+=460
200
300
400
500

Figure 7. Chromatogram of DOPAC and HVA in 7 mg rat striatal tissue.

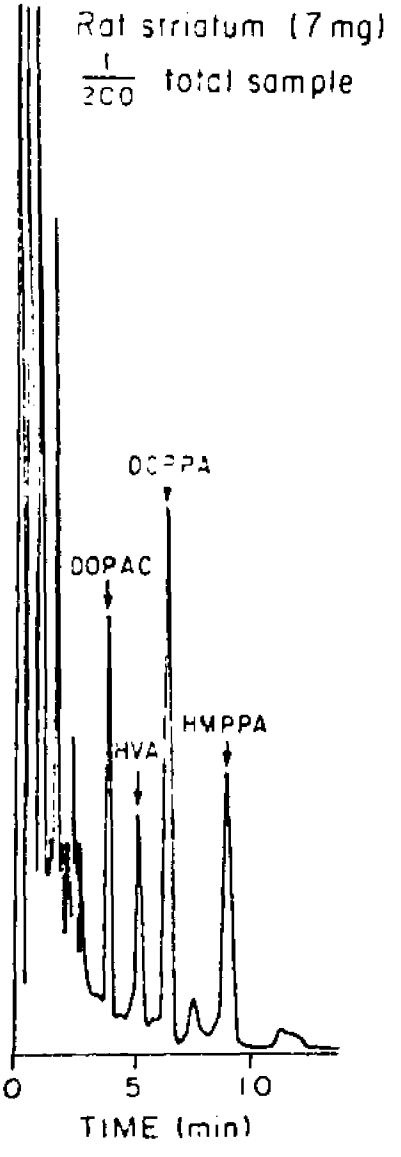

Rat striatum (7 mg)
1/200 total sample
DOPAC
HVA
HMPPA
0
5
10
TIME (min)

Figure 8. Chromatogram of DOPAC and HVA in 1 mg rat striatal tissue.

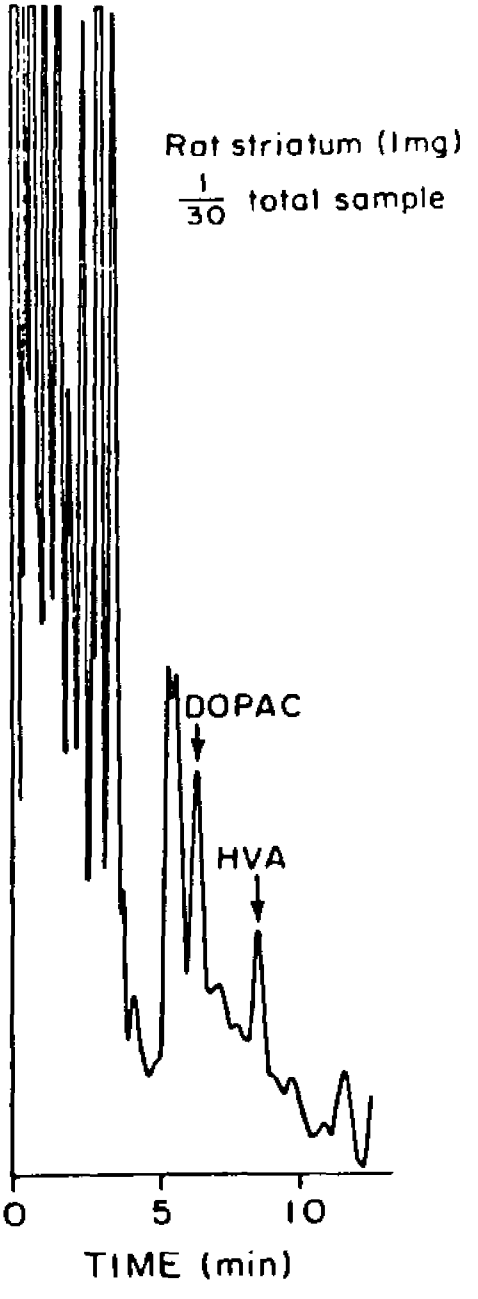
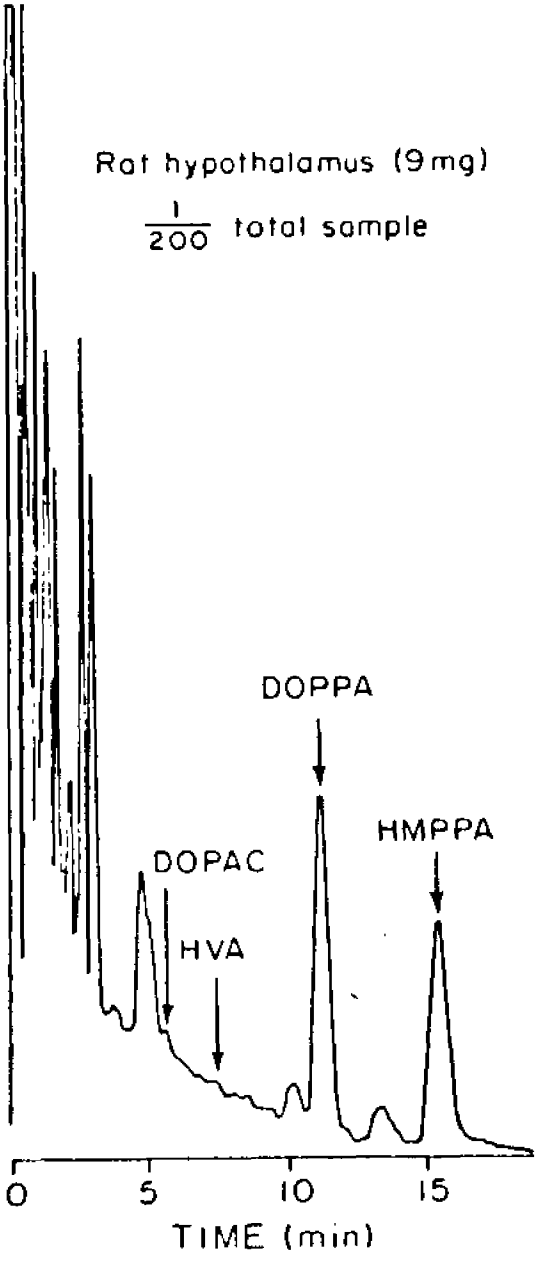

Rat striatum (1mg)
1/30 total sample
DOPAC
HVA
0
5
10
TIME (min)

Figure 9. Chromatogram of rat hypothalamus illustrating the lack of peaks for DOPAC and HVA.


Rat hypothalamus (9 mg)
1/200 total sample
DOPPA
HMPPA
DOPAC
HVA
0
5
10
15
TIME (min)

Metabolite Levels Four Weeks afer Surgery

The frontal cortex was ablated in a group of rats as already described; a sham group was also prepared. Four weeks after surgery, the animals were killed and striatal HVA and DOPAC measured by gas chromatography. Some animals received an ip injection of 200 mg/kg probenecid (Merck, Sharpe and Dohme) two hours before sacrifice. As previously discussed, probenecid has been found to block the transport of HVA out of the brain (Werdinius, 1967). By measuring the increase in HVA over time, the rate of metabolism of DA to HVA can be estimated. The drug was dissolved in 0.1N NaOH and the pH adjusted to 7.

Probenecid administration resulted in a large increase in striatal HVA levels in both sham and frontal animals (Table 3). DOPAC levels were unaffected by the dose used (Table 4). The data was subjected to an analysis of variance. Frontal animals were found to have significantly lower DOPAC levels ($p < 0.05$). No significant difference was found in HVA levels. Thus, at four weeks after surgery, when there is 36% more DA in the striatum of frontal animals, sham animals were found to have 24% more striatal DOPAC.

Early Metabolite Levels

Metabolite levels were measured in another group three days after surgery (Table 5), before the new steady-state in DA levels was achieved. No significant differences were observed.

TABLE 3

STRIATAL HVA FOUR WEEKS AFTER SURGERY

	Shams	Frontals
Baseline	0.75 ± 0.05 (N=7)	0.68 ± 0.07 (N=9)
Probenecid (200 mg/kg)	1.47 ± 0.07 (N=12)	1.32 ± 0.09 (N=10)

Values are in μg/g ± SEM

TABLE 4

STRIATAL DOPAC FOUR WEEKS AFTER SURGERY

	Shams	Frontals
No drug	0.93 ± 0.10 (N=7)	0.76 ± 0.06 (N=9)*
Probenecid (200 mg/kg)	1.01 ± 0.09 (N=12)	0.82 ± 0.07 (N=10)*

Values are in ug/g ± SEM

*$p < 0.05$, analysis of variance

TABLE 5

STRIATAL DA METABOLITES THREE DAYS AFTER SURGERY

	Shams	Frontals
HVA	0.63 ± 0.07 (N=6)	0.65 ± 0.03 (N=6)
DOPAC	0.89 ± 0.12 (N=6)	0.95 ± 0.13 (N=6)

Values are in μg/g ± SEM

Use of Pargyline to Evaluate DA Turnover

Turnover was estimated by measuring the decline in metabolite levels following MAO inhibition. Pargyline was selected to inhibit the formation of HVA and DOPAC. Differences in the rates of decline of metabolites following pargyline would suggest that there is a difference in turnover between the two groups of rats.

Rats underwent frontal cortical ablation or a sham operation and were used four weeks later. The animals were killed and the striatum removed for assay 20 minutes after an ip injection of 75 mg/kg pargyline · HCl dissolved in saline (Abbott Labs.), or 40 minutes after an ip injection of 150 mg/kg pargyline. The two doses had been shown to be equivalent in their effects. They were compared so as to ensure that the lower dose resulted in complete inhibition. Twenty minutes after the injection of 75 mg/kg pargyline, unlesioned animals had 0.17 $\pm$ 0.01 SEM ug/g DOPAC and 0.40 $\pm$ 0.02 SEM µg/g HVA in the striatum. Twenty minutes after the administration of 150 mg/kg pargyline, striatal DOPAC was 0.18 $\pm$ 0.01 SEM µg/g while HVA was 0.39 $\pm$ 0.01 SEM µg/g.

Calculations for Metabolite Data: Inhibiting MAO with pargyline results in a decline in metabolite levels which follow simple first order kinetics (Wiesel et al., 1973). The rate of decline in metabolite levels is described by the equation

$$\frac{-d[M]}{dt} = k[M] \quad . \qquad (1)$$

Equation (1) can be rearranged to give

$$\frac{-d[M]}{[M]} = k\,dt, \qquad (2)$$

and upon integration becomes

$$\ln \frac{[Mo]}{[M]} = kt, \tag{3}$$

where $[Mo]$ represents metabolite concentration at time zero, and $[M]$ represents metabolite concentration at some subsequent time. At the halftime of metabolite decline, the ratio of $[Mo]/[M]$ will be equal to 2. Thus

$$\ln \frac{[Mo]}{[M]} = \ln 2 = kt_{\frac{1}{2}} . \tag{4}$$

Converting to common logs:

$$2.3 \log 2 = kt_{\frac{1}{2}} \tag{5}$$

$$0.693 = kt_{\frac{1}{2}} \tag{6}$$

By finding the half-time of metabolite decline experimentally and inserting it into the above equation, one can calculate the fractional rate constant (k) of metabolite formation. One can then calculate metabolite turnover according to the formula

Turnover = k (steady-state concentration).

Since the decline in metabolite levels over time following pargyline is an exponential function, plotting the logs of the metabolite levels should give a straight line. The best straight lines were plotted by the method of least squares. The y-intercepts, reflecting steady-state concentrations, were found not to differ significantly when sham and frontal groups were compared. However, as determined experimentally, these differences are in fact significant for DOPAC, though not for HVA (see Tables 3 and 4).

The half-times ($t_{\frac{1}{2}}$) of metabolite decline were obtained by examining the lines. These figures were inserted into the equation

$$kt_{\frac{1}{2}} = 0.693$$

so that the fractional rate constants could be calculated. By multiplying the fractional rate constants by the steady-state concentrations, turnover figures for DOPAC and HVA were calculated.

Under steady-state conditions, the rate of DA synthesis should be equal to the rate of DA metabolism. Assuming that most of the DA in the brain is converted to either DOPAC or HVA:

DA turnover = nmoles HVA/g/hr + nmoles DOPAC/g/hr

Sham Group: The half-life ($t_{\frac{1}{2}}$) for DOPAC was 0.19 hr and the fractional rate constant (k) was 3.56 hr^{-1}. By multiplying k by the steady-state concentration of DOPAC, a turnover rate of 19.7 nmoles/g/hr was calculated. HVA had a $t_{\frac{1}{2}}$ of 0.31 hr and a k value of 2.27 hr^{-1}. The turnover was calculated to be 9.3 nmoles/g/hr. Thus total metabolism (DOPAC+HVA) was 29.0 nmoles/g/hr.

Frontal Group: DOPAC had a $t_{\frac{1}{2}}$ of 0.19 hr, a fractional rate constant of 3.67 hr^{-1}, and turnover rate of 16.6 nmoles/g/hr. The $t_{\frac{1}{2}}$ for HVA was 0.31, with a k of 2.27 hr^{-1}, and a turnover rate of 8.5 nmoles/g/hr. This made a total turnover rate of 25.1 nmoles/g/hr.

Assuming that HVA and DOPAC are the major metabolites of DA, the sum of their rates of formation can be taken to represent the rate of DA metabolism. Under steady-state conditions, this would also be equivalent to the rate of DA synthesis. Thus, the rate of DA metabolite formation should be equal to the rate of DA turnover. By dividing the rate of

turnover by the steady-state level of DA, the fraction of the DA pool that is renewed per unit time can be calculated. This is called the k_{DA}, or fractional rate constant for DA turnover. The k_{DA} in sham animals was found to be 0.81 hr^{-1} while in frontal animals it was only 0.50 hr^{-1}.

Discussion

Dopamine Metabolism in Sham Animals

In order to estimate DA turnover from the rate of formation of its metabolites, the following assumptions were made: (1) HVA and DOPAC account for most DA metabolism. (2) The two metabolites arise from separate pathways. Wiesel et al. (1973) carried out turnover studies on rat striatum on the assumption that HVA is the major DA metabolite in the brain, i.e. that almost all the DA is metabolized to HVA. These authors administered pargyline and measured changes in HVA levels by gas chromatography-mass fragmentography. From the rate of decline, they calculated a fractional rate constant k_{HVA} of 3.44 hr^{-1}. By multiplying this figure by the steady-state level of HVA (4.1 nmole/g), they estimated the turnover of HVA to be 14 nmole/g/hr. Assuming that HVA is the major DA metabolite, at steady-state conditions, the rate of DA turnover should be equal to the rate of HVA formation, that is, 14 nmole/g/hr. This figure, which does not take into account DOPAC formation, is less than half of that obtained in the present experiments. Wiesel and his colleagues cautioned that their figure was only an approximation, since other DA metabolites might be of some importance in estimating turnover.

If DOPAC is present in the brain merely as a precursor to HVA, it would not be proper to sum both metabolites in estimating DA turnover. However, there is some indication in the literature that most of the DOPAC in the striatum is not further metabolized to HVA. If DOPAC represents an alternate route of metabolism, it cannot be excluded when estimating rates of DA turnover.

Roffler-Tarlov et al. (1971) administered to mice drugs which are known to increase synthesis of HVA. When O-methylation was inhibited with tropolone, the formation of HVA in the striatum was inhibited, but only a small increase in DOPAC levels was observed. This indicated to the authors that DOPAC is not usually the precursor of HVA but that most HVA is formed from 3-MT at a site where DOPAC is not present. The small increase in DOPAC after tropolone may reflect a small amount of HVA that is formed from DOPAC. If 3-MT serves as a precursor to HVA, as Roffler-Tarlov et al. suggest, there is no need to measure it in addition to HVA in calculations of DA turnover.

Data following probenecid support the suggestion that HVA and DOPAC are formed at different sites. Probenecid inhibits the transport of HVA out of the brain, but blocks DOPAC transport only at toxic doses (Spano and Neff, 1972; Roffler-Tarlov et al., 1971). This was confirmed in the present study in which a large increase in HVA but no significant change in DOPAC levels was found after the administration of probenecid. This suggests that these metabolites are present at different sites in neural tissue so that they are not equally accessible to the drug. DOPAC is thought to be formed intraneuronally by the action of mitochondrial MAO (Roffler-Tarlov et al., 1971). Most HVA is thought to be formed extraneuronally after the initial O-methylation of DA by COMT, an extraneuronal enzyme (Broch and Fonnum, 1972).

Spano and Neff (1972) studied DA turnover in the guinea pig striatum by using radioisotopes and by measuring increases in metabolite levels following probenecid administration. They gave a pulse injection of H^3-tyrosine ip and measured changes in the specific activity of DA over time, correcting

their calculations for the specific activity of the remaining H^3-tyrosine. They estimated a turnover rate for DA of 32 nmoles/g/hr. They were able to confirm their turnover estimation by administering 600 mg/kg probenecid ip and measuring HVA and DOPAC fluorometrically. As already discussed, although both of these metabolites are organic acids, they are differentially affected by probenecid. HVA was maximally affected by a dose of 200 mg/kg. DOPAC showed no increase until 400 mg/kg was injected and increased linearly for at least 1 hour after the injection of 600 mg/kg. Higher doses were not tested because of the toxic effects of the drug. About 15 nmoles/g/hr of each metabolite was formed after the administration of 600 mg/kg of probenecid, representing a total metabolite formation of 30 nmoles/g/hr. This figure was taken to be equivalent to DA turnover under steady-state conditions. It agrees very well with the one estimated by following the conversion of H^3-tyrosine to H^3-DA. This study suggests that DOPAC is an important DA metabolite and not merely a precursor of HVA. Therefore it must be taken into consideration in any estimation of DA turnover based on metabolite levels.

The present estimation of HVA turnover may be underestimated, since it is possible that some HVA is being formed from pre-existing stores of DOPAC even after MAO inhibition. Even taking into account this possibility, the rate of formation of DOPAC (19.7 nmoles/g/hr) appears to be much greater than that of HVA (9.3 nmoles/g/hr), establishing DOPAC as a major DA DA metabolite.

HVA and DOPAC levels have been found to correlate with dopaminergic activity Drugs such as haloperidol and chlorpromazine which increase the firing rate of dopaminergic neurons (Bunney et al., 1973) result in increased HVA (Andén, 1972; O'Keefe et al., 1970) and DOPAC (Bunney et al., 1973) levels. Roth et al. (1973) reported that stimulation of DA neurons in the nigro-striatal

system results in an increase in striatal DOPAC levels, while interruption of impulse activity by lesioning the system or by injecting an anesthetic results in a decrease in DOPAC levels. Pearson and Sharman (1974) found that electrical stimulation of the preganglionic nerve resulted in a 372% increase in the concentration of DOPAC as well as a 78% increase in HVA levels in the sympathetic ganglion. Thus, both DOPAC and HVA may be indicators of dopaminergic activity.

Comparison of DA Metabolism in Frontal and Sham Animals

Although frontal animals have 36% more striatal DA than sham animals four weeks after surgery, the metabolite studies show that there is no proportional increase in the activity of the dopaminergic system, since the k values for HVA and DOPAC are identical in the two groups. There was a small but significant difference in the steady-state levels of DOPAC, with frontal animals having the lower level. The fractional rate constant for DA in sham animals was calculated to be 0.81 hr^{-1} while in frontal animals the k_{DA} was only 0.50 hr^{-1}. This means that a smaller proportion of the DA pool of frontal animals replaces itself per unit time as compared to sham animals.

In order to try to explain how the removal of the frontal cortex can permanently affect the nigro-striatal dopamine system, it is necessary to describe the present concept of striatal organization and the feedback mechanisms that are thought to operate in the system.

As has been described earlier, the striatum receives a projection from both the cortex and the substantia nigra. Many investigators believe that the cortical input to the striatum exerts an effect opposite to that of the nigral input. The striatum itself is thought to have an inhibitory effect on motor behavior. For example, Mettler et al. (1939) reported that

striatal stimulation in cats and monkeys results in the inhibition of movements induced by stimulation of the motor cortex. Similarly, Liles and Davis (1969) found that stimulation of the anteroventral region of the head of the caudate nucleus of cats inhibited cortically induced movement. This area of the caudate receives a projection from the prefrontal cortex (Webster, 1965) and other investigators have reported that prefrontal stimulation results in the inhibition of spontaneous or cortically induced movements (Tower, 1936). Thus, the prefrontal cortex may do this by activating the striatal system, as the activation of an inhibitory system would result in the inhibition of motor behavior. Electrophysiological studies indicate that cortical stimulation may indeed have an excitatory effect on striatal neurons (Rocha-Miranda, 1965; Liles, 1973; Buchwald et al., 1973). Furthermore, prefrontal lesions have resulted in hyperkinetic tendences in many species (Hannon and Bader, 1974; Iversen, Wilkinson, and Simpson, 1971; Richter and Hines, 1938), as have striatal lesions (Harmon and Bader, 1974; Mettler and Mettler, 1942; Davis, 1958).

If frontal cortical and striatal activation seem to have an inhibitory effect on motor behavior, the nigral dopaminergic system appears to have an opposite effect. Catecholamine-releasing drugs such as amphetamine will cause an increase in locomotor and stereotyped behavior (Randrup and Munkvad, 1966). Striatal implants of crystals of DOPA or apomorphine produce stereotyped behavior (Ernst and Smelik, 1966), while intrastriatal injections of DA result in hyperactivity and stereotyped behavior (Fog and Pakkenberg, 1971).

Many studies have suggested that DA released from nigral afferents inhibits the striatum; the inhibition of an inhibitory system would result in behavioral activation. The electrical stimulation of nigral cells has been found to decrease the firing rate of striatal neurons in most cases (Connor, 1970); a striatal neuron that was inhibited by nigral stimulation

was also inhibited by the microiontophoretic application of DA. McLennan and York (1967) also reported that direct application of DA to caudate cells results most often in their inhibition. Herz and Zieglgänsberger (1966) found that such DA application inhibited both the spontaneous firing of caudate cells as well as the firing produced by amino acid application or thalamic stimulation.

If the nigro-striatal and fronto-striatal systems do indeed have opposite effects, it is very likely that the removal of one will cause some change in the activity of the other in order to compensate for the imblance of afferents into the striatum. One might expect a decrease in nigro-striatal activity to compensate for the loss of frontal input. Such a decrease in activity and metabolism could result in increased levels of DA. The major weakness of this explanation lies in its failure to explain the two week delay in the rise of DA levels after frontal lesions. Frequently transsynaptic effects are invoked to help explain such an extended time-course.

There is evidence in the literature that compensatory mechanisms are operative in the nigro-striatal dopaminergic system. For example, Sharman et al. (1967) measured HVA levels in monkey striatum one to four months after making electrolytic lesions which interrupted the nigro-striatal system. They found that DA levels were reduced to a much greater extent than HVA levels, i.e. the HVA:DA ratio was increased after lesioning. The ratio in control samples ranged from 1.3 to 1.7. Following lesions, the ratio ranged from 2.1 to 9.9, a considerable increase. The fall in DA levels reflects the degeneration of DA fibers. The much smaller decrease in HVA levels suggests that the turnover of DA in the remaining

cells increases in order to compensate for the drastic removal of dopaminergic input.

Agid, Javoy and Glowinski (1973) found similar evidence one month after a 6-OH-DA lesion of the nigro-striatal system of rats. While striatal DA levels fell in a dose related fashion, DA synthesis as measured with radiochemical methods decreased to a much lesser extent. This study suggests that increased turnover or hyperactivity of remaining intact dopaminergic neurons compensates for decreased DA levels. In the present study, removing the fronto-striatal system might also result in compensatory changes in the nigro-striatal system. Removing a modulating influence on the striatum, one which may have effects opposite to that of the nigral system, may bring about a compensatory decrease in activity of the dopaminergic system to restore the balance between afferents. This might account for the lower metabolite: DA ratio and the decreased DOPAC levels found in the present study.

Dopaminergic activity can be modulated by neuronal feedback loops. Many investigators have demonstrated the existence of a fiber system running from the striatum to the substantia nigra (Voneida, 1960). Yoshida and Precht (1971) found that electrical stimulation of caudate cells, or the fiber system running from the caudate to the nigra resulted in an inhibition or hyperpolarization of nigral neurons. McNair et al. (1972) also reported that stimulation of the caudate nucleus results in the inhibition of cell firing in the substantia nigra. Thus, the striato-nigral fiber system may provide feedback inhibition of the dopaminergic system according to the level of activity of the caudate.

There are many other examples in the literature of control or compensatory changes in dopaminergic activity. If a DA receptor stimulant

such as apomorphine is administered, DA synthesis will decrease (Kehr et al., 1972) and the dopaminergic neurons will decrease their firing rate (Bunney et al., 1973). Obviously, this would tend to restore the system to its normal level of activity. Similarly, if a DA receptor-blocking agent, such as haloperidol or chlorpromazine is administered, turnover will increase so as to overcome the transmission block. Thus, there will be an increase in DA synthesis (Kehr et al., 1972; Nybäck and Sedvall, 1971), an increase in HVA levels (Andén, 1972; O'Keeffe et al., 1970) as well as increased disappearance of ^{14}C-DA (Nybäck, 1972). There will also be a large increase in the rate of firing of DA neurons (Bunney et al., 1973).

The decrease in DOPAC levels found four weeks after frontal lesions was not great. However, decreased metabolism may contribute to increased amine levels and yet be undetectable once the new steady-state has been established. The work of Roth et al. (1973) and Walter et al. (1973) has already been described in which an interruption of dopaminergic activity is associated with increased synthesis and decreased metabolism of DA. After the establishment of a new steady-state level of DA, synthesis and metabolism return to normal while amine levels remain high. The newly accumulated amine appears to be particulate-bound and protected from metabolism and release. We were still able to detect a lowering of DOPAC levels in frontal animals four weeks after the lesion. Since DOPAC levels may reflect intraneuronal metabolism of DA, the lowered DOPAC levels may indicate that a portion of the intraneuronal DA is not accessible to MAO.

Although there is much logic to the hypothesis that decreased metabolism led to an increase in DA levels, it does not explain why there is a two week delay before the increase in DA levels becomes evident. It may be that the reduction in metabolism is slight and that the process takes several weeks to reach its full extent. In order to test the hypothesis that early changes in DA metabolism contributed to the final increase in DA levels, metabolites were assessed three days after surgery. No significant differences were observed between frontal and sham animals. This finding opposes the contention that an early decrease in DA turnover contributed to a gradual increase in DA levels. However, it is not inconsistent with the two week delay. In order to arrive at a definitive conclusion, a very extensive time-course of DA metabolism following surgery would have to be undertaken. It may be that the degenerating fronto-striatal system still exerts some effect for a short period after the lesion. Ungerstedt (1971a) made a unilateral injection of 6-OH-DA and found that the lesioned nigro-striatal system exerted a stronger effect than the intact system between 24 and 34 hours after surgery, most likely because of the release of DA from degenerating terminals. Although an extensive time-course of DA turnover would be desirable, it was decided that a more promising hypothesis was that of collateral sprouting.

Removing the frontal input to the striatum produces a partial denervation. If the nigro-striatal dopaminergic system underwent collateral sprouting to reinnervate the postsynaptic sites left bare by the frontal lesion, striatal DA levels would be expected to rise gradually. This would increase the already existing imbalance between the lesioned fronto-striatal system and the intact nigro-striatal system.

In response to this growing imbalance, the turnover of striatal DA might be expected to decrease relative to the increased pool size, thus tending to keep the activity of the system within normal limits. The time-course required for collateral sprouting to occur correlates very well with the time-course of DA increase seen in the present experiment. The DA increase is first seen at two weeks is even greater at four weeks, and remains elevated at three months postoperatively.

UPTAKE OF H^3-DOPAMINE BY STRIATAL SYNAPTOSOMES

Sprouting in a catecholaminergic system is usually studied using fluorescent histochemical methods. However, as already noted by Moore et al. (1974), the fluorescence histochemical method is not sensitive enough to detect increases in innervation when the normal innervation is very dense, as is the case in the striatum. It was desirable to approach the problem by neurochemical techniques that would be more quantitative. Determination of the kinetics of the uptake of H^3-DA by striatal synaptosomes offered an alternative approach.

Physiological Significance of Uptake

The primary means of catecholamine inactivation after release into the synaptic space is re-uptake into the presynaptic terminal. After the injection of H^3-NE, the amine can be found in tissues which are sympathetically innervated (Whitby et al., 1961; Kopin, Gordon, and Horst, 1964). If the sympathetic innervation is destroyed, the ability to take up injected amine is abolished or reduced (Hertting et al., 1961; Hertting & Schiefthaler, 1964), indicating that uptake normally occurs into the postganglionic noradrenergic nerve terminal. Radioautographic studies have confirmed that H^3-NE is taken up into the preterminal portion of the axon (Wolfe et al., 1962).

Characteristics of Uptake into Sympathetically Innervated Organs

Since the catecholamines are highly polar molecules, there must exist some specialized mechanism to aid in their transport across biological membranes. H^3-NE uptake into perfused sympathetically innervated organs is energy and temperature dependent. It is decreased by inhibitors of oxidative metabolism and glycolysis (Kirpekar and Wakade, 1968; Wakade and Furchgott, 1968), and by reducing the incubator temperature to $0^{\circ}C$ (Green and Miller, 1966). Isolated organs or tissue slices can accumulate NE against a concentration gradient (Green and Miller, 1966). Amine transport follows Michaelis-Menten kinetics (Iversen, 1963), and has an absolute requirement for Na+ ions (Horst, Kopin, and Ramey, 1968; Iversen and Kravitz, 1966).

Synaptosomes

Uptake processes can be studied using synaptosomes as well as intact organs or tissue slices. When brain tissue is gently homogenized in isotonic sucrose, the nerve terminals are torn from the rest of the neuron. The membranes of these terminals reseal themselves to form rounded structure called synaptosomes. These structures, when examined by electron microscopy, look very similar to nerve terminals in intact tissue.

Gray and Whittaker (1962) subjected homogenates of guinea pig cerebral cortex to a subcellular fractionation procedure and examined the fractions by electron microscopy. The brain tissue was homogenized in 0.32 M sucrose and the homogenate was centrifuged at 1000 g for 10 minutes. The pellet (P_1) consisted of nuclei, and cell debris. The supernatant (S_1) was recentrifuged at 17,000 g for 55 minutes. The resulting pellet (P_2) was subjected

to a discontinuous density gradient separation yielding three fractions: fraction A contained myelin fragments, fraction B synaptosomes, and fraction C mitochondria. The synaptosomes in fraction B showed many of the characteristics of nerve terminals *in situ*. They contained numerous synaptic vesicles and frequently a mitochondrion. Often a postsynaptic membrane was attached to the synaptosome, forming a synaptic region similar in appearance to the synapses seen in intact tissue.

Jones and Brearley (1972) studied the ultrastructure of synaptosomes prepared from rat cerebral cortex and judged it to be very similar to that of nerve terminals of intact cerebral cortex. Fine details of the synaptic region included a postsynaptic thickening, dense material in the intracleft region, and a network within the presynaptic element.

Biochemical Studies on Synaptosomes

Isolated synaptosomes usually contain mitochondria and are capable of respiration. Thus, they use oxygen, metabolize glucose, pyruvate, and Krebs' cycle intermediates, and generate ATP and phosphocreatine (Bradford, 1969). They are capable of many biosynthetic reactions and can convert glucose into aspartate, glutamate, glutamine, alanine, and GABA (Bradford and Thomas, 1969). They also carry out protein synthesis (Autilio et al., 1968). Energy production for protein synthesis appears to take place intrasynaptosomally, since it can be blocked by the addition of inhibitors of glycolysis, the Krebs' cycle, or oxidative phosphorylation (Autilio et al., 1968; Morgan and Austin, 1969). Abdel-Latif et al. (1968) found that synaptosomes were able to incorporate $[^{32}P]$ orthophosphate into nucleotides, phosphoproteins, and phospholipids.

Substances which are thought to be neurotransmitters, such as ACh, NE, 5-HT, and DA, have been found in synaptosomal fractions (Inouye et al., 1963; Michaelson and Whittaker, 1963; Laverty et al., 1963). Furthermore, there is evidence that synaptosomes are capable of synthesizing these compounds. Karobath (1971) found evidence of tyrosine hydroxylase activity in isolated synaptosomes. Rodriquez De Lores Arnaiz and De Robertis (1964) measured 5-HTP decarboxylase activity in isolated nerve endings. Grahame-Smith (1967) found tryptophan-5-hydroxylase activity. Similarly, Haga (1971) reported that synaptosomes were able to take up ^{14}C-choline and convert it to ^{14}C-ACh.

Synaptosomal Transport Systems

Synaptosomes are capable of the active transport of a variety of ions and molecules. They have high concentrations of Na^+-K^+ ATPase, can actively extrude Na^+ (Ling and Abdel-Latif, 1968), and actively accumulate K^+ (Escueta and Appel, 1969). Logan and Synder (1971) found evidence for the uptake of several amino acids by synaptosomes plus a special high affinity system for glutamate, aspartate, and glycine.

The uptake of labeled amines into synaptosomes shares many of the characteristics of uptake into intact nerve terminals i.e. *in vivo* or into perfused organs. Synaptosomes are able to transport amines against an apparent concentration gradient (Synder and Coyle, 1969). Uptake is temperature dependent (Harris and Baldessarini, 1973a; Baldessarini and Vogt, 1971) and is maximal when Na^+ and K^+ ions are present at physiological concentrations (Colburn et al., 1968; Harris and Baldessarini, 1973b). Furthermore, it is dependent upon a source of energy such as glucose. Compounds which interfere with energy metabolism such as dinitrophenol and NaCN inhibit uptake (White and Keen, 1970).

Synaptosomal uptake of DA appears to follow Michaelis-Menten kinetics (Synder and Coyle, 1969; Harris and Baldessarini, 1973a). The equation describing the relationship between velocity of uptake and the substrate concentration is the same as the Michaelis-Menten equation.

$$v = \frac{Vmax\,[S]}{K_T + [S]}$$

where v = velocity,

Vmax = maximal velocity

$[S]$ = substrate concentration

K_T = the apparent Michaelis-Menten constant; the substrate concentration that results in half the maximal velocity.

K_T should reflect the affinity between the uptake site and the substrate molecule. A low K_T indicates that the affinity is high, that is, a low substrate concentration results in half the maximal velocity. The Vmax or maximal velocity is a reflection of the total number of uptake sites present. The K_T is independent of the number of uptake sites.

Subcellular studies indicate that, when brain homogenates are incubated with labeled amine, the amine is selectively concentrated by synaptosomes. For example, Harris and Baldessarini (1973a), after incubating brain homogenates with H^3-CA, subjected these homogenates to continuous sucrose density gradient ultracentrifugation. They found a peak of radioactivity at a density corresponding to the synaptosomal fraction. They subjected a P_2 fraction, containing synaptosomes, mitochondria, and small myelin fragments, to osmotic shock by suspension in distilled water. More than 85% of the total radioactivity was released, indicating that such radioactivity was present in a membrane enclosed particle. Mitochondria appeared to be resistant to hypo-osmotic shock. Furthermore, when a P_2 fraction was put on a discontinuous sucrose density gradient, only 6% of the radioactivity

was found to be associated with mitochondria. Coyle and Axelrod (1971) prepared 40,000 g pellets from incubated brain homogenates and subjected them to subcellular fractionations. The peak of radioactivity coincided with the peak of occluded lactate dehydrogenase activity (a marker for synaptosomal cytoplasm), and both occurred at a sucrose density associated with synaptosomes. These peaks were easily differentiated from the peak of monoamine oxidase activity (a marker for mitochondria), which occurred at a higher density of sucrose.

Methods

Reagents:

1. Krebs-Ringer solution:

122mM	NaCl
4.9 mM	KCl
0.87mM	$CaCl_2 \cdot 2H_2O$
0.13mM	Na_2EDTA
1.2 mM	$MgSO_4 \cdot 7H_2O$
1.2 mM	KH_2PO_4
10.3 mM	Na_2HPO_4
11.8 mM	glucose
1.1 mM	ascorbic acid

Two stock solutions were made up at 5 times the final concentration:

Solution a

NaCl

KCl

$CaCl_2 \cdot 2H_2O$

Na_2EDTA

$MgSO_4 \cdot 7H_2O$

Solution b

KH_2PO_4

Na_2HPO_4

Glucose, ascorbic acid, and nialamide (12.5 uM) were added fresh for each experiment. The pH was adjusted to 7.4 before bringing the solution to the proper volume with water. The final solution was oxygenated for about 10 minutes before use.

2. 0.32 M sucrose

3. H^3-DA (New England Nuclear)
   specific activity 8 Ci/mmole

The initial procedure used was a modification of the method of Snyder and Coyle (1969). Briefly, brain samples were homogenized in 10 volumes of cold 0.32 M sucrose and centrifuged in the cold at 1000 g for 10 minutes to remove heavy cell debris and nuclei. The supernatant was stirred gently, and 0.1 ml aliquots were added to 2ml Krebs-Ringer-phosphate medium containing 10^{-5}M iproniazid, an MAO inhibitor. The samples were pre-incubated at 37°C for 5 minutes with shaking, after which different concentrations of labeled amine were added and the incubation continued for an additional 5 minutes. The incubation was stopped by placing the samples on ice. Control samples contained both tissue and labeled amine, but were kept on ice at all times. Control values, which represented non-specific binding of label, were subtracted from sample values. As already discussed, active uptake is temperature dependent whereas non-specific binding is not.

After incubation, the samples were centrifuged at 22,000 g for 30 minutes in order to sediment the synaptosomes. Aliquots of the supernatants were removed for counting to determine the radioactivity present in the medium. The pellets were rinsed gently 5 times with 5 ml ice cold 0.9% NaCl. They were then resuspended in 2 ml 0.4N perchloric acid, thus disrupting the synaptosomes and releasing the radioactivity. The samples were centrifuged at 1000 g for 10 minutes, and an aliquot of the resulting supernatant removed for counting. This would determine the amount of labeled amine taken up by the tissue. From this information, a tissue/medium (T/M) ratio was calculated:

$$\text{T/M ratio} = \frac{\text{cpm/g tissue}}{\text{cpm/ml medium}}$$

A T/M ratio greater than 1 indicates that the tissue has actively incorporated the labeled amine.

Using this modified method, no uptake of labeled amine was found. In the method of Snyder and Coyle (1969), synaptosomes were sedimented at 48,000 g for 30 minutes after incubation, although 22,000 g should be sufficient. When this higher speed of centrifugation was tested, positive results were obtained. The T/M ratio was 11 at an amine concentration of 10^{-7}M after a 5 minute incubation period.

Synaptosomes should sediment at 22,000 g, but positive results were obtained only after sedimentation at 48,000 g. It was suspected that uptake was being measured in smaller subcellular particles, rather than intact synaptosomes.

The method of Baldessarini and Vogt (1971), was also evaluated. After brain homogenates are incubated with labeled amine, the synaptosomes are isolated by ultrafiltration instead of centrifugation. Millipore filters are used which have a pore size that trap synaptosomes, but allow the passage of smaller species. This method of obtaining synaptosomes appears to be more specific than preparing a 48,000 g pellet. Baldessarini and Vogt found that the radioactivity trapped by passing samples over an 0.8 μm pore size Millipore filter was only 12.3% of the radioactivity extractable from the 48,000 g pellet. Subcellular distribution studies revealed that filtration sucessfully removes the peak of radioactivity

associated with synaptosomes. Therefore, the 48,000 g pellet must include a considerable amount of radioactivity associated with particles other than synaptosomes. Such material would be too small to be trapped by a filter with a pore size of 0.8 μm.

Using filtration, there was a problem with consistently high blanks. About 1% of the labeled amine was binding to the filter, even when no tissue was present. Different filters were tested but did not improve upon the Millipore filter because the pore size was crucial. Washing the filters with large amounts of buffer after filtration did not help to reduce blank levels. Soaking the Millipore filters in cold DA to saturate the binding sites did not reduce blanks. This suggested that the high blanks were due to a contaminant in the labeled DA. The purity of the labeled amine was tested by thin layer chromatography (silica gel GF, Analtech Inc., Newark, Delaware) in a butanol: acetic acid: water (25:4:10) system. A small peak was found, equivalent to approximately 5% of the authentic H^3-DA peak. A very minor contaminant would be sufficient to produce high blanks and obscure a small amount of active incorporation. Therefore the amine was further purified by passing over alumina. An alumina column was prepared as described in the section on the fluorometric determination of catecholamines. After diluting the labeled amine to 10 ml with water and adding 0.5 ml 10% EDTA, the pH of the solution was adjusted to 8.4 with NaOH and passed over a column containing 0.5 g alumina. After washing the column with 10 ml distilled water, the labeled DA was eluted with 5 ml 0.2M acetic acid. The eluate was concentrated by lyophilization, the amine taken up in 0.15 N tartaric acid and stored in the freezer. The purification procedure eliminated the contaminating peak and reduced blank values to satisfactory levels.

Another problem encountered was variability in duplicate samples. Since only one filtering stand was available for all samples, the samples had to be kept on ice for varying periods of time before they could be filtered; this process took up to an hour. This might have been contributing to the variability. Cross-contamination had been eliminated as a factor. The use of a multi-sample filtering apparatus (Millipore 3025 Sampling Manifold) eliminated this problem. Samples were incubated in staggered fashion and filtered immediately after the incubation period without being kept on ice.

When time of incubation was varied, incorporation was linear over 10 minutes at 37°C, with decreased incorporation after 15 minutes. This may be a reflection of the fragile nature of synaptosomes which can break down if kept at high temperatures for long periods of time. This is similar to the phenomenon noted by Philippu and Beyer (1973) for caudate vesicles. When incubated with ^{14}C-DA at 25°C in the presence of ATP and magnesium, the vesicles showed maximum uptake after 20 minutes, and this level remained fairly constant for another 20 minutes. At 37°C, uptake reached a peak after 2.5 minutes and then gradually declined. The authors attributed this to the thermo-lability of the vesicles. This may be the reason that in the present experiment greater incorporation was found when no pre-incubation period was used. The synaptosomes may begin to break down during the pre-incubation period. Since a longer incubation period would make the handling of many samples more convenient, the incubation temperature was decreased to 27°C and the incubationtime increased to 15 minutes. No pre-incubation period was used.

Final Procedure

The final method used was a modification of the filtration method of Baldessarini and Vogt (1971). Rats with frontal or sham lesions were decapitated four weeks postoperatively. The striatum was quickly dissected and homogenized in 20 volumes of ice-cold 0.32 M sucrose in Thomas AA59 homogenization tubes, using 8 up-and-down strokes.

The homogenates were centrifuged in the cold at 1000 g for 10 minutes to remove nuclei and heavy cell debris. The supernatants (S_1), containing synaptosomes, among other cellular structures, were stirred gently and diluted with Krebs-Ringer solution to make a concentration of 2 mg wet weight tissue/0.8 ml. Aliquots of this solution were removed for incubation.

The total incubation volume was 1 ml and consisted of (a) 0.8 ml crude synaptosomal suspension containing synaptosomes derived from 2 mg wet weight tissue, (b) 5.0×10^{-11} moles H^3-DA equivalent to 0.4 μCi delivered in a volume of 0.1 ml Krebs-Ringer solution, (c) an appropriate concentration of unlabled DA delivered in a volume of 0.1 ml Krebs-Ringer solution so as to make the final total DA concentration 1×10^{-7}M, 2×10^{-7}M, 5×10^{-7}M, or 9.5×10^{-7}M.

Samples were kept on ice until incubated; the H^3-DA was added just before incubation. Samples were incubated with constant shaking for 15 minutes at 27°C. Control tubes containing both tissue and labeled amine were treated identically, but kept on ice.

After incubation, samples were filtered immediately using Millipore filters, 25mm in diameter with a pore size of 0.8 μm (Millipore Corp., Bedford, Mass.) which had been pre-moistened by soaking in Krebs-Ringer solution. After filtration, each filter was washed with 10 ml Krebs-Ringer solution, transferred to a counting vial, and dissolved by the addition of 1 ml Cellosolve (Fisher). Ten ml scintillation fluid (Bray's) was added to each vial, and samples were counted.

Results

Results were analyzed using the Lineweaver-Burk equation, which is a linear transformation of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_T}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

In this plot, the slope is $\frac{K_T}{V_{max}}$ and the y intercept represents $\frac{1}{V_{max}}$

For each animal, the velocity of uptake was measured at four different substrate concentrations. The method of least squares was used to calculate the best straight line describing the plot of $\frac{1}{v}$ against $\frac{1}{[S]}$. Thus, a value of K_T and Vmax was found for each individual animal.

As can be seen in Table 6, the K_T for the striatal synaptosomal uptake of DA was found to be 1.2×10^{-7}M in sham animals. This agrees quite well with values found in the literature. Harris and Baldessarini reported a K_T of 1×10^{-7}M in one paper (1973a), and 2×10^{-7}M in a second paper (1973b). Snyder and Coyle (1969) found a higher K_T of 4×10^{-7}M.

The Vmax in the present experiment cannot be compared with others in the literature since the temperature of incubation differed. This would have a large effect on the velocity of uptake. K_T may also show temperature dependency.

There was no significant difference between frontal and sham animals in either the Vmax or K_T of synaptosomal uptake.

TABLE 6

KINETIC CONSTANTS OF STRIATAL SYNAPTOSOMAL UPTAKE

	Sham	Frontal
Vmax*	13.1 ± 2.0 (N=7)	13.6 ± 1.7 (N=8)
Km x 10^{-7}M	1.2 ± 0.2 (N=7)	1.8 ± 0.2 (N=8)

*Vmax is in pmoles/mg/15 min at 27°C

Values are shown ± SEM

Discussion

Kinetic analysis of the uptake of H^3-DA into striatal synaptosomes did not reveal any significant differences between frontal and sham animals four weeks after surgery. If Vmax is indeed a measure of the number of uptake sites and if the number of uptake sites is correlated with the number of nerve terminals, then the data do not support the hypothesis concerning the collateral sprouting of the dopaminergic system in frontal animals. Still, because of problems in methodology, this hypothesis cannot be discarded with certainty.

The most direct method of demonstrating the collateral sprouting of a catecholaminergic system is via histochemical fluoresence. In this method, tissue specimens are treated with formaldehyde gas resulting in fluorescent monoamine products (Falck, 1962). Nerve terminals containing monoamines can then be visualized under the fluorescence microscope. However, this method cannot be used to detect increases in innervation when control levels are very dense (Moore et al., 1974). Since the dopaminergic innervation of the striatum is very dense, a method was chosen that could be quantitated and would be more likely to detect differences between experimental groups.

There are many precedents in the literature for the use of synaptosomal uptake as an index of the number of nerve terminals present. However, the differences examined were usually much greater than the one expected in the present study.

Many investigators have used synaptosomal uptake to trace regeneration of nerve terminals. For example, Jonsson and Sachs (1972), after producing

a chemical sympathectomy in mice by the administration of 6-OH-DA, used this method to study the regeneration of adrenergic nerve terminals in irises and atria. They found a very good correlation between terminal regeneration measured by fluorescence histochemistry and the development of H^3-NE uptake. H^3-NE uptake was very low immediately following sympathectomy and increased to 80% of control levels after four weeks. K_T values were the same in control and experimental groups, while the Vmax differed.

Coyle and Axelrod (1971) traced the development of rat CNS adrenergic systems from 14 days of gestation to adulthood using synaptosomal H^3-NE uptake as an index of the number of nerve terminals. Kinetic analysis revealed that the Vmax of uptake reached adult levels by 28 days of age.

Kuhar, Aghajanian, and Roth (1972) made lesions in the midbrain raphe nuclei of rats, thus interrupting serotonergic pathways which innervate forebrain areas. They reported a very good correlation between reduction in 5-HT levels, tryptophan hydroxylase activity, and synaptosomal uptake of H^3-5HT in different regions of the brain. Fluorescence histochemical estimates of 5-HT terminals paralleled the biochemical results.

These examples in the literature suggested that the method of synaptosomal uptake would be sensitive enough to allow detection of a small increase in DA innervation. In most other investigations, comparisons were made between groups with normal innervation and groups with little or no innervation. Since in the present study, there was only a 36% increase in DA levels, the expected difference between groups was comparatively small. Furthermore, since the normal striatal DA innervation is very high, it might make it more difficult to detect differences. It may be that synaptosomal uptake, like histochemical fluorecence is not sensitive enough to detect increases when baseline levels are high.

Synaptosomes are very fragile, and even when great care is used in their preparation, the yield may be quite variable. Thus, it has been the experience in this laboratory and others (Barker, Johnson, personal communications) to find considerable variability from day to day in the determination of Vmax, whereas estimations of K_T remain quite consistent. Naturally, this would make moderate differences difficult to detect.

The possibility that synaptosomal uptake is not specific enough to detect changes in one amine system must be considered. For example, in some instances, the CA uptake mechanism may not be able to discriminate between DA and NE. Synder and Coyle (1969) did a regional analysis of the uptake of H^3-DA and H^3-NE into rat brain synaptosomes. In extrastriatal areas, DA competitively inhibited the uptake of NE with an inhibitory constant (Ki) equal to its own affinity constant (K_T), suggesting that the two amines are using the same transport system. Paradoxically, the specific uptake system for NE appears to have a greater affinity for DA (reflected by a lower K_T) than for NE. The K_T for DA was lower than that for NE in all regions tested, even in predominantly noradrenergic areas such as the hypothalamus. The striatum showed a lower affinity for DA than the other areas tested. NE appeared to use the DA uptake system in the striatum, since it competitively inhibited the uptake of DA into striatal synaptosomes with a Ki equal to its K_T. Probably because of factors such as these, the Vmax for NE was not correlated with the amount of endogenous NE present. Thus, the highest Vmax for NE was found in the striatum, and there was not much difference between areas high in NE and areas comparatively low in this amine. The striatal uptake

system showed a much greater affinity for DA than for NE, and thus appears to be specific for DA. Furthermore even though NE nerve terminals appear to concentrate DA very efficiently, the contribution of such terminals in a population of striatal synaptosomes would be minimal.

DA and NE are very similar in structure. This probably accounts for the relative nonspecificity between them. In other instances, synaptosomal uptake appears to be rather specific when low concentrations of amines are used. Many investigators have found evidence of two uptake mechanisms at the synaptosomal membrane. The high affinity mechanism is specific and operates at low amine concentrations. The low affinity system is nonspecific and will take up many different amines if they are present in high concentrations.

Coyle and Snyder (1969) found evidence of high and low affinity mechanisms for DA in synaptosomes taken from different brain regions. Double-reciprocal plots of uptake vs DA concentration revealed two linear components differing widely in their affinity constants. However, only one component was evident in synaptosomes prepared from the striatum.

Kuhar, Roth and Aghajanian (1972) found a selective decrease in the synaptosomal uptake of low concentrations of 5-HT after making lesions in a serotonergic pathway of the brain. This decrease was not as great if the concentration of 5-HT in the incubation medium was increased, suggesting that at high concentrations, the 5-HT was being accumulated by non-serotonergic terminals. Furthermore, the uptake of DA or NE was not affected by these lesions. If lesions were made in a non-serotonergic pathway of the brain, the uptake of 5-HT was not reduced. These results indicate that at low incubation concentrations, 5-HT is selectively taken up by synaptosomes originating from serotonergic neurons.

Similarly, Zigmond et al. (1971) made lesions in the lateral hypothalamus of rats and found a decrease in H^3-NE uptake by telencephalic synaptosomes, along with a decrease in NE levels. The lesion did not affect the uptake of H^3-serine. Furthermore, interruption of a serotonergic pathway by lesioning the central grey area of the mesencephalon resulted in a decrease in forebrain 5-HT levels, but not in the degree of synaptosomal H^3-NE uptake.

The nature of collateral sprouts may make them more difficult to detect than normal nerve terminals. For example, they may be more fragile than other terminals and therefore their yield may be less. Similarly, they may not function as normal terminals and thus not contribute an appropriate number of uptake sites. Furthermore, Raisman (1969), in studying the collateral sprouting of the medial forebrain bundle input to the septal nuclei after a lesion of the fornical input, found an increase in the number of double synapses and multiple contacts. This would not involve growth of new terminals, but extensions of already existing ones. Thus, although a new system is reinnervating a denervated structure, such an extension of synaptic area would not result in new terminals and could not be detected in an experiment on synaptosomal transport. However, such reinnervation might result in larger synaptosomes. If this were the case, the larger synaptosomes, having different sedimentation properties, might have been lost. Laverty et al. (1963) found that this could happen when they studied the distribution of synaptosomes upon fractionation of dog caudate nucleus. Apparently the striatal synaptosomes of the dog have a very wide range of sizes. The first pellet (P_1), obtained by centrifuging striatal homogenates at 1000g, was found by electron microscopic examination to contain large synaptosomes.

Thus, these large synaptosomes would be absent from the crude synaptosomal fraction after this initial centrifugation. This might occur with synaptosomes that are enlarged due to the extension of synaptic contacts.

There is the question of whether endogeneous levels of DA affect uptake. That is, will a synaptosome with higher initial DA levels have the same uptake properties as normal synaptosomes, or would there be less uptake due to the partial saturation of storage sites. Some investigators think it is likely that endogenous stores of amine are partially depeleted when the tissue is homogenized in amine-free sucrose (Baldessarini and Vogt, 1971). Raiteri and Levi (1973) have found that some amines are depleted when synaptosomes undergo sudden cooling, as they certainly do when the tissue is homogenized in ice-cold sucrose. A related phenomenon was studied by Philippu and Beyer (1973). They found that when striatal subcellular vesicles were incubated at 37°C with no exogenous amine, the endogenous DA stores were sharply reduced. When they were incubated in the presence of ^{14}C-DA, they actively transported the exogeneous DA, but the endogenous stores still fell to very low levels. Thus, at least at the vesicular level, endogenous stores are not maintained and would therefore appear unlikely to affect maximal uptake.

Collateral sprouting is not a specific process. Raisman (1969) found that more than one system sprouted to reinnervate the septal area after removal of the hippocampal input. If there are many afferent systems innervating the target, there may be competition for the denervated sites and this could make it more difficult to detect changes in one specific

system. It is likely that many systems that have favorable positions in relation to the denervated sites will make new contacts.

On the other hand, not all afferents to a partially denervated structure are in a position favorable for sprouting. There are examples of negative results in the literature and possible reasons. Moore et al. (1974) caution that there may be several prerequisites for the phenomenon to occur. First, the projection removed must be considerable. Removing the hippocampal input to the lateral preoptic area does not result in the sprouting of the adrenergic innervation (Moore et al., 1971). This may be because the hippocampal innervation is not heavy enough. A second rule is that the sprouting system must also be of a significant density. The removal of the heavy hippocampal projection to the lateral mammillary nucleus does not result in the sprouting of the sparse adrenergic input (Moore, Björklund, and Stenevi, 1974). However, in our system, the dopaminergic projection to the striatum is considerable.

Proximity may also be an important factor. The sprouting system must have a favorable position in the synaptic arrangement in relation to the denervated sites and the other systems present. For example, adrenergic sprouting is not found in the dorsal lateral geniculate nucleus after retinal afferents are removed, but is found after the cortical afferents to this nucleus are removed (Stenevi et al., 1972). Perhaps the adrenergic terminals are more proximal to the corticogeniculate synapses whereas other systems are in a better position to reinnervate the retinogeniculate synapses.

If the nigrostriatal system does not sprout after frontal lesions, it may be because of the synaptic arrangement in the striatum. Important factors may be which afferents terminate in the same region of the nucleus,

which end on the same neurons, and whether they make axodendritic or axosomatic contacts.

If DA were not preferentially localized in the nerve terminal, increased DA levels would not necessarily be correlated with an increased number of DA terminals. Laverty et al. (1963) concluded from their experiments on the dog caudate nucleus that DA has primarily a cytoplasmic localization. DA was found predominantly in the high speed supernatant (63%) as opposed to the particulate fraction, and had a distribution similar to that of lactate dehydrogenase, a cytoplasmic marker. In contrast, caudate ACh and 5-HT, and hypothalamic NE were recovered primarily in the particulate fraction.

The geometry of synapses may be important in determining whether or not synaptosomes form upon tissue homogenization. Synaptic endings arising from the axon terminal, called boutons terminaux, are the type most frequently seen in electron micrographs. When presynaptic elements occur along the length of the axon, they are called boutons en passant. This latter form may not shear and seal as well as boutons terminaux and thus may not form synaptosomes. Were reinnervation to occur by the formation of boutons en passant, such structures might be undetectable by measuring the synaptosomal transport of DA.

GENERAL DISCUSSION

Even though there are reservations concerning the negative results of the synaptosomal uptake experiment, these negative results will have to be accepted in formulating the final conclusions. Perhaps more sensitive methods could answer the question more definitively in the future. Histological examination would be the most direct and the most desirable. A study on the electron microscopic level might reveal whether there is a change in the synaptic arrangement of the striatum, such as an increase in multiple synaptic contacts. Lesioning the nigro-striatal system four weeks after frontal cortical ablation might reveal an increased number of degenerating terminals upon electron microscopic examination. This would be strong evidence that the nigro-striatal system had undergone collateral sprouting.

Further examination might reveal that other striatal systems had sprouted. Nonspecific sprouting could be measured histologically or by measuring occluded lactic acid dehydrogenase (LDH). LDH is a cytoplasmic marker. That fraction of LDH enclosed within synaptosomal particles reflects size or quantity of synaptosomes. If other systems had sprouted, the animals might display changed sensitivity to drugs which release these transmitters, though not necessarily to direct agonists.

At present the increase in striatal DA can best be seen as the result of an attempt to compensate for the imbalance in afferents to the striatum after the frontal projection is removed. Frontal animals were found to have slightly lower levels of DOPAC than sham animals four weeks post-operatively. Reduced metabolism could have contributed to a moderate increase in DA levels. Transsynaptic mechanisms could be invoked to account for the extended time-course of the phenomenon. One could hypothesize

that the striato-nigral fiber system is involved in mediating the effect. As already discussed, this system is thought to play a role in modulating nigral activity in relationship to the level of activity in the striatum. The hypothesis could be tested by lesioning the striato-nigral system and studying the effect of this or DA levels both before and after frontal lesions.

A definitive answer to this question would require a very complete time-course of DA levels, rate of synthesis, and rate of metabolism. No significant differences in metabolite levels were found three days after surgery. A direct approach to the question would be to measure electrophysiological activity in the nigro-striatal system both before and at various times after frontal lesions. Complimentary experiments would involve stimulating the frontal cortex in non-lesioned animals and examining the effect of this on DA turnover and on electrical activity in the nigro-striatal system. Some studies of this type have already been described. For example, cortical stimulation has been found to have an excitatory effect on striatal neurons (Liles, 1973; Buchwald et al., 1973).

Apparently end-product inhibition does not prevent the rise in DA levels although it is believed to contribute to the control of DA synthesis. This mechanism acts to decrease amine synthesis in response to increased amine levels, but is not sensitive enough to prevent levels from rising in the first place. For example, after the administration of an MAO inhibitor, endogenous levels of DA rise, and the rate of DA synthesis is reduced (Javoy et al., 1972). The decrease in DA synthesis after MAO inhibition does not prevent DA levels from rising high above normal levels.

A related phenomenon has already been described in the work of Roth et al. (1973). They found an increase in DA levels associated with a decrease in dopaminergic activity, and the increased levels do not appear to be affected by end-product inhibition. They attribute this either to the relative insensitivity of end-product inhibition or to the accumulation of DA in pools which do not have access to tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis.

Of course, there is probably more than one process occurring after frontal lesions. The theory that denervation supersensitivity occurs might be explored further by administering drugs that stimulate the receptor directly, rather than indirectly by releasing endogeneous amine stores. Something similar to this was done by Fuxe and Ungerstedt (1970). These investigators made transections at different levels in front of bregma, thus separating frontal from posterior area of the brain. They then administered both apomorphine, a direct DA agonist and cataprezan, a direct NE agonist. Lesioned animals displayed increased sensitivity to the behavioral effects of the drugs, i.e. increased exploratory behavior (rearing and running) and increased aggressive behavior. This behavior could be explained by a denervation supersensitivity to DA and NE resulting from the lesion. However, the authors interpreted it as the result of removing inhibitory cortical projections to other brain areas, especially the limbic system. Studying drug sensitivity at several time points after lesioning might help to distinguish between the two possibilities. Denervation sensitivity has been found in many systems to take around four weeks to reach its maximum. The effect of

removing a projection would be expected to reach its end-point after a much shorter period of time. Again electrophysiological examination would be more direct. DA could be injected into the striatum via microiontoelectrophoresis and the response of striatal neurons could be compared in frontal and sham groups.

If the transmitter of the fronto-striatal system were known, it would suggest a whole series of experiments similar to the ones performed by Ungerstedt (1971a) and others afer lesioning the nigro-striatal system. For example, animals could be tested for the development of denervation supersensitivity to agonists of the transmitter after frontal lesions. Furthermore, it would be very interesting to see whether such drugs antagonize the effects of amphetamine and the functions of the nigro-striatal DA system. Chronic administration to frontal animals might antagonize the development of hypersensitivity to amphetamine. This would suggest that a nonspecific denervation supersensitivity plays a role in the changed response to amphetamine. The agonistic agent, by mimicking the normal state, might also antagonize the increase in DA levels that takes place after frontal lesions.

In order to explain how frontal lesions affect the nigro-striatal system, it has been necessary to postulate the operation of feedback mechanisms such as neuronal feedback loops. This is because the system as described is not equivalent to a peripheral denervation. Although the striatum is partially denervated, changes are noted in a system which originates in the substantia nigra. In order to examine the way in which the dopaminergic nigro-striatal system reacts to being denervated, it would be necessary to lesion an area which projects heavily to the pars compacta

of the substantia nigra and subsequently study DA function in the striatum. This would be equivalent to making a preganglionic lesion and studying the postganglionic neuron.

Although steady-state levels of DA were higher in frontal animals, metabolite studies showed that the activity of the system is kept within normal limits or below. Thus, under non-drug conditions, the DA-mediated behavior of the animal is kept near normal. However, when the CA-releasing drug amphetamine is administered, the increased stores are released. This could account for the hypersensitivity to some of the effects of the drug shown by frontal animals.

This would not explain all the changes shown by frontal animals to amphetamine. As already discussed, frontal animals may display hypo- or hypersensitivity to the drug according to the task involved. There may be many reasons for this. First of all, amphetamine affects both noradrenergic and dopaminergic systems (Carlsson, 1970; Fuxe and Ungerstedt, 1970), causing release of CA and inhibiting re-uptake. Furthermore, there are many different NE and DA systems in the brain, presumably serving different functions. The effects of amphetamine are not limited to the release and inhibition of re-uptake of the catecholamines. It also inhibits monoamine oxidase (Glowinski, Axelrod, and Iversen, 1966). This may cause it to effect other biogenic amines in the brain. It may also have a direct post-synaptic effect (Feltz and DeChamplain, 1973). The direct effect is minor but might increase after the development of denervation supersensitivity. However, striatal DA has been implicated in the locomotor and stereotypic effects of amphetamine (Creese and Iversen, 1975). Increased levels of striatal DA in frontal animals could be expected to modify their response to the drug.

SUMMARY

Frontal cortical lesions are known to result in hypersensitivity to the locomotor effects of the catecholamine-releasing drug amphetamine. This hypersensitivity increases with time after surgery, asymptoting approximately four weeks post-operatively. It was therefore hypothesized that frontal lesions would effect catecholamine systems in the brain.

Frontal lesions resulted in a temporary lowering of hypothalamic NE levels one week after surgery; NE levels returned to control values by two weeks after surgery. Striatal DA levels first showed an increase two weeks postoperatively and increased still further four weeks after the lesion.

It was suggested that a small decrease in the rate of DA metabolism could lead to a gradual increase in DA levels. Frontal animals were found to have less striatal DOPAC four weeks after surgery. However, metabolite levels three days after surgery showed no significant differences between frontal and sham animals. It was decided that an extensive time-course of DA metabolism would be necessary to confirm or deny the hypothesis.

Denervation supersensitivity was also considered. If the partially denervated striatum became more sensitive to DA, it would result in a decrease in DA metabolism by feedback mechanisms. This could cause DA levels to rise as supersensitivity gradually progressed. Testing this hypothesis would also require a complete time-course of postoperative DA metabolism as well as drug sensitivity studies using DA agonists.

The major weakness of the above hypotheses is that they do not account for the two week delay before the increase in DA levels becomes evident. Collateral sprouting would account for a two week delay followed by increases at least until four weeks after surgery. It was suggested that the nigrostriatal system sprouted in response to the partial denervation of the

striatum by frontal lesions. This hypothesis was tested by measuring the striatal synaptosomal uptake of H^3-DA. The resuls were inconclusive, but the results of experiments using a more sensitive method might be more definitive.

If the increase in DA levels is seen as the result of a decrease in DA metabolism, transsynaptic mechanisms could be invoked to account for the delay. Several systems leading indirectly from the frontal cortex to the substantia nigra were described and would have to be investigated.

It was concluded that the increased levels of striatal DA in frontal animals may help to explain their hypersensitivity to the locomotor effects of amphetamine. However, under non-drug conditions, the decreased turnover of striatal DA relative to the larger pool size tends to keep the DA-mediated behavior of the animal within normal limits.

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