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The effect of L-dopa on levels of uric acid in the striatum of the rat as measured by in vivo electrochemical detection and its implications for dopamine-adenosine interactions

Togasaki, Daniel Minoru, Ph.D.

City University of New York, 1991

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THE EFFECT OF L-DOPA ON LEVELS OF URIC ACID IN THE STRIATUM OF THE RAT AS MEASURED BY IN VIVO ELECTROCHEMICAL DETECTION AND ITS IMPLICATIONS FOR DOPAMINE-ADENOSINE INTERACTIONS

by

DANIEL MINORU TOGASAKI

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment for the requirements for the degree of Doctor of Philosophy, The City University of New York.

1991

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Abstract

THE EFFECT OF L-DOPA ON LEVELS OF URIC ACID IN THE STRIATUM OF THE RAT AS MEASURED BY IN VIVO ELECTROCHEMICAL DETECTION AND ITS IMPLICATIONS FOR DOPAMINE-ADENOSINE INTERACTIONS

by

Daniel Minoru Togasaki

Adviser: Peter J. Knott, Ph.D.

This study used in vivo electrochemical detection to examine the level of extracellular uric acid in the striatum and its relationship to circling behavior in male Sprague-Dawley rats. Electrochemical measurements were made with carbon paste electrodes and a BAS DCV-5 voltammetry controller using the technique of semidifferentiated linear sweep voltammetry to examine "Peak 2". Preliminary experiments optimized electrode construction techniques and methodology.

Electrodes were bilaterally implanted into the striata of rats that had undergone unilateral lesioning of their dopaminergic nigrostriatal neurons using 6-hydroxydopamine. Three days after surgery, the rats were monitored by simultaneously measuring circling behavior and Peak 2 height during 9 ten-minute sessions before and after injection of L-dopa (three hours total). L-Dopa induced circling behavior away from the lesioned side and also an increase in the height of Peak 2 that was larger on the lesioned side.The

amount of circling behavior correlated highly with the asymmetry in the Peak 2 changes, suggesting a strong relationship between them.

Experiments using HPLC techniques to monitor striatal neurochemistry showed, in lesioned rats, that L-dopa increased turnover of dopamine in the striatum, but not serotonin.

Other experiments used an electrode/cannula assembly to monitor Peak 2 while simultaneously microinjecting substances into adjacent striatum. These experiments identified Peak 2 as uric acid and showed that its height was increased by microinjection of adenosine, suggesting that the uric acid is derived from local adenosine metabolism. L-Dopa microinjection also caused an increase in Peak 2, indicating that it affects uric acid levels by a locally mediated mechanism. Further experiments using 2'-deoxycoformycin, carbidopa, Ro4-4602, haloperidol, and apomorphine were inconclusive.

The use of in vivo electrochemical detection and electrode/cannula assemblies is discussed. The role of adenosine in striatal physiology and circling behavior is discussed regarding these results and possible clinical implications for Parkinson's Disease and Lesch-Nyhan Syndrome. The appendix includes a preliminary report of a study investigating the effect of dietary caffeine on Parkinson's Disease.

In summary, this study shows a relationship between striatal uric acid levels and circling behavior and suggests it is mediated by adenosine/dopamine interactions.

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SECTION 1 — INTRODUCTION

The relationship between neurotransmitter and behavior has been the subject of many investigations. One of the major systems studied has been the dopaminergic nigrostriatal system. This pathway has a clear relationship to certain clinical syndromes, so it has been extensively analyzed. Nevertheless, precise definition of the connection between dopaminergic dysfunction and motor abnormalities in man remains an elusive goal. One of the problems is the complexity of the interconnections of the circuitry of the striatum (Bolam 1984, Dray 1980, Kitai 1981, Pasik and Pasik 1981, Pasik, Pasik and DiFiglia 1979, Pycock and Phillipson 1984), and the large number of neurotransmitters, neuromodulators, and other assorted putative neuroactive substances associated with it (Dray 1980). The precise effects dopamine has in the striatum are still being defined.

In recent times, methodological advances have made it possible to investigate the relationships between neuronal activity, neurotransmitter release, and behavior. This makes possible the discovery of new and more complex interrelationships between these components and allows for a deeper and richer understanding of the workings of the nervous system. The work described here uses one of these techniques, in vivo electrochemical detection (IVED), to gain a fuller understanding of these relationships. This study centered on using rats for investigating the effects of drug treatments on animal behavior and on the physiology of striata that are both intact and that have been denervated by destruction of the dopaminergic nigrostriatal pathway.

I will first review some historical background in order to place this research in the proper context. A complete description of the methodology of in vivo electrochemical detection will be presented. I will then describe the experimental protocols and results. And finally, I will discuss how this work fits into the existing body of scientific literature,

and its clinical implications, including the preliminary results of a clinical investigation of patients with Parkinson's disease undertaken as a direct result of this work.

PARKINSON'S DISEASE

James Parkinson first described the disease that bears his name in his *Essay on the Shaking Palsy* (1817). Parkinson's disease, also known as paralysis agitans, is a neurological movement disorder that has three characteristic, "classic", symptoms: resting tremor, muscular rigidity, and bradykinesia. Other associated symptoms include a stooped posture, micrographia, difficulty speaking and swallowing, and a shuffling gait in which the patient takes many rapid short steps (known as festination). The disease, in its idiopathic form, usually has a gradual onset, with symptoms initially apparent only as a mild feeling of weakness or slight with difficulty walking. In order to make the diagnosis of Parkinson's disease with certainty, the presence of the classic triad of symptoms must be present. In most cases, tremor is first to appear, followed by rigidity and bradykinesia. Frequently, the problems start first in one arm or hand, later spread to the other extremities, and finally involve the trunk and face.

This process usually evolves over a number of years. In later stages, the patient often becomes demented as well. The patient gradually becomes more and more debilitated until he is confined to being bedridden and is unable to perform any motor activities. Death usually occurs from a complication, most commonly aspiration pneumonia due to loss of motor control of swallowing.

Epidemiology

Parkinson's disease usually occurs in the elderly; the average age of onset is approximately sixty years old, and it is rare for a patient to be less than forty. It is estimated to affect one percent of the U.S population over fifty years of age. Its precise etiology is unknown.

As a matter of nomenclature, parkinsonism refers to a disease including some or all of the symptoms above, whereas Parkinson's disease refers to a specific subset of parkinsonism in which there is no immediately identifiable etiology. Other types of parkinsonism include drug-induced parkinsonism, such as from neuroleptic treatment, post-encephalitic parkinsonism, which followed an epidemic of flu cases in 1918, and pugilistic parkinsonism, a probable cause of Muhammed Ali's parkinsonism. In all of these cases, the underlying lesion is a loss of the nigrostriatal neurons.

Pathology

The involvement of the substantia nigra in parkinsonism has been known for many years. This was easily done, before the discovery of many of the neuroanatomical and neurochemical techniques of today, because the nigral cells are pigmented. The loss of pigment in this brain nucleus, and thus loss of nigral cells, was easily seen in gross autopsy investigation. Another pathologic feature is the histologic demonstration of Lewy bodies, which are eosinophilic cytoplasmic inclusion bodies.

A major increase in our understanding of parkinsonism resulted from the demonstration of an association between the disease and the loss of dopamine from the striatum. This became possible in the 1950s, when neuroanatomical and neurochemical techniques were developed for examining catecholaminergic pathways in the brain. The demonstration of the loss of dopamine in the striatum was reported in 1960. This indicated that a major pathological sign of this disease, perhaps the one responsible for the movement disorders, is the death of the dopaminergic nigrostriatal neurons. (For a historical perspective see Duvoisin 1984, references in Hornykiewicz 1974.)

L-Dopa Treatment

The loss of dopamine suggested that replacement of this neurotransmitter from exogenous sources may prove to be a useful mode of treatment. Attempts to treat the disease by administering dopamine were unsuccessful, however, because the catecholamine does not cross the blood-brain barrier. The administration of a precursor

was the next step, and, after a series of trials, it was discovered that L-dopa (L-dihydroxyphenylalanine), dopamine's immediate precursor, alleviated the symptoms. Its clinical utility was hinted at in early studies (Birkmayer and Hornykiewicz 1961) and finally unequivocally demonstrated in 1967 (Cotzias, Van Woert and Schiffer 1967). Current treatment with L-dopa (Bianchine 1980, Duvoisin 1984) usually involves co-administration of a peripheral decarboxylase inhibitor, carbidopa, which prevents conversion to dopamine outside the brain. This has the two-fold benefit of reducing the required dose, and eliminating unwanted side effects from peripheral dopamine production. This combination is administered as a commercial preparation known as Sinemet™. The proposed mechanism of action is that L-dopa crosses the blood-brain barrier and enters the brain, where it is decarboxylated by the enzyme dopa decarboxylase (L-amino acid decarboxylase); the dopamine formed compensates for the loss in the striatum (Hornykiewicz 1974).

There have been extensive studies examining how and where the L-dopa is decarboxylated to dopamine (Melamed, Hefti and Wurtman 1980b). It is still not clear where the reaction occurs, although there are indications (and counter-indications) that it occurs in the remaining dopaminergic terminals (Hornykiewicz 1974), the serotonergic nerve terminals that are present in the striatum (Hornykiewicz 1974), the local capillary endothelial cells (Ungerstedt 1971b), or the intrinsic neurons of the striatum (Melamed et al. 1981).

With time, however, L-dopa loses its effectiveness, and an "on-off" phenomenon appears, in which the patient experiences abrupt shifts between clinical improvement and full blown symptomatology. Another frequent side effect is the development of dyskinesias and athetoid movements. The exact cause of these phenomena is unknown (Bianchine 1980, Melamed et al. 1983, Spencer and Wooten 1984a). It has been hypothesized that the loss of efficacy of L-dopa is due to the progression of the disease, and the continuing loss of nigrostriatal cells; only the patient's symptoms are being treated

with the drug, and not the underlying disease process (Markham and Diamond 1981). Another school of thought is that L-dopa induces an undefined change in the patient leading to the loss of effectiveness (Yahr 1976). In any case, it is clear that, although L-dopa is effective, its benefit is limited to a period of 3-5 years for an average patient before side-effects begin to develop (Yahr 1984). This is something of a point of controversy in that if L-dopa induces changes that lead to a loss of effectiveness, then the use of the drug should be delayed as long as possible in the course of the disease. If the loss of effectiveness is a result of the continuing progression of the disease, then it would make sense to start the drug at an early stage of the disease, so that the patient can derive the maximum length of time of benefits.

There are other drugs used to treat parkinsonism. Anticholinergics are commonly used, especially in the early stages and as a adjunct to L-dopa treatment. This class of drugs, however, is less effective than L-dopa.

Further improvements in the treatment of Parkinson's disease are the subject of much current clinical research. I will now turn to a discussion of the most recent advances in the understanding of Parkinson's disease.

Recent Research Advances

MPTP

In the early 1980s a number of neurologists in the San Francisco area noticed a sudden spate of patients who had developed symptoms of parkinsonism, despite their youth (Langston et al. 1984). They were found to have unusually severe symptoms of relatively acute onset. Two of the patients were brothers who were found in a state close to complete immobility because of their bradykinesia and rigidity. These patients responded exceptionally well to treatment with L-dopa, and went from a state of immobility to being ambulatory.

Investigation of the cause of these unusual cases led to the connection that all were drug abusers who had taken a "designer drug" that was a "synthetic heroin". These

designer drugs are made by enterprising chemists who synthesize substances that are structurally related to existing illicit substances, and possessing the desired properties of the illegal drugs. For a drug to be illegal, the chemical substance must be specifically outlawed. Because of the difference in chemical structure, however, designer drugs are legal. All of the patients suffering from this parkinsonian syndrome were deduced to have obtained their drug from a single supplier. By chance, a colleague of the investigators remembered a case similar to these that had been reported in 1979 (Davis et al. 1979) in which a graduate student had ingested a home-synthesized meperidine analogue known as MPPP. This patient subsequently died from a drug overdose and was found, at autopsy, to have degeneration of the substantia nigra, and a loss of the dopaminergic nigrostriatal system.

When the investigators attempted to search the literature on MPPP, they discovered that all of the pertinent articles in the Stanford library had been carefully removed from the journals. The enterprising chemist had evidently removed them for his own information and to prevent the development of any competition. The chemical actually responsible for the disease was uncertain, however, because the meperidine analog itself did not induce parkinsonism. It was deduced that the chemist had attempted to alter the synthesis and purification procedures by increasing the temperature in order to speed up the reaction and increase his yield. In doing so, he accidentally synthesized a by-product: 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Further investigation showed that monkeys injected with MPTP developed a syndrome resembling parkinsonism, which responded to treatment with L-dopa (Langston et al. 1984). Thus, MPTP was identified as the causative agent. Strangely enough, MPTP was discovered to be a previously known chemical that had been synthesized by Roche and investigated a number of years ago. It had even been screened in clinical trials as a treatment for Parkinson's disease, and was discovered to be ineffective.

This was a very exciting development because it meant that the scientific investigation of Parkinson's disease finally had a clue in the investigation of the etiology and treatment. The development of an animal model that closely approximated the disease was a major advance (Heikkila, Hess and Duvoisin 1984, Langston et al. 1984). This has greatly stimulated research in the field, and a spate of activity started and the field rapidly expanded. (The background information in this section has been condensed from news reports in Science: Lewin 1984a,b.)

Amyotrophic Lateral Sclerosis-Parkinsonism-Dementia

The Chamorro people, the indigenous natives of Guam, have a high incidence of developing an unusual syndrome that includes symptoms of amyotrophic lateral sclerosis, parkinsonism, and dementia. Early studies suggested a connection with the ingestion of an "unusual" non-protein amino acid that is found in a seed of the plant Cycas circinalis, which was traditionally used as a medicine and food source prior to the Americanization of the island following World War II. This amino acid is β-*N*-methylamino-L-alanine (L-BMAA). Investigators, however, were unable to prove this connection. Recently, Peter Spencer and his colleagues at Albert Einstein were able to produce a similar syndrome in monkeys by prolonged feeding of L-BMAA (Spencer et al. 1987). A recent report indicated that in a medium containing bicarbonate ion, L-BMAA is neurotoxic (Weiss and Choi 1988).

As with MPTP, the implication is that an environmental agent can cause parkinsonism. In this case, other neurological diseases accompany the parkinsonian symptoms.

Environmental Etiology

The cause of Parkinson's disease (idiopathic parkinsonism) has been a mystery. The discovery of MPTP suggested that a toxin could be a possible etiologic factor. This was reinforced by the uncovering of the connection of Guam amyotrophic lateral sclerosis-parkinsonism-dementia and a plant neurotoxin. Another interesting fact is that the first

description of the disease was in 1817 by James Parkinson. Even though the symptoms are quite distinctive, no one had previously put them together as a specific disease. The suggestion here is that Parkinson's disease is a relatively recent phenomenon. Perhaps it is a by-product of the industrial revolution and the many man-made substances that it unleashed into the environment.

Some investigators hypothesized that Parkinson's disease may be a result of the exposure to a toxin that destroys some of the cells of the nigrostriatal system, and that cumulative exposure, coupled with the normal cell loss due to aging, tips the brain over into Parkinson's disease at a point late in life. This would explain several facts about the disease: the lack of genetic correlations, the late onset, and the progressive nature of the symptoms which could be caused by the continuing neuronal losses.

A number of epidemiological studies have been undertaken, with some rather surprising results. Because of a structural similarity between MPTP and some industrial chemicals, specifically paraquat, a study was undertaken by a group of Canadian researchers in which pesticide use and the incidence of Parkinson's disease was compared in various geographical regions (Barbeau et al. 1987). It was discovered that a high degree of correlation existed. The suggestion was that the pesticides could be a cause of the disease. Perhaps more disturbing is the anecdotal reports that the incidence of early onset Parkinson's (i.e., beginning before age fifty) has been increasing dramatically for the last few decades, possibly by up to 50% (Lewin 1987e). Again, the suggestion is that an environmental toxin which has become more prevalent is causing Parkinson's disease.

These results, viewed overall, suggest an environmental cause for Parkinson's disease. The implications of this may extend to other degenerative neurological diseases, such as Alzheimer's and motoneuron diseases. It is possible that neurotoxins may be involved and that a potentially preventable cause could be discovered.

Transplantation

There has recently been a great deal of interest in the use of neural grafts for treating Parkinson's disease, spurred on by the report of dramatic results of the treatment by a team from Mexico City headed by Ignacio Madrazo and Rene Drucker-Colín (Madrazo et al. 1987a). In this procedure, the patient received an autograft of tissue from his own adrenal medulla. The tissue was placed in a small cavity cut into the caudate nucleus on the surface exposed to the lateral ventricle. The patients reportedly experienced a dramatic amelioration of symptoms.

The procedure has been performed on a number of patients in the United States at a number of medical centers. Because of the relative simplicity of the operation, it was attempted by many centers. The results of these surgeries, however, has been so disappointing that the validity of the results of the Mexican team have been called into question. News reports of a meeting last summer indicated that many doctors working in the field have been unable to duplicate the results. The Mexicans have yet to publish anything beyond a brief description of the procedures they use. In addition, the initially well-publicized paper in The New England Journal of Medicine reported the results of only two patients, although a much greater number had been operated on. Dr. Ignacio Madrazo, the neurosurgeon of the team, was questioned at a workshop earlier this year in Chicago, and many investigators felt that they did not receive satisfactory answers (Lewin 1988c, New York Times 1988c).

The Mexican team has been continuing their work, however, and have reported the implantation of fetal tissue, from either the substantia nigra or from the adrenal medulla, into the brains of two patients (Madrazo et al. 1988), an approach that has been felt to hold great promise based upon animal work. Work with fetal tissue in this country is a controversial area, so that this procedure has not been extensively tested in the United States. Such tissue would have to come from an abortus, and the political pressures are very strong with regard to this issue (Annas and Elias 1989, Culliton 1988c). The future

is unclear as to whether any such work will be extensively investigated (Culliton 1988b), although some preliminary attempts have been made using fetal implants at the University of Colorado (New York Times 1988b) and at Yale (New York Times 1988a).

A group of Swedish investigators has been exploring the use of neural grafts for treating Parkinson's disease, and had performed two implants in the early 1980s, basing their procedures upon an extensive amount of animal work (Backlund et al. 1985). The surgical methods that they used differed from those of Madrazo and his colleagues, in that they implanted a dissociated cell suspension by injecting into the substance of the striatum, rather than attaching a piece of tissue to the surface. These patients, however, did not show any dramatic improvement, and the work was not pursed further. After the reports came out from the Mexico City workers, the Swedish workers also performed additional surgeries utilizing fetal nigral tissue. Preliminary results suggest that the patients again have not shown any therapeutically useful improvement (Lewin 1988d).

The continuation of research into neural grafts has undoubtedly been stimulated by the interest in its clinical applications. Because of the skepticism engendered by the recent results, however, there is the possibility of a backlash that will inhibit further research and human treatment. The current consensus in the biomedical community is to continue work in this area, but to temper it with a degree of caution and prudence (Lewin 1988b, Sladek and Shoulson 1988). Slower but surer work might be a more tenable approach politically. Hopefully, future research will show that today's optimism is justified.

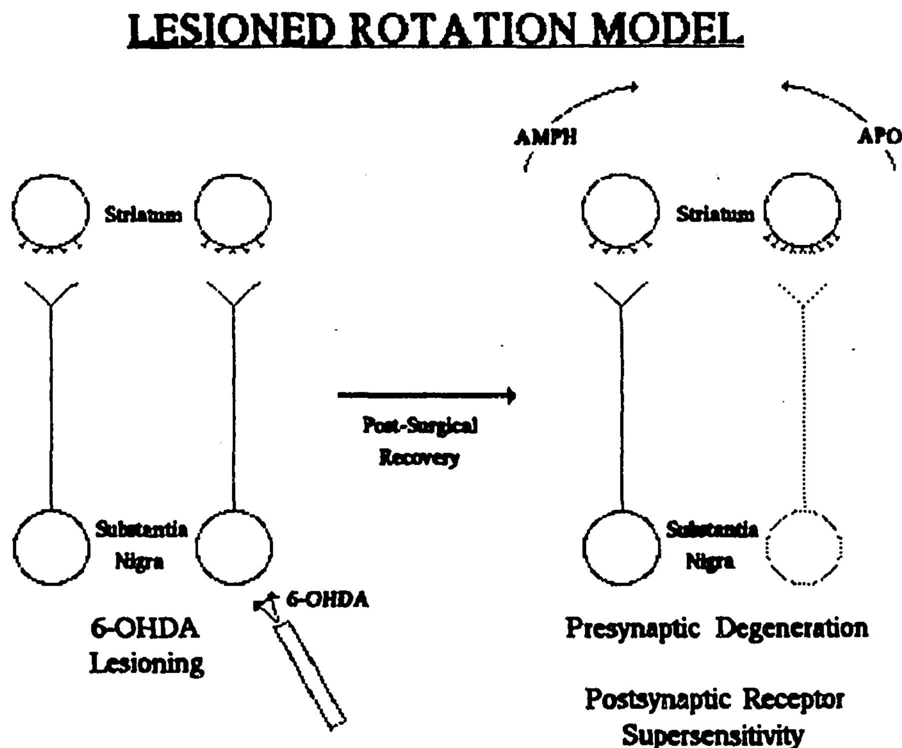
UNGERSTEDT'S CIRCLING RAT

In 1971, Urban Ungerstedt, from the Karolinska Institute in Sweden, published an extensive series of papers describing an animal model for parkinsonism in which the neurotoxin 6-hydroxydopamine was used to selectively destroy the nigrostriatal system in rats. The lesions were made unilaterally, because bilateral lesions rendered the rats adipsic and aphagic (Ungerstedt 1971a). When appropriately stimulated, the unilaterally lesioned

rats would show a marked side preference in which direction they would turn while walking, i.e., they would circle. The preferred direction showed a close correlation with which side of the brain was lesioned.

Ungerstedt demonstrated that receptor supersensitivity developed on the lesioned side, presumably a homeostatic mechanism of the system to compensate for the loss of the dopaminergic nerve terminals (Ungerstedt 1971b). The direction of the circling is away from the side receiving the greater postsynaptic stimulation. Thus, for a rat lesioned on the right side, circling in response to a receptor stimulating drug like apomorphine is toward the left. If the rat is injected with an indirect agonist, like amphetamine, circling is towards the right, because the drug releases dopamine from the intact terminals on the unlesioned side (Ungerstedt 1971c). (See Figure 1.) In each case, there is a relative enhancement of dopamine receptor activation on the side contralateral to the direction of circling.

Figure 1



Ungerstedt also showed that when unilaterally lesioned rats were injected with L-dopa, they showed circling similar to that induced by apomorphine—away from the lesioned side (Ungerstedt 1971b). It is not intuitively obvious that this should be expected. L-Dopa itself is practically inert pharmacologically; its known effects indicate that it is converted to dopamine and then acts on dopamine receptors (Bianchine 1980, Hornykiewicz 1974). As a precursor, L-dopa undergoes decarboxylation to dopamine before becoming active (Ungerstedt 1971b, Spencer and Wooten 1984b). It could be considered unusual that L-dopa was functionally more active on the side of the brain that lacked the normal apparatus for this conversion. However, this does indicate that the denervated striatum can still synthesize extracellular dopamine that can activate postsynaptic receptors.

Since Ungerstedt's classic papers appeared, the circling model has been subjected to extensive examination (for review see Pycock 1980). Although the underlying neuronal mechanism in the circling model was once believed to be simply an asymmetry in dopaminergic activity, a considerable body of research has accumulated suggesting that it is much more complex (Shapiro 1985). Most prominent is the contention that there is topographic heterogeneity within the striatum and in its connections, such that different areas (medial vs. lateral) mediate turning in opposite directions (Dunnett and Iversen 1982). This finding is interesting in light of investigations that the dopaminergic loss in Parkinson's disease is more extensive in the putamen than the caudate (Kish, Shannak and Hornykiewicz 1988). Also, the topographic distribution of the connections of the striatum to different cortical areas has led to the hypothesis that the putamen is involved in controlling movement, whereas the caudate is involved in higher complex integrative functions (DeLong, Georgopoulos and Crutcher 1983).

Nevertheless, Ungerstedt's model has proven very useful in evaluating the efficacy of potential new drug treatments for Parkinson's disease, and in gaining an understanding of the mechanisms of striatal physiology. In my research, I used this model for investigating

the actions of L-dopa in the function of the caudate, and its actions in inducing changes in uric acid.

IN VIVO ELECTROCHEMICAL DETECTION

History

Voltammetry is a powerful technique commonly used by analytical chemists for detecting and analyzing compounds in solution and is used for both quantitation and identification (Skoog and West 1976). It is sensitive to concentrations in the micromolar to nanomolar range. Many compounds important to brain chemistry are electroactive and are present in the brain at concentrations that can be detected electrochemically. In 1973, Ralph Adams's lab at the University of Kansas described the technique of in vivo electrochemistry, in which implanted voltammetric electrodes are used to measure electroactive compounds in the brains of living animals (Kissinger, Hart and Adams 1973). This idea was exciting because electrochemical detection causes only minor perturbation of the micro-environment, so that if the electrodes are permanently implanted, they can be used to make repeated measurements in the same animal, and the animal can be awake and freely moving, so behavior may be measured simultaneously.

It should be emphasized that this technique measures the extracellular levels of the compounds being monitored. The electrode used is quite large relative to neuronal cell bodies, and should not be thought of as monitoring intracellular levels. Rather than being a drawback, however, extracellular measurement is a remarkable complement to concurrent behavioral observation, because it allows a measurement reflecting levels of released neurotransmitter (see discussion below), as opposed to having to infer the amount of release from biochemical measurement of total tissue levels, and allows the construction of behavior-release correlations for different time points with the same animal.

Since the introducion of in vivo electrochemistry, it has been used and modified in a number of labs (Marsden 1984a, Stamford 1985). There is still disagreement over the interpretation of the results that this technique yields, caused to a large extent by the many varieties of electrodes that are used, and differences in the specific methods of performing the electrochemical measurements. There are some inherent shortcomings within the technique itself (discussed below). Despite these problems, it has become regarded as an important advance, and has been the subject of several recent reviews (Adams and Marsden 1982, Hutson and Curzon 1983, Stamford 1985), as well as being the subject, along with in vivo perfusion techniques, of several major conferences (see proceedings published in Myers and Knott 1986) and a methods textbook (Marsden 1984a). The state of the art has recently been described in a workshop consensus summary paper (Marsden et al. 1988). For a more complete discussion of the theory underlying electrochemistry, see Appendix 1.

Practice

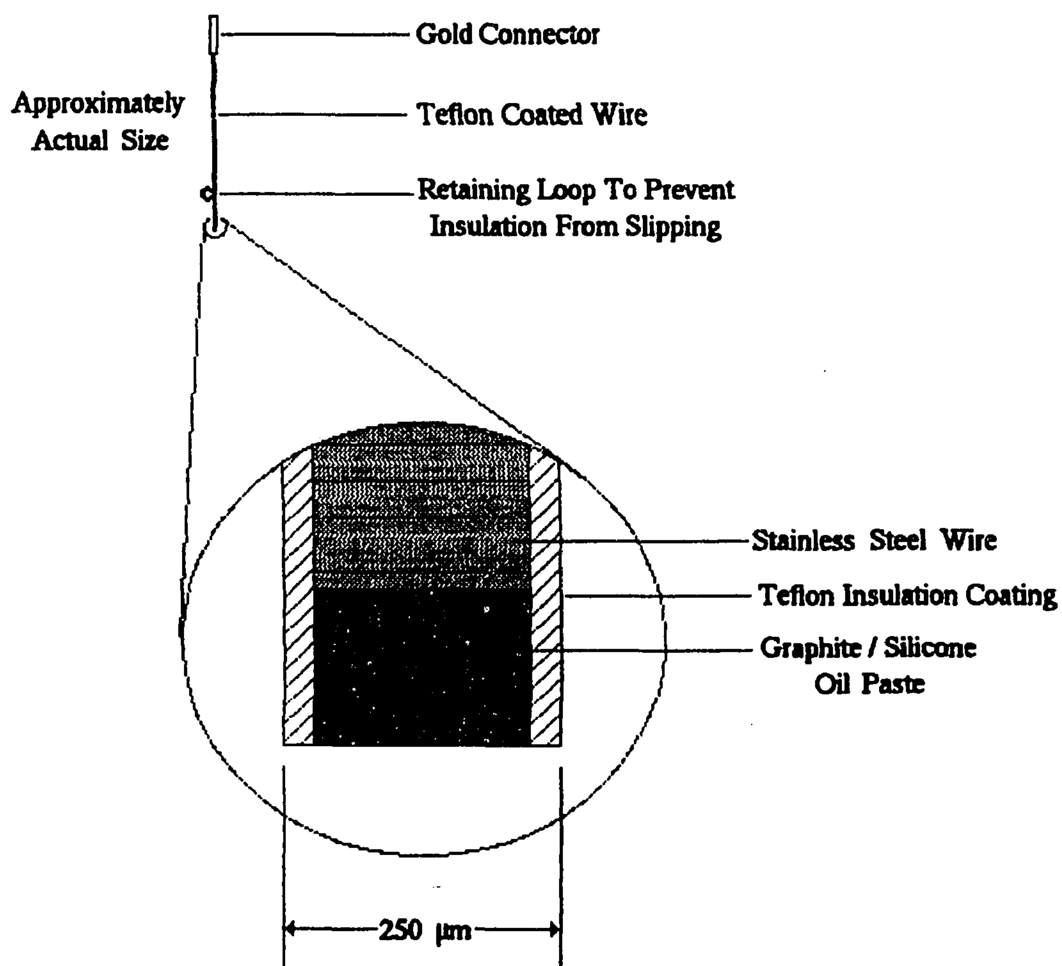
The electrodes used for work in vivo differ from those used for normal electrochemistry. The difference, however, is mostly in smaller size and construction, and the theoretical treatment of them is surprisingly similar. With electrode diameters in the single micrometer range, however, some differences do manifest themselves, as has been described in a recent article (Wightman 1988). The electrodes used in the present work were of larger dimensions.

The construction of the working electrode greatly influences the measurements made. There are a number of different types of working electrodes in use by different groups in this field (Marsden, Brazell and Maidment 1984b, Stamford 1985, Marsden et al. 1988), and different types of electrodes have distinct characteristics, some causing shifts in reaction potentials, others excluding certain substances from measurement, although some of these points have been subject to some controversy. The specific electrochemical method used can cause the electrode to exhibit different responses as well. I shall restrict

my discussion here to the method we use: linear sweep voltammetry with carbon paste electrodes.

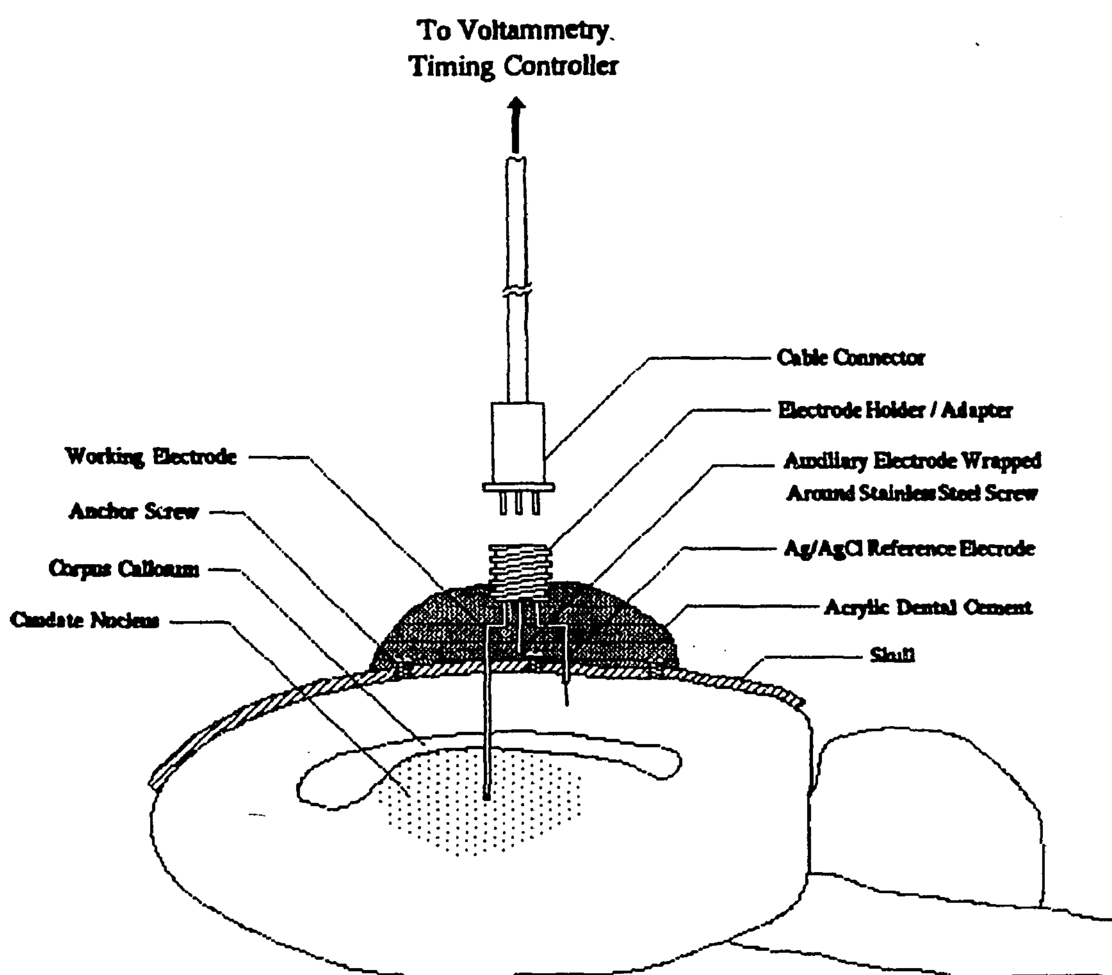
For my experiments, I hand-made all of the carbon paste electrodes. (See Figure 2.) They are constructed from stainless steel wire that has a teflon insulation coating (Medwire) of a diameter of 250 μm. The straightened wire is cut to length and one of the two ends is bared and soldered into a connecting sleeve. The teflon insulation is loosened and slid past the end of the wire to form a small well. A small loop is made in the wire to prevent any further slippage of the insulation. The well is then tightly packed with a

Figure 2



carbon paste. This paste is composed of a mixture of 1.5 g of Ultra-F grade graphite powder (Ultra Carbon Corp.) and 1.0 ml of silicone oil (Aldrich). The consistency has been described as resembling "slightly dried out peanut butter". The end surface of the electrode is then smoothed and polished by gently and repeatedly spinning the wire tip on a teflon tape, until it appears flat and smooth under magnification. Polishing the electrode

IMPLANTED HEADSET Figure 3

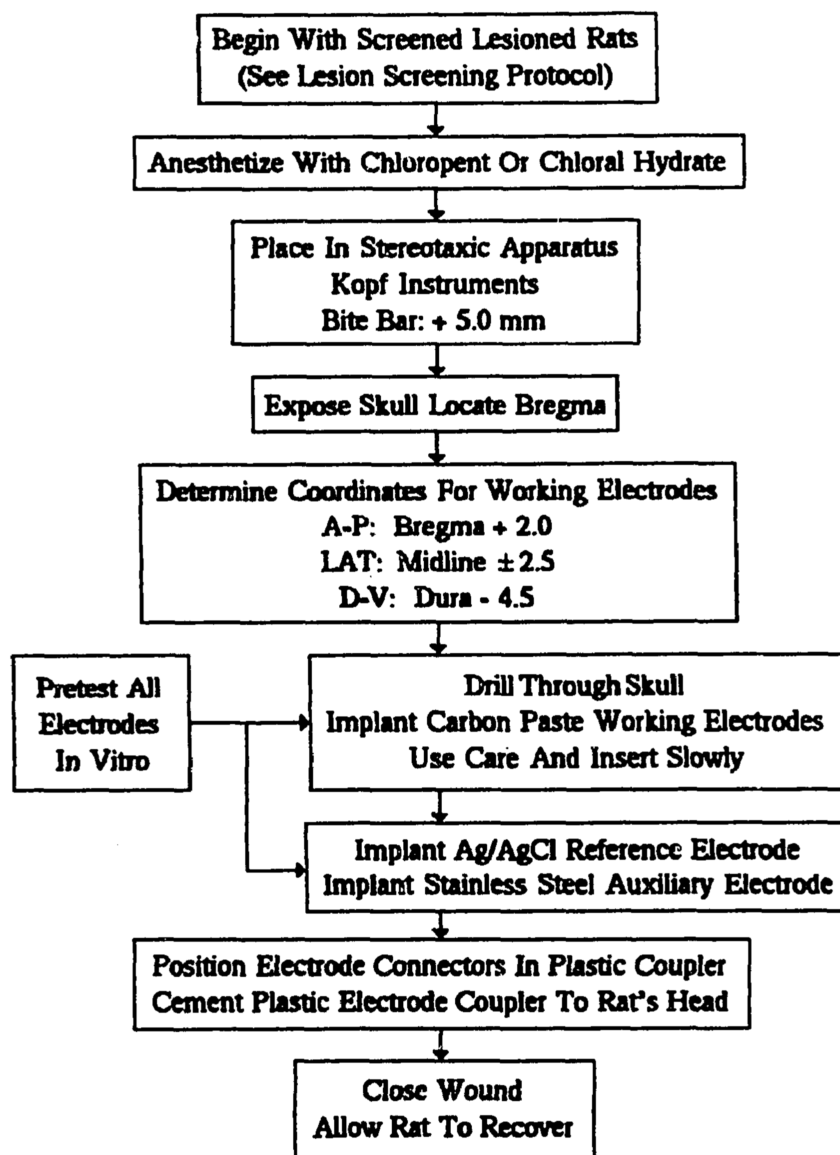


One working electrode is implanted in each caudate for a total of two working electrodes per rat. Only one of the two is shown in the diagram for clarity.

is important because the surface's physical characteristics can influence electron-transfer reactions occurring there. This electrode is known in the literature as an "unmodified carbon paste electrode".

The reference electrode generally used is a silver-silver chloride (Ag | AgCl) electrode. It is constructed in a manner similar to the working electrode. Silver wire is used

Figure 4



(Medwire); the tip is bared and electrocoated with a thin layer of silver chloride by application of 3.0 volts for 10 seconds while immersed in 1 M HCl.

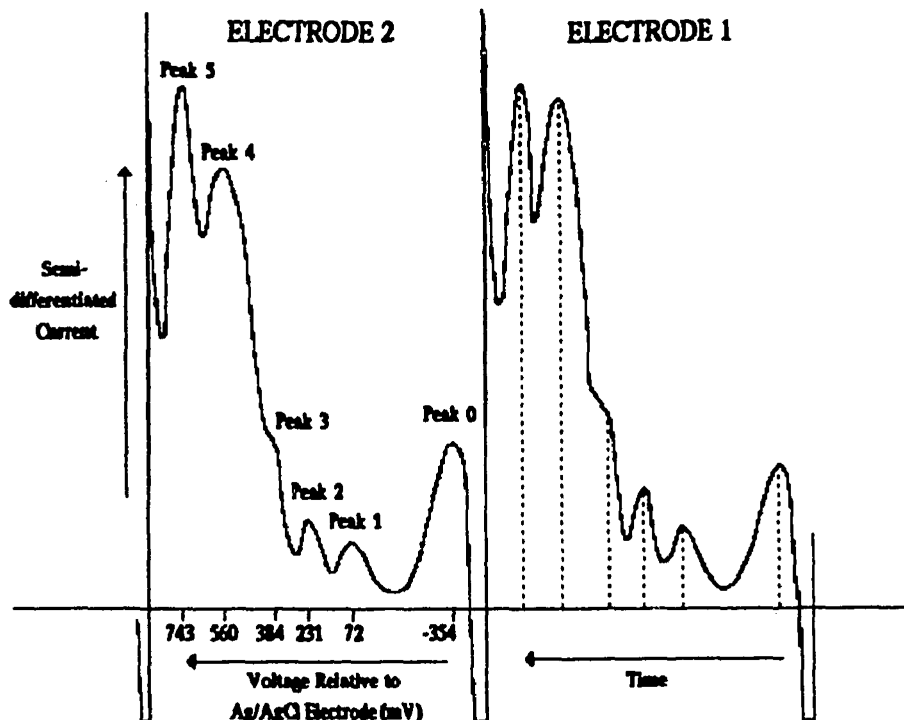
The auxiliary electrode is the least complex of the three, and need only conducts electricity to complete the circuit. We use a stainless steel wire wrapped around a stainless steel screw that is in contact with the brain surface.

The electrodes are surgically implanted into the rat's brain as illustrated in Figure 3. Just prior to implantation, electrodes are tested until a complete working set is assembled. The solution that was used for testing the electrodes was 100 μM dopamine or, in the later microinjection experiments when Peak 2 became the primary focus, 100 μM uric acid. The working electrode is stereotaxically implanted into the caudate nucleus using the coordinate system of Pellegrino, Pellegrino, and Cushman (1979) aiming for the center of the nucleus at the level of the anterior commissure (bite bar: +5.0 mm, A-P: bregma + 2.0 mm, LAT: midline ± 2.5 mm, D-V: dura – 4.5 mm). The reference electrode is implanted into the brain at an unspecified location (not critical) in the posterior cortex. And the auxiliary electrode is wrapped around a screw as illustrated. The free ends of the electrodes are fixed into a plastic electrode holder that serves as a pin adapter to a leash-like cable that will allow the rat to move freely while measurements are being made. The whole assembly is cemented in place with acrylic dental cement. (See Figure 4 Electrode Implantation Protocol.)

When an electrode is scanned, a voltage is applied over the range –430 mV to +900 mV. The applied voltage increases with time at a rate of 10 mV per second. Over this voltage range, the semidifferentiated current curve shows six different peaks, that we have termed Peaks 0 to 5. (See Figure 5) Peak 1 occurs at the voltage believed to be associated with ascorbic acid, dopamine, dihydroxyphenylacetic acid (DOPAC), and norepinephrine. It has generally been accepted that it probably reflects levels of ascorbic acid, and that dopamine is normally undetectable (O'Neill et al. 1982b). Peak 2 occurs at the voltage believed to be associated with uric acid, serotonin, and 5-hydroxyindoleacetic

REPRESENTATIVE VOLTAMMOGRAM

Figure 5



This is a representative voltammogram for a single session (two electrodes). Time and voltage increase from right to left. The dashed lines in the trace for Electrode 1 indicate the peak heights. The peak positions in millivolts (mV) are indicated. In some voltammograms, other peaks appear, but these are the ones most consistently seen.

acid (5-HIAA) (Marsden, Brazell and Maidment 1984b). Many investigators believe, however, that it reflects levels of uric acid (see below) (Knott, Mueller and Young 1986, Marsden et al. 1988, Mueller et al. 1985, O'Neill et al. 1984). Peak 3 is associated with homovanillic acid (HVA) (O'Neill and Fillenz 1985). Peak 0, Peak 4, and Peak 5 have not been described in the literature previously, or have been described only in passing, and may or may not have any relevance (Stamford 1985).

Electrode Systems

The use of different voltammetry systems can be illustrated by a partial listing of those used by different groups. These studies used what is known as the unmodified carbon paste electrode, made with silicone oil and carbon paste, for linear sweep voltammetry at a scan rate of 10 mV/second. The methodology closest to that used in the present

investigation is that of Marianne Fillenz and Robert D. O'Neill at Oxford (O'Neill is now at University College Dublin, Ireland). They also use unmodified carbon paste electrodes, but with a slightly different composition (2.8 g carbon powder to 1.0 ml silicone oil) and a different scan rate of 5 mV/second. In addition, their data analysis is slightly different insofar as they utilize a digitized data collection system that subtracts out the baseline (O'Neill, Fillenz and Albery 1983a). Another related system is that developed by Ross F. Lane and Charles D. Blaha, the stearate-modified carbon paste electrode, in which the carbon paste is made with Nujol (a mineral oil commonly used in chemistry laboratories), instead of silicone oil, with stearic acid dissolved in it (Blaha and Lane 1983). The reason for this modification is to impose a negative charge on the electrode surface from the carboxyl moities of the stearate. Theoretically, this should repel negative ionic substances from the electrode surface, increasing the resolution of the detector by shifting the oxidation potentials of these "unwanted" compounds, such as ascorbic acid and uric acid, thus improving electrode sensitivity to dopamine and serotonin. The reports of Blaha and Lane (1983, 1984) show convincing evidence that these electrodes do indeed measure extracellular levels of dopamine and exclude ascorbic acid. The use of these electrodes, however, is somewhat limited for practical reasons. The exact procedure for making them has not been fully described in the literature, and aside from those groups that have worked directly with Ross Lane, no one has been able to reproduce their manufacture. Curt Freed also claims that these electrodes do not require stearic acid for modification but rather that the exposure of the electrode to brain parenchyma alone somehow modifies the surface (presumably by absorption of unidentified factors), increasing the electrode sensitivity for dopamine, DOPAC, norepinephrine, and serotonin (Echizen and Freed 1986). There is a question of comparability to our electrodes, as Freed does not detect a peak for ascorbic acid with these electrodes, even without brain modification.

The carbon fiber electrode is another electrode system. The electrode is a graphite fiber that has been insulated in a pulled glass micropipette and cut to a specified length.

This is a popular system because the carbon fibers can be made in very small sizes, down to less than 10 μm in diameter, so that very little distortion of the anatomy is required for implantation. This system is used, in various forms, by F. G. Gonon, C. A. Marsden, R. M. Wightman, and others (see Marsden et al. 1988). This electrode, however, has a limited stability in the brain, deteriorating in the course of a single experiment within hours after implantation. Nevertheless, the small size makes it an attractive alternative, especially for those brain nuclei that are too small for monitoring in any other way. A fiber electrode 1 μm in diameter has even been used for intracellular monitoring in Aplysia neurons (Meulemans et al. 1986), an exception to the statement above that IVED only measures extracellular substances.

There are also different voltammetric measurement techniques. As mentioned above, we use linear sweep voltammetry. Another popular technique is cyclic voltammetry. Cyclic voltammetry applies an increasing voltage ramp, but differs from linear sweep voltammetry in that it is followed by a decreasing voltage ramp that returns to the original value. This technique has been used to great advantage in analyzing events with a time resolution of less than a second (Millar et al. 1985) because it can be used with high scan rates (300 V/sec).

Differential pulse voltammetry is commonly used with carbon fiber electrodes, and has the characteristics of yielding a sharper signal, similar to the signal from linear sweep voltammetry after semidifferentiation processing. The disadvantage with this technique is the requirement for more complex electronics needed to create the voltage waveform applied.

Chronoamperometry has the ability to make rapid measurements for studies in which it is desirable to closely monitor events. The scan can be completed in one second, and it is not uncommon for measurements to be repeated at one minute intervals. The trade-off made is that chronoamperometry gives up some information with regard to the specificity of the substance measured. The short scan times are possible because only one voltage is

applied, not a range that is swept, so that the signal monitored is composed of currents from a number of reactions at the working electrode, and the interpretation of the results requires an especially careful attention to control experiments.

More complete discussion of these types of techniques is available in the appendix on electrochemistry theory. (See Appendix 1.)

Peak 2

The theoretical difficulties of in vivo electrochemical detection are illustrated by the controversy surrounding the identity of the compound measured in the striatum by Peak 2. Initially, it was believed that this peak represented 5-hydroxyindoles. Pharmacological manipulations with drugs that affect serotonin metabolism and release was observed to affect the peak height in a way consistent with the peak representing serotonergic activity. The use of different electrode systems by different research groups made it difficult to compare observations. In addition, initially, the possible contribution of uric acid to this peak was not appreciated; thus no consideration was given to its possible role.

The lack of attention given to uric acid parallels the initial lack of attention paid to the involvement of ascorbic acid's contribution to Peak 1. Early experiments identifying "dopamine" as being measured by Peak 1 relied on pharmacological manipulation by drugs known to modify dopaminergic function (i.e., the signal increased in response to administration of amphetamine). Negligible consideration was given to the possibility that such manipulations may also change ascorbic acid levels, either directly, or indirectly, secondary to changes in dopamine. Ascorbic acid was just thought to be a component of the extracellular milieu, and thus should not change in response to neuroactive drugs. Investigation of extracellular ascorbate in brain determined that it is present in relatively high concentrations of approximately 125-300 μM (Brazell and Marsden 1982, Gonon et al. 1981). It was shown that ascorbate is released in the striatum in response to amphetamine administration (Grünewald et al. 1983). Later reports have shown that ascorbate can modify receptor function and behavioral response to amphetamine,

suggesting it may be pharmacologically active (Ewing et al. 1983a, Rebec et al. 1985, Heikkila, Cabbat and Manzino 1981). Other studies indicate that ascorbate release may be linked to reuptake of excitatory amino acids, thus reflecting cortico-striatal activity (Grünewald and Fillenz 1984). When ascorbate oxidase was microinjected in proximity to the electrodes in the brain, Peak 1 virtually disappeared, indicating that it consisted mostly of ascorbic acid (Brazell and Marsden 1982), at least when using unmodified carbon paste electrodes. Since then, a number of electrode modifications have been devised in order to decrease the response of the electrodes to ascorbate and increase them to dopamine, or at least to shift the electrode potentials of these two reactions so as to resolve them into two separate peaks.

Similarly, the identity of Peak 2 is questionable, except that in this case it was uric acid that was not considered because it was merely a waste product that existed in the extracellular milieu, and the signal was presumed to measure 5-hydroxyindoles. A series of investigations indicated that pharmacologic manipulations affecting serotonin changed Peak 2 as predicted, or at least in an explicable way. About this time Ungerstedt's group reported the detection of uric acid in extracellular fluid in brain using microdialysis (Zetterström et al. 1983). This prompted suspicions that uric acid may make a contribution to the voltammogram, possibly interfering with one of the known peaks. The enzymatic elimination of uric acid with uricase microinjected at the electrode tip demonstrated the true identity of Peak 2 (Crespi et al. 1983, Mueller et al. 1985, O'Neill et al. 1984). In this case, uric acid, per se, has not been shown to be neuroactive, but the possibility has arisen that its level may change in response to neuronal activity. This will be discussed in greater depth later.

SECTION 2 — PRELIMINARY INVESTIGATIONS

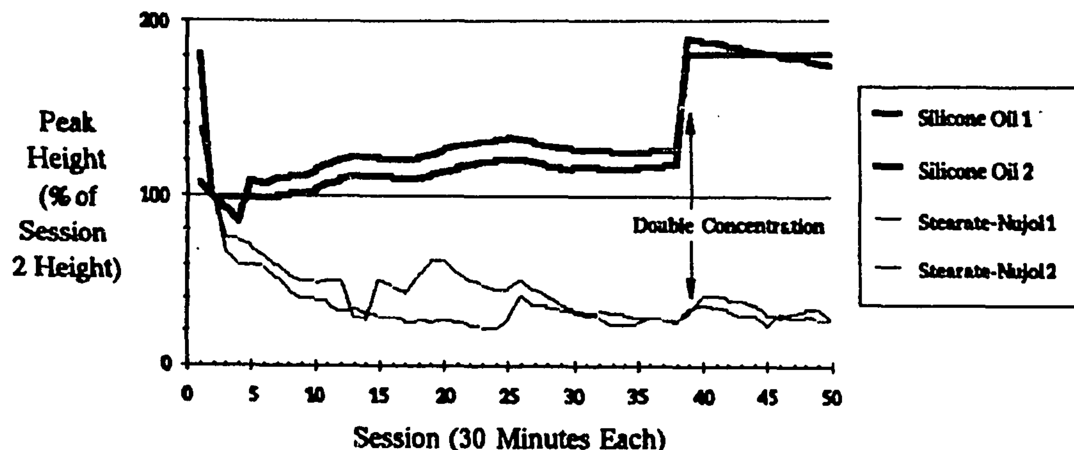
NUJOL/SILICONE OIL: STEARATE-MODIFIED ELECTRODES

The groups of Ross Lane and Curt Freed (who worked with Ross Lane), have reported that the stearate-modified electrodes have the ability to resolve the dopamine-ascorbate problem by excluding the measurement of ascorbate from Peak 1, as mentioned above. We attempted to make electrodes similar to theirs.

The stearate-modified electrodes are reported to be similar to the unmodified carbon paste electrodes we use, except that instead of using silicone oil as the suspension medium for the graphite powder, Nujol with stearic acid dissolved in it is used. It became apparent that construction of these electrodes is not straight-forward. Stearate-modified Nujol carbon paste electrodes were made first by combining 0.75 g of Ultra-F grade graphite powder (Ultra Carbon Corp.) and 0.50 ml of Nujol that had 50 mg of stearic acid dissolved in it. This paste was used to make electrodes similar to the ones described earlier. These electrodes were then examined for their response to various solutions. The signal response of these electrodes, however, was not stable over time, and after a few hours in solution, the signal had deteriorated to the point that it was questionable whether the electrode was responding at all to the compound in solution. A brief experiment was run in which a silicone oil electrode was directly compared with a stearate-Nujol electrode over a long period of time in the same solution, with the same equipment and the same reference and working electrodes. The results can be seen in Figure 6. As can be seen, there is some decrease in response of the stearate-Nujol electrodes over time in the solution, in that the peak height decreases, especially within the first few hours. In order to determine whether the electrodes had lost responsiveness to dopamine, or had only decreased responsiveness, an aliquot of concentrated dopamine was added to the solution

Figure 6

Stearate-Nujol/Silicone Oil Working Electrode Comparison
Tested In 200 Micromolar Dopamine



at the end of the run in order to see if the electrodes respond to the increase in concentration. As can be seen, the silicone oil electrodes were still able to register an increase in peak height, indicating that in this case, the electrodes had only a decrease in responsiveness. The stearate-Nujol electrodes, in contrast, showed almost no change in peak height, indicating that the electrodes had lost the ability to respond at all. Because of this observation, it was felt that stearate-Nujol was not an acceptable suspension medium for the carbon paste.

Given that Nujol was ruled out, an attempt was made to use stearic acid to modify the silicone oil electrodes. All attempts to dissolve the stearic acid in the silicone oil, however, were unsuccessful, even with heating to high temperatures. An attempt to grind up the stearic acid and mix it with the silicone oil before making the carbon paste were unsuccessful.

As was indicated above, the exact procedure for making these electrodes has never been fully described in the literature. Questions the inability to replicate these electrodes were referred to a former member of Ross Lane's group, who advised that much of the problem stemmed from the handling of the reagents. While some of the suggestions

seemed reasonable (i.e., keep the carbon powder away from possible contaminants), the rationale for others was unclear (i.e., the carbon powder must be refrigerated immediately upon receiving it, but the paste was stable at room temperature indefinitely). None explained the differences observed between the Nujol electrodes and the silicone oil electrodes.

Subsequently, it has become apparent that essentially no one outside of those working directly with Lane has been able to reproduce these electrodes. This problem highlights an aspect of in vivo electrochemistry research that needs emphasis: the role of the art of technique. As with any highly technical operation, the fine points of making stearate-modified Nujol electrodes may require personal observation. The problems may arise, in part, due to a lack of consensus among the researchers using these electrodes, and to the still incomplete understanding of surface phenomena in chemistry. The underlying basis for some conflicting observations are still obscure, so that it is possible that the details for making stearate-modified Nujol electrodes are each of utmost importance. An adequate explanation will have to wait until full publication of the methodology for making these electrodes.

These problems do not seem to be as prominent with the unmodified carbon paste electrodes, and the observations seem to be more consistent from group to group according to the literature.

REFERENCE ELECTRODE COMPARISON

Another preliminary investigation examined differences in the construction of the reference electrode. Different groups use slightly different variations of the Ag | AgCl reference electrode, which are, in order of increasing complexity: a plain silver wire implanted directly into brain tissue, a silver wire that has been electrocoated with silver chloride implanted into brain tissue, and an electrocoated silver wire connected to brain

tissue by a salt bridge. This last electrode is made from a plastic disposable pipette tip that has been cut to length and filled with gelatin in 1M NaCl. The Ag/AgCl wire is inserted into this gel and the top is sealed with acrylic cement. Prior to filling with gelatin, the open end of the pipette tip was occluded by cotton soaked with solution, to retard leakage of constituents while allowing the passage of ions for current flow. This partially occluded tip is placed in contact with the surface of the brain and cemented in place. This construction keeps the microenvironment around the Ag/AgCl tip more constant than either of the other two methods, but has the drawback of a more complex and time-consuming manufacture.

To determine the practical differences between the three types of reference electrodes, one of each type was implanted in a single rat, along with an auxiliary electrode and two working electrodes, using the same methods as for normal implantations. After allowing the rat to recover, a series of voltammograms were recorded according to the methods described, except that the reference electrode used for each scan was rotated among the three. The resulting voltammograms indicated that the type of reference electrode used made little difference in the voltammogram, except that the entire tracing was slightly offset along the voltage axis from one reference electrode to the next. The voltammograms were otherwise essentially identical in appearance, indicating that the reactions at the working electrode were not affected by the reference electrode. This result also indicated that the locus of implantation of the reference electrode was of minimal importance, insofar as each electrode was in a different location. Both of the wire electrodes were simply inserted into posterior cortex, whereas the salt bridge electrode was placed in contact with the brain surface. The silver wire electrode was offset by being 180 mV more positive than the salt bridge electrode, while the Ag | AgCl electrode was 25 mV more negative. Within the scans for each individual reference electrode, peak placement was completely consistent.

Because of this lack of difference, it was felt that the extra effort needed to make and implant the salt bridge reference electrodes was unnecessary, as long as it was understood that the potential of the peaks might not exactly match that expected for a standardized Ag | AgCl reference electrode. It was more practical to identify the peaks by the characteristic appearance of the voltammogram, using the potential as an additional guide.

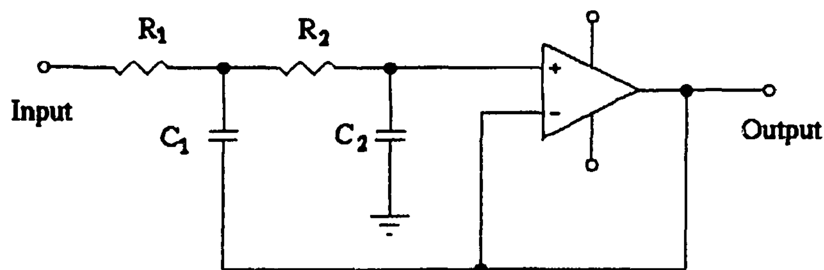
INSTALLATION OF LOW-PASS FILTER

Another difficulty that had to be surmounted was the appearance of noise in the voltammograms recorded in vivo. Recordings made in vitro were reasonably noise-free. Measurements in vivo, however, had a great amount of signal variability. This noise was not due to an external source, such as nearby electrical equipment, because recordings in vitro in the same location were noise-free. It was also not due to a loose connection, insofar as the amount of noise did not increase with the rats' activity. A rat with a noisy voltammogram when alive did not have a noisy recording when scanned after death, even when the body was shaken vigorously. Inspection of the recordings led to the suggestion that the origin of the signal variability may be brain electrical activity, and that what we were seeing was an electroencephalogram superimposed upon a voltammogram. This phenomenon was not explored further, as it was more important for this study to eliminate the noise by filtering, so that the voltammogram peaks could be examined.

The BAS DCV-5 voltammetry controller incorporates a low-pass filter into its circuitry; evidently it was inadequate to process the signal. To eliminate the noise, an external second order low-pass filter was constructed based upon a published design (Brown, Franz and Moraff 1982). (See Figure 7). The filter was installed between the DCV-5 output and the chart recorder input. This circuit was constructed to allow switching between three cutoff frequencies as needed: 0.1 Hz, 1.0 Hz, 10 Hz. (See Figure 8). Empirical observation indicated that a setting of 1.0 Hz was sufficient to

Figure 7

LOW-PASS FILTER CIRCUIT DESIGN



Second Order Low-Pass Butterworth Filter

Z = Impedence of Circuit = 1.0 M Ω
f_c = Cutoff Frequency = 0.1, 1.0, 10 Hz

$C_1 = \frac{5}{2 \pi f_c Z}$ farads = 8.0, 0.80, 0.080 µF

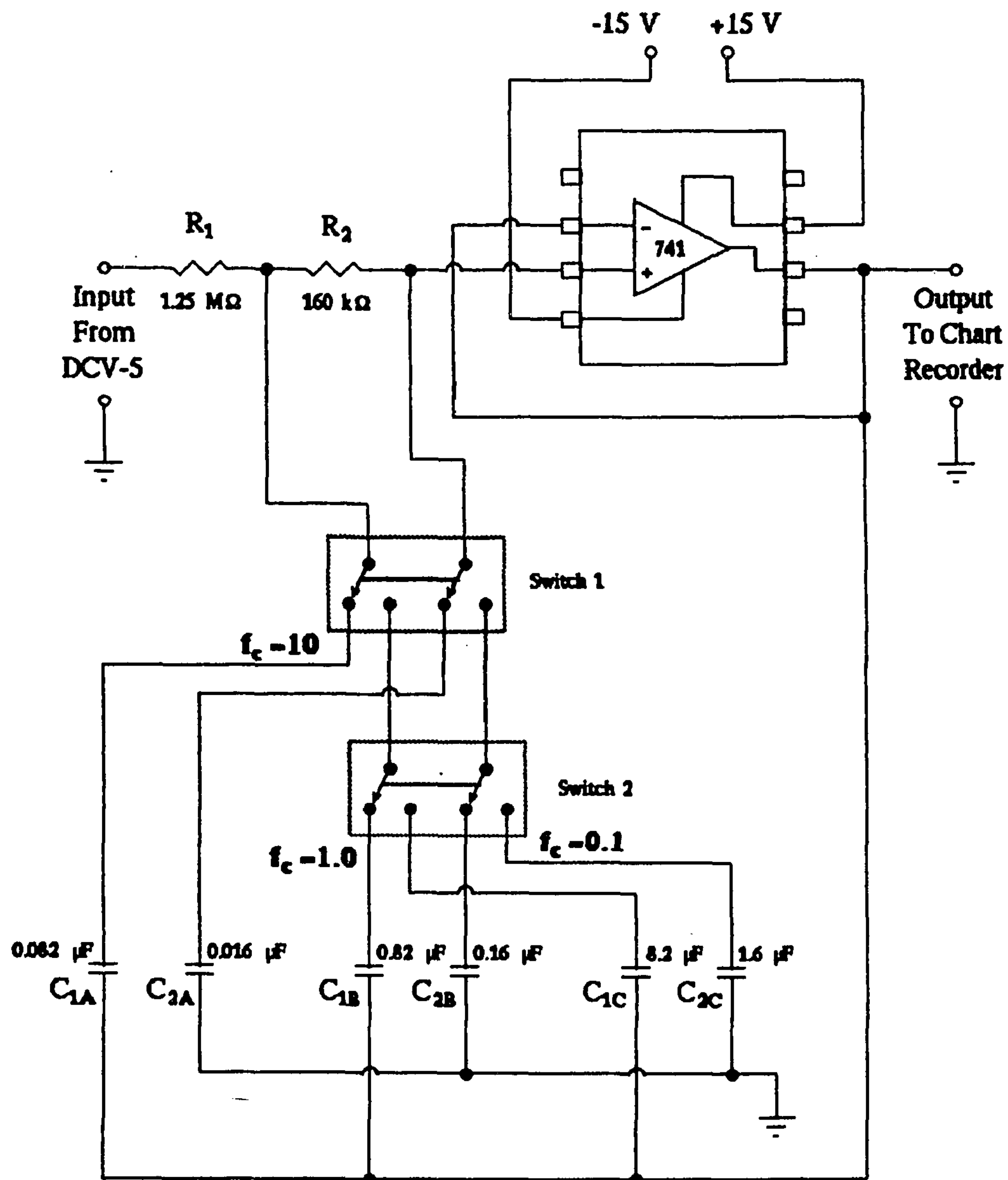
$C_2 = \frac{1}{2 \pi f_c Z}$ farads = 1.6, 0.16, 0.016 µF

R_1 = 1.25 Z ohms = 1.25 M Ω
R_2 = 0.16 Z ohms = 160 k Ω

eliminate the noise and yield a relatively smooth voltammogram with no apparent loss of signal quality. This filter, at this setting, was used for all of the experiments reported here, except for the preliminary studies in vitro already described.

Figure 8

LOW-PASS FILTER CIRCUIT CONSTRUCTION



SECTION 3 — INITIAL EXPERIMENTS

INTRODUCTION

In the striatum, Peak 2 has been shown to result from the oxidation of uric acid with our unmodified carbon paste electrodes (Mueller et al. 1985) and other similar electrodes (O'Neill et al. 1984). Some investigators contend that, with their electrodes, this peak also measures serotonin and/or its metabolite 5-hydroxyindoleacetic acid (5-HIAA).

Peak 1 measures a combination of ascorbic acid, dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC). Under normal conditions, the bulk of this peak is probably ascorbic acid (O'Neill et al. 1982b), although dopamine can make substantial contributions after certain treatments such as acute cerebral ischemia (Phebus et al. 1986, Brannan et al. 1987).

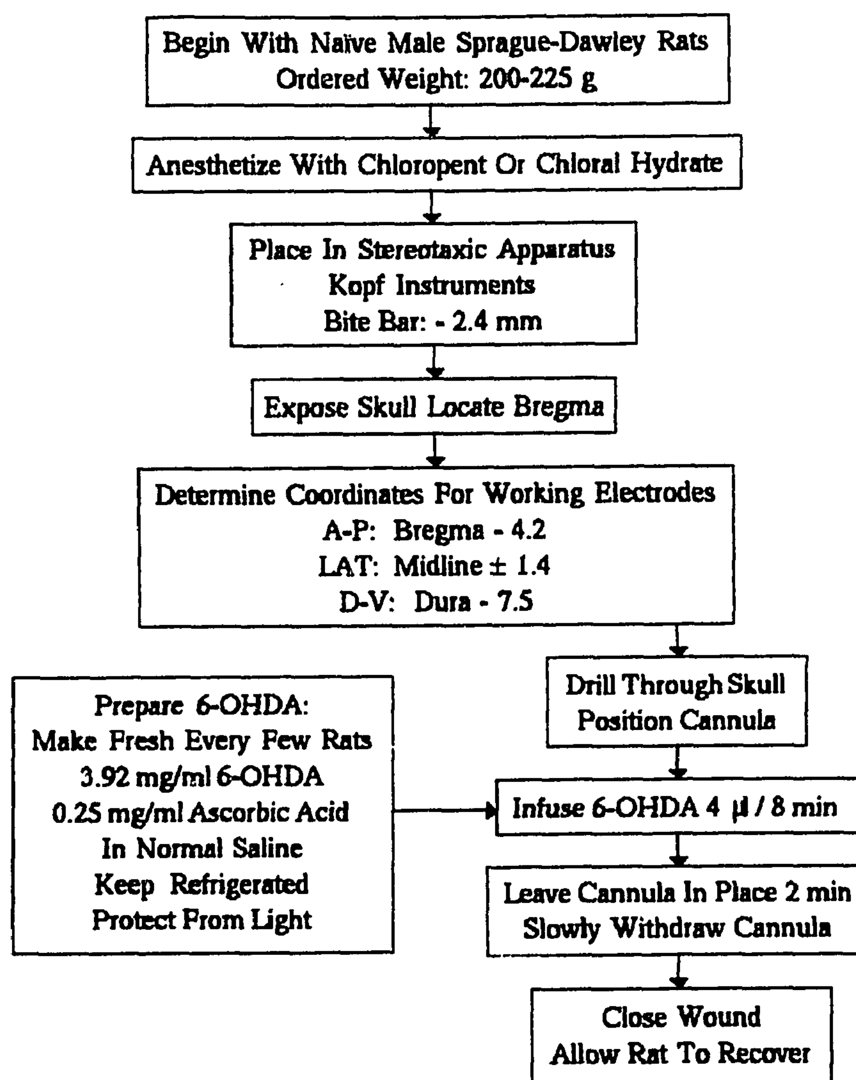
METHODS

Male Sprague-Dawley rats with initial weight 200-225 g were used for these studies. They were housed in individual cages on a 12-hour light/dark cycle (on 06:15, off 18:15). Temperature and humidity were monitored. The temperature was 23.5 ± 1.5°C throughout the time the rats were housed. All behavioral measurements were made during the hours 10:00-18:00.

Dopaminergic nigrostriatal neurons were destroyed with 6-hydroxydopamine (Sigma) by microinjecting 15.7 μg in 4 μl of 0.9% saline over 8 minutes into either the left or right substantia nigra (bite bar: –2.4 mm, A-P: bregma – 4.2 mm, LAT: midline ± 1.4 mm, D-V: dura – 7.5 mm) of rats anesthetized with chloral hydrate / pentobarbital. (See Figure 9 Lesioning Protocol.) After a recovery period of 6-8 days, the rats were screened for effectiveness of the lesioning by measuring rotation for one hour after administration of

LESIONING PROTOCOL

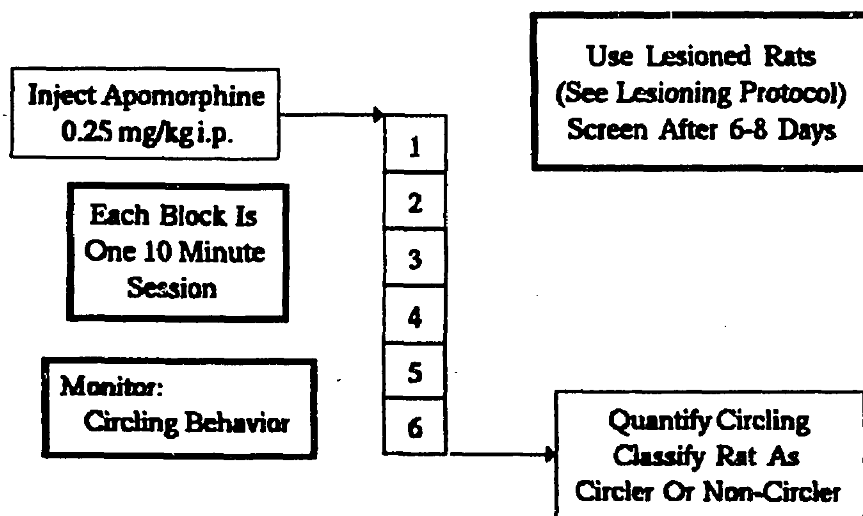
Figure 9



apomorphine (Sigma) 0.25 mg/kg i.p. (See Figure 10 Lesion Screening Protocol.) Behavior was recorded by videotaping the rats from above. Rotations were counted by reviewing the tapes on fast scan mode. One rotation was recorded for every four consecutive quarter turns in the same direction. Rotations were tabulated as left turns and right turns over 10 minute sessions. Rats that did not show clear circling behavior after apomorphine (about 40% of the animals) were not used further.

Figure 10

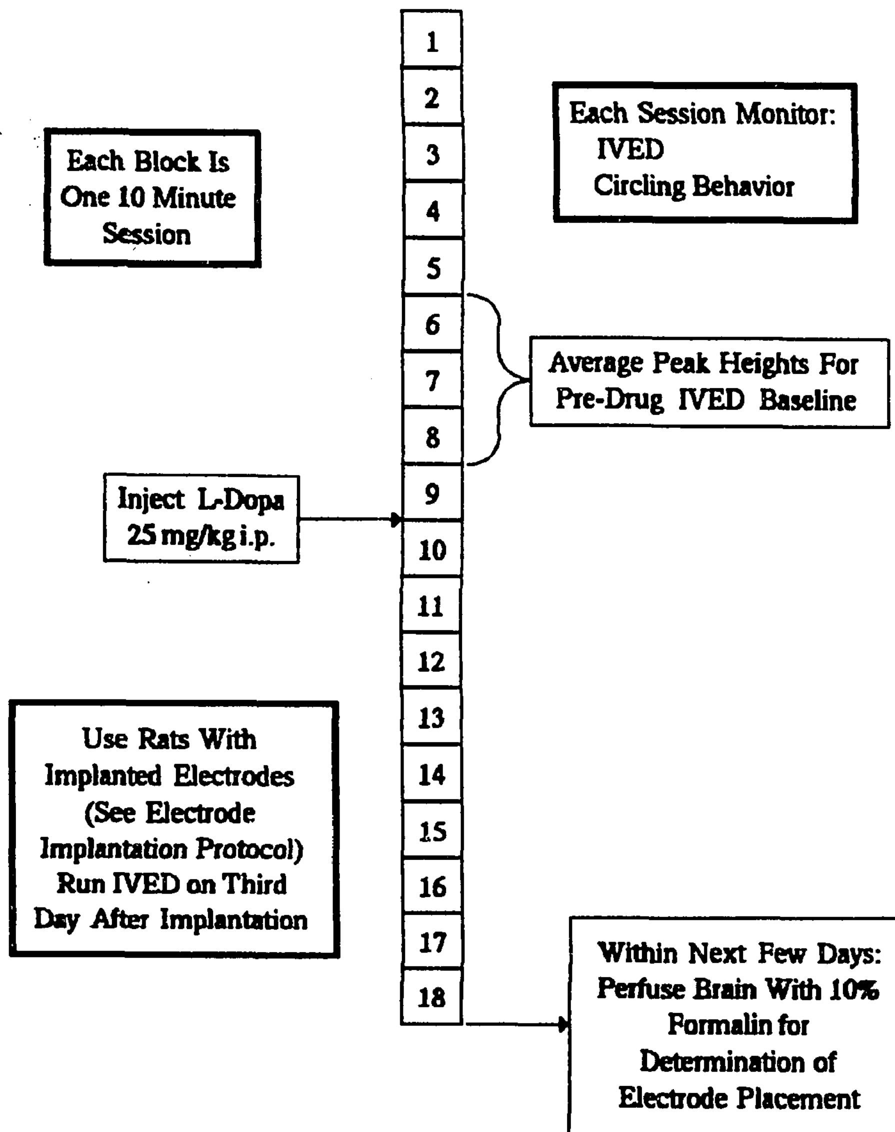
LESION SCREENING PROTOCOL



Successfully lesioned rats were bilaterally implanted with striatal carbon paste electrodes (bite bar: +5.0 mm, A-P: bregma + 2.0 mm, LAT: midline ± 2.5 mm, D-V: dura – 4.5 mm). (See Figure 4 Electrode Implantation Protocol.) Construction of electrodes was described in detail above. The placement of the electrodes was confirmed at the completion of the study by sectioning the fixed brain.

Three days after implantation rats were injected with L-dopa (Sigma) 25 mg/kg i.p. Semi-differentiated linear sweep voltammograms were recorded from each working electrode every 10 minutes over the voltage range –430 mV to +900 mV at a scan rate of 10 mV/s using a DCV-5 voltammetry controller (Bioanalytical Systems). The output passed through an external second-order low-pass filter before being displayed on a chart recorder. (See Figure 11 IVED Protocol.) Circling behavior and voltammograms were recorded for nine sessions before and nine sessions after injection. The voltammograms

Figure 11

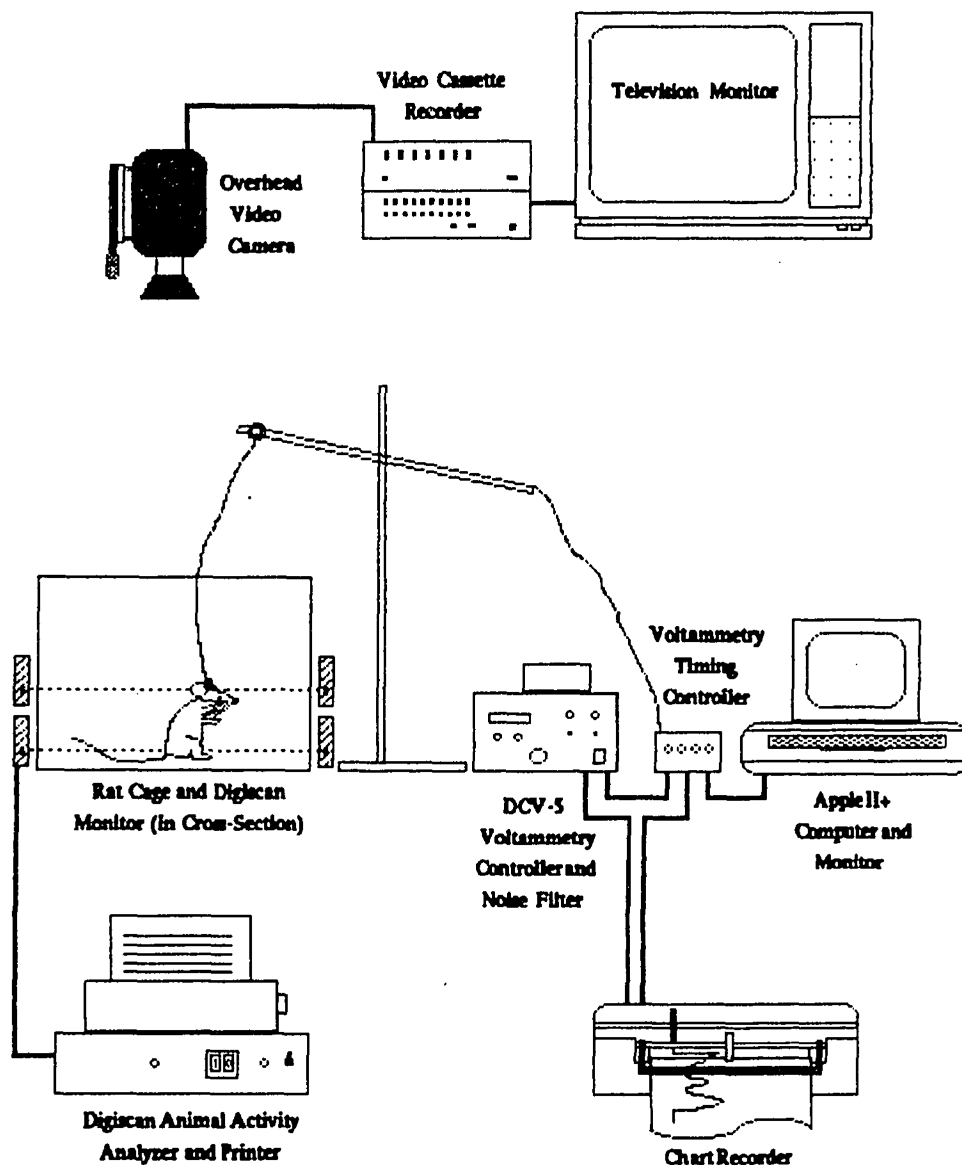


were recorded during the middle of each behavioral session. (See Figure 12 for a depiction of the equipment configuration.)

The heights of Peaks 1 and 2 were measured from an absolute zero baseline. The three sessions just prior to drug administration were averaged to determine the pre-drug peak height. Peak heights post-drug were expressed as a percentage of this average.

Figure 12

IVED EQUIPMENT SET-UP



Some rats did not show distinct peaks in the voltammogram from one side of the brain, and were excluded from the study.

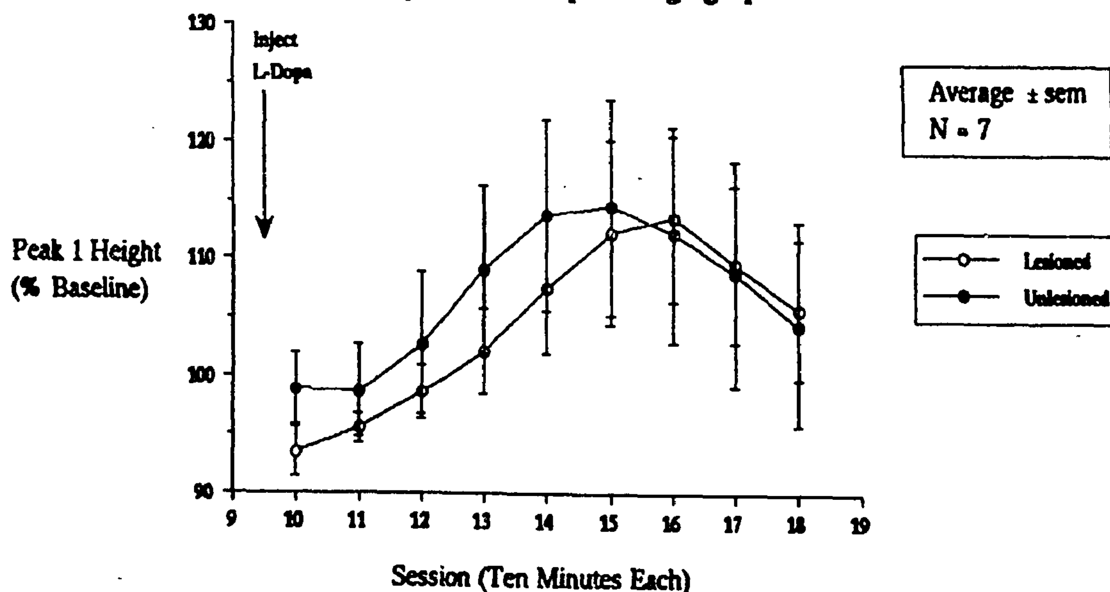
The time courses of the post-injection changes of Peaks 1 and 2 were analyzed by repeated measures analysis of variance. The repeated measure variable is time and the independent variable is lesioned vs. unlesioned side. The difference in the Peak 2 heights

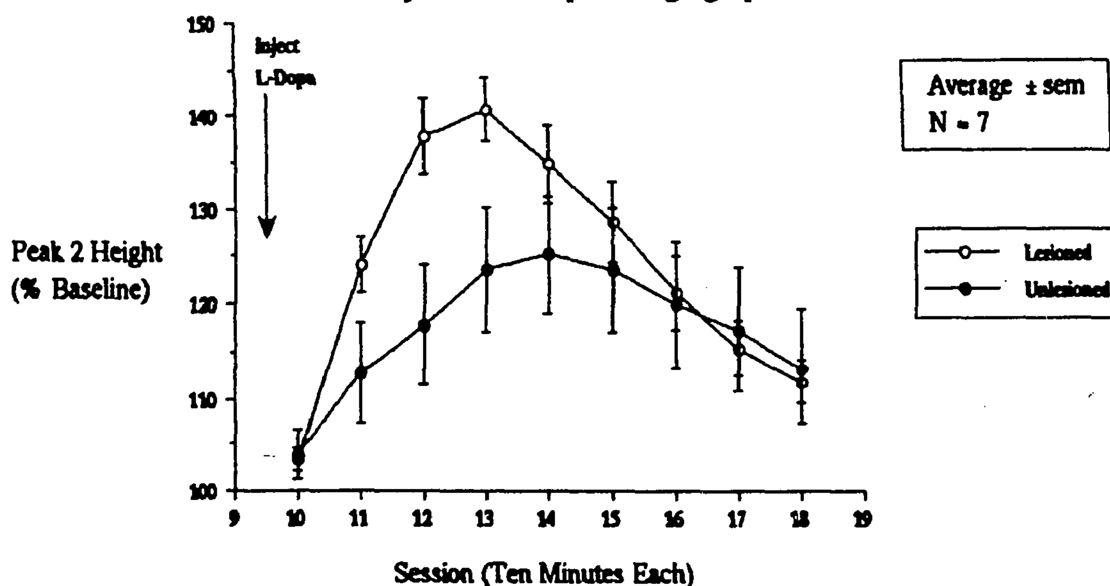
on the two sides was calculated by subtracting the height on the lesioned side from the height on the unlesioned side. This was correlated with the net rotations for that session or the preceding session.

RESULTS

The use of apomorphine as a screening device indicated that about 40% of the rats had questionable lesions. The circling induced by the drug in the rats with good lesions was marked, and the separation between circlers and non-circlers was obvious. When these rats were later injected with L-dopa, they again circled in the same direction as they did to apomorphine. The time course for the two drugs was slightly different: apomorphine-induced rotation began at about 10 minutes, peaked at about 20 minutes, and ended at about 40 minutes; L-dopa-induced rotation began after only 3 minutes, peaked within 10-15 minutes, and ended by about 30 minutes. With both drugs, after circling ended the rats went into a quiescent phase in which they would sit or sleep in one corner of their cage without stirring.

The time courses of Peaks 1 and 2 are illustrated in Figures 13 and 14. Peak 1 showed similar changes over time in both the lesioned and unlesioned caudate. It increased gradually to a maximum level of about 114% of baseline at 60-70 minutes, and then gradually decreased. Peak 1 did not quite return to baseline by the end of the experiment at 90 minutes. Peak 2 also increased in both the lesioned and unlesioned caudate. In the lesioned caudate, however, the increase was greater than in the unlesioned caudate (141% of baseline, as opposed to 125%). The maximum levels were reached at 40 minutes in lesioned caudate and 50 minutes in unlesioned caudate. By 90 minutes the height of Peak 2 was equal on the two sides, but had not quite returned to baseline.





Statistical analysis leads to the following conclusions: (a) the heights of both Peaks 1 and 2 show significant changes over time (1: $p=0.0003$, 2: $p=0.0000$); (b) the heights of Peaks 1 and 2 do not show significant differences between the lesioned and unlesioned sides when compared over the entire nine post-injection sessions (1: $p=0.7378$,

2: p=0.2977); and (c) there is a significant interaction of time and side (lesioned vs. unlesioned) in the height of Peak 2 but not Peak 1 (1: p=0.7482, 2: p=0.0061). In other words, the injection of L-dopa induces changes in the heights of both Peaks 1 and 2 in scans of both the lesioned and unlesioned caudates. Only Peak 2, however, shows a difference in the time course of the change on the lesioned side when compared to the unlesioned side; the changes in Peak 1 are the same on the lesioned and unlesioned sides.

As indicated above, L-dopa induced circling in the same direction as apomorphine. The circling began within a few minutes of injection and lasted about one hour. The time course is shown in Figure 15. Almost no circling occurred before injection. Circling is reported as net turns, which is the difference between the number of turns away from the side of the lesion and the number of turns toward the side of the lesion. Very few, if any, turns were made toward the side of the lesion while the drug exerted its effect.

Figure 15

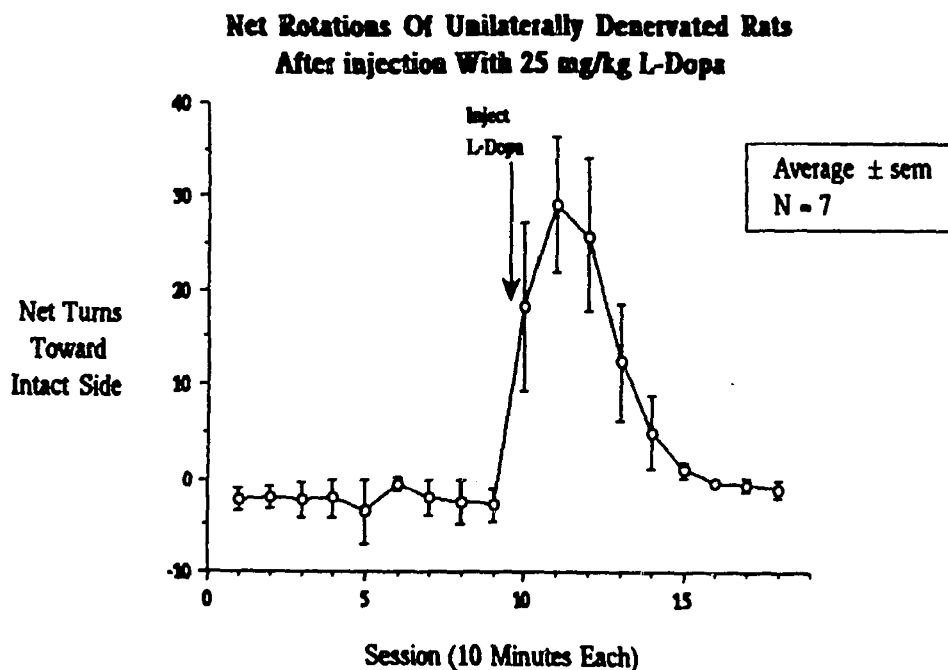
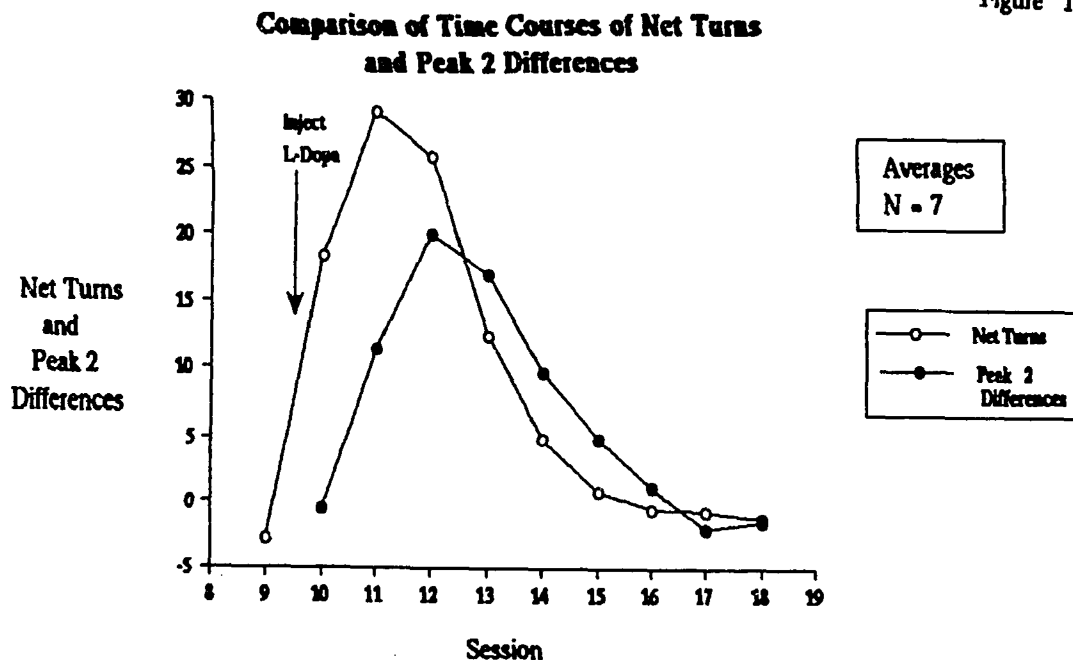


Figure 16



The difference in the Peak 2 heights on the two sides was calculated by subtracting the height on the lesioned side from the height on the unlesioned side. (See Figure 16 for time course.) This was correlated with the net rotations for that session. The values of the correlation coefficients are reported in Table I. There is a statistically significant positive correlation between the two parameters. A calculation was also performed in which the peak height differences were correlated with the net turns from the session immediately preceding the one in which the electrochemical measurements were made. In other words, the peak height differences from Session 2 were matched with the net turns from Session 1, the peaks from Session 3 were matched with the net turns from Session 2, and so on. (See Table I.) This association was stronger than that obtained by matching the same sessions. This suggests that the change in Peak 2 is somehow linked to the circling change, but that the electrochemical measure lags behind the behavior. (See Figure 16.) The two rats that had low correlations were rats that did not show good rotation in response to L-dopa, for an unknown reason, as opposed to having an unusual IVED time course.

Table I
CORRELATION OF PEAK 2 DIFFERENCES WITH NET TURNS

Rat Number	Peak 2 (t) : Net (t)		Peak 2 (t) : Net (t-1)	
	N=9		N=8	
	r	p	r	p
L2	0.86063	**0.0016**	0.93497	**0.0006**
L8	-0.66975	-	0.05532	0.4325
L11	0.36073	0.1709	0.83085	**0.0054**
L63	-0.50794	-	0.47221	0.1183
L66	0.65012	**0.0282**	0.96641	**0.0002**
L68	0.89501	**0.0008**	0.88188	**0.0022**
L70	0.69981	**0.0174**	0.82753	**0.0057**

Pearson's product moment correlations of Peak 2 height differences with net rotations for the sessions following L-dopa administration. For PEAK 2 (t) : NET (t), the correlation coefficient is based upon 9 points. For PEAK 2 (t) : NET (t-1), the correlation coefficient is based upon 8 points. Statistical significance calculated from a one-tailed t test with the null hypothesis $\rho \leq 0$. Statistically significant values are shown in bold print.

DISCUSSION

These results indicate a relationship exists between the height changes of Peak 2 and the circling behavior induced by L-dopa administration. This was quite unexpected, as Peak 2 is believed to reflect uric acid levels. That Peak 2 is changing as a result of serotonin release is possible but unlikely; similarly treated rats had decreases in the total tissue levels of both serotonin and its metabolite 5-HIAA (see section on biochemical results), suggesting that serotonin release and metabolism are decreasing, not increasing. Another potential source of the Peak 2 changes is the L-dopa itself directly causing changes in the voltammogram. Separate studies using microdialysis to monitor extracellular concentrations of amines and metabolites in the striatum suggest this is

unlikely. L-Dopa levels in microdialysates, following parenteral administration of twice the dose used in the present study (50 mg/kg i.p.), coadministered with a peripheral decarboxylase inhibitor to facilitate L-dopa accumulation by brain, were too low to account for the Peak 2 changes observed here. Furthermore, these studies suggest that extracellular concentrations of L-dopa were similar in both lesioned and unlesioned striata, indicating differences in the voltammogram do not reflect asymmetric accumulation of extracellular L-dopa (Brannan et al. 1989). Further definition of the identity of the substance measured by Peak 2 is presented in a later section.

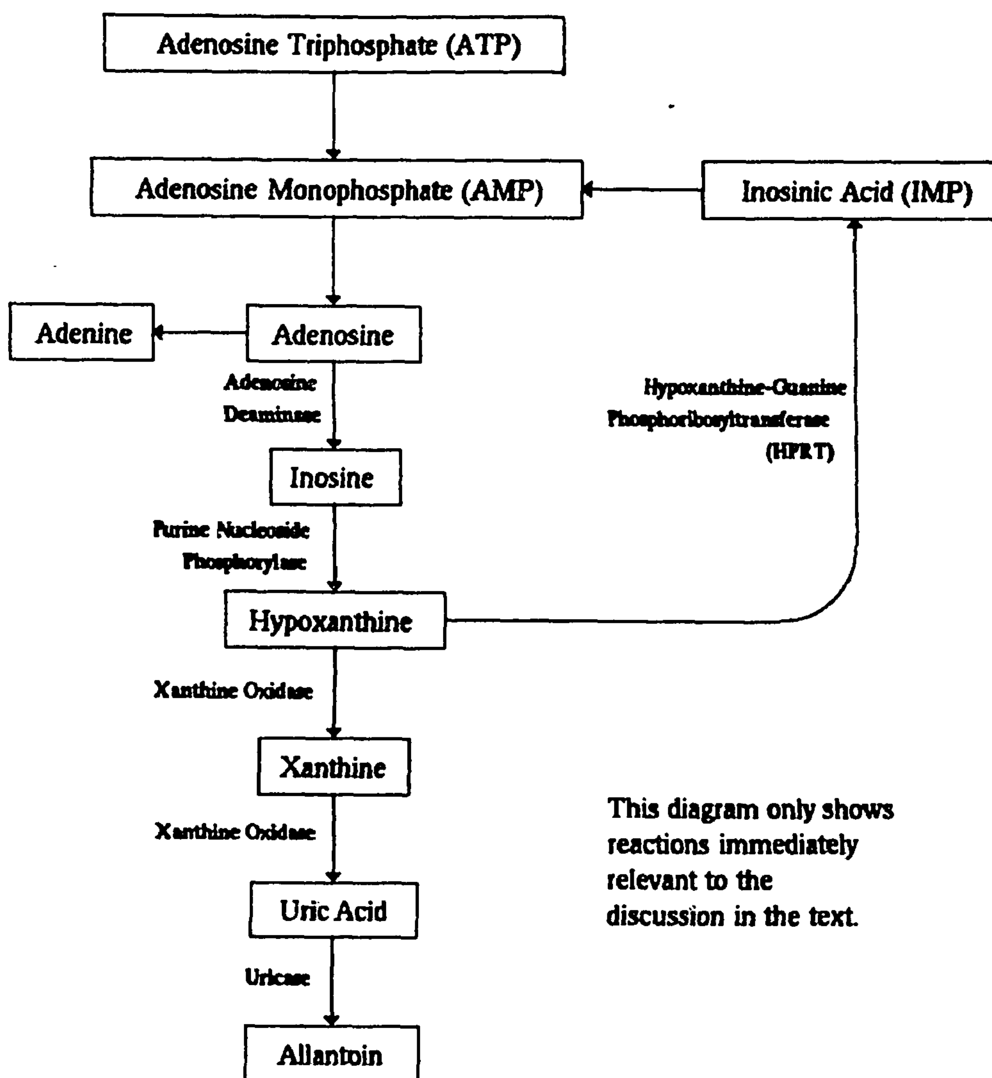
The strong association of the changes in Peak 2 with the rotational behavior indicates that this is not a nonspecific or artifactual result. This association is the most striking finding of this study. Regardless of the ultimate source of the Peak 2 changes, it is clear that they reflects a process that is strongly associated with circling, and, by extension, striatal activity. One other study has reported a statistical correlation between electrochemical responses and behavior (O'Neill and Fillenz 1985). In that research the investigators described a relationship between Peak 3 (corresponding to homovanillic acid) and locomotor activity during circadian monitoring, but with a lower correlation coefficient (mean $r = 0.363$, range 0.502 to 0.234). The changes in Peak 1 are not asymmetric, so the possibility that Peak 2 is related to the behavioral response is strengthened. We are unable to determine from this study whether this relationship is direct or indirect.

The lag of Peak 2 behind net rotations suggests two possibilities: (a) Peak 2 changes may be a result of the behavioral changes; or (b) there may be a time delay between what occurs at the site of action and at the electrode tip for the substance measured by Peak 2. This latter possibility has a basis in theoretical models in which a second diffusion compartment exists around the electrode (Albery, Fillenz and O'Neill 1983, Cheng et al. 1979). The time lag is not necessarily an unusual finding, as the study mentioned above

reported that the highest correlation is obtained if the electrochemical measure is considered to lag 30 minutes behind the behavior (O'Neill and Fillenz 1985).

It is interesting to speculate that the L-dopa effect on Peak 2 may indicate an interaction between dopaminergic neurotransmission and adenosine release/turnover in the striatum. It is possible that brain uric acid levels reflect purine metabolism. Most released adenosine is believed to be removed by a high-affinity reuptake system, but it may be that once it is taken back up it is degraded. The reaction pathway from adenosine to uric acid is:

ADENOSINE REACTION PATHWAY Figure 17



adenosine is deaminated to inosine by the enzyme adenosine deaminase (ADA, E.C.3.5.4.6); inosine is degraded to hypoxanthine by purine nucleoside phosphorylase (E.C.2.4.4.1); hypoxanthine is oxidized in two oxidation steps, via the intermediate xanthine, to uric acid by xanthine oxidase (E.C.1.2.3.2). (See Figure 17.) There are other, more circuitous routes, but this is the major one (Fox 1978).

There are indications that uric acid is derived from local adenosine metabolism. If xanthine oxidase is inhibited by microinjection of allopurinol into the striatum in the vicinity of the electrode tip, Peak 2 decreases (Mueller et al. 1985). This indicates that uric acid is being formed from local xanthine and hypoxanthine. Inhibition of adenosine deaminase by the systemic administration of an adenosine deaminase inhibitor resulted in an increase in extracellular levels of adenosine in the striatum, and an elimination of extracellular inosine (Zetterström et al. 1982). This suggests that deamination is a significant way of decreasing extracellular adenosine, and that extracellular inosine comes almost exclusively from adenosine deamination. In brain slices, inhibition of adenosine deaminase decreased the metabolism of labelled adenosine to inosine, hypoxanthine, and uric acid that was recoverable from the perfusion medium (Fredholm et al. 1983). Subjecting rats to hypoxia causes an 3–4-fold increase in extracellular adenosine, inosine, and hypoxanthine in the striatum (Zetterström et al. 1982). Other experiments indicate that inhibition of adenosine deaminase can mimic the sedative response to adenosine (Radulovacki et al. 1983), suggesting that the enzyme may play a role in deactivation of adenosine, or at least in inactivation, analogous to monoamine oxidase's role with the monoamines.

Adenosine is a candidate for neurotransmitter status (Snyder 1985), and there is evidence for an interaction between adenosine and dopaminergic neurons, in which adenosine presynaptically inhibits the release of dopamine (Michaelis, Michaelis and Myers 1979). In addition, adenosine (A1 and A2) receptors affect adenylate cyclase activity (Phillis and Barraco 1985), and so may interact with the effects of dopamine-

sensitive adenylate cyclase in the post-synaptic cell (Prémont, Perez and Bockaert 1977). Unilateral microinjection of adenosine analogs into the striatum of normal, naïve rats elicits circling upon systemic apomorphine challenge, further suggesting that adenosine may play a substantial role in this behavior (Green, Proudfit and Yeung 1982). O'Neill reported that modulation of adenosine neurotransmission affected Peak 3, and that Peak 2 could be increased by microinjection of adenosine deaminase, further evidence for the hypothesis that uric acid (Peak 2) is derived from adenosine metabolism (1986a).

It has been suggested that adenosine plays an inhibitory role with regard to turning behavior (Fredholm et al. 1983). It has previously been shown that adenosine inhibits striatal cell firing (Kostopoulos and Phillis 1977). This fits in very well with the notion that striatal cell firing is somehow correlated with turning behavior. It has also been reported that cell depolarization in the striatum affects adensine release (Barberis et al. 1984), further strengthening the case for a physiological role for adenosine in the striatum.

SECTION 4 — BIOCHEMICAL EXPERIMENTS

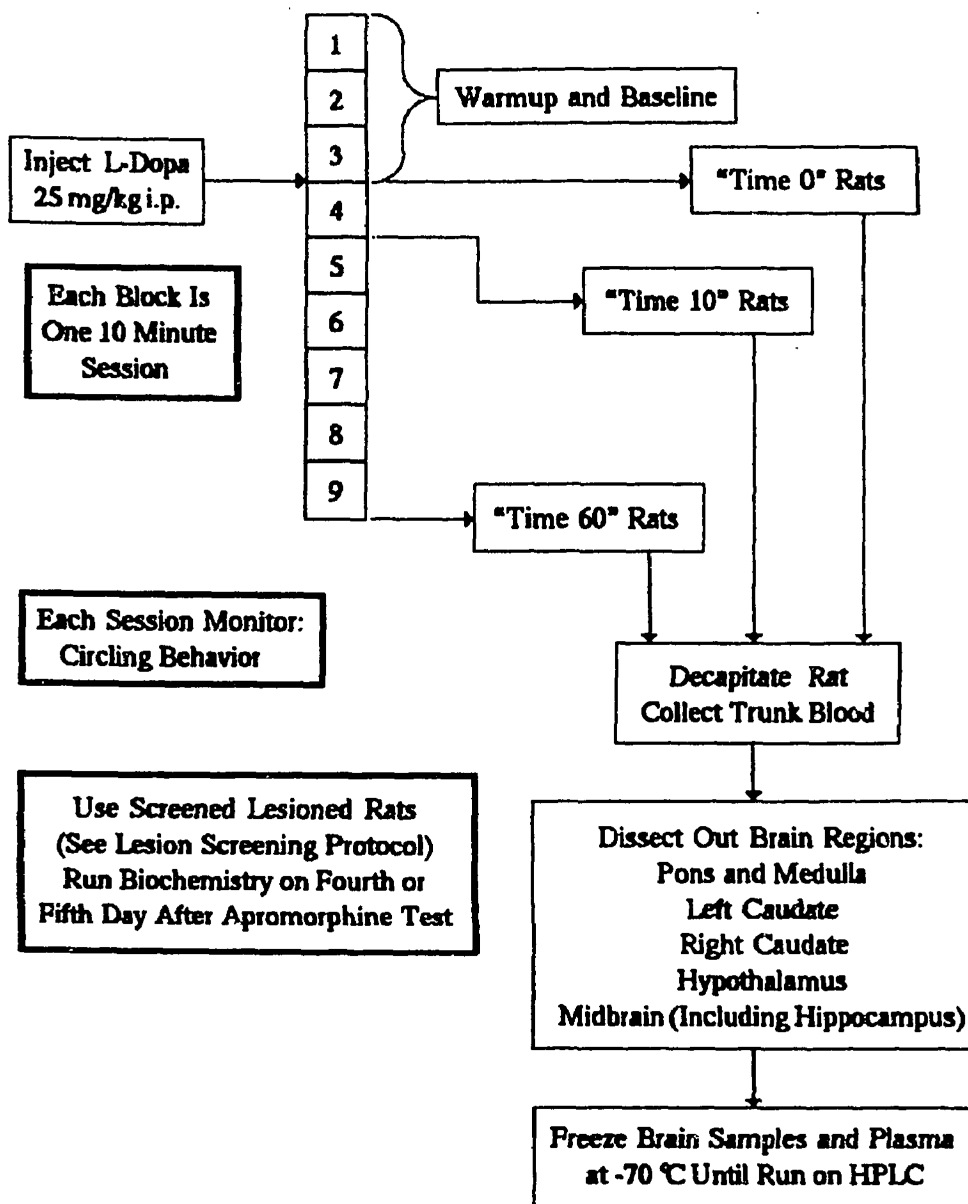
INTRODUCTION

Accompanying the IVED studies above were a series of experiments conducted in parallel that investigated the total tissue levels of neurochemical substances using a different methodology. By using an unrelated method for measuring chemical levels, the validity of both approaches can be assessed. The assay used was high performance liquid chromatography with electrochemical detection. This technique is very sensitive and has been extensively used so that the assays have become standardized and are fairly simple to perform.

These studies collected a great deal of data. Although the values of interest were primarily the levels of dopamine and its metabolites in the striatum, several other brain regions and plasma were also examined, and a number of other substances were assayed, including 5-hydroxyindoles. Because these other measurements were of peripheral interest in this study their results will not be discussed here and are shown in a series of figures in Appendix 4.

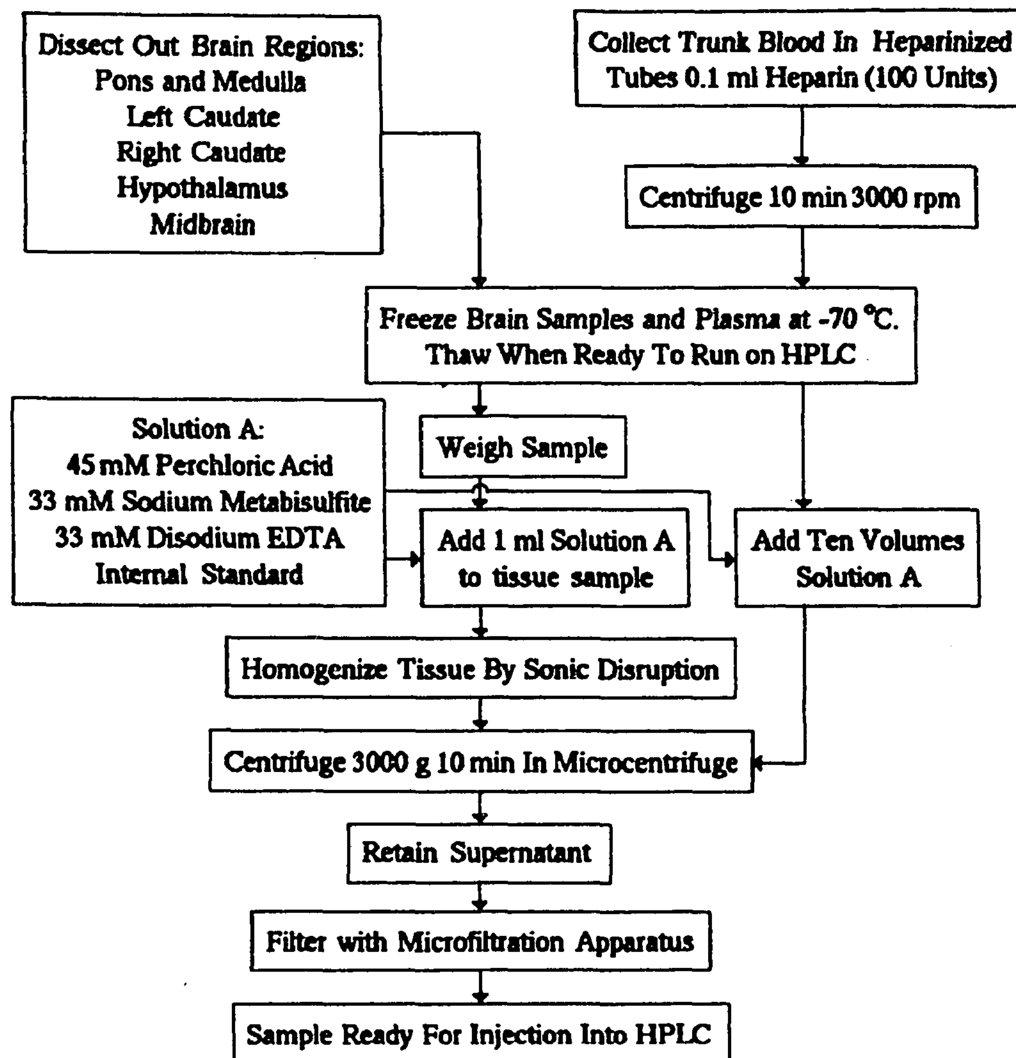
METHODS

Male Sprague-Dawley rats with unilateral nigrostriatal lesions similar to the ones used for the in vivo electrochemistry studies were used to determine striatal tissue levels of a variety of neurochemical substances as measured by high performance liquid chromatography (HPLC). Based upon the time course of the circling behavior after L-dopa (see Figure 15), it was decided that for these biochemical studies the rats would be killed at three time points: just before they would have been injected with L-dopa, at 10 minutes after injection (peak circling), and at 60 minutes after injection (no circling). Ideally, the neurochemical changes associated with the behavioral changes will show the



greatest differences at these time points. The rats were killed by decapitation and their brains dissected at the appropriate times before or after injection. In addition, the trunk blood from each of these rats was collected, and the plasma retained and frozen for later analysis. (See Figure 18 Biochemistry Protocol.) The samples were prepared by sonic disruption of the tissue in buffer, centrifugation to remove cellular debris, and microfiltration of the supernatant. (See Figure 19 HPLC Sample Preparation Protocol.)

Figure 19



RESULTS

In the caudate, the substances measured were: norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), serotonin (5-HT), 3-methoxytyramine (3-MT), and tryptophan (TRYP). See Figures 20 to 27 for graphical presentation of the results.

Figure 20

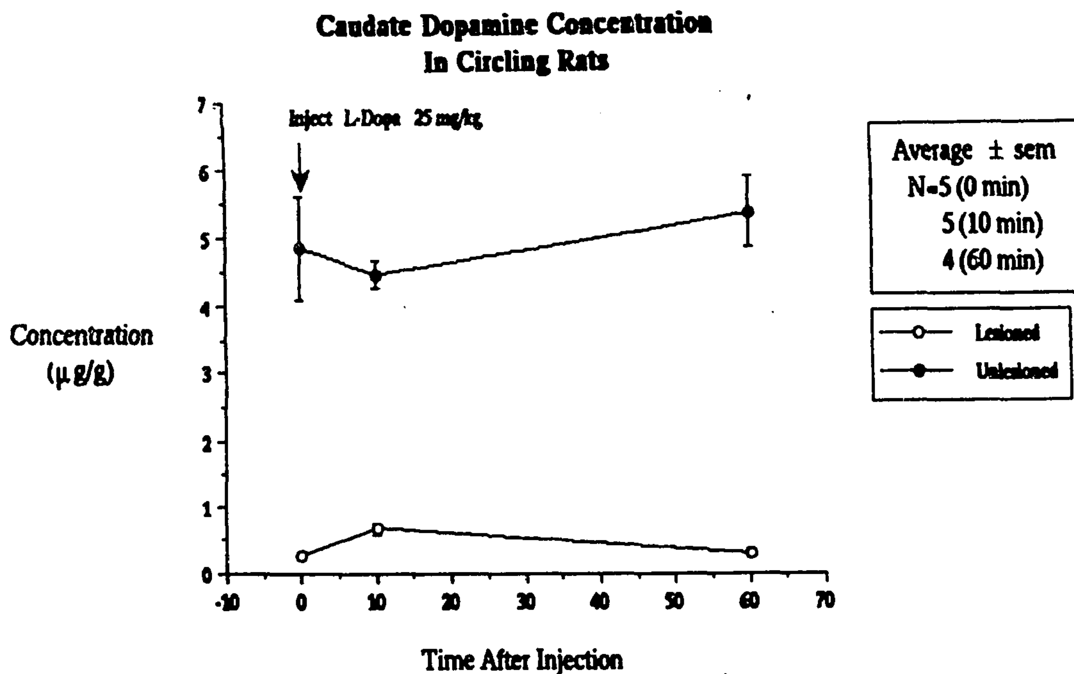


Figure 21

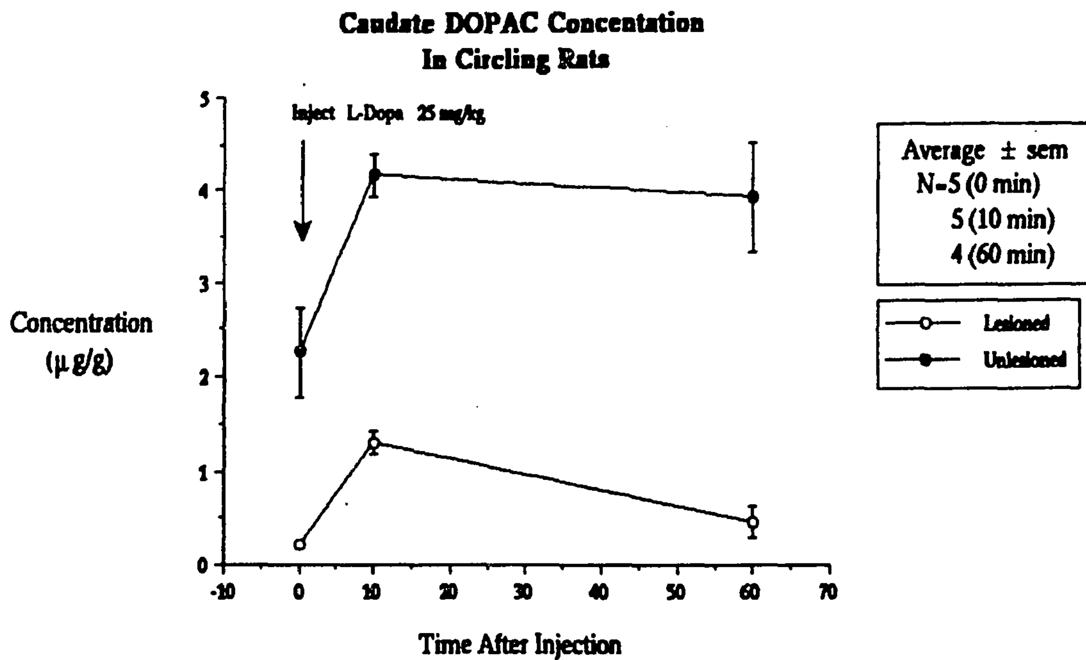


Figure 22

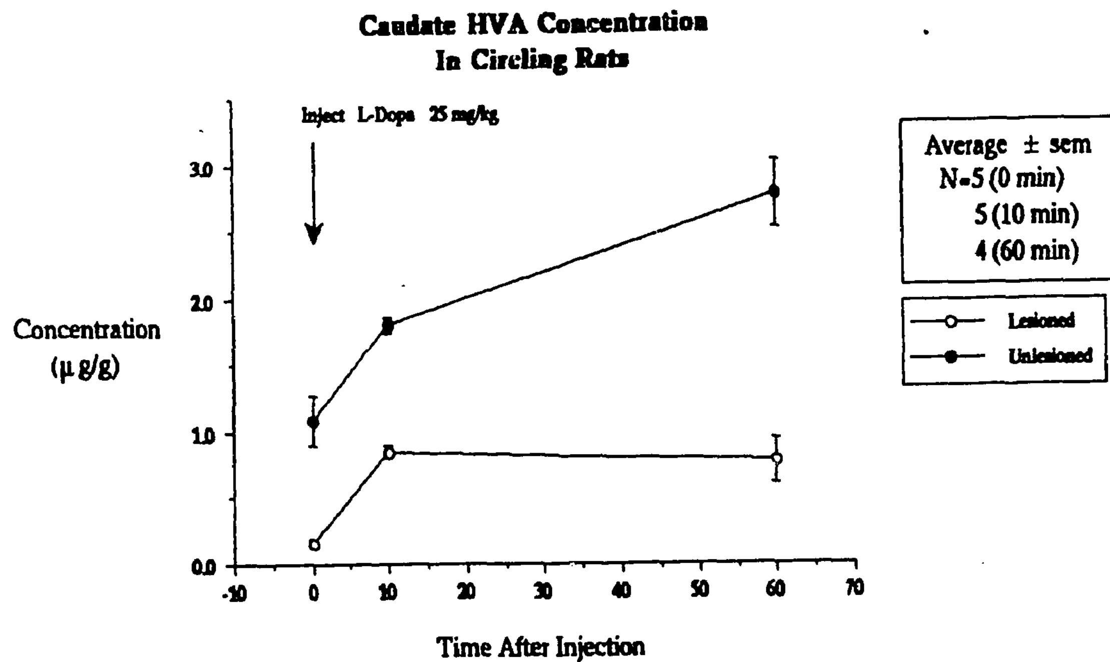


Figure 23

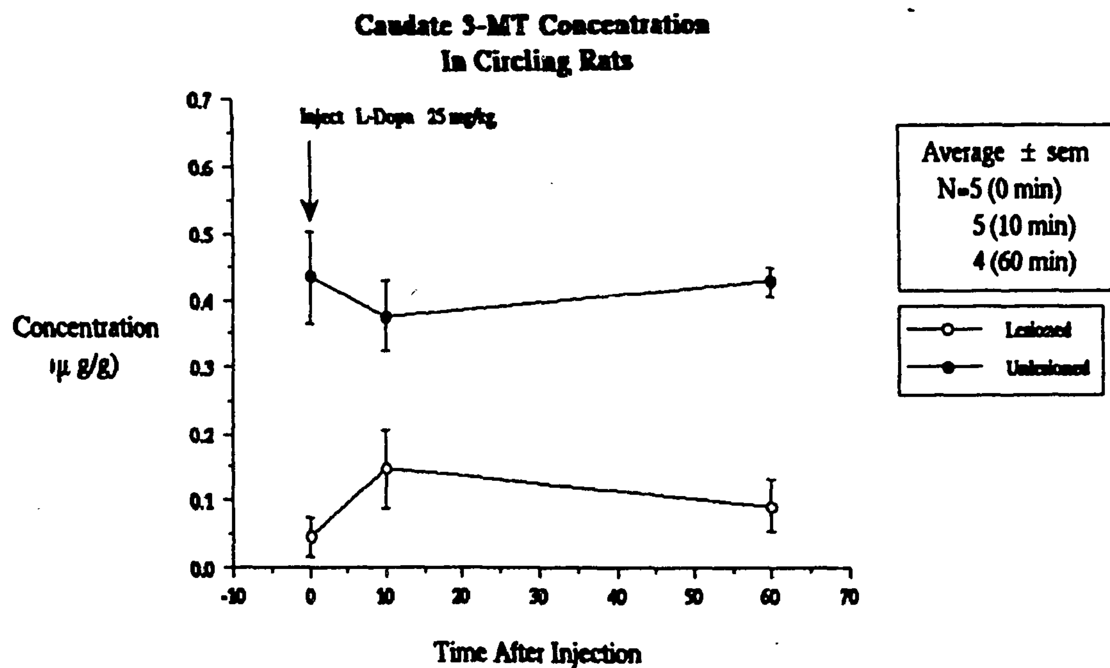


Figure 24

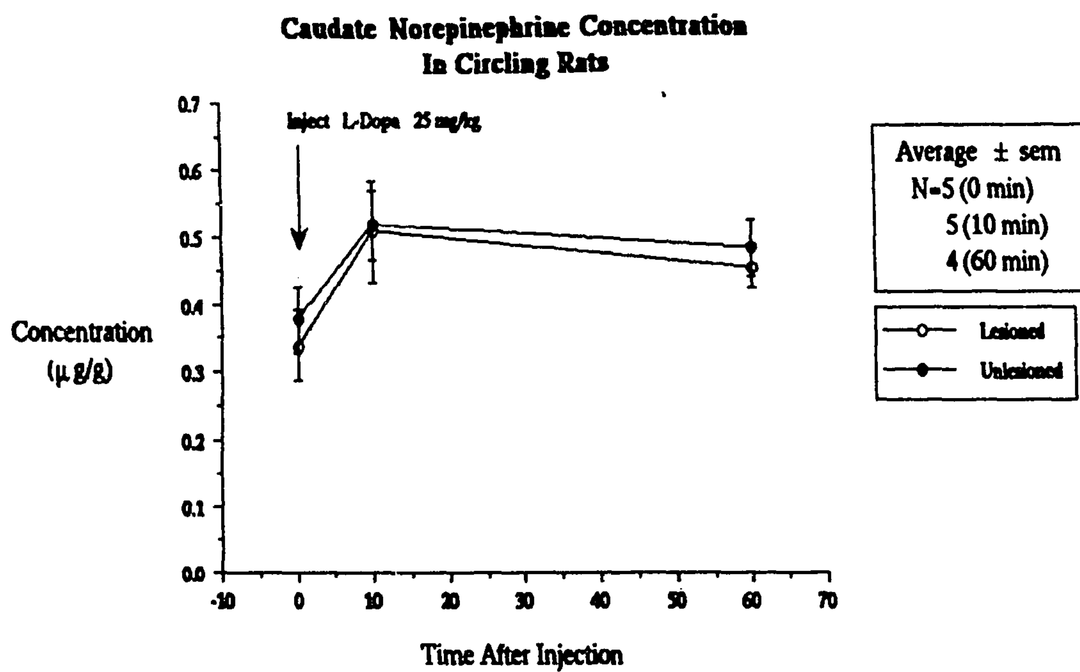


Figure 25

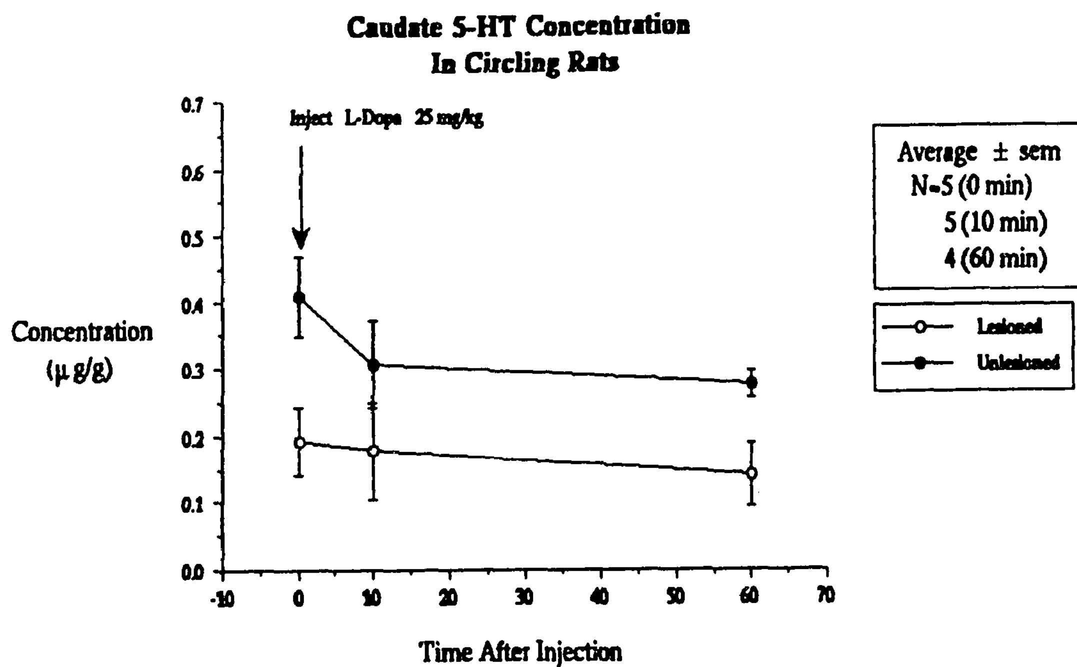


Figure 26

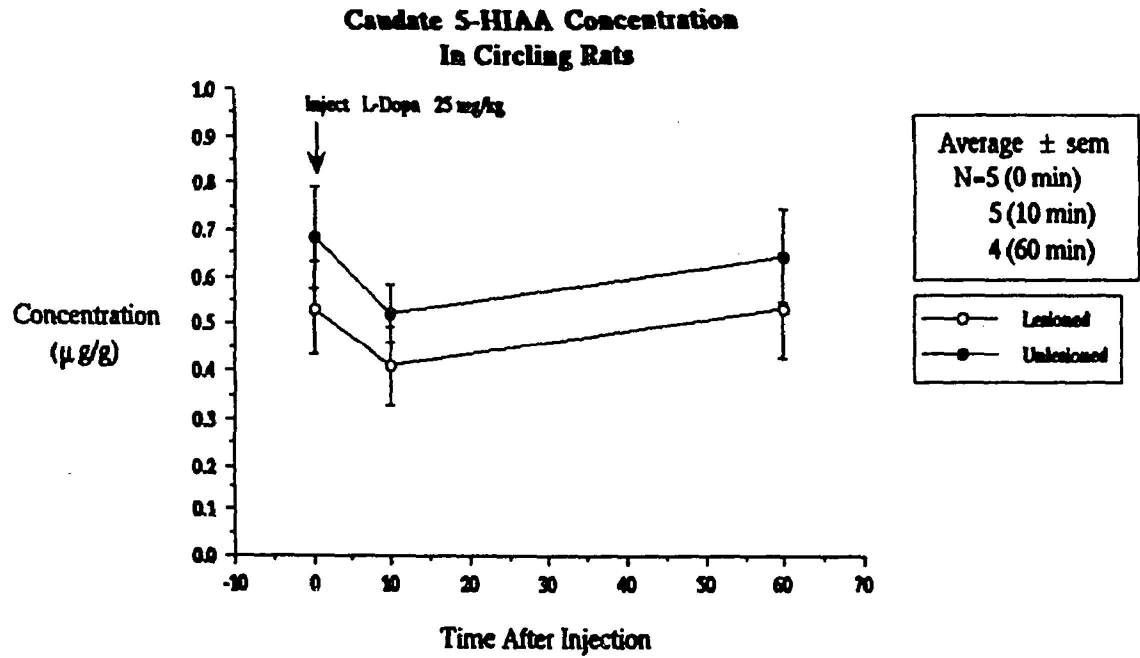
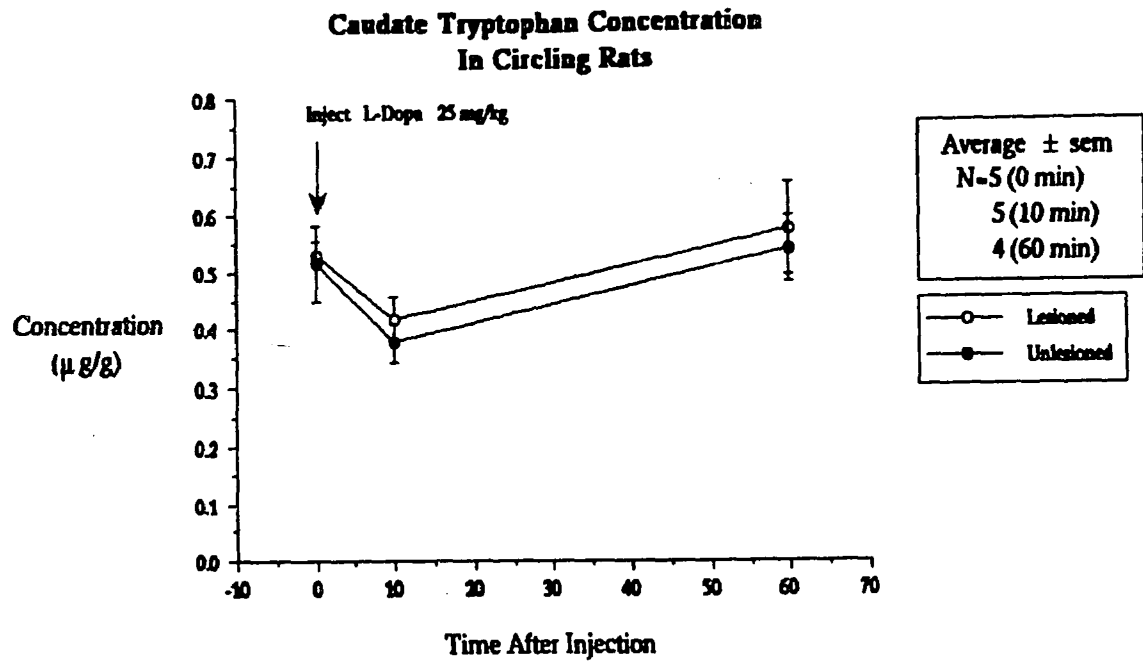


Figure 27



Statistical analysis was performed to compare the lesioned and unlesioned caudate levels in the rats that did not receive L-dopa. The sides were compared with two-tailed paired t-tests. The time courses of the different levels of the measured substances was analyzed by repeated measures analysis of variance with time as the repeated measure being time and side as the inter-group factor. Dopamine and its metabolites all showed significant reductions in the denervated caudate when compared to the intact caudate (See Table II). Serotonin also showed a significant reduction, although the decrease is not as extensive or as dramatic as for dopamine (53% reduction, p=0.0183). There were no significant changes in the levels of norepinephrine (p=0.5911), tryptophan (p=0.7376), and 5-HIAA (p=0.3707).

After administration of L-dopa, dopamine levels do not change over time (p=0.7375). In contrast, the levels of the dopamine metabolites DOPAC and HVA showed significant

Table II
BIOCHEMICAL LEVELS IN LESIONED AND UNLESIONED CAUDATES

	Caudate Concentrations (μg/g)		% Reduction	p
	Unlesioned	Lesioned		
Norepinephrine	0.3769	0.3395	9.9	0.5911
DOPAC	2.2666	0.2178	90.4	**0.0116**
Dopamine	4.8480	0.2837	94.1	**0.0037**
5-HIAA	0.6850	0.5322	22.3	0.3707
HVA	1.0897	0.1580	85.5	**0.0060**
Serotonin	0.4094	0.1923	53.0	**0.0183**
3-MT	0.4352	0.0454	89.6	**0.0104**
Tryptophan	0.5135	0.5311	-3.4	0.7376

Comparison of the concentrations of neurochemical levels in the denervated and intact caudates of circling rats. The negative value for tryptophan indicates that the levels in the lesioned caudates were higher than in the intact caudates. The two sides were compared by two-tailed paired t-test, and the p values are shown. Statistically significant values are shown in bold print.

changes over time, although 3-MT did not (DOPAC: p=0.0003; HVA: p=0.0001; 3-MT: 0.891). Although only HVA showed an interaction of time and side (p=0.0014). DOPAC and HVA both increased bilaterally after L-dopa injection. Norepinephrine also increased bilaterally (p=0.0222), but there was no difference in concentration between the two sides. Serotonin and 5-HIAA did not change over time (serotonin: p=0.3038; 5-HIAA: p=0.2704). Tryptophan decreased after L-dopa (p=0.0146), probably due to competition for transport into the brain.

DISCUSSION

The interpretation of this data in comparison with the data from the in vivo electrochemical detection experiments is somewhat challenging because the brain compartments being sampled are different for the two techniques. It must be kept in mind that IVED monitors extracellular levels in the area of the electrode tip, whereas biochemical assays with HPLC measure whole tissue levels for the entire volume of the striatum. HPLC samples the intracellular space, as well as the extracellular. Thus the two methods are not directly comparable. Despite the differences, however, the biochemistry results are consistent with the electrochemistry conclusions.

These experiments verified the procedure of nigral lesioning with 6-hydroxydopamine: those rats that passed the apomorphine screening test had depletions of striatal dopamine that exceeded 94% compared to the intact striatum. This is consistent with previously reported results that unilaterally lesioned rats do not circle following L-dopa administration unless the depletion is at least 90% (Hefti, Melamed and Wurtman 1980).

The injection of L-dopa 25 mg/kg i.p. did not induce a change in the concentration of dopamine in the striatum, but it did increase the concentration of its metabolites. This suggests that L-dopa does not increase the storage pool of dopamine, and that the

dopamine stimulating the post-synaptic receptors is a relatively small fraction of the whole tissue levels. The increase in the metabolites indicates that L-dopa does induce an increase in dopaminergic activity and turnover.

The increase in norepinephrine, which was sustained for at least 60 minutes, suggests that L-dopa can induce a change in the storage pool of this transmitter. The lack of a change in serotonin and its metabolite 5-HIAA suggests that L-dopa does not affect serotonergic activity. As mentioned previously, this argues against the possibility that the changes in Peak 2 reflect an increase in 5-hydroxyindoles. Thus the hypothesis that Peak 2 is measuring an increase in extracellular uric acid is strengthened. As was suggested earlier, the decrease in tryptophan may be a result of the decrease in the transport of the amino acid across the blood-brain barrier because of competition from L-dopa. Again, if anything, this change would be expected to decrease levels of 5-hydroxyindoles, rather than increase them.

The next step in this study was to develop the methodology for further investigation of the Peak 2 increases. Techniques were developed for simultaneous microinjection of drugs and in vivo electrochemical detection. Initial unsuccessful attempts were made to use anesthetized and acutely implanted rats, after which a chronic implantation protocol was instituted. This work is described in the next section.

SECTION 5 — URIC ACID / ADENOSINE EXPERIMENTS

INTRODUCTION

The Peak 2 changes warranted further investigation. Under normal circumstances, Peak 2 is almost completely due to the oxidation of uric acid. The questions are: are the observed alterations in Peak 2 due to changes in the levels of uric acid or do they reflect changes in the levels of a totally different substance that coincidentally oxidizes at the same potential? If the change is due to uric acid, why does it occur? Uric acid is the end-product of purine metabolism, so there are several possible sources. It could result from the breakdown of ATP, due to an increase in local energy metabolism when intrinsic striatal neurons are stimulated by L-dopa. Or it could come from the breakdown of either ATP or adenosine that has been released into the extracellular space to act as a neurotransmitter and/or neuromodulator.

Recently, much evidence has accumulated that adenosine acts as a neurotransmitter and interacts with specific adenosine receptors in the brain (Snyder 1985). Many studies suggest an interaction between adenosine and dopaminergic neurons in the striatum (Harms, Wardeh and Mulder 1979, Michaelis, Michaelis and Myers 1979, Prémont, Perez and Bockaert 1977, Prémont et al. 1983). In addition, there is a report that an adenosine agonist microinjected directly into the striatum of a rat treated with a dopamine agonist causes the animal to circle (Green, Proudfit and Yeung 1982). These observations in the literature prompted an investigation into the hypothesis that the uric acid in these experiments was derived from extracellular adenosine released as a neurotransmitter. Because adenosine is an intermediate reaction product in the metabolism of ATP to uric acid, however, the other possible sources of uric acid mentioned above would be difficult to rule out.

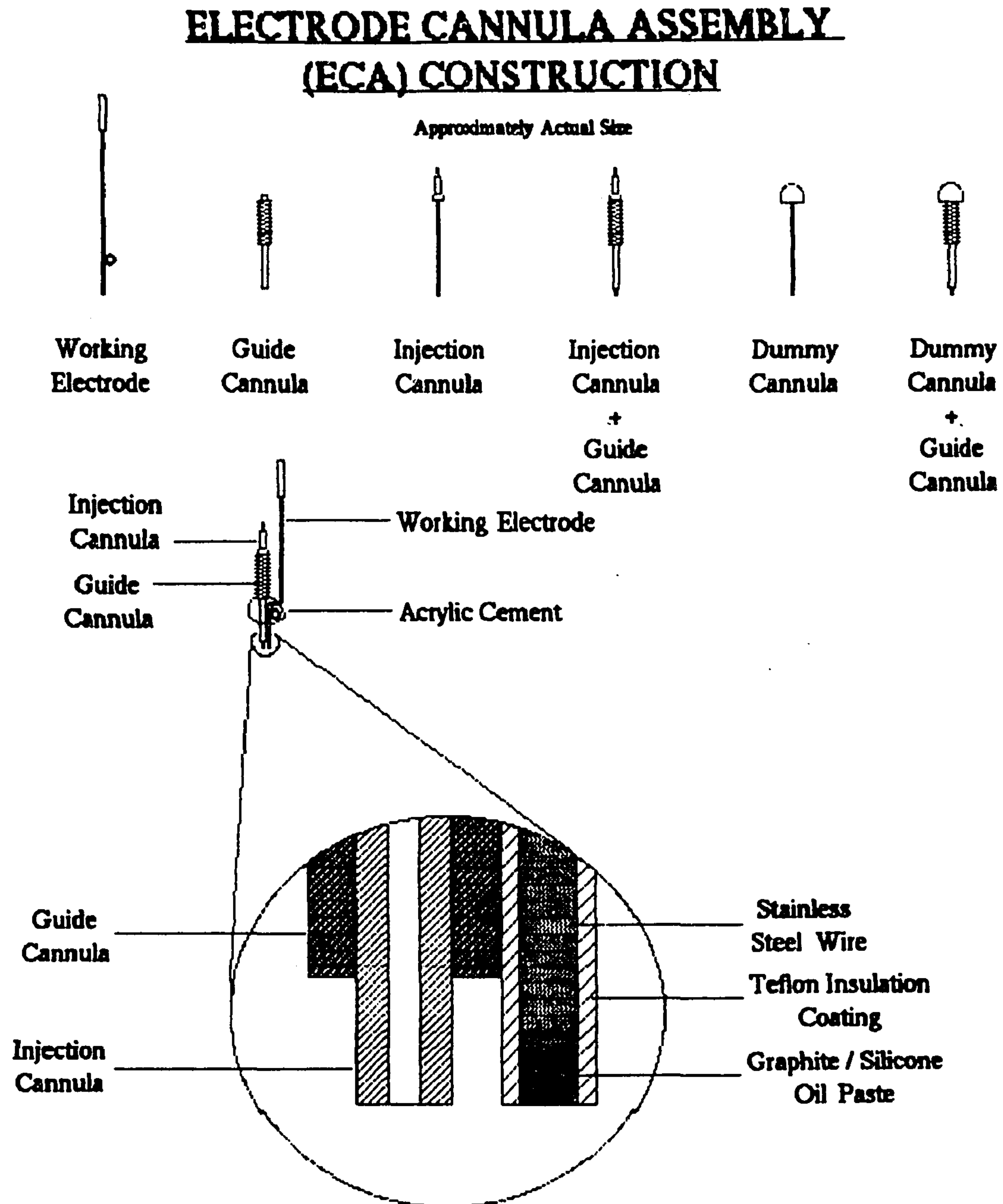
The working hypotheses for this study were: (1) the previously seen changes in the electrochemical signal due to L-dopa administration are due to changes in extracellular uric acid, (2) the uric acid is derived from adenosine release, and (3) the adenosine release is a result of L-dopa being transformed into dopamine and stimulating dopamine receptors. Only the first hypothesis and part of the second are directly addressed in the experiments reported here. The third hypothesis is explored in the next section.

NEW METHODS

The experimental protocols, as initially proposed, called for microinjection of various drugs into the striata of anesthetized rats that were acutely implanted with electrode/cannula assemblies (ECAs). This protocol was modified after some preliminary studies, because it was apparent that the voltammograms were different in anesthetized rats when compared to previous experiments. It was unclear whether this difference was due to the anesthesia, or whether it was due to changing from a chronic to an acute procedure. The nature of this difference was not extensively explored. The protocol was modified to use unanesthetized rats that had received permanent ECA implants for chronic study. Thus, each experiment took more time, but the new protocol had the advantages of allowing each rat to be used in multiple experiments.

Electrode/cannula assemblies (ECAs) were constructed by cementing together a working electrode and a guide cannula for the microinjection. (See Figures 28 and 29) The electrode was positioned so that the active surface at the tip would be at the same level as the tip of the injection cannula. Whenever the rat was not being microinjected, including during implantation, the guide cannula was kept patent with a solid dummy cannula. The injection cannula was inserted only for the actual microinjection, and removed shortly thereafter. Cannula components were obtained from Plastic Products.

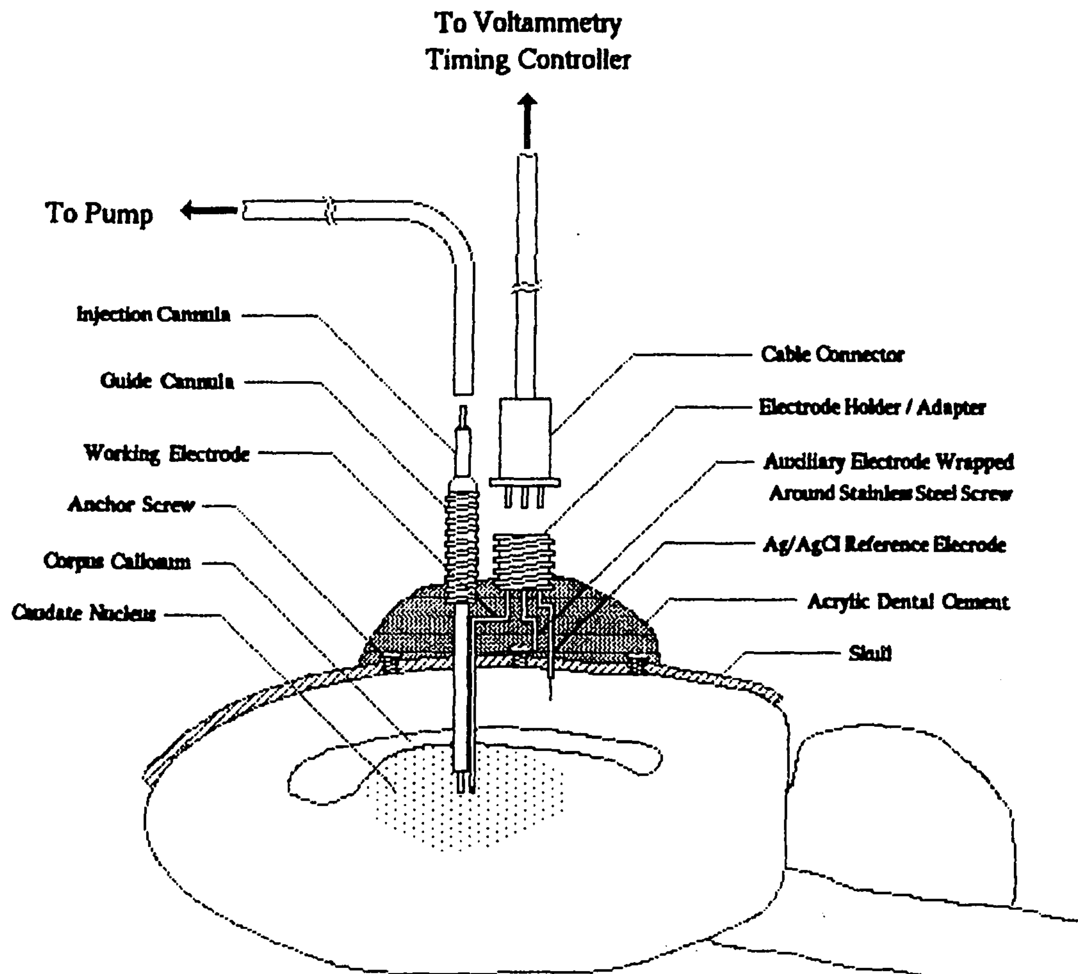
The rats had one ECA implanted into each caudate. (See Figure 29 Implanted ECA Headset and Figure 30 ECA Implantation Protocol.) The rats used were not lesioned with



6-hydroxydopamine, except in one set of experiments. After recovery from surgery, the animals were used in a series of microinjection experiments. Each experiment consisted of a warm-up period of 8 ten-minute sessions, following which the dummy cannulae were

Figure 29

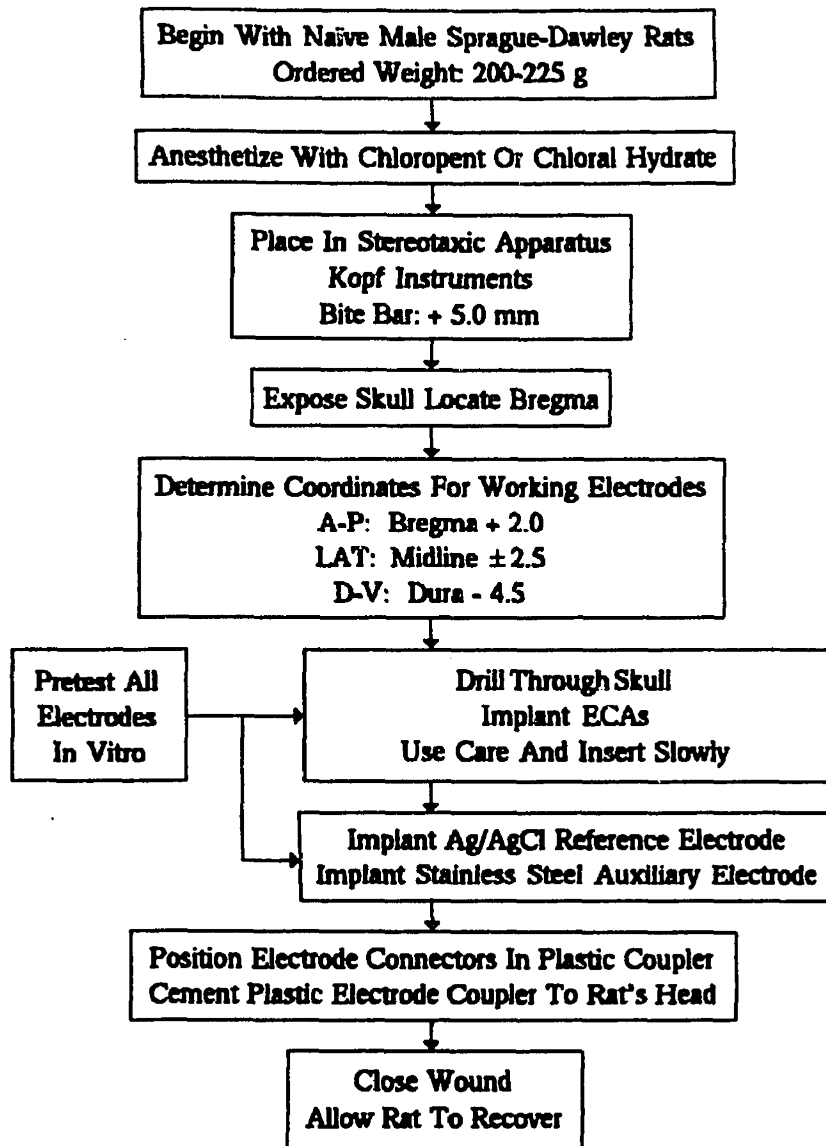
IMPLANTED ECA HEADSET



One ECA is implanted in each caudate for a total of two ECAs per rat. Only one of the two is shown in the diagram for clarity.

replaced with injection cannulae. One session after the injection cannulae were inserted, a drug in saline or plain saline solution was microinjected into each caudate simultaneously. The cannulae were loaded with the solution to be injected during the 10 minute session just prior to being inserted. The solutions were run through the cannula system and all

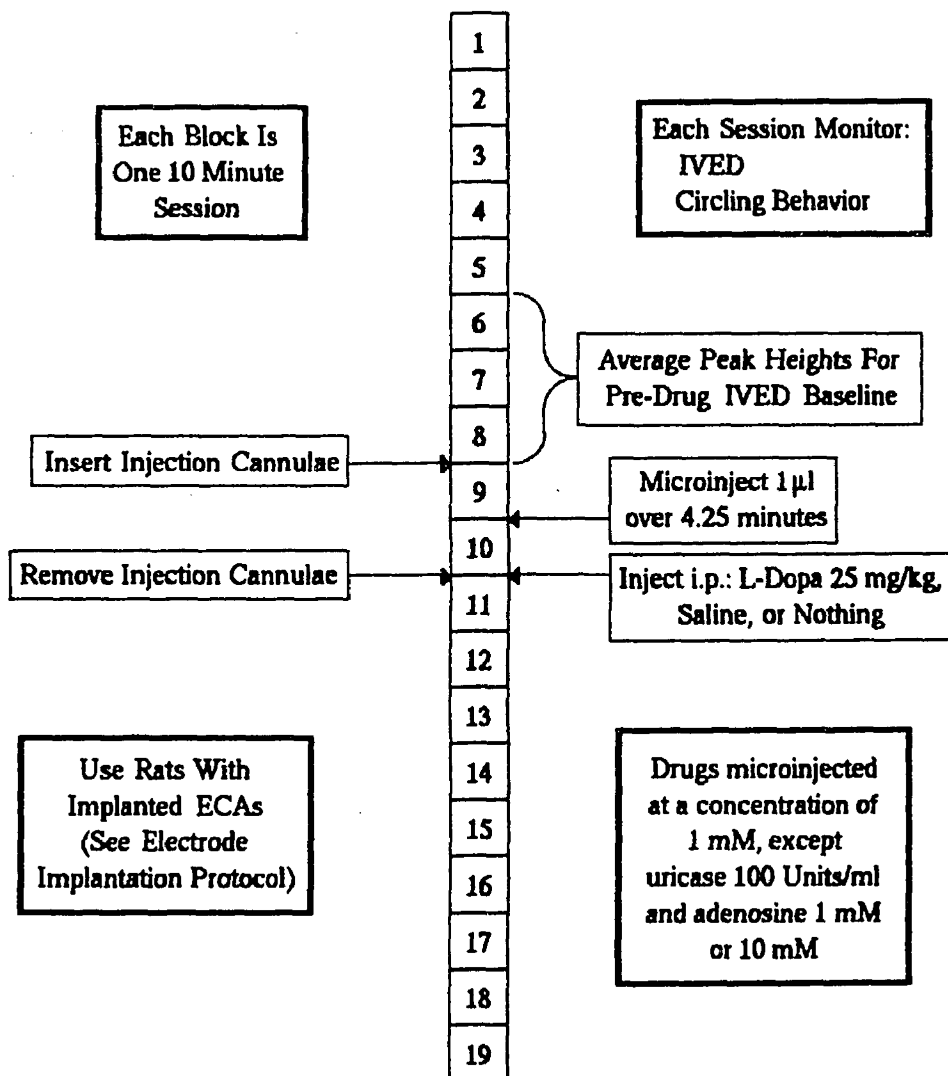
Figure 30



dead space was eliminated. This procedure, however, resulted in the solutions being present at the tip when the injection cannulae were inserted, so that prior to the start of the microinjection, some of the solutions may have already diffused into the surrounding tissue, albeit a small amount. All microinjections were made bilaterally with instillation of

Figure 31

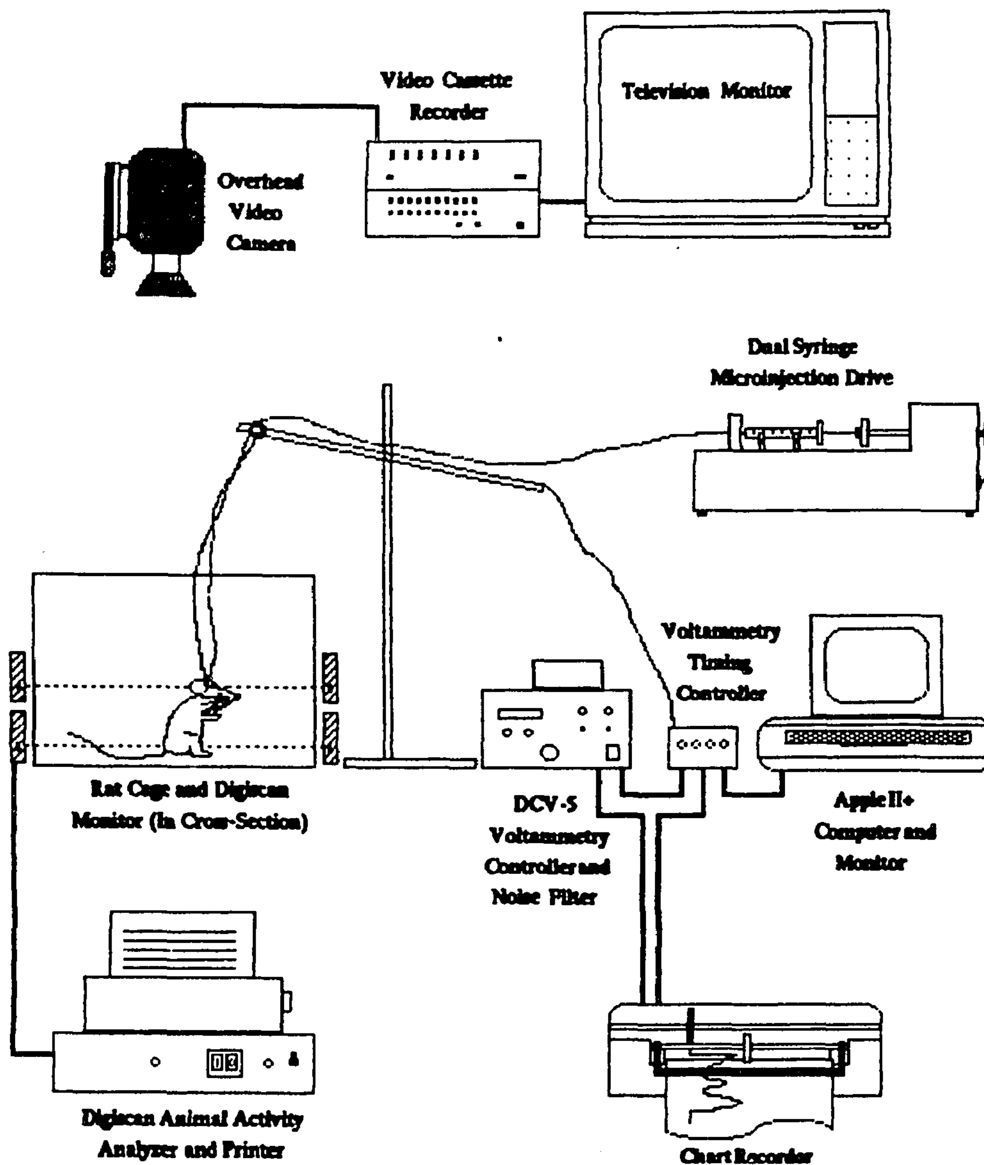
IVED / MICROINJECTION PROTOCOL



1 microliter of solution over a time period of 4.25 minutes. The volume of microinjection was chosen based upon a report of the volume of spread of dye injections in the brain that indicated that this volume was sufficient for spread from the cannula tip to the electrode tip (Myers 1974). The cannulae were left in place until the end of the next session when they were replaced by the dummy cannulae. Any systemic drugs were administered

Figure 32

IVED/MICROINJECTION EQUIPMENT SET-UP



intraperitoneally at this time. The rat was then monitored for 9 more sessions, for a total of 19. During each session, concurrent measurements were made of circling behavior and linear-sweep IVED of electroactive substances. (See Figure 31 IVED / Microinjection Protocol.) The equipment configuration used is depicted in Figure 32.

The voltammograms were analyzed as described in the appendix. The treated and the control sides were compared with repeated measures analysis of variance with time the repeated measure and brain side the second factor. In some cases, where some problem occurred in the measurement of one or more Peak 1 heights, due to noise or equipment problems, only the Peak 2 data were used for an experiment, and not the Peak 1 data. (This is to explain the occasional difference in the "n" between Peak 1 and Peak 2 within the same study for the experiments reported below.)

In all cases, the data from each experimental striatum were accompanied by corresponding data from the contralateral control striatum. In some cases, to increase the sample size, and to provide an additional level of control, rats were used twice under the same experimental protocol, except with the sides switched. Thus, some studies used a different striatum for each experiment, rather than a different animal.

At the conclusion of each animal's final experiment, it was killed under ether anesthesia by intracardiac perfusion with saline followed by 10% formalin, and the brain dissected out. Electrode-cannula assembly placement was verified by sectioning the brain. For all the animals, the electrode was found to be placed in the striatum. One animal was discarded because of the development of a large abscess at the tip of the right ECA, severe enough to cause distortion of the gross anatomy.

EXPERIMENTS

Control Saline Microinjections

The effects of the microinjection procedure itself had to be evaluated. Each rat received bilateral control microinjections of normal saline. Two sets of experiments were run; the first set of rats received no injections i.p., whereas the second set received saline i.p., similar to the injections in other studies. The voltammograms were analyzed as described previously. The results from each set of rats were averaged, combining both

striata from each animal, since there was no basis for making a distinction of ipsilateral versus contralateral.

The results of these experiments are shown in Figures 33 to 36. Both Peaks 1 and 2 were apparently affected by the procedure of replacing the dummy cannulae with the injection cannulae. This is seen by the increase in the peak heights in all cases, after the replacement of the cannulae, but before the microinjections. The increase can be quite substantial, as seen in Peak 1 in the rats that did not receive any injections i.p.; the peak increased to 250% of baseline during the session that followed switching the cannulae. In all cases, however, the increase was transitory, and the peak heights returned to baseline levels.

The increase could be due to any of several causes. The handling of the rats needed when changing the cannulae could cause sufficient discomfort and agitation to alter brain activity resulting in release of electroactive substances. Alternatively, the physical disturbance of the brain tissue surrounding the cannula tip during the procedure could cause the increase in peak heights. Because both Peaks 1 and 2 were increased, it seems likely that this is a nonspecific phenomenon. One possibility is that the surrounding tissue adheres to the dummy cannulae over a period of time. When the cannula is removed, the adherent tissue is disturbed, resulting in an increase in detectable levels of electroactive substances. This possibility could account for the lack of an increase in the peaks when the cannulae are changed the second time during the experiment at the end of session 10. In later experiments, I tried to minimize the effect by slightly loosening and then tightening the dummy cannulae prior to hooking the rat up to the electrochemistry equipment, in the hopes that any perturbations would have returned to baseline levels before the experimental manipulations began. Regardless, the influence of these peak height changes should make little difference, because, in all experiments, each rat served as its own control, so that these effects should cancel out. The possible effect of the cannula replacements was taken into consideration in further experiments.

Figure 33

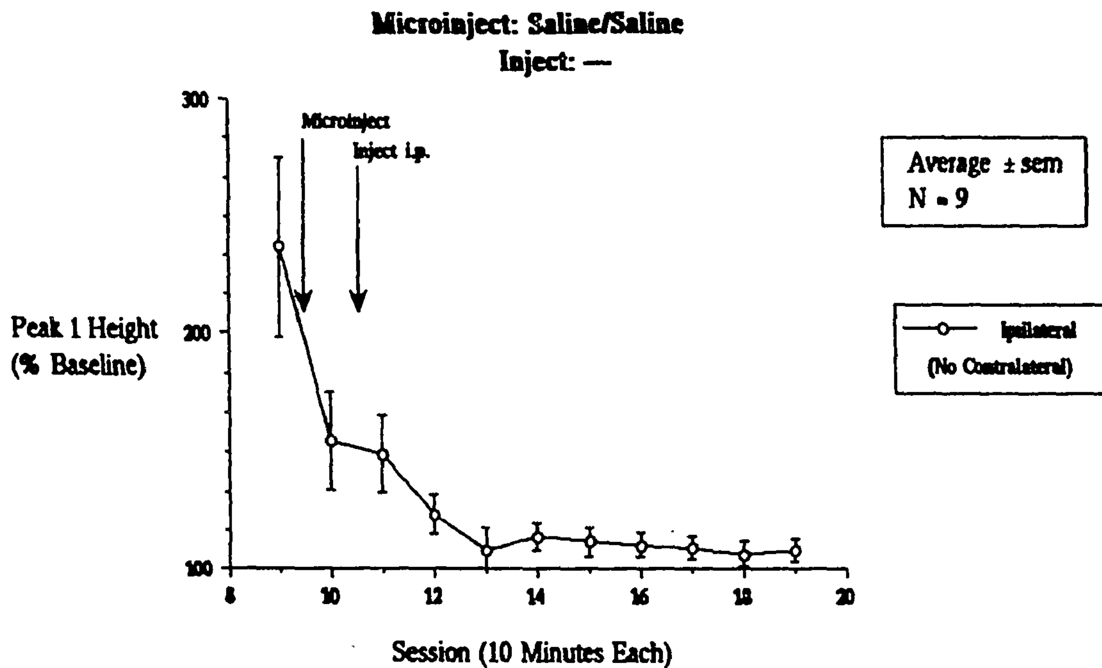


Figure 34

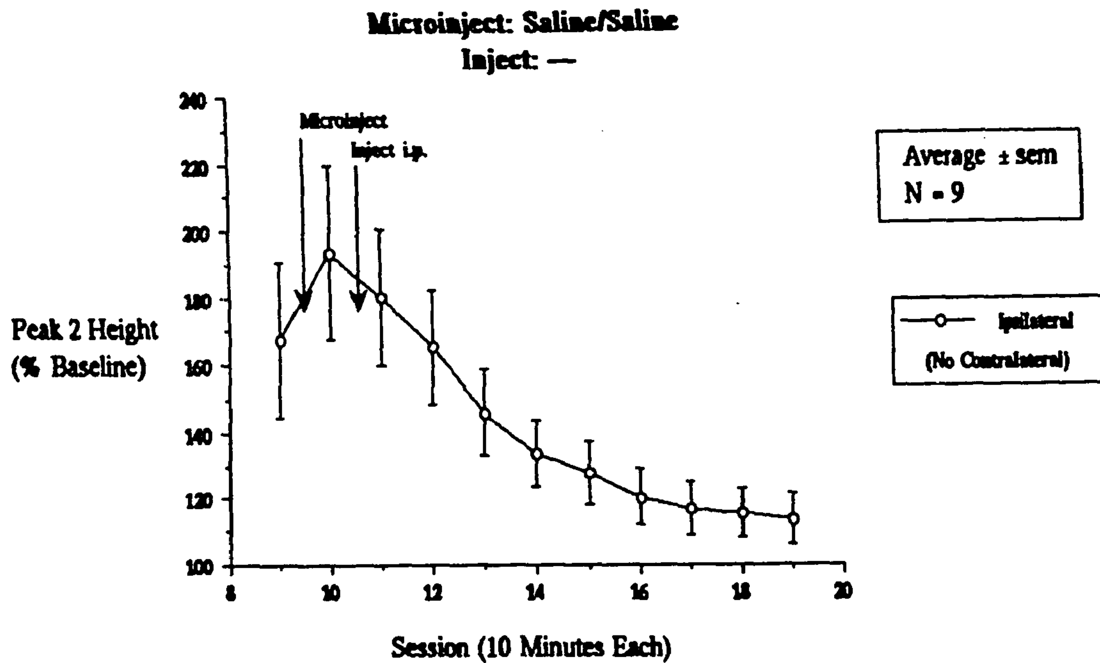


Figure 35

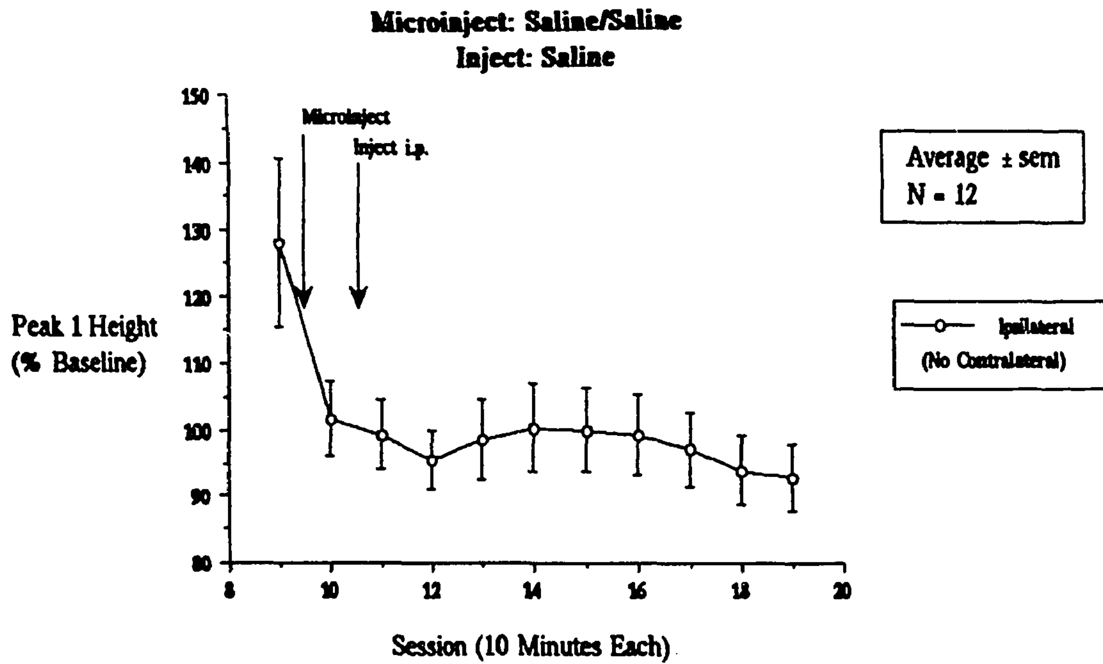
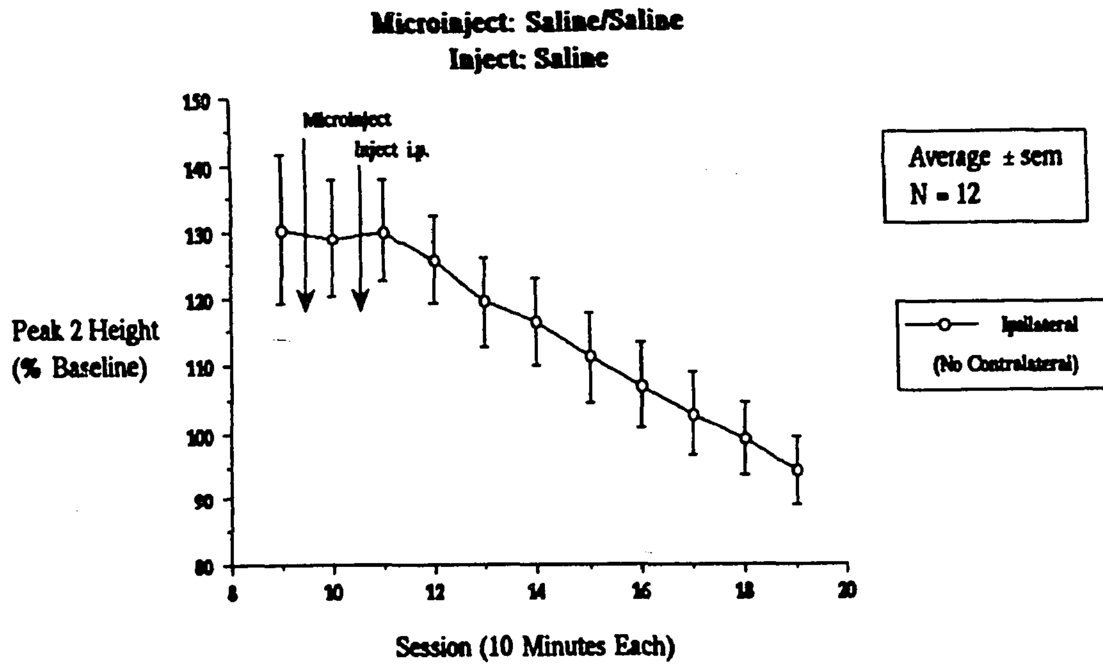


Figure 36



Investigation of the Unlesioned Striatum

The Peak 2 changes were previously demonstrated in experiments with lesioned rats. Using unlesioned rats in experiments to investigate the Peak 2 changes required first proving that the phenomenon was comparable in the two different experimental protocols. This meant showing: (1) that the administration of L-dopa to unlesioned rats induced a change in Peak 2 comparable to that seen in the unlesioned striatum in lesioned rats; (2) that the changes in Peak 2 in unlesioned rats is due to uric acid in both the lesioned and unlesioned striata; and (3) that the changes in Peak 2 in unlesioned rats is also due to uric acid. These experimental results would correlate Peak 2 with uric acid under three conditions: (1) lesioned striata in lesioned rats, (2) unlesioned striata in lesioned rats, and (3) unlesioned striata in unlesioned rats. If any of these hypotheses were shown incorrect, then Peak 2 changes in unlesioned rats might not necessarily reflect those seen in lesioned rats.

Effects of L-Dopa in Unlesioned Rats

These experiments examined the effects of systemic L-dopa administration in rats that did not have striatal lesions, and in which control microinjections of normal saline were made. The experimental protocol was as described above, with microinjection of saline bilaterally, followed by the systemic administration of L-dopa 25 mg/kg i.p. The results are shown in Figures 37 and 38.

The voltammograms showed that Peak 1 underwent no changes as a result of the L-dopa administration. In contrast, Peak 2 increased in height reaching a maximum of about 120% at the third session after drug injection (Session 12), that gradually decreased over the rest of the experiment. The Peak 2 changes here are similar to those seen previously in the unlesioned striatum in the rats treated with L-dopa. This suggests that the Peak 2 changes in unlesioned rats are the same as those seen in the previous experiments, indicating that contralateral lesioning is not a requirement for an increase in Peak 2 height on the intact side.

Figure 37

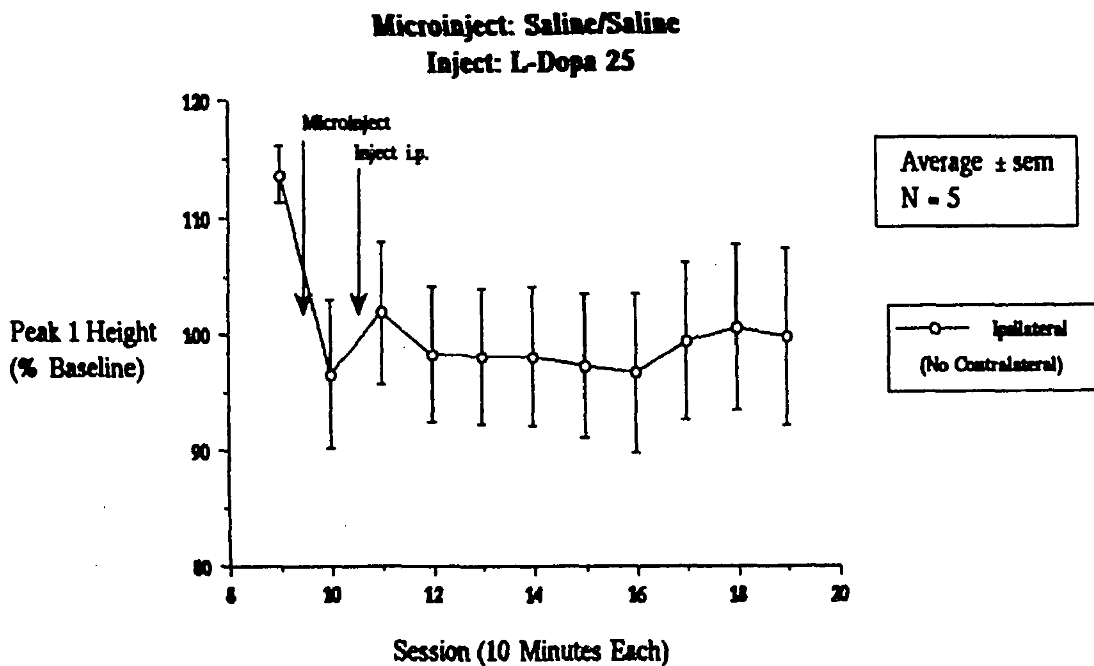
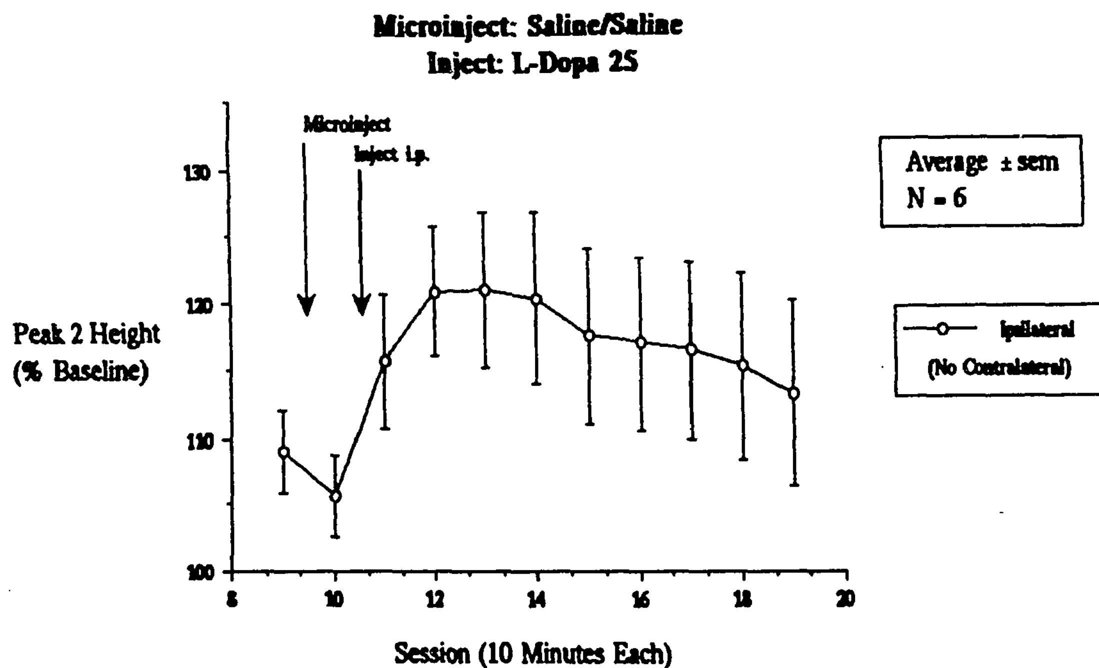


Figure 38



Uricase/Uricase in Lesioned Rats

The next step was to show that the Peak 2 changes seen are due to uric acid on both the lesioned and the unlesioned sides. The most direct way to do this was to use lesioned rats and show that the changes in Peak 2 are abolished by uricase, an enzyme that degrades uric acid to allantoin, a non-electroactive substance. Normally, this reaction does not occur in rats (or humans) because the species lacks this enzyme. Allantoin, however, is the final waste product of purine metabolism in other species. Uricase solutions were prepared with Uricase Type IV from Candida utilis (Sigma) dissolved in normal saline at a concentration of 10 units in 100 μl, which resulted in the microinjection of 0.1 units in 1 μl. The experimental protocol was as above, except that these rats were unilaterally lesioned with 6-hydroxydopamine to denervate the striatum and were screened for circling according to the previous procedures. (See Figure 9 Lesioning Protocol and Figure 10 Lesion Screening Protocol.) The results of these experiments are shown in Figures 39 and 40.

Figure 39

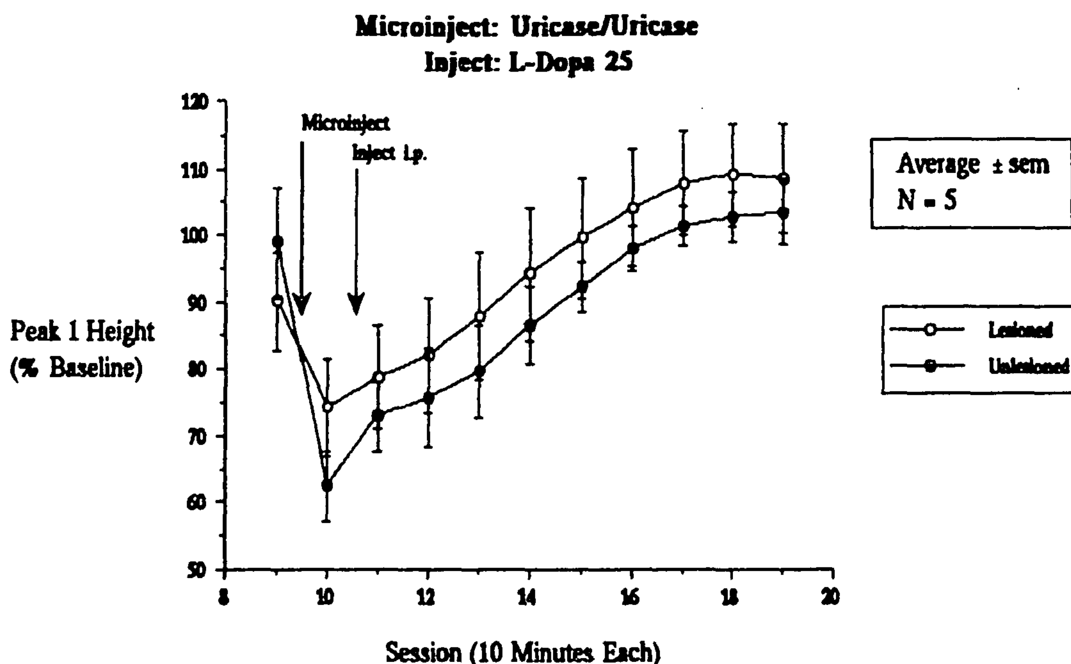
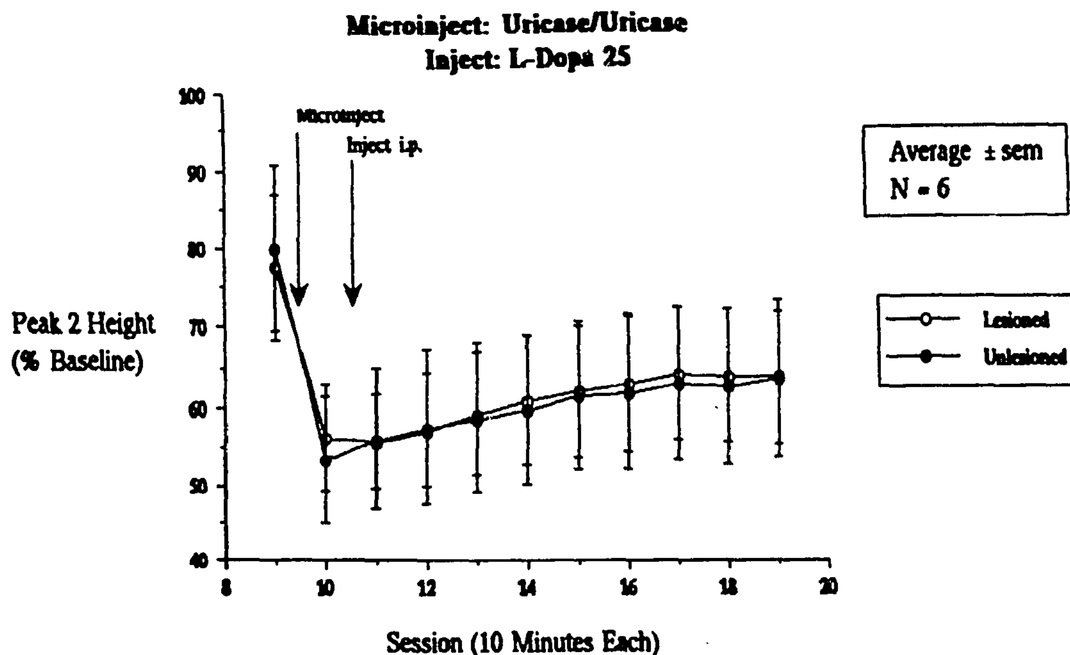


Figure 40



Peak 1 underwent a drop in signal after the administration of uricase, following which it steadily increased, reaching a level exceeding 100% by the end of the experiment (p=0.0001, n=5). The lesioned and unlesioned sides showed no difference between them (p=0.542, n=5).

Peak 2 also underwent a decrease in height after uricase, but the drop was much greater than in Peak 1, going down to 55%, and it was sustained throughout the experiment, with only a slight increase to 60% (p=0.0001, n=6). The lesioned and unlesioned sides showed identical responses (p=0.9612, n=6).

This experiment showed that the changes in Peak 2 that were observed in the previous investigation were definitely due to uric acid, and not to some other substance, such as serotonin or 5-HIAA as other investigators have suggested. Although the height of Peak 2 did not drop to zero, this could reflect the possibility that the defined zero point is not the same as the point corresponding to zero concentration (See Appendix 3). The Peak 2

changes, however, are completely abolished, as no change in Peak 2 was seen after the initial drop after infusion of uricase. This indicates that no increase in Peak 2 is induced by L-dopa if uric acid is eliminated. The equivalence of the responses in the lesioned and unlesioned sides indicates that the asymmetric response of Peak 2 in the earlier experiments is completely attributable to uric acid, and not to some direct effect of the denervation of the striatum that somehow results in the production of a new electroactive substance at the oxidation potential of Peak 2.

The change in Peak 1 is somewhat puzzling. The drop is not as large in magnitude as the decrease in Peak 2, but it is substantial. Peak 1 does not represent uric acid, and it should not be affected by uricase. The possibility exists that the huge drop in Peak 2 affects the height of surrounding peaks, but this seems unlikely, especially when the data from the next set of experiments is taken into account. More likely, this represents a dilution effect. Another possibility is that this is an accentuation of the drop seen after the peak height increase induced by cannula manipulation. In any case, Peaks 1 and 2 differ in their time course. After the initial drop, Peak 1 gradually returns to, and then exceeds, baseline height, while Peak 2 continues to remain at low levels. It is possible that the initial decrease in Peak 1 could have been related to the change in Peak 2, which is affecting surrounding peaks by a nonspecific overlap process. The subsequent increase might then be due to the normal increase in Peak 1 that follows L-dopa administration. Further investigation of the changes in Peak 1, unfortunately, was not continued, because the primary objective was investigating the changes in Peak 2.

Confirmation of Uric Acid in Unlesioned Rats

In order to show that Peak 2 and its L-dopa-induced changes represent uric acid in unlesioned rats, the next series of experiments involved microinjecting uricase into rats that did not have nigrostriatal lesions. These rats had uricase microinjected into only one striatum. The contralateral striatum received a saline microinjection as a control. Two sets of experiments were performed. In the first, unilateral uricase microinjection was

followed by injection of normal saline i.p. as a control. In the second, uricase was followed by L-dopa 25 mg/kg i.p. The results of these experiments are shown in Figures 41 through 44.

Figure 41

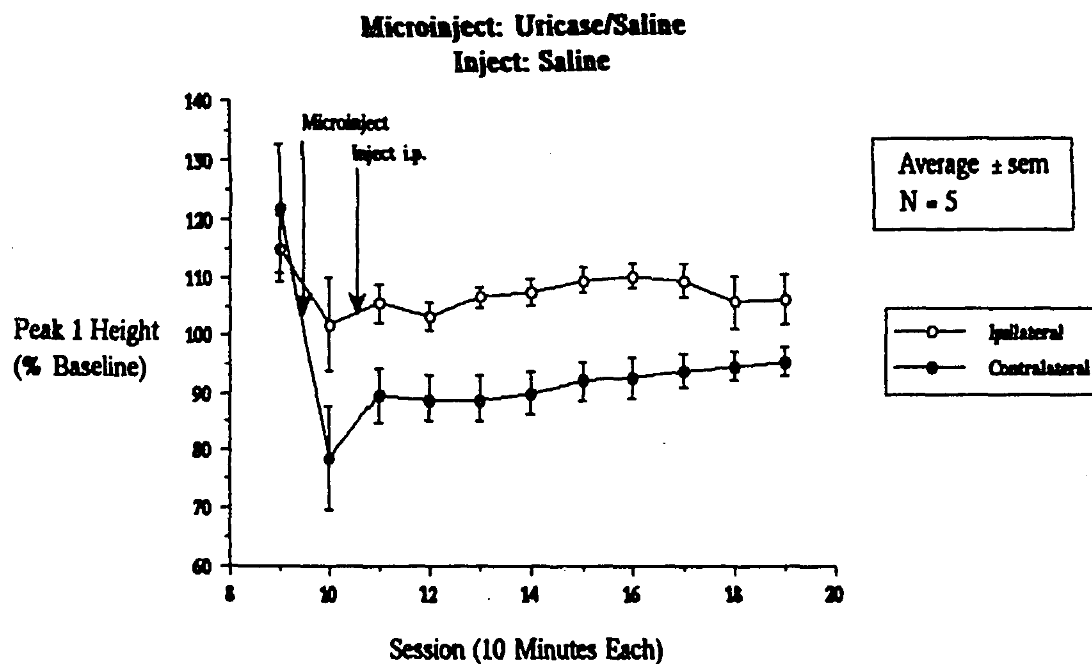


Figure 42

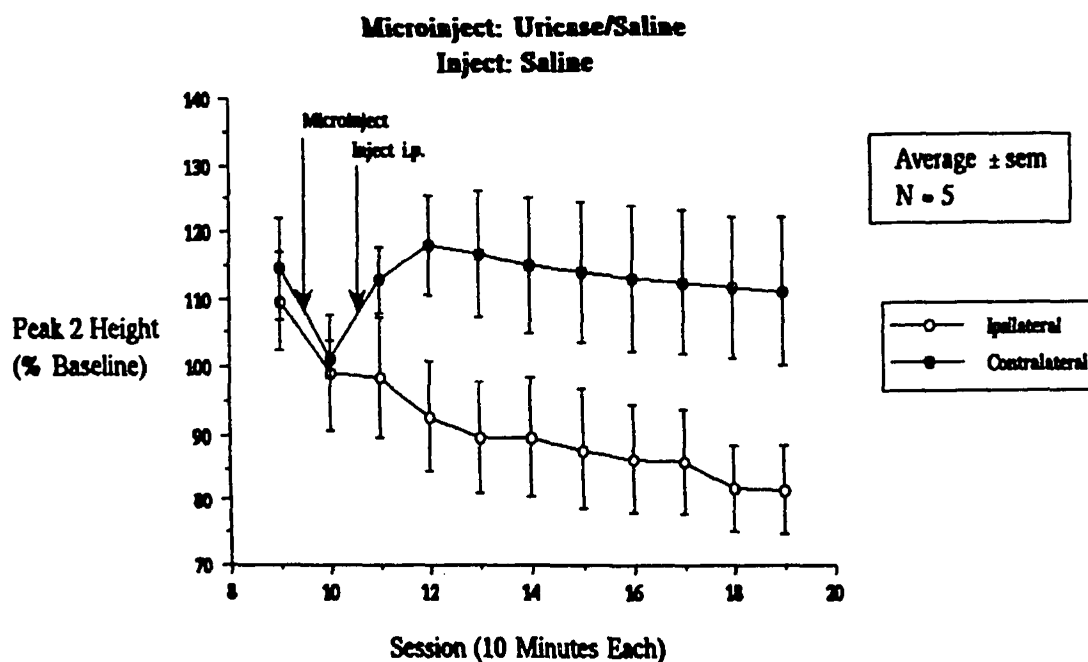


Figure 43

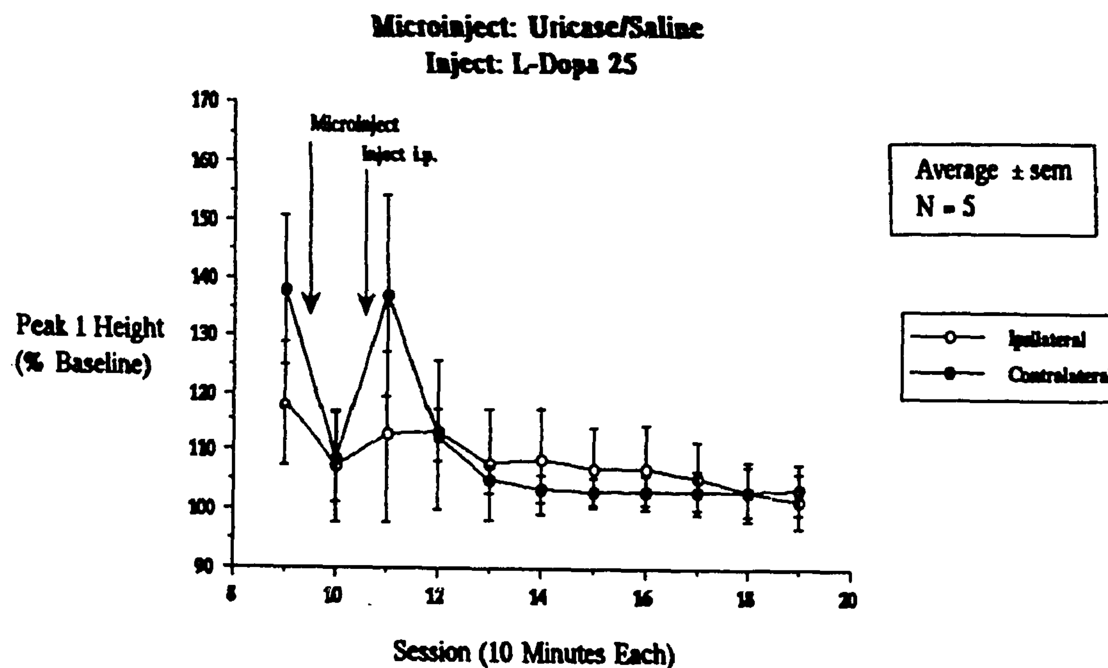
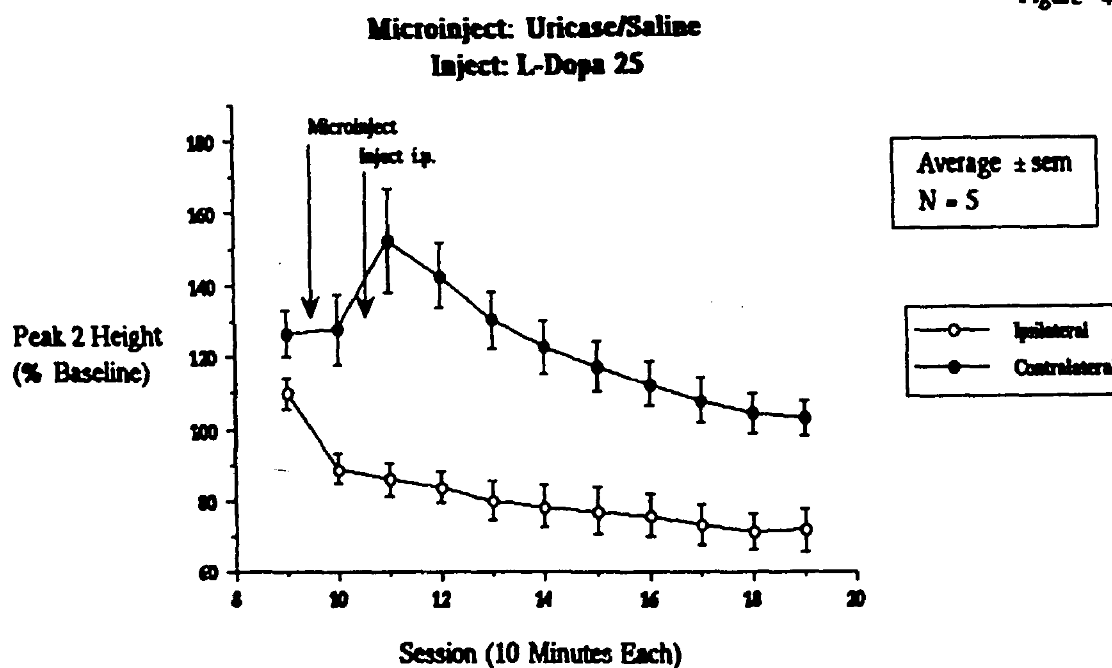


Figure 44



The results of both of these investigations showed that the microinjection of uricase caused the height of Peak 2 to decrease. In the case of the saline injections i.p., the difference between the two sides approached statistical significance (p=0.0657, n=5), and

the injection of L-dopa 25 mg/kg i.p. caused the appearance of a significant difference (p=0.0004, n=5), possibly by accentuating the height of Peak 2 in the striatum not receiving uricase. In both cases, there was a significant interaction between time and side, indicating that the time course of the two sides was different (p=0.0427 for saline injections i.p., p=0.0002 for L-dopa injections i.p.).

Peak 1 also showed a difference over time for both of these treatments (p=0.0001, n=5 for both saline and L-dopa injections i.p.). For reasons that are not clear, the rats receiving saline i.p. also showed a difference between the side receiving uricase and the side receiving saline (p=0.0019). This is an especially unusual result in light of the results reported above and the fact the the rats receiving L-dopa did not show any difference (p=0.7859). It is not due to a misidentification of Peak 2 as Peak 1, because the direction of the change is opposite to that predicted for Peak 2, with the uricase-microinjected side higher than the control side. This result could not be explained.

These results support the contention that Peak 2 and its L-dopa-induced increase represent uric acid. Although the microinjection of uricase did not significantly lower the height of Peak 2 in rats injected with saline i.p. as a control, there was a definite trend in that direction, and the low p value of 0.0657 suggested that it would reach statistical significance if further experiments were conducted to increase the sample size. Taken together, these last three studies support the hypothesis that uric acid is tied to Peak 2 under all of the conditions being examined, whether the striatum was (1) lesioned, (2) intact and opposite a lesioned striatum, or (3) intact and opposite an intact striatum; and whether it was subjected to systemic L-dopa or not.

Adenosine Microinjection

The next step was an investigation into the possible origin of the uric acid. Because uric acid is an end product of purine metabolism, adenosine was microinjected into the striatum to determine if its local increase would lead to an increase in the height of Peak 2.

In these experiments, no systemic injections were performed. Thirteen rats were microinjected with adenosine at a concentration of 1 mM, and two at 10 mM. The results of these experiments are shown in Figures 45 to 48.

Figure 45

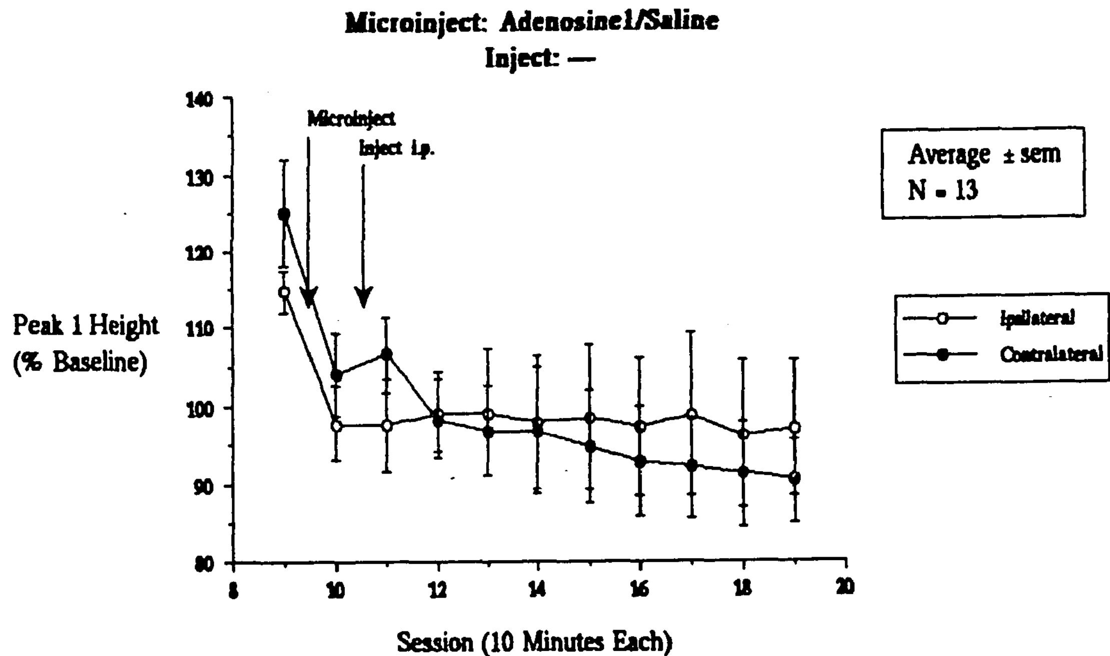
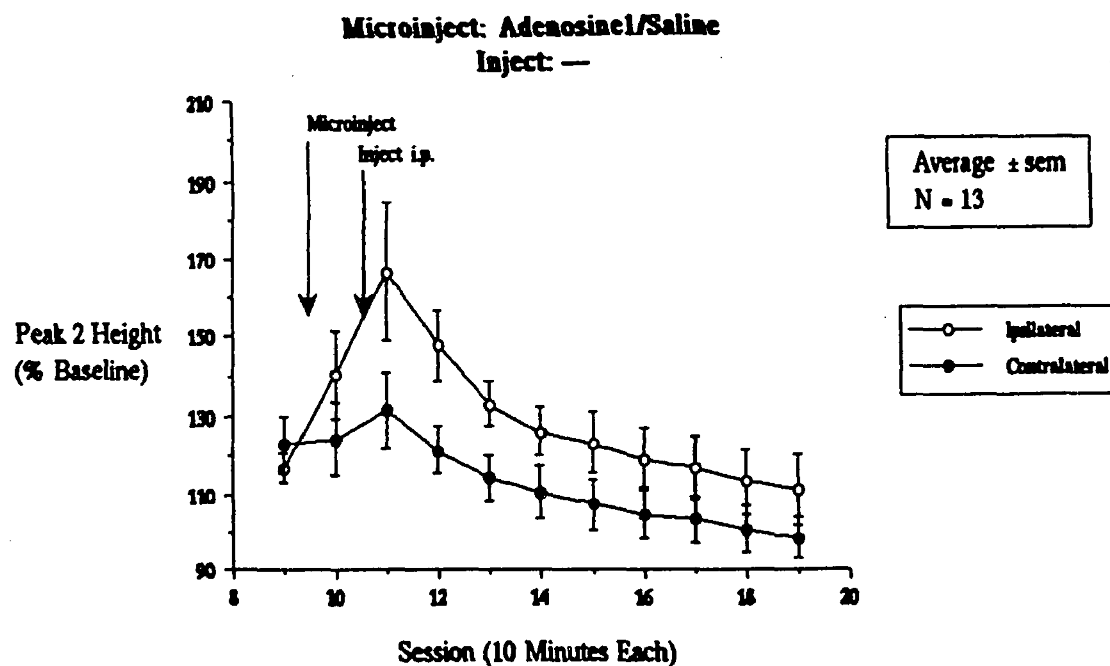
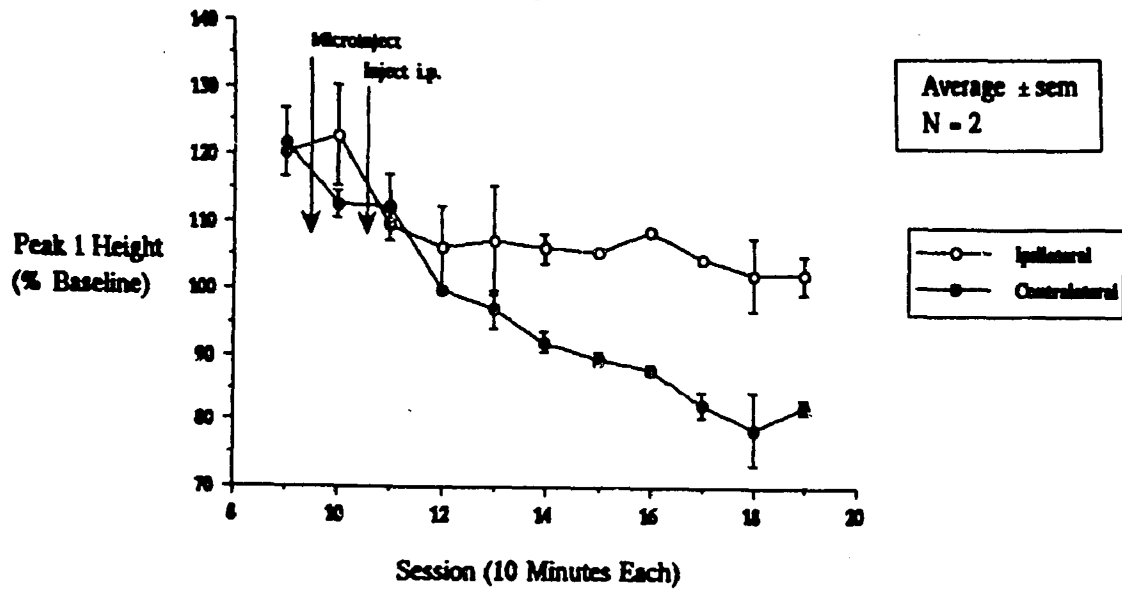
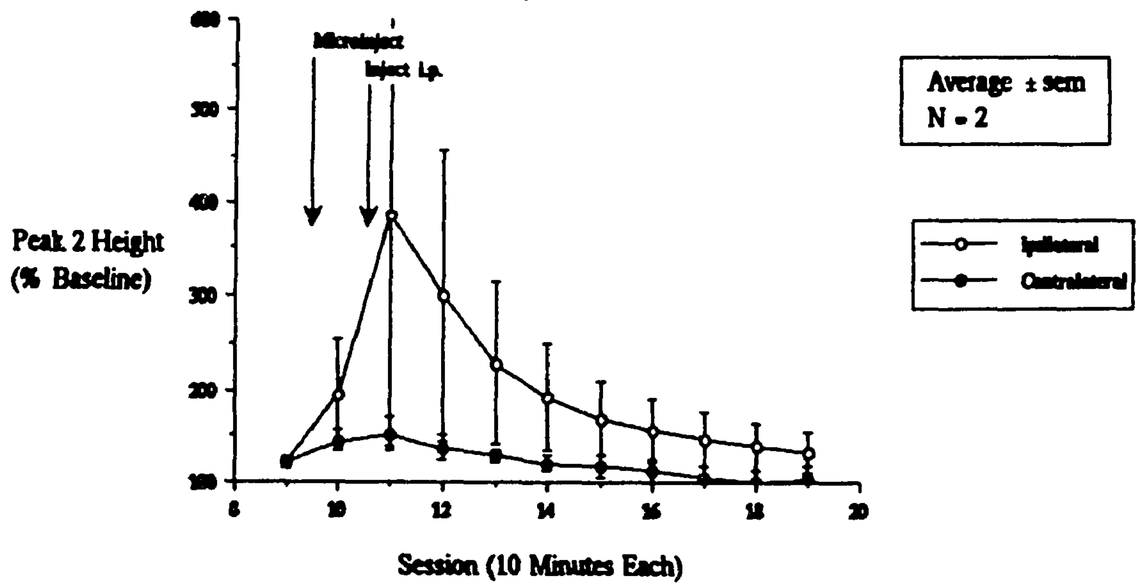


Figure 46







Adenosine at a concentration of 1 mM caused an increase in Peak 2 greater than the saline side (p=0.0338, n=13). Adenosine at 10 mM caused a huge increase in Peak 2 by a

huge amount that was not significant, probably due to the small sample size. The increase was very impressive and unequivocal, however, and no further injections were done.

Peak 1 did not show an increase in response to 1 mM adenosine, but there was a trend for the contralateral side to decrease slightly that was not significant (p=0.9483, n=13), although 10 mM adenosine caused a significant contralateral decrease (p=0.0232, n=2). The cause of this decrease is unclear, although the existence of communication between the two caudates may be a mediating pathway.

These experiments indicate that extracellular adenosine, locally instilled into the striatum, can increase the height of Peak 2. The confirmation of uric acid as the mediating agent was not specifically tested with concomitant microinjection of uricase. In conjunction with the experiments described above, however, the evidence is compelling. That adenosine itself may oxidize causing the increase in Peak 2 is a possibility, but it seems unlikely insofar as adenosine in vitro oxidizes at a voltage (900-1000 mV vs Ag/AgCl reference electrode, Pachla and Kissinger 1979) far above the level of Peak 2, and, in fact above the voltages for Peaks 3, 4, or 5.

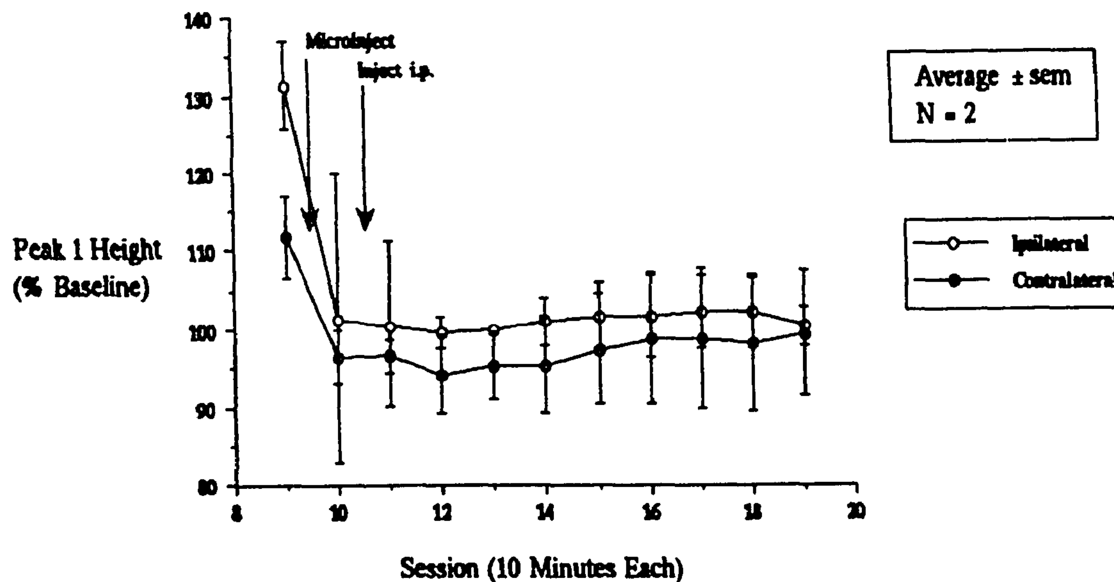
2'-Deoxycoformycin

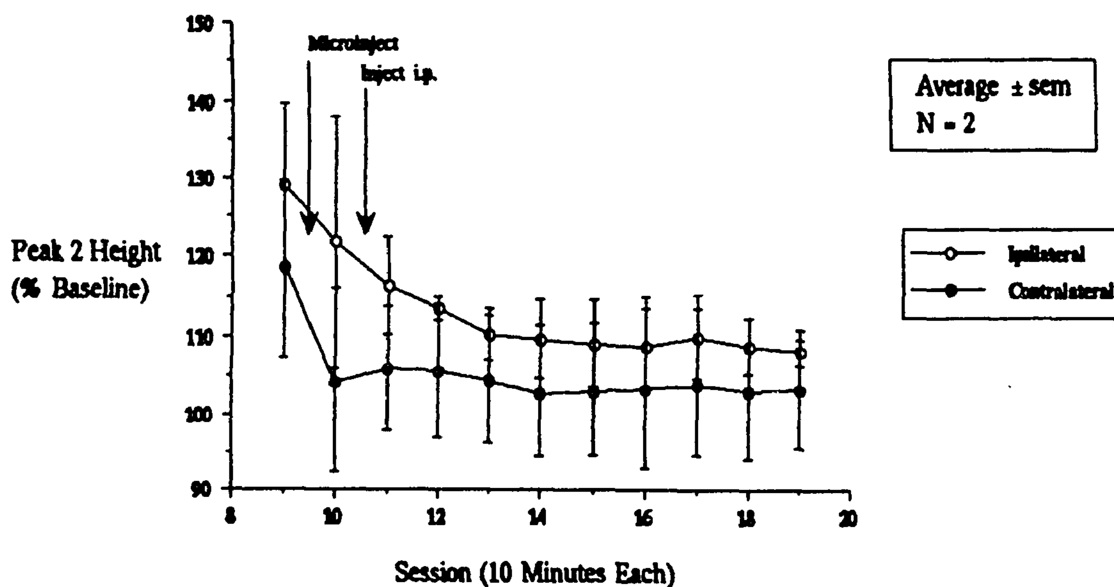
After showing that the L-dopa-induced increase in Peak 2 is caused by uric acid, and that this increase could be mediated by an increase in extracellular adenosine. The next set of experiments investigates whether the Peak 2 changes can be prevented by inhibiting the enzyme adenosine deaminase, which catalyzes the degradation of adenosine to inosine, the first reaction in the pathway of degradation of adenosine to uric acid. (For a more complete description of the reaction pathways, see Figure 17.) This inhibition can be accomplished with the drug 2'-deoxycoformycin, also known as pentostatin, an agent used in chemotherapy for T-cell lymphoid malignancies (Major, Agarwal and Kufe 1981a).

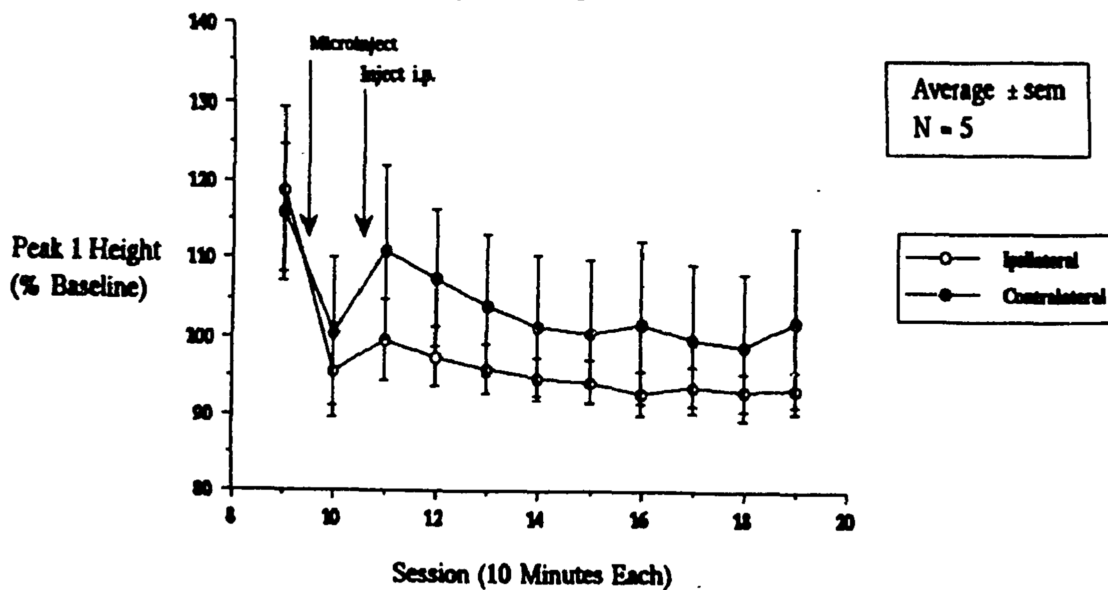
The initial experiments determined whether 2'-deoxycoformycin itself causes a change in Peak 2 (i.e., increasing Peak 2 by oxidizing at that potential in vitro). The drug was

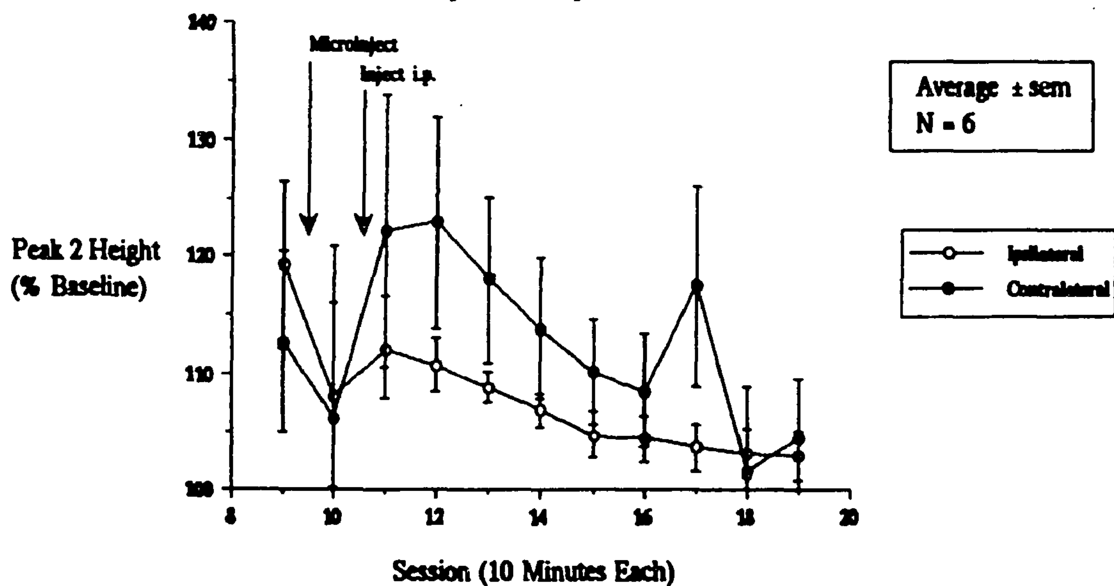
dissolved in normal saline and microinjected at a concentration of 1 mM. Saline was injected i.p. as a control for the later experiments.

Neither Peak 1 nor Peak 2 showed a significant difference between the side microinjected with drug and the side with saline (Peak 1: p=0.4760, n=2, Figure 49;







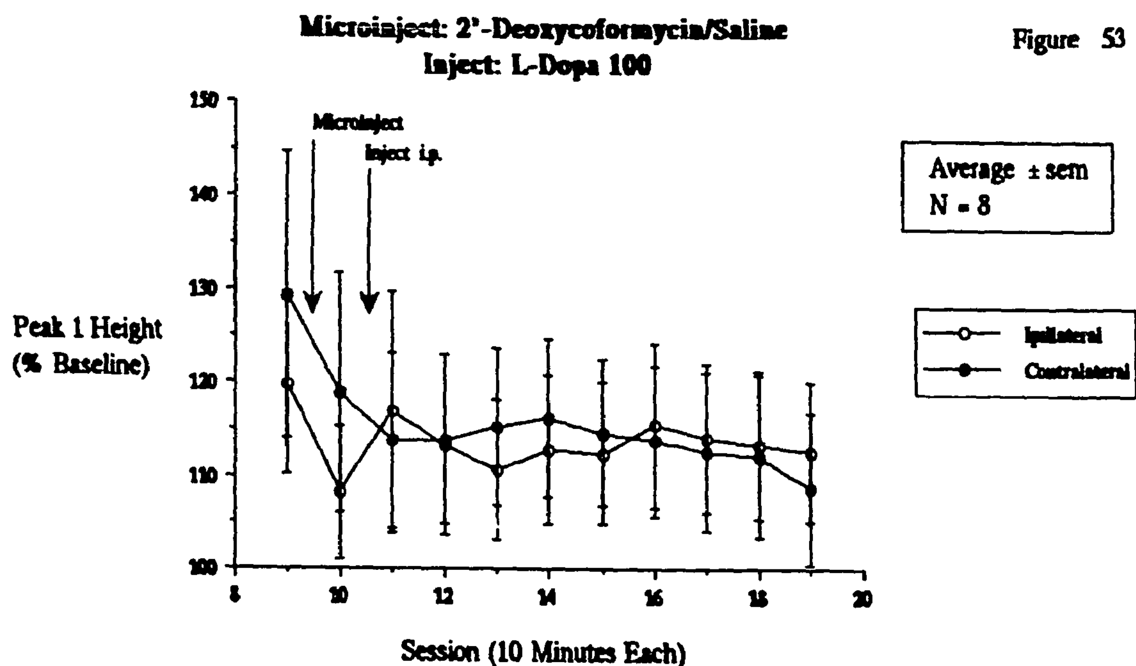


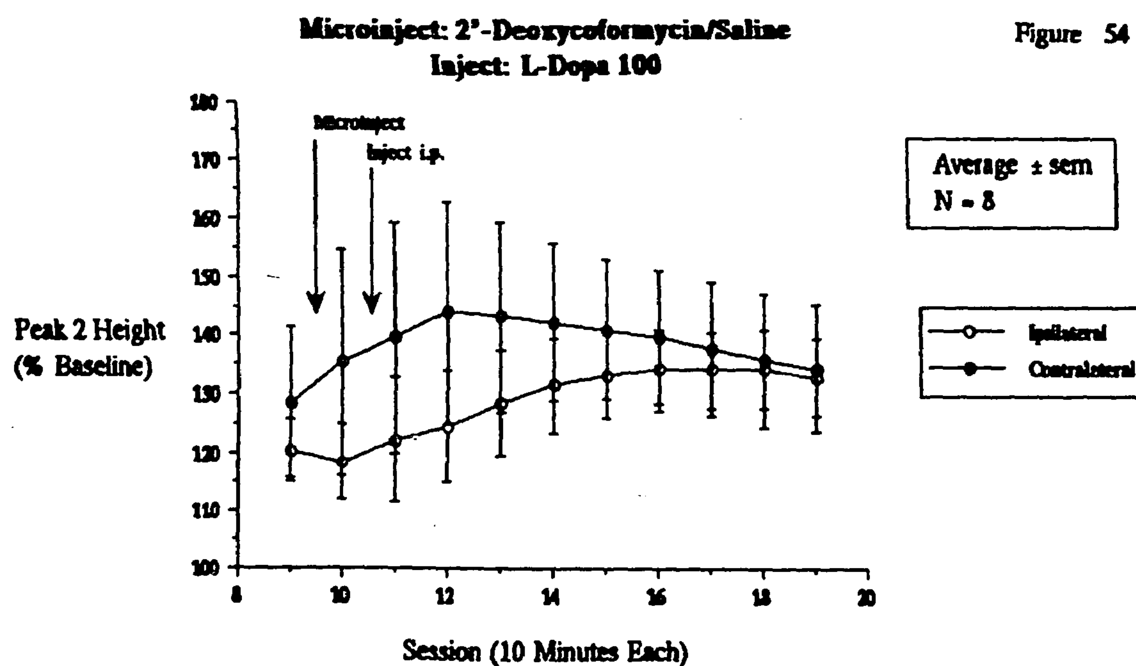
Peak 2: p=0.4786, n=2, Figure 50), although the number of animals microinjected was not large.

The injection of L-dopa 25 mg/kg i.p. also showed no significant differences between the drug and control sides in Peak 1 (0.5204, n=5, Figure 51) and Peak 2 (p=0.4705,

n=6, Figure 52). There was, however, a non-significant trend for the suppression of the increase in Peak 2 on the drug-treated side compared to the control side (See Figure 52).

Similar experiments were tried with the administration of an increased dose of L-dopa of 100 mg/kg i.p., instead of 25.mg/kg, in an attempt to accentuate the changes in Peak 2





on the control side. The results of this series of experiments was essentially the same with both Peaks 1 and 2 showing no significant differences between the 2'-deoxycoformycin and the saline sides (Peak 1: p=0.8804, n=8, Figure 53; Peak 2: p=0.5205, n=8, Figure 54). Again, there was a non-significant trend for suppression of an increase in Peak 2 height. Also evident here was a general increase in the height of Peak 2 overall as compared to the lower dose of L-dopa. The height on the control side reached a maximum of almost 145%, whereas with 25 mg/kg the maximum height was less than 125%. This suggests that by increasing the dose of L-dopa, the process leading to the increased levels of uric acid can be driven harder, and that it is not at saturation at a dose of 25 mg/kg.

These experiments, in total, are not supportive of uric acid deriving from adenosine degradation, at least not at a site where the enzyme adenosine deaminase is accessible to microinjected 2'-deoxycoformycin. It is possible that the pharmacokinetics of the drug interactions are not properly matched, such that the dosage of enzyme inhibitor are not in the proper range, or that the timing of administration leaves insufficient time for full inactivation of the enzyme. Alternatively, penetration of the drug into the cells could be insufficient for the experimental conditions, if the adenosine is degraded in an intracellular process. Adenosine deaminase is considered a cytoplasmic enzyme (Pull and McIlwain 1974), although evidence does not contradict an extracellular location also. The trends of a suppression of the Peak 2 increase seen in Figure 52, however, suggest that this area might still benefit from further investigation; the large standard errors for the saline-microinjected side suggest that by increasing the number of experiments, the non-significant trend could turn out to be genuine.

SUMMARY AND DISCUSSION

The experiments described in this section address the issue of the identity of Peak 2 in this research project, and the chemical origin of the increase in its height. Investigating these questions required the development of a new set of techniques for microinjecting

drugs directly into the striatum of the awake rat. Electrode-cannula assemblies were designed, constructed, and implanted into the striatum bilaterally, and a new experimental protocol was instituted in which the rats received both local microinjections and systemic drugs. The initial set of control experiments indicated that this was a feasible approach, but that there were some changes in both Peaks 1 and 2, as a result of the manipulations, that needed to be recognized and considered when interpreting the results.

The experiments involving uricase microinjections clearly show that all of the changes associated with Peak 2 are due to uric acid, and that the peak is qualitatively similar in the striatum in all of the different experimental conditions used in these studies.

The question is where does the uric acid come from? The possibility that it is derived from extracellular adenosine is raised by the experiments showing that local microinjection of adenosine causes an increase in Peak 2. If Peak 2 had not increased, it would indicate that an increase in extracellular adenosine does not lead to an increase in uric acid levels. The results support the idea that the uric acid is increasing due to the metabolic breakdown of adenosine.

The next step was to show that the administration of L-dopa is increasing uric acid by affecting adenosine degradation. An adenosine deaminase inhibitor, 2'-deoxycoformycin, was used to prevent the conversion of adenosine to uric acid. The results of these studies did not support the hypothesis. The non-significant trends, however, did suggest that with further experiments, this area of investigation might show a statistically significant difference consistent with 2'-deoxycoformycin preventing the L-dopa-induced increase in uric acid. The pharmacokinetics of the various processes would need to be worked out more completely for these studies.

The possibility also exists that uric acid is arising from a process involving adenosine deaminase that is not accessible to 2'-deoxycoformycin microinjected into the striatum. This may be due to one of several possibilities. The enzyme may be in a location inaccessible to the inhibitor, perhaps in an intracellular compartment that the drug cannot

penetrate. The kinetics of the inhibitor may be such that it is unable to effectively neutralize the enzyme in the time period when the Peak 2 changes are occurring. The concentration of the microinjected drug may be ineffective, although this seems to be unlikely because 1 mM should be a relatively high dose. Alternatively, the uric acid could be coming from a peripheral source, and is not arising from local degradation of purines. This option seems unlikely because of the experiments described above in which Peak 2 changes asymmetrically in the two striata, and because of the close correlation of the asymmetry with circling behavior. If the uric acid originated in the periphery, the changes would be expected to be the same on the two sides of the brain, and there would not be a correlation with behavior. Additionally, previous studies have shown that Peak 2 can be decreased by using allopurinol to inhibit xanthine oxidase production locally (Mueller et al. 1985).

Thus, in summary, these experiments demonstrate that the L-dopa-induced Peak 2 changes are due to changes in uric acid levels in the striatum, and that an increase in extracellular adenosine could be an intermediate step in the process.

SECTION 6 — DOPAMINE EXPERIMENTS

INTRODUCTION

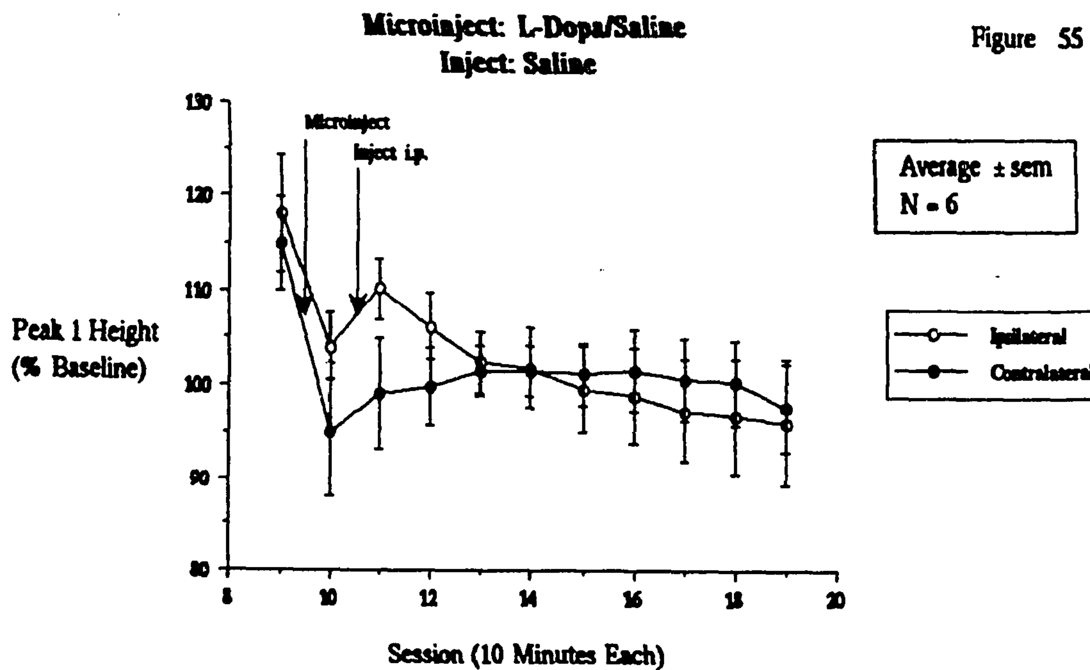
Another avenue of investigation approaches the changes from the L-dopa side, examining the interactions of the drug with the striatum and the dopaminergic system. The goal of this approach is a greater understanding of the pathway by which L-dopa interacts with striatal physiology to produce the increase in Peak 2. The hypothesis is that L-dopa is decarboxylated to dopamine, which then stimulates dopamine receptors, either D1, D2, or both, resulting in cellular changes that ultimately cause an increase in uric acid. In order to test this hypothesis, L-dopa metabolism was manipulated with dopa decarboxylase inhibitors to prevent decarboxylation. Dopamine receptor involvement was examined by attempting to block the receptors with the antagonist haloperidol, and by stimulating the receptors with the agonist apomorphine.

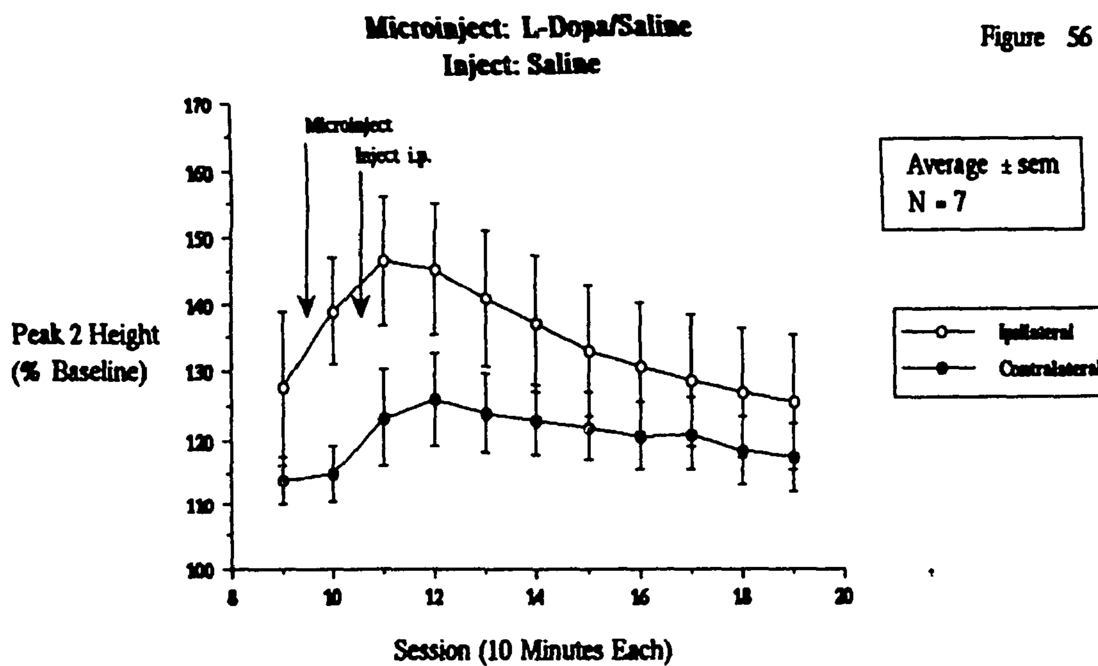
The methodology used here was the same as that used for the previous microinjection experiments. (See Figure 30 ECA Implantation Protocol and Figure 31 IVED / Microinjection Protocol). In all cases unlesioned rats were used. Data analysis was done as before.

EXPERIMENTS

L-Dopa Microinjection

In these experiments, L-dopa was microinjected into the striatum to determine whether local application would induce the same changes seen in the earlier experiments in which the drug was administered intraperitoneally. This was done to confirm that the changes did not require systemic administration of the L-dopa, suggesting that the L-dopa was acting at the level of the striatum, and not at a distant location that was indirectly resulting in changes in uric acid metabolism.





L-Dopa was dissolved in normal saline at a concentration of 1 mM, and microinjected into the striatum. Saline was microinjected into the contralateral striatum as a control. The rats also received injections of saline i.p. The results of these experiments are shown in Figures 55 and 56.

As can be seen in Figure 55, Peak 1 was slightly higher in the L-dopa striatum than in the control striatum, although the increase was transitory and not statistically significant ($p=0.7569$, $n=6$). The slight increase could be from the L-dopa itself oxidizing, since L-dopa is expected to oxidize at the voltage of Peak 1 (Marsden, Brazell and Maidment 1984b, Figure 2 on p. 130).

Peak 2 was also higher on the side microinjected with L-dopa. It increased to 147% at the second session after microinjection. The control side also increased, but to a lesser degree and reached only 126% at the third session after microinjection. (See Figure 56). This difference, however, was also not significant ($p=0.1751$, $n=7$). If the analysis of variance was limited to the earlier sessions after microinjection, however, when the difference was greatest, the two sides did show a significant difference ($p=0.0338$ for sessions 10-11, $n=7$).

The increase in Peak 2 is the expected result for L-dopa in accordance with L-dopa causing an increase in uric acid by a mechanism involving interaction in the striatum. A short duration of action is suggested by the lack of statistical significance when the analysis of variance is carried out over the entire period of measurement following microinjection, whereas a statistically significant difference is obtained when only the first few sessions are compared. Possibly, the L-dopa is quickly compensated for by homeostatic mechanisms active in the intact striatum, such that the dopaminergic nerve terminals take up the drug and rapidly convert it into stored dopamine, with only a short period when neurotransmitter "overflows" to sites where it can induce an increase in uric acid.

These results suggest that L-dopa need not be given systemically to increase uric acid, but that it can have an effect when microinjected locally, supporting the hypothesis that neuronal activity induced in the striatum is an intermediate step in the process.

Decarboxylase Inhibitors

Once the role of L-dopa in the striatum was established, the next step was to determine if the increase in Peak 2 required decarboxylation of the drug to dopamine. This hypothesis was tested by attempting to block the reaction with a dopa decarboxylase inhibitor. The inhibitor was microinjected directly into the striatum at a concentration of 1 mM. Both carbidopa and Ro4-4602 were used in these studies. The predicted response was prevention of the increase in uric acid.

Carbidopa

Carbidopa was microinjected according to protocol. The rats were systemically treated with either saline or L-dopa 25 mg/kg i.p. The results of these experiments are not shown. Carbidopa caused the appearance of a new peak in the voltammogram at a potential between Peaks 1 and 2. Carbidopa, along with other catechol-related drugs, would be expected to oxidize at a voltage near Peak 1 (Marsden, Brazell and Maidment 1984b, Figure 2 on p. 130). Because carbidopa itself was appearing in the voltammogram in the voltage range of interest, the experiments had to be discontinued and the data collected could not be considered a reliable indicator of changes in either Peak 1 or Peak 2.

Ro4-4602

In an attempt to overcome the difficulties of microinjecting carbidopa, a similar set of experiments was run using a different dopa decarboxylase inhibitor, Ro4-4602. This drug was the same decarboxylase inhibitor used by Ungerstedt in his initial experiments describing the circling rat (1971b). Unfortunately, the same problem was encountered with the use of this drug as was seen with carbidopa. A new peak emerged after microinjection of Ro4-4602, again at a potential between Peaks 1 and 2, interfering with height measurement.

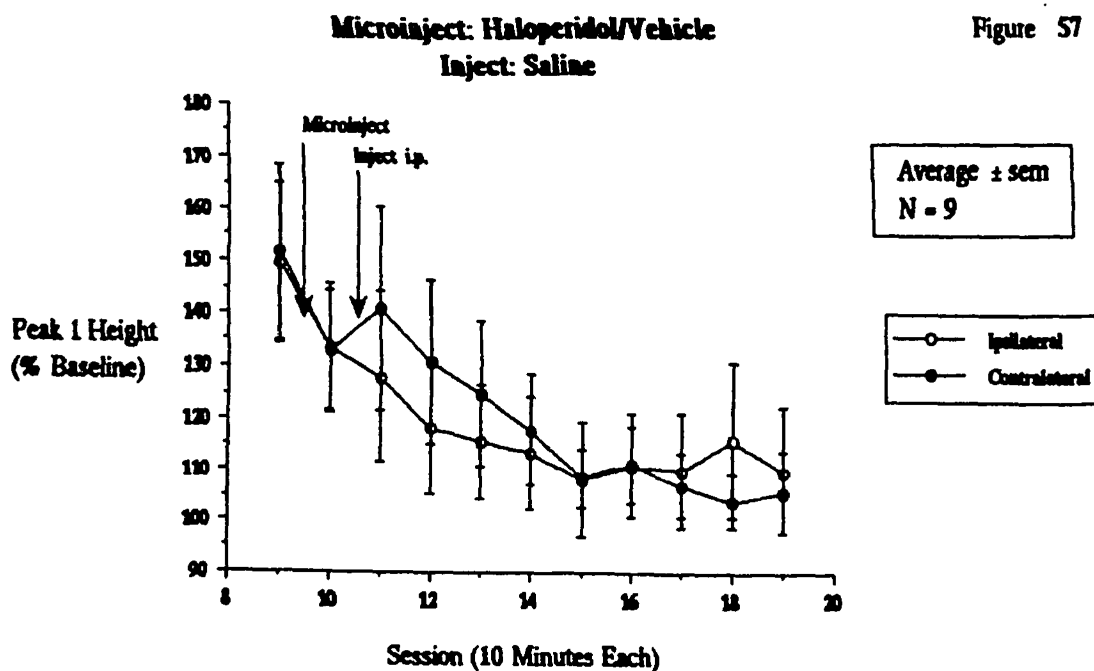
Because of this problem, the investigation of the effects of a decarboxylase inhibitor remains inconclusive. This would be an area for further investigation in the future, possibly with the use of an alternative technique. In this case, in vivo dialysis would be

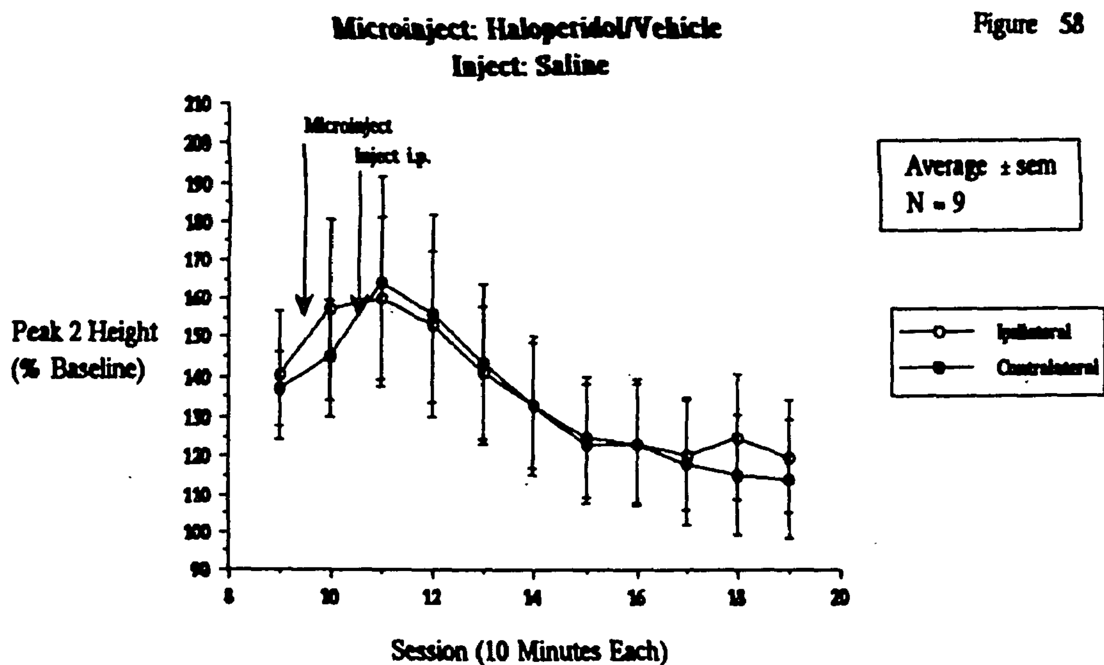
an ideal choice for examining the effects of either carbidopa or Ro4-4602. This is because the problem of overlapping oxidation peaks would not cause any interference. This technique will be discussed later.

Haloperidol

Another way of manipulating the dopaminergic system is to modify receptor response with dopaminergic antagonists and agonists. An antagonist should be able to prevent the increase in Peak 2 by blocking the stimulation of dopamine receptors caused by L-dopa after it has been decarboxylated. In order to accomplish this, the antagonist haloperidol was used. Haloperidol is not specific for D1 or D2 receptors, and so seemed well suited as an initial drug for inhibiting response, since it was unknown which of class of receptors might be responsible for mediating the increase in uric acid. Unlike previous drugs used for microinjection, however, haloperidol did not dissolve well in normal saline, and a special vehicle had to be used to microinject it. The haloperidol solution consisted of 17 ml distilled water, 2 ml of 10 mM haloperidol dissolved in 10% volume/volume acetic acid, and 1 ml of saturated Tris base to adjust the solution to a pH of 5.74. Vehicle solution that omitted the haloperidol was used for control microinjections in the contralateral striatum.

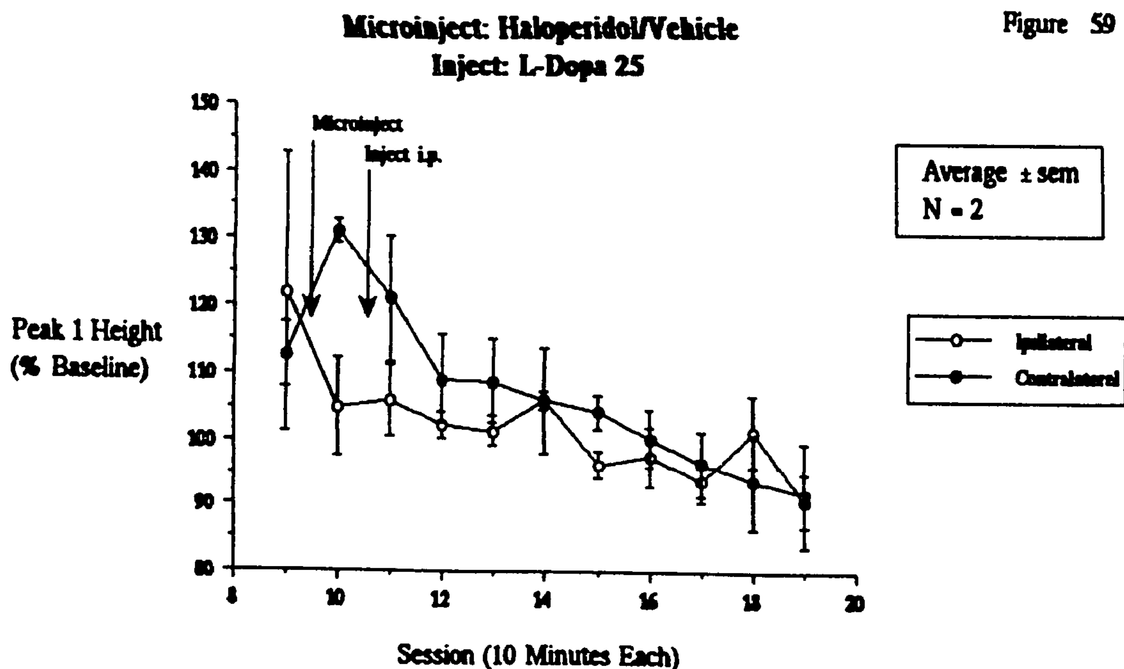
Microinjection of haloperidol without systemic L-dopa did not result in any difference in either Peaks 1 or 2 when compared to vehicle (Peak 1: p=0.8876, n=9, Figure 57; Peak 2: p=0.9212, n=9, Figure 58). There was a tendency for Peak 2 to increase bilaterally in response to the microinjection. Possibly, the vehicle causes a response in the striatum that increases Peak 2. The slight acidity of the solutions could irritate the neurons, provoking a change in uric acid levels. Again, the important point here is to consider this, and to draw conclusions only from comparisons of the haloperidol-treated side with the control side. Haloperidol also had no effect on Peak 1 (p=0.4331, n=2, Figure 59). It also had no statistically significant effect on the increase in Peak 2 induced

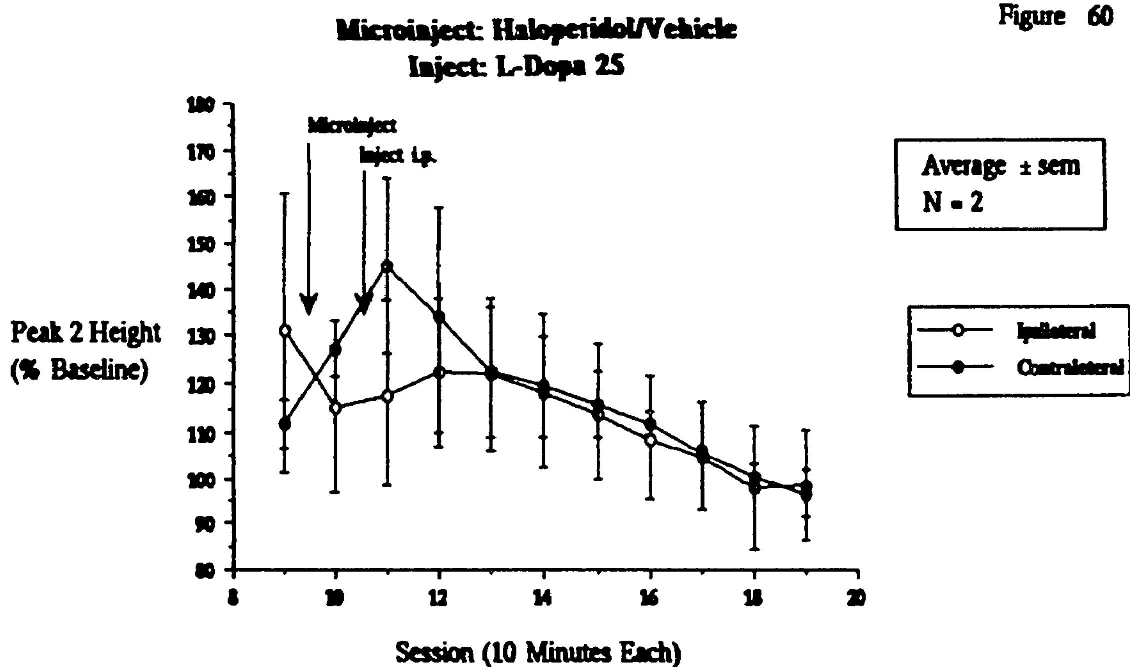




by L-dopa 25 mg/kg i.p., although there was a non-significant trend for inhibition of the response ($p=0.8501$, $n=2$, Figure 60).

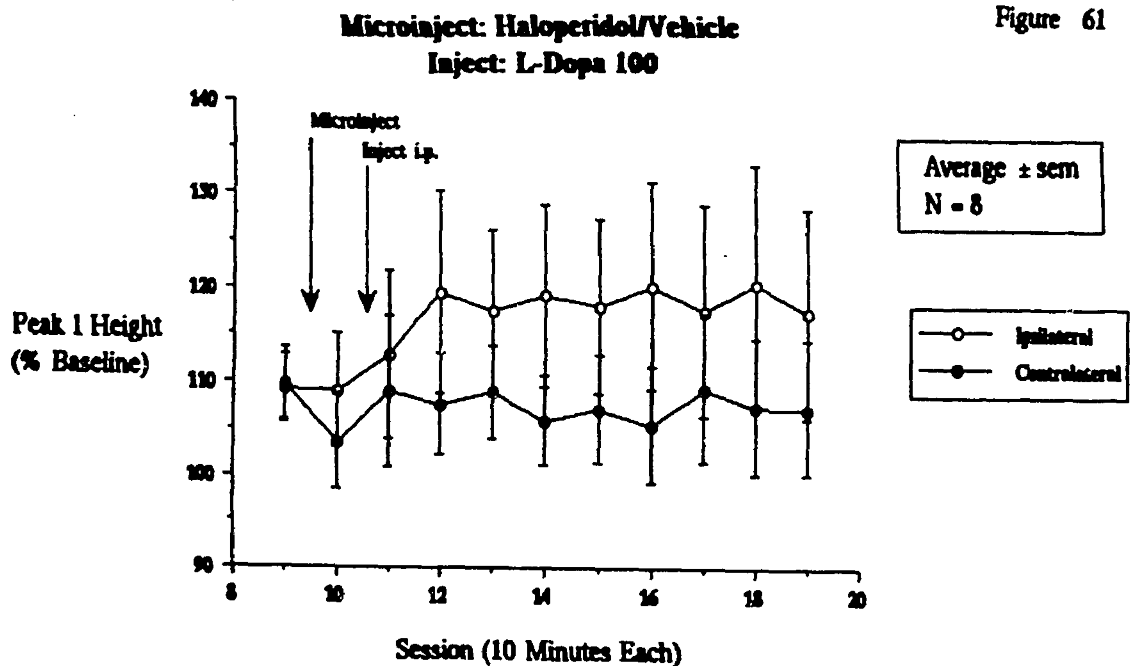
A similar experiment was performed with a dose of L-dopa of 100 mg/kg. The increased dose was an attempt to accentuate the response of Peak 2, as was done in the

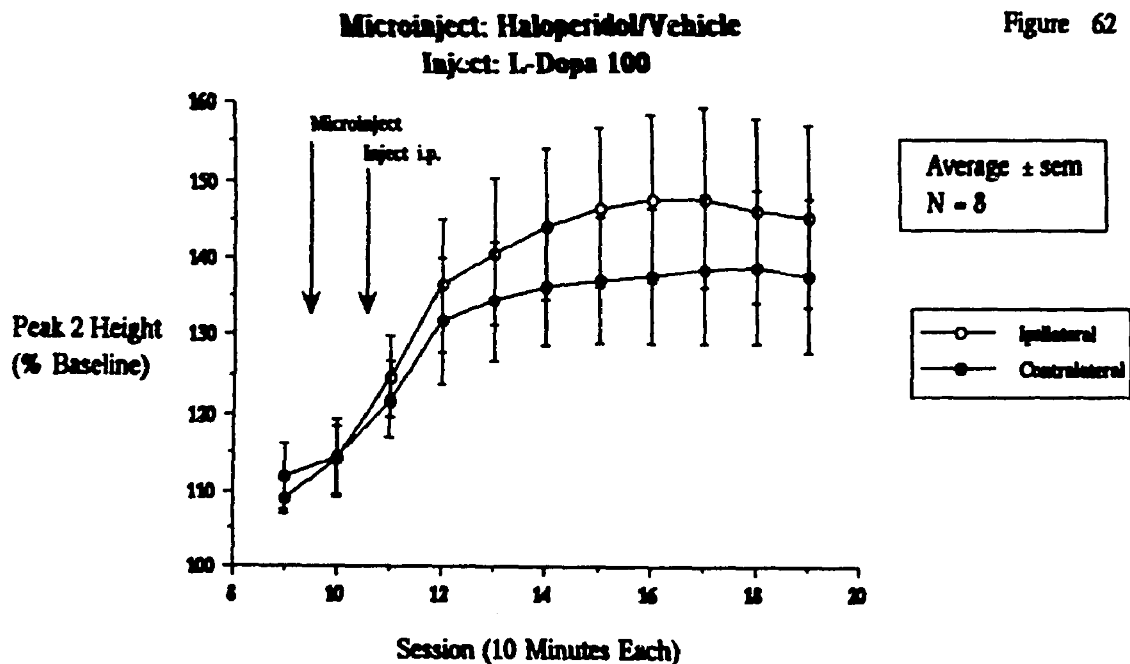




2'-deoxycoformycin experiments. Haloperidol had no effect on either Peaks 1 or 2 at this dose of L-dopa (Peak 1: p=0.4147, n=8, Figure 61; Peak 2: p=0.6144, n=8, Figure 62).

Peak 2 showed a bilateral increase that was sustained throughout the experiment. Haloperidol did not have any effect on this increase. The sustained nature of the increase





in Peak 2 has not been seen in any other experiments with the lower dose of L-dopa at 25 mg/kg. By administering a dose four times as great, the dopamine created may be too overwhelming for the antagonist to inhibit, and the response may be at the saturation point

in both the haloperidol- and vehicle-treated striata. This is consistent with the previous experiments with 2'-deoxycoformycin in which it was determined that L-dopa at 25 mg/kg did not maximally drive the system (see above).

The results of these experiments do not support the hypothesis that the Peak 2 response to L-dopa is mediated by dopamine receptors that can be blocked by haloperidol. The conclusion that can be drawn from this is unclear. The dose of L-dopa was increased for these experiments to accentuate the effects on Peak 2, so as to enhance the difference between antagonist and control. This dose, however, as described above, may have been too large for meaningful comparisons to be made. Further exploration of dosage adjustment in these experiments is an area for future investigation.

Apomorphine

Following the natural progression of investigation, the next step was to attempt to reproduce the Peak 2 increases with a dopamine receptor agonist, in this case apomorphine. The drug was microinjected directly into the striatum at a concentration of 1 mM, with saline control microinjections in the contralateral side. The protocol was the same as described for the other microinjection experiments, except that no injections were given intraperitoneally (no L-dopa injections were going to be administered, so no control saline injections were needed).

Apomorphine did not affect either Peaks 1 or 2 as compared to the control side (Peak 1: $p=0.3635$, $n=4$, Figure 63; Peak 2: $p=0.5477$, $n=4$, Figure 64). This result does not support the involvement of dopaminergic receptors in the increase in Peak 2, although there was, again, a non-significant trend for Peak 2 on the apomorphine side to be higher than on the saline side. As with haloperidol, the problem may be related to the dosage of drug administered. Further investigation of different dosages is an area for future research.

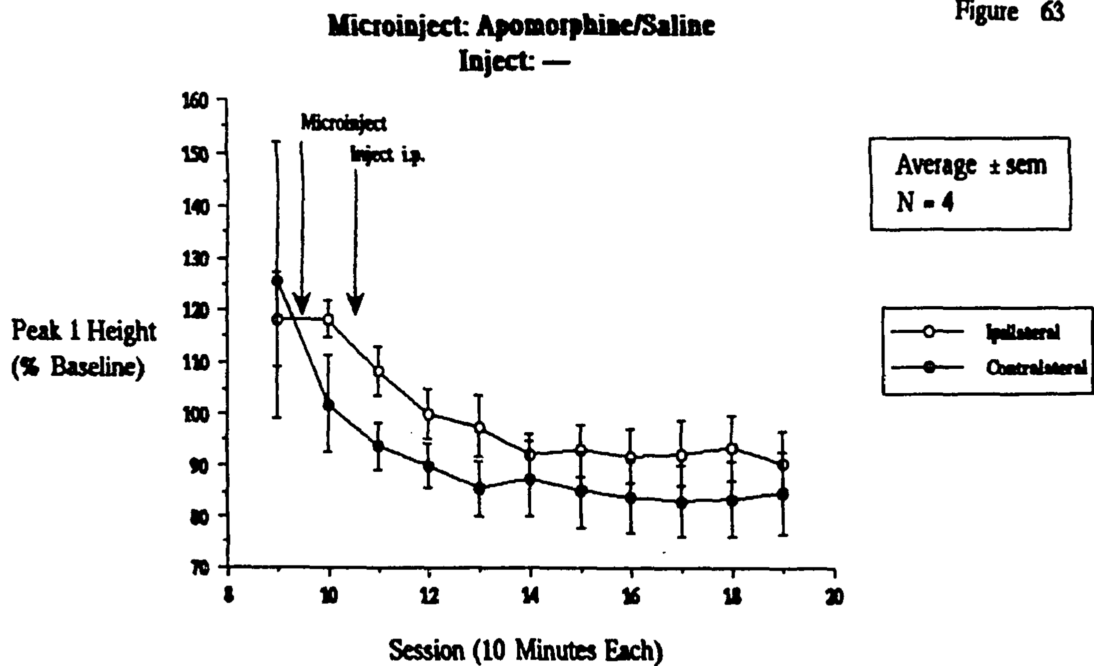


Figure 63
Microinject: Apomorphine/Saline
Inject: —
Microinject
Inject i.p.
Average ± sem
N = 4
Ipsilateral
Contralateral
Peak 1 Height
(% Baseline)
160
150
140
130
120
110
100
90
80
70
8
10
12
14
16
18
20
Session (10 Minutes Each)

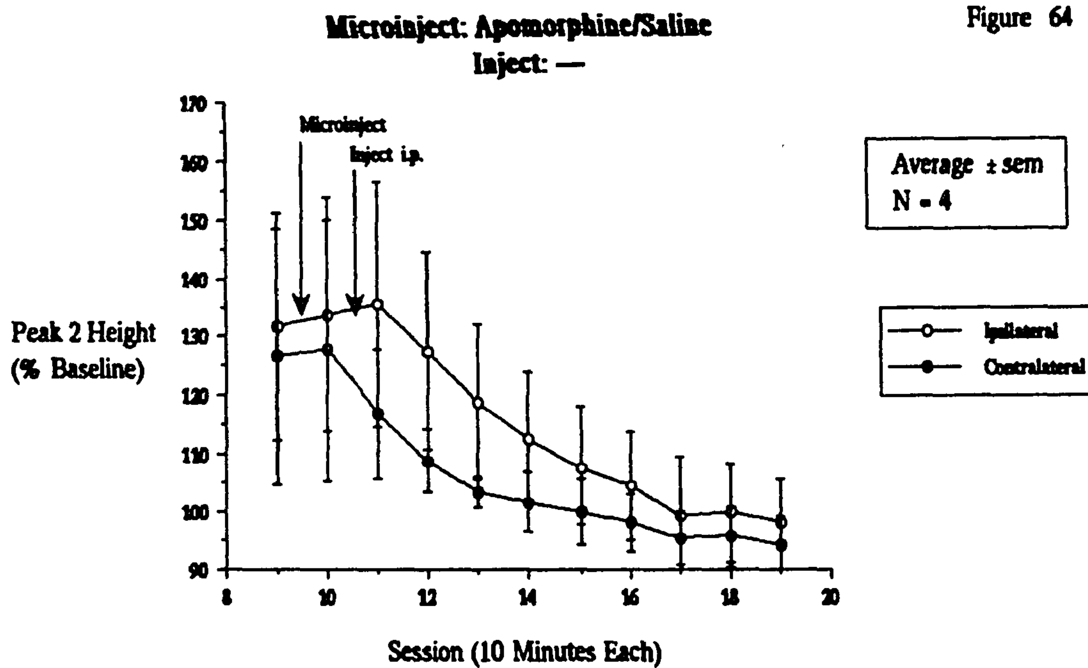


Figure 64
Microinject: Apomorphine/Saline
Inject: —
Microinject
Inject i.p.
Average ± sem
N = 4
Ipsilateral
Contralateral
Peak 2 Height
(% Baseline)
170
160
150
140
130
120
110
100
90
8
10
12
14
16
18
20
Session (10 Minutes Each)

SUMMARY AND DISCUSSION

The experiments described in this section suggest that the increase in Peak 2 is caused by the presence of L-dopa locally in the striatum. Further investigation of the role of L-dopa, however, was somewhat inconclusive. The attempts to investigate the effects of inhibition of dopa decarboxylase were hampered by disruption in the electrochemical measurements by carbidopa and Ro4-4602, which were both found to be electroactive at a voltage that interfered with the measurement of both Peaks 1 and 2. The results of these studies were too indefinite for any conclusions to be drawn with confidence. Further experiments that examined the involvement of dopamine receptors were also inconclusive. Both a dopamine antagonist, haloperidol, and an agonist, apomorphine, were microinjected. Both these studies were difficult to interpret. The predicted results were not seen, but it could not be determined whether the lack of difference between the drug-treated striatum and the control striatum was attributable to a lack of effect of the drug, or whether the drugs were delivered at suboptimal doses. These studies delineate areas that are open for further investigation by alternative methodological approaches (see later discussion).

Consideration must be given to the possibility that the changes in Peak 2 are not mediated by dopamine receptors and/or do not involve decarboxylation of L-dopa to dopamine. The studies described in the previous section indicated that the Peak 2 changes could be attributed to increases in uric acid. As such, it is difficult to envision how L-dopa could lead to uric acid changes without involving dopaminergic receptors, especially with the initial observation of correlation with circling, a behavior intimately associated with dopamine receptor stimulation. One explanation could involve the concentration of dopamine into storage vesicles. This is an ATP-dependent process, and possibly the increased metabolism of ATP is responsible for the increase in uric acid. It is also possible to envision a similar explanation involving dopamine reuptake. In both of these

alternatives, however, it is difficult to visualize how they operate in the denervated striatum., where dopaminergic nerve terminals have been eliminated.

It should be noted that the identity of Peak 2 is disputed in the literature. Cespuglio et al. has reported that the components comprising Peak 2 at times less than one week after electrode implantation differ from the components at times greater than one week (Cespuglio et al. 1985). This group indicates that before 7 days, Peak 2 reflects both 5-HIAA and uric acid, whereas after 7 days, only 5-HIAA is measured. In the experiments described here, however, the evidence indicates that this is not the case. The microinjection of uricase caused a great decrease in Peak 2, even though the rats had had electrodes implanted for longer than 7 days.

Despite the ambiguous nature of these results, the best explanation for the increase in uric acid induced by L-dopa remains the catabolism of adenosine. This conclusion, however, still cannot be stated with certainty. As indicated above, a number of experiments still remain as areas for future research in addressing this question. In the next section, possible directions for these investigations will be discussed.

SECTION 7 — DISCUSSION

SUMMARY OF RESULTS AND CONCLUSIONS

The experiments in this project were conducted to further understand the relationship between behavior and biochemistry. The studies began with the serendipitous observation of a correlation between circling behavior and Peak 2 changes, moving on to the identification of the underlying chemical substance involved, then progressing to the exploration of its possible origin, and to the investigation of the physiological significance of these changes.

The initial (preliminary) experiments were conducted to define the methodology more precisely, and to demonstrate the consistency and reliability of the technique of in vivo electrochemical detection. These studies showed that the results are reliable within the constraints of the protocols used. Only silicone oil electrodes were used, because Nujol electrodes and the stearate-modified electrodes were considered too problematic. In the studies described here, and in the hands of other investigators, the silicone oil electrode has been a very reliable electrode for both short-term and long-term use.

Having explored and solved some of the technical obstacles involved in the methodology, the next step was the application of this technique to a scientific problem. The next series of experiments employed in vivo electrochemical detection to investigate the pharmacology and physiology of circling behavior in rats in which dopaminergic nigrostriatal neurons were destroyed unilaterally. As explained above, L-dopa administered systemically causes these rats to circle. The underlying physiology involved in this behavior have not been fully elucidated. In vivo electrochemical detection seemed an ideal tool for examining this question. A series of experiments were carried out to explore the pharmacokinetics of L-dopa, dopamine, their metabolites, and several other neurotransmitters of interest. It was hoped that using the complementary techniques of in

vivo electrochemical detection and HPLC would afford a comprehensive view of the intracellular and extracellular compartments and thus allow conclusions to be drawn regarding the dynamics of neurotransmitter formation, release and action at receptors. The final results, however, proved to be more complex than anticipated.

The plans for observing the changes in Peak 1 were superseded by the striking changes that were seen in Peak 2. Unexpectedly, Peak 2 showed an increase in height following L-dopa administration, and this increase had a different time course on the lesioned and unlesioned sides. Even more surprising, the difference in the increase of the peak height on the two sides was highly correlated with the magnitude of the circling that the rats exhibited. The implication of these results is that Peak 2 directly reflects the physiological processes involved in circling behavior. The implication is that the substance (or substances) responsible for Peak 2 is biologically important. Unfortunately, Peak 1 did not show any comparable results that were interesting enough for further investigation.

These experiments also provided further information about the methodology. It was apparent that the discrimination of Peak 1 from Peak 2 was sufficient for the differences in their time courses to be detected. It also helped to validate the utility of using in vivo electrochemical detection for examining correlations between behavior and biochemistry.

Because the anticipated changes in Peak 1 were not seen, the HPLC experiments were not as informative as had been hoped. The dopaminergic changes were not clearly defined by in vivo electrochemical detection. The HPLC results indicated that the administration of L-dopa does little to alter the levels of dopamine itself in the striatum, but the increase in the levels of its metabolites suggested that that dopaminergic activity and turnover was augmented. The conclusion drawn from this is that L-dopa causes an increase in dopaminergic activity, without increasing the storage pool of dopamine, and that the amount of dopamine that stimulating the receptors involved in circling behavior is a relatively small proportion of the total tissue dopamine.

The other measurements of total tissue levels were important for contributing to the understanding of the microenvironment in the striatum. One other important result was that serotonin, its metabolite 5-HIAA, and its precursor tryptophan were all either unchanged or decreased by L-dopa treatment, further strengthening the probability that Peak 2 was increasing as a reflection of uric acid and not of 5-hydroxyindoles, as others have contended.

The next set of experiments explored the possible origin of the increase in Peak 2, first by demonstrating that uric acid is unequivocally the substance responsible for the increase, and second by attempting to define the source of the uric acid, which would hopefully illuminate the significance for the increase as well. A series of microinjection studies with uricase established the identity of Peak 2 as uric acid under a variety of circumstances: in the lesioned striatum as well as in the intact striatum both with and without L-dopa injected systemically. These experiments established the adequacy of the unlesioned rat for investigating the changes in Peak 2. Otherwise properly controlled experiments would have been difficult because it was preferable to use the contralateral striatum as the control. To use rats with both striata lesioned would have presented technical difficulties related to the animals becoming adipsic and aphagic, so that the use of intact animals greatly facilitated further studies.

Having definitively established that uric acid accounted for the changes seen in Peak 2, the next set of experiments examined its biochemical and metabolic origin. Because uric acid is the physiologic end product of purinergic metabolism, the possibility that extracellular adenosine could be a source for the increase was tested by the microinjection of adenosine in the vicinity of the electrode tip. The increase in Peak 2 that was seen following these injections indicated that extracellular adenosine can be converted to uric acid in the striatum. The fact that the increase was dose-dependent further supported this hypothesis.

The role of adenosine in the normal physiology of the striatum was somewhat less clear. The attempt to block the increase in Peak 2 by inhibiting the conversion of adenosine to uric acid was unsuccessful. 2'-Deoxycoformycin was microinjected to inhibit the enzyme adenosine deaminase. Although the experiments showed a trend toward suppressing the L-dopa-induced increase in Peak 2, this suppression was not statistically significant. Adenosine deaminase may be in a site inaccessible to drug inhibition, or the pharmacokinetics of the microinjected 2'-deoxycoformycin may be inappropriate for the timing in the protocol used for these studies.

The results from these experiments demonstrate the role of uric acid in Peak 2, and support the possibility that extracellular adenosine could be an intermediate in the process. The negative results for the attempts to block conversion of endogenous adenosine to uric acid do not support this hypothesis, but they also do not refute it; more extensive investigation of the intervening steps is needed to determine the significance of these results.

Because the local infusion of 2'-deoxycoformycin was unsuccessful in establishing the pathway from L-dopa to Peak 2, the next step was to determine the locus of action of L-dopa. Microinjection of the drug directly into the striatum would reveal whether it was acting locally or whether this was an indirect effect, possibly mediated peripherally insofar as these investigations were carried out without the co-administration of a peripheral decarboxylase inhibitor. The possibility exists that L-dopa could be acting via an indirect mechanism, for example causing a hyperuricemia that increases striatal uric acid by peripheral uric acid penetrating the blood-brain barrier. The result of this study indicated that the microinjection of L-dopa caused an increase in Peak 2, just as the systemic administration of L-dopa does. This established that L-dopa is acting locally within the striatum to increase the levels of uric acid. This result brings the Peak 2 changes and the behavioral changes into a closer affinity, and increases the probability that they are mechanistically related, rather than artifactually related.

In addition, the short duration of action of the intrastriatal L-dopa indicates that within the brain there is a relatively efficient homeostatic mechanism that is able to quickly compensate for the effect of the exogenous perturbation. The difference in the time course that is seen with the systemic L-dopa is different from the microinjection time course. They may differ because of the difference in drug concentrations administered, or the underlying reason may have to do with the pharmacokinetics of drug entry into the striatum, whether it occurs all at once in a single bolus, as with the microinjection, or whether it occurs with a longer time course, as with the intraperitoneal injection. The slower processes of peritoneal uptake, circulation within the blood, transport across the blood-brain barrier, and finally diffusion into the parenchyma of the striatum, would all be expected to delay entry into the striatum and to prolong the time over which L-dopa is available.

The next set of experiments attempted to investigate the role of dopa decarboxylase in these observation. Specifically, the question was whether L-dopa had to be decarboxylated to dopamine in order for it to stimulate the increase in uric acid. In other words, is the increase in uric acid mediated via dopamine receptors? Unfortunately, this set of experiments was entirely inconclusive. Both of the inhibitors utilized, carbidopa and Ro4-4602, caused a new peak to appear in the voltammogram at a voltage between that of Peak 1 and Peak 2, interfering with the readings so that neither peak height could be determined reliably. This problem illustrated one of the limitations of the method of in vivo electrochemical detection. Exploration of this question will require the application of a different methodology.

And the final series of experiments more directly examined the involvement of dopamine receptors as an intermediate step. A dopamine receptor antagonist, haloperidol, and an agonist, apomorphine, were microinjected in separate studies to determine their effects on Peak 2 height.

Haloperidol was microinjected into the striata of rats that were treated with systemic L-dopa in an attempt to block the increase in Peak 2. These experiments were unsuccessful in demonstrating that receptor blockade was effective in preventing uric acid formation. These experiments used a higher dose of L-dopa, 100 mg/kg, than previous experiments in an attempt to heighten the response at the dopamine receptor and to accentuate any effect that haloperidol may have. Two factors may play a role here. First, the technical difficulty in dissolving the haloperidol for delivery by microinjection required the use of a specially prepared vehicle incorporating acetic acid and Tris buffer (pH 5.74). This vehicle could itself induce perturbation of the striatal microenvironment, as is suggested by the observation that both haloperidol and vehicle induced an increase in Peak 2, without the systemic administration of L-dopa. This alteration in the striatal physiology could interfere with the normally expected response to L-dopa. Second, the use of the increased dose of L-dopa may have driven the striatal system to a point of maximal response that the haloperidol might not be able to overcome. In retrospect, it may have been wise to continue the experiments with the lower dose of L-dopa (25 mg/kg), insofar as there was a trend, albeit non-significant, for inhibition of the response.

Microinjection of apomorphine also did not yield the expected result. There was no statistically significant difference between the Peak 2 responses on the apomorphine and the control sides, although there was, again, a non-significant trend for Peak 2 on the apomorphine side to be higher than on the control side.

Results with haloperidol and apomorphine were equivocal. Although neither the haloperidol nor the apomorphine results support the involvement of dopaminergic receptors in the mediation of the Peak 2 response to L-dopa, they do not disprove such a relationship. In each case, however, there is a non-significant trend for the response to be consistent with the hypothesis. In addition, the technical problems associated with delivery of the haloperidol may have been a factor in the results. Because these negative results do not refute the possibility of dopamine receptor involvement, more elaborate

pharmacokinetic and concentration studies using alternative methodology would be useful.

In summary, the results of this project indicate that systemic L-dopa can induce circling in rats with a unilateral nigrostriatal lesion caused by 6-hydroxydopamine, and that this circling behavior is closely correlated with an asymmetric increase in Peak 2 as measured with the technique of in vivo electrochemical detection. Furthermore, the identity of Peak 2, in the circumstances pertinent to this investigation, has been demonstrated to be uric acid, and thus it is uric acid that is asymmetrically changing in response to L-dopa. The close correlation of the uric acid changes with the circling behavior indicates a mechanistic connection between the two. This connection is strengthened by the observation that Peak 2 increases with the local microinjection of L-dopa into the striatum. The hypothesis is advanced that uric acid reflects the metabolism of extracellular adenosine in the striatum, where purinergic receptor activity has been linked to circling behavior, and that this adenosine is being released as a response to dopaminergic activity. The microinjection of adenosine into the striatum caused a striking increase in the height of Peak 2. Further experiments, however, were not supportive, although non-significant trends were observed that were consistent with the hypothesis. The use of dopa decarboxylase inhibitors was fruitless, due to the technical limitation of the methodology, precluding the possibility of determining the necessity of conversion of L-dopa to dopamine. The results of the experiments with an adenosine deaminase inhibitor did not support the hypothesis that uric acid was being formed from extracellular adenosine, although there was a non-significant trend consistent with the drug blocking the increase in uric acid seen after L-dopa, at least partially. The experiments attempting to manipulate the dopamine receptor were also not supportive, although, again, both the receptor antagonist and the agonist caused non-significant trends consistent with the hypothesis. Each of these results is difficult to interpret because of the problems inherent

in negative results. The question is left open for further investigation as to the effects of modified protocols utilizing different doses and/or different time courses.

The overall conclusion of these studies is that L-dopa induces changes in striatal activity that results in circling behavior and in an increase in extracellular uric acid. This increase in uric acid may be mediated by an increase in extracellular adenosine.

IN VIVO ELECTROCHEMICAL DETECTION METHODOLOGY

The experiments described here used the technique of in vivo electrochemical detection to make observations about biochemical activity in the brain and its relationship with circling behavior. The results of these experiments also yielded information about the methodology.

These experiments confirmed the utility of in vivo electrochemical detection as a method for following biochemical changes in awake, alert, freely-moving animals. The ability to correlate these measurements with circling behavior further advances the usefulness of this technique.

These studies were conducted with unmodified silicone oil paste electrodes using linear sweep voltammetry, and may not apply to in vivo electrochemistry using other electrodes and other voltammetric techniques. The reliability and stability of the unmodified paste electrodes is seen in the literature (Marsden et al. 1988, Discussion 1986), and has been confirmed here, first by the stability of the measurements in these rats, and second by an experiment in which unmodified paste electrodes were compared with Nujol electrodes and with stearate-modified electrodes. Although the results support the stability and reliability of the unmodified paste electrodes, they suggest that the Nujol electrodes are too unstable for making measurements for any longer than a few hours, and that sensitivity declines precipitously in solution after only a short period of time, progressing to insensitivity after an overnight run in vitro.

The reference electrode is an important part of the three-electrode voltammetric system. The results of the comparison experiment suggest, however, that the specific details of reference electrode construction is of lesser importance than is consistency. The three different types of reference electrode had little effect on the voltammogram, except to cause displacement of the peaks such that the voltammogram from each reference electrode looked similar except for being offset from each other by a constant amount. This implies the absolute voltage of any peak is of lesser importance in its identification than its position in the context of the overall voltammogram shape. A permanently implanted Ag | AgCl wire electrode, kept in contact with brain tissue over an extended period of time, such as several weeks to months, might be exposed to a changing microenvironment. Both the electrode surface and the surrounding tissue may undergo a gradual alteration in chemical composition due to slow redox reactions, reactive gliosis, or other unknown processes. The identification of the peaks by position in the overall shape of the voltammogram allows some degree of flexibility in the reaction conditions around the Ag | AgCl electrode, because it is assumed that there may be some shift of the peak positions due to variation in the reference electrode composition and/or soluble reactant concentrations.

Other results in this study confirm that uric acid is the substance measured by Peak 2. The use of uricase established the identity of Peak 2 in the lesioned striatum, the unlesioned striatum, and both with and without systemic L-dopa treatment. This is supportive of the contention that Peak 2 is uric acid, and argues against the alternative that Peak 2 represents, partially or wholly, 5-hydroxyindoles.

Another result (described in Appendix 3) demonstrated the validity of the measurement method used in this study, in which the peak heights are measured from an absolute zero baseline. A comparison with a different analysis in which the peak heights are measured from a trough-to-trough tangent baseline indicated that qualitatively, the two methods are equivalent. Other groups use a depletion baseline with digital storage of the

data and computation of subtraction voltammograms to calculate peak heights; unfortunately this was not feasible for these studies. The consistency of the results seen in these studies, however, indicates that the data analysis techniques are sufficiently sensitive for these purposes.

The use of permanently implanted electrode/cannula assemblies (ECAs) is another contribution of this project. These results demonstrate the long-term use of permanently implanted cannulae in conjunction with in vivo electrochemical detection. In these animals, few problems were encountered with regard to the surgical procedure, or with the long-term viability of the ECAs. It did become apparent that the problem of the pharmacokinetics of drugs delivered via the microinjection cannula needs to be addressed more extensively in terms of time course, dosages, and physical spread within the brain parenchyma. The injection volume chosen was based upon the spread of dye by diffusion within the brain, but the injection of substances such as L-dopa or adenosine, both of which are found naturally in the brain, would be influenced by other factors as well, such as tortuosity, ionic charge, cellular uptake, and the action of enzymes, so that determining the extent to which these substances spread from the cannula tip to the electrode tip is a complex problem (Rice et al. 1985). This is an area for further investigation.

ADENOSINE / DOPAMINE INTERACTIONS

Adenosine has become a major candidate for neurotransmitter status in recent years (for reviews see Burnstock 1985, Fredholm and Hedqvist 1980, Marangos and Boulenger 1985, Phillis and Wu 1981, Snyder 1985, Williams 1984). The existence of several adenosine receptors has been demonstrated in the brain (Daly 1985, Snyder 1984, Ribeiro and Sebastião 1986), and a variety of drugs have been shown to have properties as agonists and antagonists at these receptors (Daly 1983, 1985). Caffeine is well known for its behavioral stimulating properties (Rall 1980), and adenosine has been shown to have sedative effects (Radulovacki et al. 1984). In addition, there is evidence that

adenosine may interact with a number of other neuronal systems, including cholinergic, adrenergic, and dopaminergic neurons (Corrodi, Fuxe and Jonsson 1972, Harms, Wardeh and Mulder 1979, Michaelis, Michaelis and Myers 1979, Waldeck 1971).

Another interesting observation has been that the methylxanthines have an effect on turning behavior in unilaterally lesioned rats (Fredholm et al. 1983, Satoh et al. 1976, Ungerstedt, Herrera-Marschitz and Brugue 1981, von Voigtlander and Moore 1973). Administration of caffeine or theophylline causes marked rotation away from the lesioned side. The mechanism by which caffeine induces turning is unclear. It is not due to phosphodiesterase inhibition, as other phosphodiesterase inhibitors do not induce turning (Fredholm et al. 1983). It has been proposed that caffeine and theophylline may have a direct effect on dopamine receptors (Fredholm et al. 1983). Another, perhaps more likely, explanation is that their effects are mediated by their antagonism of the adenosine receptor.

The interaction of adenosine with dopamine may help explain the effects of methylxanthines on turning behavior. Adenosine is able to presynaptically inhibit the release of dopamine (Harms, Wardeh and Mulder 1979, Michaelis, Michaelis and Myers 1979). In addition, A1 and A2 receptors affect adenylate cyclase activity (Anand-Srivastava and Johnson 1980, Phillis and Barraco 1985, Prémont et al. 1979), and so may interact with the effects of dopamine-sensitive adenylate cyclase in the post-synaptic cell (Prémont, Perez and Bockaert 1977, Prémont et al. 1983). Microinjection of adenosine analogs into the striatum of normal, naïve rats elicits circling upon apomorphine challenge, further suggesting that adenosine plays a substantial role in this behavior (Green, Proudfit and Yeung 1982). In connection with this, it has been shown that adenosine production/content, and its depolarization-dependent release are associated with intrinsic neurons of the striatum (kainic acid-sensitive). The dopaminergic nerve terminals also seem to contain some of this adenosine, since it is decreased after 6-hydroxydopamine lesion. The kainic acid lesions of the striatum also decrease adenosine-

sensitive adenylate cyclase 99% (Prémont et al. 1979, Wojcik and Neff 1983b), leading the investigators to suggest that essentially all the A2 receptors in this brain area are located on intrinsic neurons.

The following is a hypothesized mechanism of action for adenosine.

Adenosine-releasing neurons are feedback inhibitors of the actions of dopamine. The striatum has efferent neurons that are mediating the turning behavior induced by dopamine, although the role of different subclasses of the neurons is not established, and it is unresolved whether the relevant ouput projects to the globus pallidus, the substantia nigra, or both (Pycock 1980, Ungerstedt et al. 1985). The efferent neurons of the striatum are medium spiny neurons (Kitai 1981, Pasik and Pasik 1981). There are two types of these neurons: SI neurons, which use gamma-amino butyric acid, or possibly enkephalin, as their transmitter, and SII neurons, which use substance P as their transmitter (Pasik and Pasik 1981, Pycock 1980). Both receive nigrostriatal dopaminergic synaptic inputs, which are believed to be excitatory (Kitai 1981, Kocsis, Sugimori and Kitai 1977, Pasik and Pasik 1981, Richardson, Miller and McLennan 1977).

The hypothesized model has three points. (1) Adenosine has an effect on these efferent neurons that is opposite to the effect that dopamine has upon them, or that adenosine decreases dopamine's effect. As such, adenosine is inhibitory to dopamine's actions. (2) The adenosine neurons are activated to release adenosine via a feedback loop that is stimulated by the actions of dopamine. In other words, if dopamine stimulates striatal efferent neurons, then the activation of those efferent neurons will cause the release of adenosine. (3) This adenosine will then be inhibitory to the efferent neurons, or it may decrease the effect of dopamine, either pre- or post-synaptically.

Adenosine-releasing neurons are intrinsic striatal neurons. The most likely candidates for the adenosine neurons are intrinsic striatal neurons (Wojcik and Neff 1983b). Adenosine may be released from either inhibitory interneurons or the efferent

neurons themselves. If it is the efferent neurons, then the possibility exists that the adenosine may be released by axon collaterals, or it may originate from the cell body itself subsequent to dopamine receptor stimulation. The spiny neurons have been shown to have numerous local collaterals, including ones that may form autapses, which are thought to mediate recurrent inhibition (Park, Lighthall and Kitai 1980, Richardson, Miller and McLennan 1977). These collaterals may exert their effects through interneurons. These interneurons may also release adenosine.

Obviously, the possibile circuitry models are numerous, and can get more and more complicated. The main concept, however, is simple: adenosine acts as a mediator of negative feedback for the effects of dopamine on the striatal output neurons. One conclusion that can be deduced from this model is that the levels of released adenosine will follow the activity of the striatal output neuron.

CLINICAL IMPLICATIONS

The results of this basic science research, in conjunction with reports in the literature, have clinical implications for patient care. Most obviously, there is a connection with Parkinson's disease. In addition, the increases in the levels of uric acid and the involvement of purinergic metabolism suggest that these studies may have some implications for Lesch-Nyhan syndrome as well. The sections below discuss how these studies fit together with the literature on these diseases.

Parkinson's Disease

As discussed above, this investigation started as a study of the dynamics of L-dopa and its effects in the striatum. The implication of purinergic metabolism in the physiology of circling behavior immediately suggested an area for clinical study. Because caffeine, one of the most used drugs in the world, is an antagonist at the adenosine receptor, it was important to examine the effects of caffeine on the disease process. Adenosine is hypothesized to act in a way opposite to dopamine, and since caffeine augments the

effects of dopamine, the working hypothesis for a clinical study would be that exogenous caffeine may affect Parkinson's disease in a way similar to that of drugs that increase dopaminergic activity. With this hypothesis, an epidemiological study was undertaken to explore the interaction between dietary caffeine and the course and treatment of Parkinson's disease. A copy of the report, "The Effect of Dietary Caffeine on the Course of Parkinson's Disease", is included in Appendix 6. The results and conclusions of this study are summarized below. (References and additional discussion are available in the report in Appendix 6.)

This study showed that dietary caffeine intake may accelerate the gradual worsening of the patient's symptoms normally seen as the disease progresses. However, the data did not support the hypotheses that dietary caffeine, in the shorter-term, affects the dose of levodopa needed to achieve optimal clinical effectiveness against Parkinson's disease, that it affects the clinical stage or the symptoms of Parkinson's disease, or that it exacerbates the side effects of levodopa treatment. By failing to demonstrate an interaction between caffeine and levodopa dose, it supported the idea that caffeine is not an effective therapeutic agent for Parkinson's disease. The positive finding of this study suggested that dietary caffeine may accelerate the degenerative processes of Parkinson's disease. This finding, however, needs to be interpreted cautiously, until more data can be gathered.

With that consideration, the implication is that dietary caffeine consumption should be restricted in Parkinson's patients. A mechanism to explain this finding is not obvious. One way adenosine is believed to affect dopaminergic transmission is by inhibition of dopaminergic neurotransmitter release presynaptically. It is possible that caffeine, by antagonizing adenosine, could enhance dopamine turnover in the nigrostriatal neurons that remain in the parkinsonian patient. This could enhance formation of free radicals formed as a result of dopamine metabolism, leading to an increase in the rate of dopaminergic cell injury, thus accelerating the progression of the disease. A recent report examining the

possible effects that early life dietary factors have on Parkinson's disease concluded that certain foods with a high content of the antioxidant vitamin E are associated with the absence of Parkinson's disease, suggesting that the presence of a free radical scavenger in the diet may have a protective effect for nigrostriatal neurons (Golbe, Farrell and Davis 1988).

Another clue to understanding what processes may underlie the accelerated degeneration might be found in some of the literature of Lesch-Nyhan syndrome and a proposed animal model of it.

Lesch-Nyhan Syndrome

A further connection between dopamine, turning and purinergic metabolism may be provided by Lesch-Nyhan Syndrome and its proposed animal models. Lesch-Nyhan Syndrome is an X-linked genetic disorder characterized by mental retardation, hyperuricemia, spasticity, choreoathetosis, and compulsive self-mutilation (Lesch and Nyhan 1964). The metabolic basis for this disease has been determined to be a deficiency of the enzyme hypoxanthine guanine phosphoribosyltransferase (HPRT) (Seegmiller, Rosenbloom and Kelley 1967). The lack of this enzyme leads to a loss of the salvage pathway for purines, causing a great increase of purine turnover and the hyperuricemia. It is easy to visualize that this metabolic disturbance could affect adenosine neurotransmission.

The movement disorder seen in this syndrome is suggestive of basal ganglion involvement. It has been reported that nigrostriatal dopaminergic neurotransmission is greatly impaired (Baumeister and Frye 1985, Lloyd et al. 1981). Although the dopaminergic cell bodies in the substantia nigra are intact, functional loss of 65-90% of the nigrostriatal and mesolimbic dopamine terminals is indicated. This loss is specific for dopamine. Loss of noradrenergic and serotonergic function is not seen. It has also been reported that normally the basal ganglia have a higher level of HPRT than any other tissue

(Kelley et al. 1969), and that the caudate has one of the lowest levels of de novo purine biosynthesis (Watts et al. 1982).

The animal models that have been proposed for Lesch-Nyhan Syndrome indicate that there may be involvement of both purinergic and dopaminergic neurotransmission. Nyhan proposed that chronic administration of high doses of caffeine be used as an animal model, and it was found that this induces self-mutilation behavior (Discussion 1968, Mueller et al. 1982). This self-mutilation behavior in rats can be greatly potentiated by unilateral 6-hydroxydopamine lesions of the nigrostriatal system (Casas-Bruge et al. 1985). Unilateral lesions alone can cause self-mutilation after apomorphine administration (Ungerstedt 1971b). A similar phenomenon has been reported in monkeys (Goldstein et al. 1985). Rats with unilateral microinjections of an adenosine analog into the striatum also show self-mutilation after apomorphine (Green, Proudfit and Yeung 1982). A further refinement has been proposed in which rats are treated with 6-hydroxydopamine neonatally, and have their catecholaminergic systems totally destroyed (Breese et al. 1984a,b). This procedure increases the self-mutilation behavior over that seen if the rats are lesioned as adults, perhaps indicating a developmental difference that could explain differences in the movement disorders seen in Lesch-Nyhan patients and parkinsonian patients. The self-mutilation behavior in the neonatal rat studies have been shown to be specific to depletion of dopamine, and does not occur with selective depletion of either norepinephrine or serotonin.

The investigations of Lesch-Nyhan strengthen the contention that there is a connection between dopaminergic neurotransmission and purine metabolism, and perhaps adenosine as a transmitter. In light of the results of the study of caffeine and Parkinson's disease, it is interesting to speculate that loss of adenosinergic activity could have a toxic or degenerative effect on nigrostriatal neurons. Possibly, the loss of adenosine receptor stimulation leads to a loss of neurotrophic stimulation of the dopaminergic terminals. In the case of Lesch-Nyhan syndrome, this could lead to a developmental deficiency in the

nigrostriatal system; with chronic caffeine, perhaps there is a degenerative change due to caffeine blocking the adenosine receptors on the presynaptic terminals. This is especially attractive in conjunction with the animal model of Lesch-Nyhan syndrome that uses neonatal 6-hydroxydopamine. The implication is that Parkinson's disease and Lesch-Nyhan syndrome may be both connected with nigrostriatal deficiency, with some of the symptomatic differences arising from the immature brain's potential for developmental compensation. Further investigation of these hypotheses would be interesting.

SECTION 8 — FUTURE DIRECTIONS FOR THIS RESEARCH

STUDIES WITH IN VIVO ELECTROCHEMICAL DETECTION

The studies described above using in vivo electrochemical detection suggest several new avenues of research. First and foremost, the pharmacokinetic studies using the 2'-deoxycoformycin, and the dopaminergic receptor agents haloperidol and apomorphine are a good starting point for follow-up studies. Characterization of the actions of these drugs at varying concentrations would be useful for determination of the expected responses, and would also yield important information as to the proper timing of the systemic administration of L-dopa in relationship to the microinjection of these drugs.

Additional investigation of the relationship of changes in the animal's circling behavior with the changes in the voltammogram would be interesting. For example, investigating the effects of systemic apomorphine on unilaterally lesioned rats, and determining how it affects the height of Peak 2. Also, it would be worthwhile to examine the effects of microinjection of substances that affect adenosinergic physiology, such as adenosine receptor agonists and antagonists, on circling behavior. This, however, would require modification of the microinjection protocols so that the drug would have a wider area of spread to include a greater proportion of the striatum. It may also require the coadministration of dopaminergic agents such as apomorphine, as in previous studies (Green, Proudfit and Yeung 1982).

FOLLOW-UP OF CAFFEINE AND PARKINSON'S STUDY

The issues raised by the epidemiological study of caffeine and its effects on Parkinson's disease (see Appendix 6) require further study. The widespread use of caffeine and its correlation with an accelerated progression of the disease needs replication, preferably with a better controlled group of patients and a larger sample size.

Extension of the previously used methodology to a larger number of patients would be a relatively simple undertaking. With a larger sample size, it would also be possible to evaluate the effects of concomitant drug therapies upon the parameters examined in the study. It is also possible for a study to be undertaken in which the patients came from the national registry of Parkinson's patients who have been maintained drug-free, so as to rule out all confounding drug effects.

The possibility that caffeine has an adverse effect in patients with Parkinson's disease has important clinical implications. The restriction of caffeine use would thus be another area for study, as it would be relatively simple to place patients on a caffeine-free diet. This type of prospective study, however, would require the commitment of much greater resources than a simple epidemiological study. It would also have to be conducted over the course of a number of years to yield information about the effects of caffeine upon the rate of progression of Parkinson's disease.

The possible effects of short-term caffeine administration could also be more definitively tested by performing a prospective study on patients that are non-caffeine users or who have had their caffeine intake restricted so as to eliminate any possible effects of tolerance to chronic use. Results of such a study, if positive, might have future clinical implications.

All of these studies could have additional implications for the general population, in that any adverse effects of caffeine may also affect normal users, but not be manifest until later in life. To be more specific, it is possible that the use of caffeine could cause an acceleration in the normal wear-and-tear loss of dopaminergic neurons that does not become manifest until later in life when the deficit reaches the threshold for becoming symptomatic parkinsonism.

Because the use of caffeine is ubiquitous, reports on its effects could have widespread ramifications.

INTRACEREBRAL DIALYSIS

Another technique for examination of extracellular levels of substances in the brain has been gaining popularity recently: intracerebral dialysis. This is another technique pioneered by Urban Ungerstedt (1984). It is an extension of the methodology of the push-pull cannula, except that the intracerebral ends of the push cannula and the pull cannula, and the channel connecting them, are shielded from the parenchyma of the brain by a semi-permeable membrane. This keeps the medium flowing in the microdialysis probe separate from the extracellular milieu, while allowing exchange of chemical substances between the two. The dialysate is then assayed, most often using the techniques of HPLC, for the substances of interest. By enclosing the flowing perfusate within dialysis tubing, the brain tissue is partially protected from mechanical damage due to either erosion or hydrostatic pressure at the cannula tip producing expansion lesions.

This method has advantages and disadvantages in comparison with in vivo electrochemical detection. The advantage is that the biggest problems of in vivo electrochemical detection can be overcome. First, other substances can be measured besides those that are electroactive. In addition, the problems of specificity and discrimination of the voltammetric peaks are avoided, because actual samples of the substance are obtained, so that separation and measurement procedures can be individually tailored. The limiting factor here is that the sample sizes are generally small, so that the assays must have great sensitivity to small amounts. One disadvantage is that the collection of a sufficient amount of sample for an assay to be run usually requires a longer period of time than voltammetric scanning, so that the temporal discrimination is less sensitive. Also, the technical requirements are much greater with microdialysis than with in vivo electrochemical detection.

With these considerations, it is apparent that microdialysis will be a useful technique for further studies of dopamine-adenosine interactions and the role of uric acid in them. Adenosine can be measured in the dialysates, as can uric acid and the intermediates

xanthine and hypoxanthine. This would be important for understanding the kinetics of uric acid formation and for more directly observing the levels of extracellular adenosine. In addition, the decarboxylase inhibitor experiments could be carried out without the problem of the peak overlap interference seen in the experiments with in vivo electrochemical detection because chromatographic separation techniques can be adjusted so that carbidopa, Ro4-4602, L-dopa, and dopamine and its metabolites can be resolved from each other. Furthermore, the additional flexibility afforded by HPLC separation would allow concurrent measurement of adenosine metabolites. As mentioned above, however, there would be a loss of some temporal discrimination, with samples being taken over longer session intervals, and an increased requirement for technical resources.

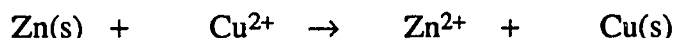
Experimental studies along these lines are presently being contemplated and planned. Once again, technological innovations should make possible a greater understanding of the mysteries of the brain and the relationship between the biochemical events in the brain and the behavioral output of the animal.

APPENDICES

APPENDIX 1: THEORY OF IN VIVO ELECTROCHEMICAL DETECTION

Electrochemistry is based upon the investigation of chemical reactions known as redox reactions. Redox is short for reduction-oxidation. In these reactions, one or more electrons are transferred from one chemical species to another. The loss of an electron by a reactant is oxidation, the gain of an electron is reduction. These transfers occur because different chemical species have different propensities to exist in their various ionized and unionized forms. Reactants with higher "electron affinities" will oxidize those with lower "affinities" by taking electrons from them, thus reducing themselves in the process.

A typical redox reaction is that between zinc and copper ion:



$$Zn(s) + Cu^{2+} \rightarrow Zn^{2+} + Cu(s)$$

which can be rewritten as two half-reactions:

$$2e^{-} + Cu^{2+} \rightarrow Cu(s) \quad \text{reduction}$$

$$Zn(s) \rightarrow Zn^{2+} + 2e^{-} \quad \text{oxidation}$$

In this case, two electrons are transferred from each zinc atom to each copper ion. Writing the reactions as two half-reactions is more than just a mental construct, because the reaction can actually be separated so that each half runs in a separate beaker. This is accomplished by having the electrons involved flow through a wire from one reaction site to the other as illustrated. By convention, the electrode at which oxidation occurs is called the anode; reduction occurs at the cathode (See Figure 65). A salt bridge is included to complete the circuit. This arrangement is called an electrochemical cell.

If the current is reversed with an external voltage source, the reactions will also reverse. (Some reactions, however, are irreversible due to the chemical properties of the reactants and products). As with any reaction, the laws of thermodynamics apply and the

ELECTROCHEMICAL CELL

Figure 65

Voltmeter

e^- e^-

Salt Bridge
KCl-Agar

Zinc Electrode Anode

Cathode Copper Electrode

$ZnSO_4$ 1M

$CuSO_4$ 1M

$$Zn(s) + Cu^{2+} \rightarrow Zn^{2+} + Cu(s)$$

$$Zn(s) \rightarrow Zn^{2+} + 2e^-$$

$$2e^- + Cu^{2+} \rightarrow Cu(s)$$

reaction will proceed according to the change in Gibbs free energy of the system, ΔG. The reaction will tend to progress to conditions of equilibrium.

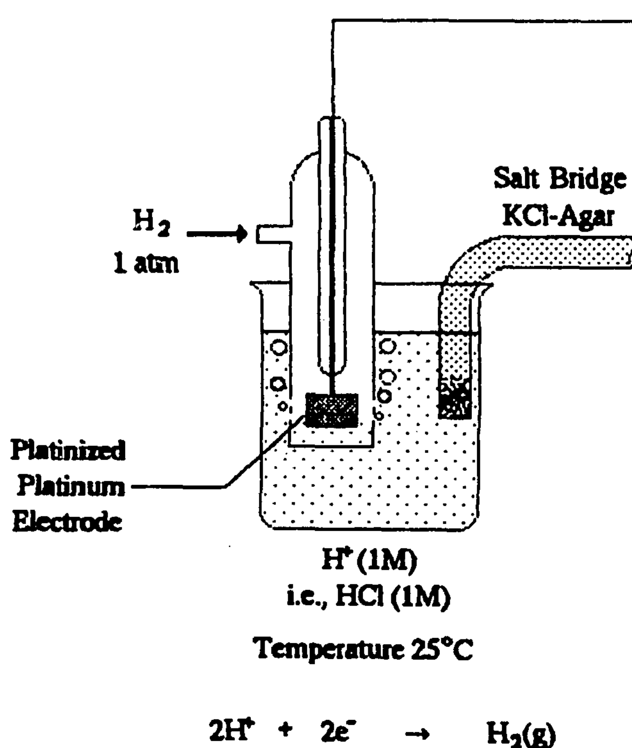
When an oxidation half-reaction occurs at an electrode surface, the oxidized product deposits electrons on the electrode. A difference in electrical potential develops between the electrode and the solution, although it is impossible to directly measure this difference. The oxidation half-reaction can be coupled to another half-reaction, however, and the resulting potential difference between the anode and cathode can be measured. This potential difference is known as electromotive force (emf). The potential associated with each half-reaction is known as the electrode potential.

The emf that exists across the external circuit depends upon how much driving force there is for the reaction. This depends upon the difference in the electrode potentials of the reactants, and, as with any chemical reaction, upon the reaction conditions, including the concentrations of the species involved, and the temperature. Under the same conditions, the voltage measured will be the same.

An electrode potential is measured for a specific half-reaction by determining the voltage difference between its electrode and the electrode of a standard half-reaction against which all other half-reactions are compared, under certain specified conditions. In other words, in order to easily compare the electrode potentials of half-reactions involving many different chemical species, it made sense to measure the emf against a single

Figure 66

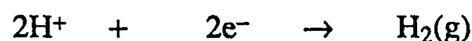
STANDARD HYDROGEN ELECTRODE (SHE)



$$2H^+ + 2e^- \rightarrow H_2(g)$$

reference half-reaction. By convention, as agreed by the International Union of Pure and Applied Chemists (IUPAC), this reference reaction is the reaction that occurs at the Standard Hydrogen Electrode (SHE). The standard conditions that the electrode uses are illustrated in Figure 66.

The half-reaction that occurs in the Standard Hydrogen Electrode is:



$$2H^+ \quad + \quad 2e^- \quad \rightarrow \quad H_2(g)$$

The electrode potential associated with the Standard Hydrogen Electrode is defined as being 0 volts at all temperatures.

By measuring the voltage generated when a half-reaction is connected to the Standard Hydrogen Electrode, its "standard electrode potential" can be determined. The half-reaction must be measured at a set of conditions in which the activity of each of the involved reactants and products is one unit (i.e., 1 molar or 1 atmosphere). In addition, by a convention adopted by the IUPAC, each half-reaction is written as a reduction, and the electrode potential is given as if that were the direction the reaction proceeded in. Because some half-reactions will proceed as oxidations when coupled with the SHE, these potentials are given negative values, because the electromotive force is in the opposite direction in the external circuit.

The measurement of electromotive force can be used to gain understanding of the thermodynamics of the chemical reaction, and the equilibrium constant of a reaction can be computed from the electrode potentials. In addition, the expected electromotive force for two half-reactions not at standard conditions (i.e., unit activity) can be computed by the use of the Nernst equation. Details of this procedure are not germane to the types of measurements involved in this work and will not be discussed further.

Voltammetry uses the principles explained above to measure the concentration of a reactant in solution. Measurements are made of the potential in the external circuit and the amount of current flowing. The current flow is determined at different voltages that are applied between the anode and cathode, and at different times. Current measures the

number of electrons being transferred in the redox reaction, which is directly related to the number of molecules reacting, and which varies with the voltage applied to the electrodes by the external circuit. A specific compound will undergo a significant amount of reaction only when the applied potential exceeds a voltage that is characteristic for that compound. If the applied potential exceeds a certain level, the reaction proceeds essentially to completion.

When the current flowing in the system is relatively high, an electrode phenomenon is seen known as polarization. The occurrence of polarization depends upon the size, shape, and composition of the electrode, the composition of the solution, the temperature of the system, and the amount of mechanical disturbance (stirring) that is present. If the electrode reaction is rapid and reversible, a layer forms around the electrode in which the concentration of the ionic species is determined by the potential applied at the electrode. The applied potential controls the amount of reaction occurring at the electrode-solution interface, and sets up equilibrium conditions that the reaction tends to move toward. Reactant species become depleted and product species accumulate. The layer of solution immediately adjacent to the electrode thus assumes a composition different from that of the bulk of the solution. In other words it becomes polarized with respect to the rest of the solution, and a concentration gradient is formed.

As mentioned above, when the voltage is sufficiently high, the reaction proceeds essentially to completion, and the concentration of reactant immediately adjacent to the electrode surface is zero. This sets up a concentration gradient between the region at the surface and the bulk of the solution, such that molecular movement is promoted. Movement of ions to and from the electrode surface is determined by three factors: (1) diffusion, (2) electrostatic forces, and (3) mechanical/convection forces.

In order to get information about the concentration of reactant in the solution, it is desirable for ion movement to depend solely upon diffusion, with the effect of electrostatic and mechanical forces minimized. This is because the rate of diffusion is

dependent upon the magnitude of the concentration gradient between the tip of the electrode and the bulk of the solution. The concentration of reactant at the electrode tip is zero, as described above. Thus, the gradient is entirely dependent upon the concentration in the bulk of the solution. The rate of electron transfer, and therefore the amount of current in the external circuit, is directly proportional to the rate of delivery of reactant to the electrode tip, which is directly proportional to the rate of diffusion, which is directly proportional to the concentration gradient. The end result is that a linear relationship exists between the measured current and the solution concentration. This relationship is dependent upon the elimination of the electrostatic and mechanical contributions.

To minimize the mechanical factor the solution should be kept as still as possible, with no stirring or physical perturbation. Mechanical disturbance is not a major factor in vivo.

Electrostatic forces come into play because the electrode has a electrostatic charge as a result of the applied potential. Many of the species involved in the reactions are charged; they exist as ions at physiologic pH. The role of these forces is decreased in vitro by the addition of a supporting electrolyte to the solution. This electrolyte is a salt that separates into ions that do not undergo redox reactions in the voltage range of interest. By introducing a large number of charges into the supporting medium, the effect of electrostatic interactions between the electrode and the reactant is diminished. Electrode modification by inclusion of stearic acid in the carbon paste is an attempt to enhance the repulsion of negatively charged ascorbate and DOPAC ions from the electrode surface by incorporating negative carboxyl groups (Blaha and Lane 1983).

In linear sweep voltammetry, the voltage applied is gradually increased with time. The current that flows through the working electrode is continuously monitored, indicating the magnitude of the redox reaction occurring at the electrode tip. This information is plotted on a current-potential curve, called a voltammogram, in which voltage is the independent variable and current is the dependent variable. When the voltage scan exceeds the potential at which an electroactive compound begins reacting, the oxidation reaction causes an

increase in the current flowing, and a peak appears in the voltammogram. The voltage at which this peak appears depends upon the conditions at the electrode tip, such as pH and temperature, but should be consistent within one series of measurements with any one electrode. The height of the peak relates to the concentration of reactant that exists in the vicinity of the electrode tip.

For much voltammetric work, the use of the SCE is somewhat impractical, because it requires the presence of an acidic medium, and the presence of hydrogen gas at 1 atmosphere. Because of these limitations, a substitute reference electrode is commonly used. The characteristics of such a reference electrode need to be: ease of assembly, known half-cell potential, relative stability in electrode potential in the range of conditions expected, and relative insensitivity to the solution studied. The electrode most commonly used for in vivo electrochemistry is the silver-silver chloride (Ag | AgCl) electrode. The half-reaction involved is:

$$AgCl(s) \quad + \quad e^- \quad \rightarrow \quad Ag(s) \; + \quad Cl^-$$

At 25°C, the electrode potential of this half-reaction is +0.197 V versus the SHE.

In the brain, there are many electroactive substances. Among these are ascorbic acid, uric acid, dopamine, norepinephrine, DOPAC, HVA, serotonin, and 5-HIAA. On a voltammogram, unfortunately, it is not possible to discriminate between some of these compounds, but the approximate voltages at which their peaks appear are known. Because the redox potentials of dopamine and ascorbic acid, for example, are so close together, the peaks associated with each overlap enough to make it difficult to separate them.

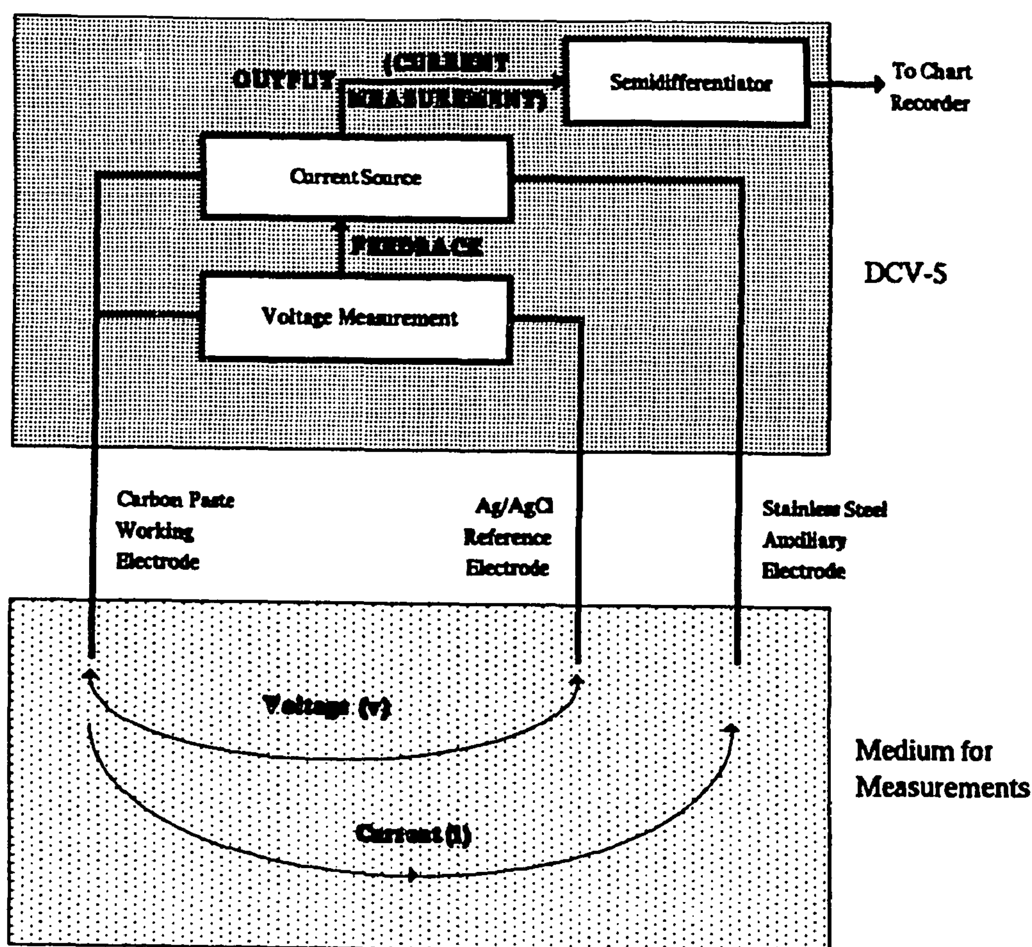
There is a mathematical device that helps to delineate some peaks: semidifferentiation. This is a transformation from the fractional calculus that yields a curve that is halfway between the original curve and its derivative, $d^{1/2}i/dt^{1/2}$. (A full discussion of semidifferentiation is available in Oldham and Spanier 1974.) The transformed curve has the advantage of better separation of the different peaks. Semidifferentiation is commonly

used in electrochemical analyses (Dalrymple-Alford, Goto and Oldham 1977, Lane, Hubbard and Blaha 1979), and it is easily carried out, since it can be performed electronically with a resistor-capacitor network, and is a standard feature of the DCV-5 voltammetry controller that was used for these investigations. (See Figure 67)

The voltammetry controller employs a standard three electrode system for applying the

Figure 67

ELECTRODE CONFIGURATION



The current passing through the working and auxiliary electrodes is controlled by feedback from the voltage measurer. The current level is adjusted so that the voltage between the working and reference electrodes is maintained at a specific level thta increases linearly with time. The DCV-5 is connected to a chart recorder that plots the semiderivative if the current levels against the applied voltage (actually time).

voltage and measuring the current. (See Figure 67) The reason for this is to minimize deviations in the applied voltage caused by electrical resistance in the circuit, known as the IR drop. The applied voltage should be utilized for causing the oxidation reaction to occur at the electrode surface. Because current flows, however, some of the applied voltage will be used to overcome the electrical resistance of the brain matter that exists between the two electrodes of the circuit. By using a feedback mechanism to connect separate circuits for voltage measurement and current flow, the voltage lost to overcome tissue resistance can be minimized. This arrangement also minimizes the amount of current that needs to flow through the Ag|AgCl reference electrode, thus reducing the perturbation to the conditions around its tip, and keeping the reference electrode potential constant.

In linear sweep voltammetry, the voltage applied is increased at a constant rate. As the voltage reaches that which causes a redox reaction to occur at the working electrode, the electron transfer causes an increase in the current flowing in the external circuit. As the voltage continues to increase, the magnitude of the reaction increases until all of the reactant available at the electrode tip is consumed (i.e., the concentration of reactant at the reaction surface is zero). At this point, the current decreases because further reaction depends on replenishment of reactant from the surrounding tissues. Generally, this depends on diffusion of the reactant.

As the voltage continues to increase, additional reactions also occur at the working electrode. Each new reaction contributes another peak as described above. The earlier reactions continue to proceed, but their rate should be relatively constant, so that any new peaks are due to another reaction beginning as the potential increases. Therefore, what appears on the output voltammogram is a series of peaks, each of which represents a different chemical species that is beginning to undergo reaction at that potential.

For a more complete explanation of the theory of electrochemistry and its various techniques, a chemistry textbook should be consulted. (Skoog and West 1976, Marsden 1984a).

APPENDIX 2: VOLTAMMOGRAM ANALYSIS

The analysis of the voltammograms that are recorded from these experiments requires some discussion because of the complexity of some of the interpretations. This section will outline the step by step method used for examining the output of the chart recorder.

At the beginning of each experiment, the chart recorder pen was set at an absolute zero baseline from which all heights were measured. Generally, the baseline was stable, but it would show slight drifting in some experiments. Therefore, at various points throughout an experiment, the baseline was reevaluated, and any drift was corrected. Those scans recorded with a non-zero baseline were corrected when the peak heights were measured.

The analysis of the chart recorder output required several passes. The first pass would locate and identify all peaks that were readily apparent and obvious. Not all peaks were present in all scans, partly because of the differences from rat to rat and partly because the experimental manipulations could sometimes obscure or accentuate a peak (as noted in the sections describing the results of the studies). The second pass would determine expected peak locations in sessions where a peak was not obvious, or was equivocal. In the third pass the peak heights were measured. These steps are explained in greater depth below.

The first step in analyzing a voltammogram was to determine the position (i.e., the voltage at the maximum height) of all distinguishable peaks. The general appearance of the voltammogram was fairly consistent from session to session, so that the peaks could be readily identified as Peak 1 or Peak 2. The voltage values identified with the same peaks throughout the experiment could be grouped together and averaged in order to determine the "peak position". In sessions in which the peak was not easily identifiable a voltage was not measured and the session was not included in computing the average peak position. Once computed, the average peak position was used to determine where the peak should be expected to appear, and the peak height was then defined as the height from the baseline and the voltammetric tracing at the projected location.

This allowed for the measurement of peak height in all sessions. The third pass through the chart recorder output was when the heights of all the peaks was determined and recorded. In a very few cases, noise in the voltammogram in some of the sessions prevented an accurate determination of peak height. The treatment of these experiments is discussed below.

The peak heights obtained were still considered raw data that could not be directly compared from one electrode to another. Because each electrode has a different sensitivity, the current recorded by different electrodes in vitro would often differ, even with the same concentrations of solution. This was evident when the electrodes were pre-tested prior to implantation. Although the working electrodes gave voltammetric responses to test solutions that had similar appearances, the peak heights varied from electrode to electrode. This difference in response is a normal and expected phenomenon. This difference can be accounted for by experimental variability in electrode construction. The exact size and shape of the exposed surface of the electrode leads to slight differences in the amount of reaction that can occur. Differences in the conductive interfaces within the electrode, for example between the carbon paste and the steel wire or at the soldered contacts, would be expected to affect the voltammogram, also. Each electrode should be consistent when compared to itself, however, so that if the peak heights were normalized, they could then be compared to peak heights measured using other electrodes.

This normalization was accomplished by expressing the heights as a percentage of the pre-treatment height. The three peak heights at the end of the warm-up period (sessions 6, 7, and 8) were averaged together to determine the "baseline peak height", which was defined as 100%. All subsequent peak heights were expressed as percentages of this value. These normalized peak heights were used for comparisons and for subsequent graphical and statistical analyses.

The identification of a specific peak in a voltammogram as Peak 1 or Peak 2 or any other specific peak was determined, largely, by visual inspection of the voltammogram

and assignment of peak identities by comparing the individual voltammograms with the appearance of an ideal voltammogram. By this method, the identity of the peaks was usually readily apparent. There was variation in the position of the peak from one rat to another, and from one experiment to another in the same animal, but on different days. The only peaks consistently identified in the voltammograms were Peak 1 and Peak 2.

APPENDIX 3: DISCUSSION OF PEAK MEASUREMENT METHOD

There are some complexities in the way the data have been analyzed that bear further explanation. These relate to technical problems in evaluating and measuring peaks on the voltammograms that have been recorded, and interpreting their meaning.

Because peak heights were measured from an absolute baseline, it is possibile that a peak may include a component comprising substances other than that with which the peak is normally associated. For example, the administration of uricase causes Peak 2 to flatten out and become indistinct as a peak, but the signal at that voltage may still have a positive value with respect to an absolute baseline. In other words, something else may be oxidizing at the same potential as uric acid, but that substance is just part of a constant background level of "noise", upon which the uric acid reaction is superimposed. The problem is how to estimate the background signal, how to determine the "blank" value. Use of an absolute baseline assumes that the "blank" value is zero.

Other groups measure peak heights from a baseline defined by drawing tangents to the troughs on either side of the peak. This generally results in a peak height somewhat smaller than with the method used in these studies. Any changes observed in the peak height would therefore be magnified, as they are expressed as a percentage of the pre-treatment peak height. This method also has problems, however, because the troughs can be affected by the peak just preceding it, so that a change in the height of either the peak being measured or one immediately preceding it, could affect the baseline without any actual change. This sort of interaction seems to be minimized by using an absolute baseline. Each method has its shortcomings, and what seems to be of more importance is to use one of the two methods in a consistent manner throughout the study, as long as the results reasonably reflect what is occurring.

The difference between the two methods outlined above was evaluated by using both of them to analyze the same data and comparing the results. The voltammograms from

two sets of experiments, the 2'-deoxycoformycin microinjection with L-dopa 100 mg/kg i.p., and the haloperidol microinjection with L-dopa 100 mg/kg i.p., were used. The peak heights were measured from two different baselines, an absolute baseline and a trough-to-trough tangent baseline. The resulting averaged Peak 2 heights can be compared by examining Figures 54 and 68 for the 2'-deoxycoformycin results and Figures 62 and 69 for the haloperidol results.

The major difference between the results is the greater proportionate changes that occur in the peaks measured from a trough-to-trough baseline. This is expected, because the "blank" values are subtracted, giving the pre-drug peaks a lower height. Surprisingly enough the different methods yield qualitatively similar graphs. The shapes of the time courses are almost the same, and the statistical results are equivalent. This information indicates that the assumption that the method used is of lesser importance than the consistent employment of one or the other is valid. Except for Figures 68 and 69, all results in these studies were processed by measuring peak heights from an absolute baseline.

Figure 54

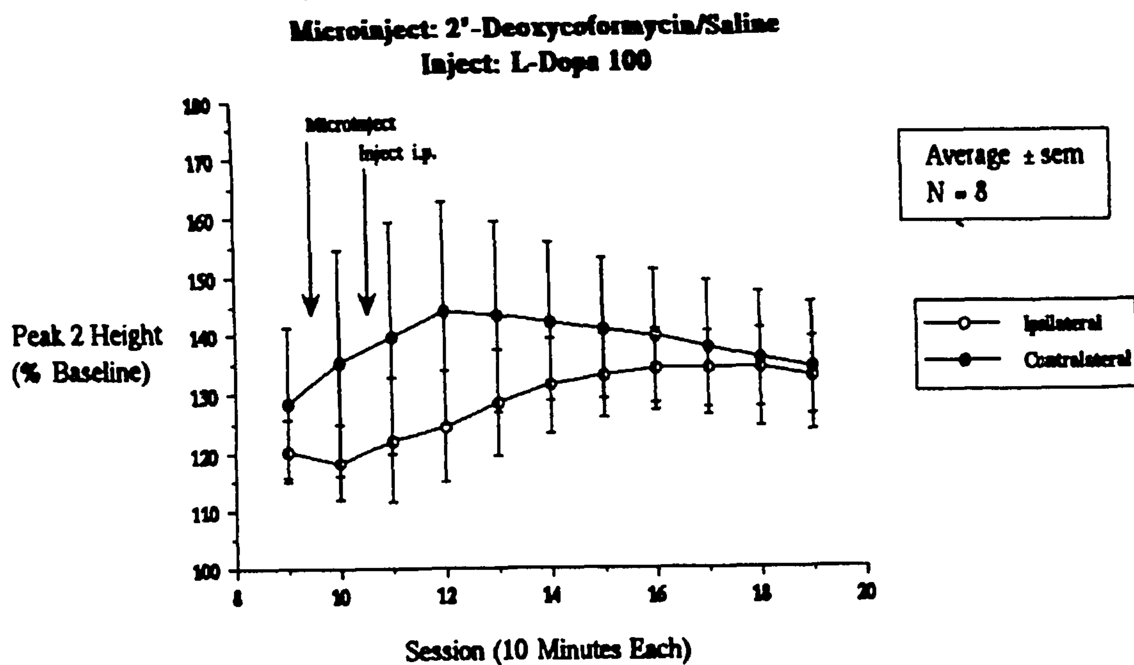


Figure 68

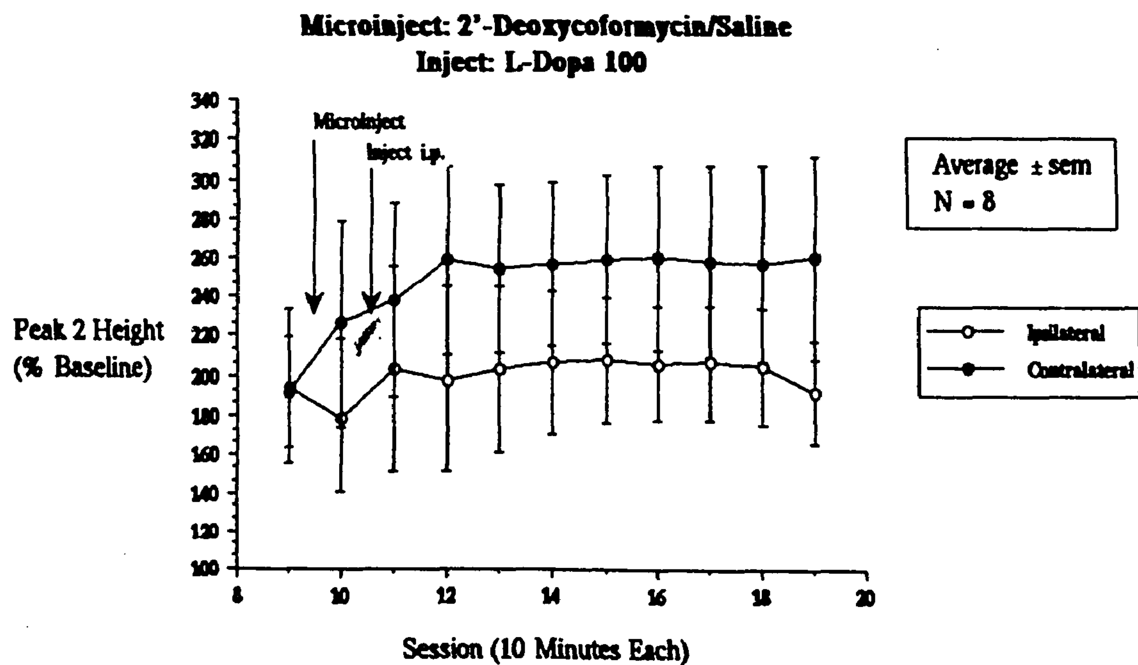


Figure 62

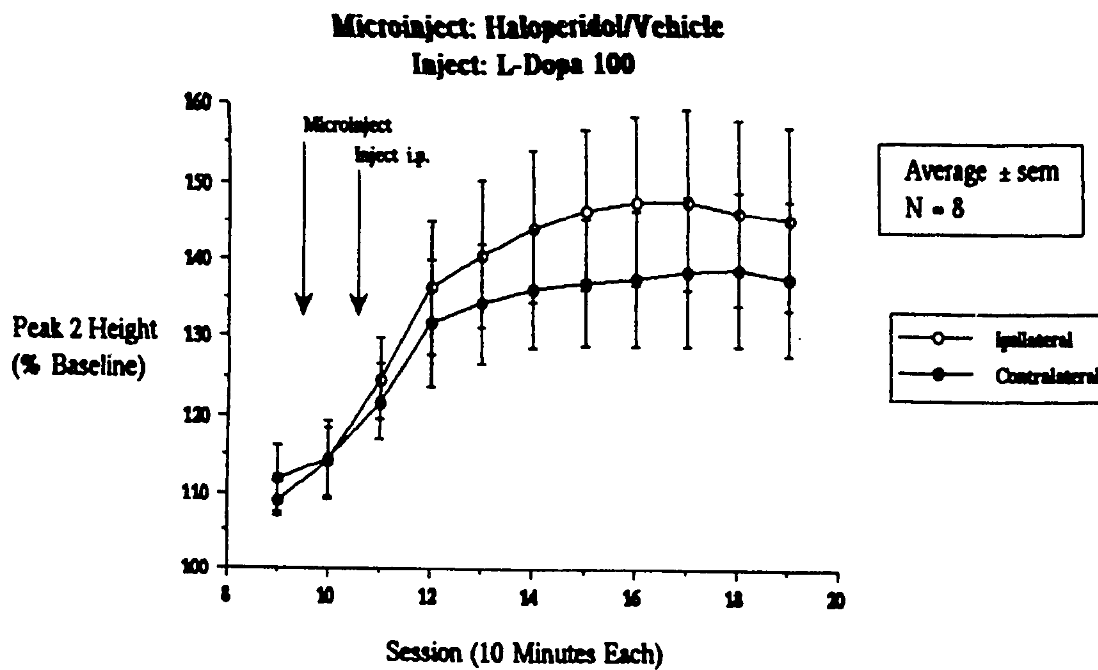
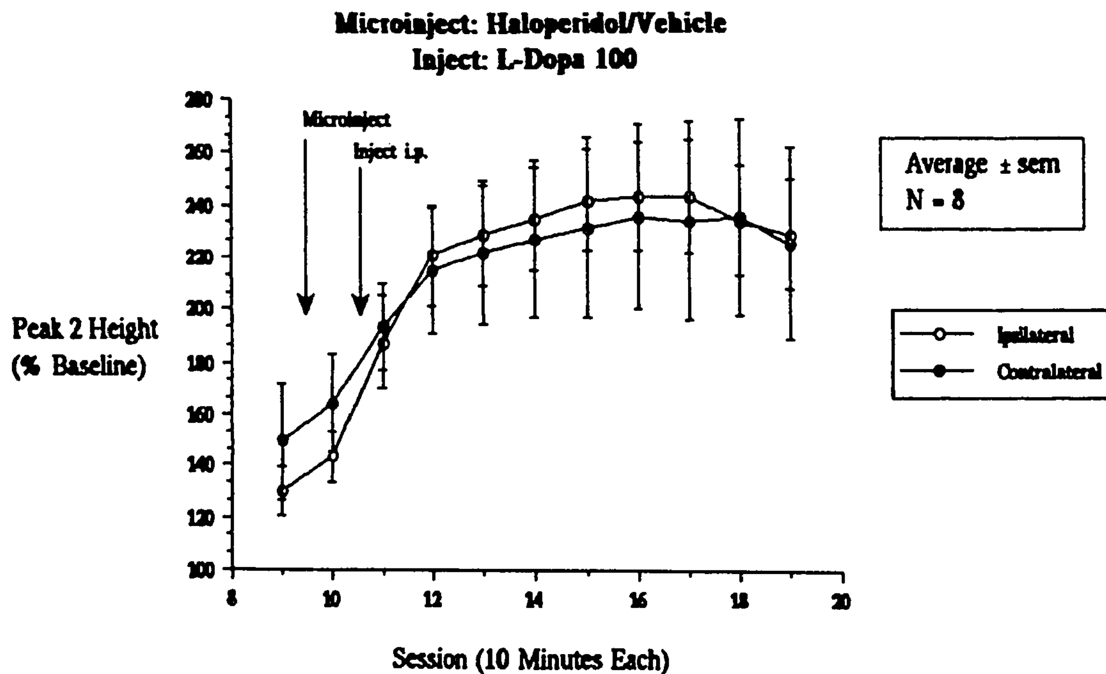


Figure 69



APPENDIX 4: BIOCHEMICAL RESULTS OF OTHER BRAIN AREAS

The figures in this appendix show the results of the measurement of total tissue levels of norepinephrine, L-dopa, DOPAC, dopamine, 5-HIAA, HVA, 5-HT, and tryptophan. The brain regions examined were the hypothalamus, midbrain (including the hippocampus), and the pons/medulla. In addition, plasma levels of the same substances were assayed. Those results are included here also. The experimental protocol used for the experiments that gathered these data is the same as that described in the text in the biochemistry section. The protocol is illustrated in flow chart form in Figure 18 Biochemistry Protocol. Briefly, male Sprague-Dawley rats with unilateral nigrostriatal lesions were screened for successful lesions. (See Figure 9 Lesioning Protocol and Figure 10 Lesion Screening Protocol.) The rats were killed by decapitation and the brain dissected out at the appropriate times before or after injection with L-dopa. Three time points were used: just before they would have been injected with L-dopa, 10 minutes after injection, or 60 minutes after injection. In addition, trunk blood from each of these rats was collected, and plasma retained and frozen for later analysis. (See Figure 19 HPLC Sample Preparation Protocol.) The tissues were homogenized by sonic disruption in buffer and centrifuged to remove cell debris. The supernatants were assayed by high performance liquid chromatography (HPLC). Plasma samples were injected directly onto the column. The results of the assays were graphed with the averages and standard errors of the mean for the different time points, and are shown in Figures 70 to 105.

Figure 70

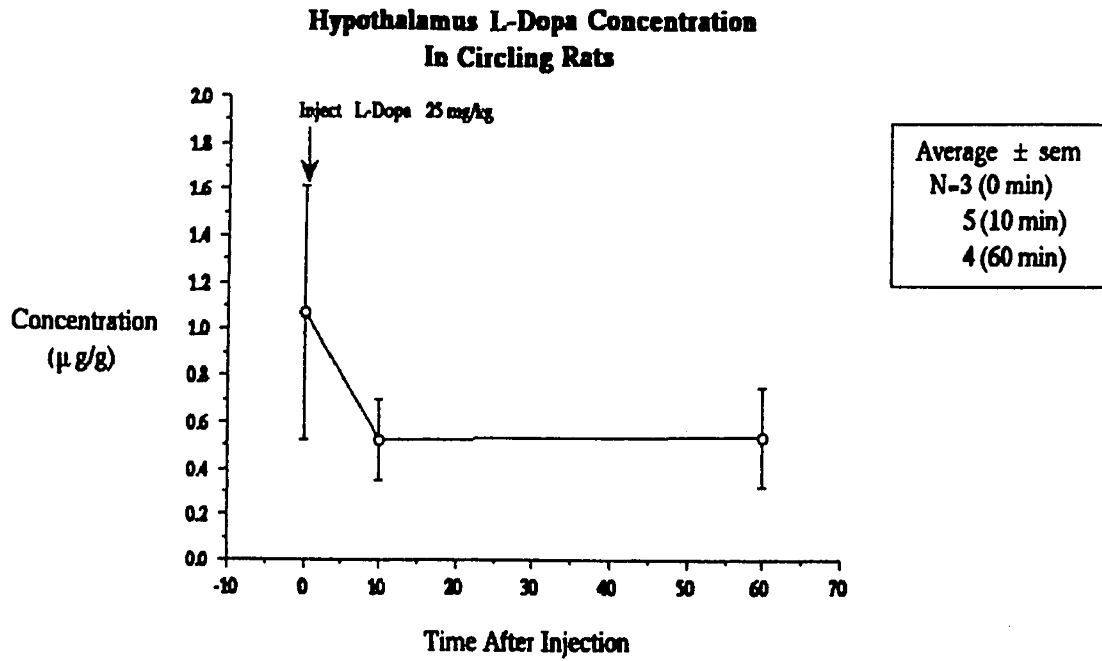


Figure 71

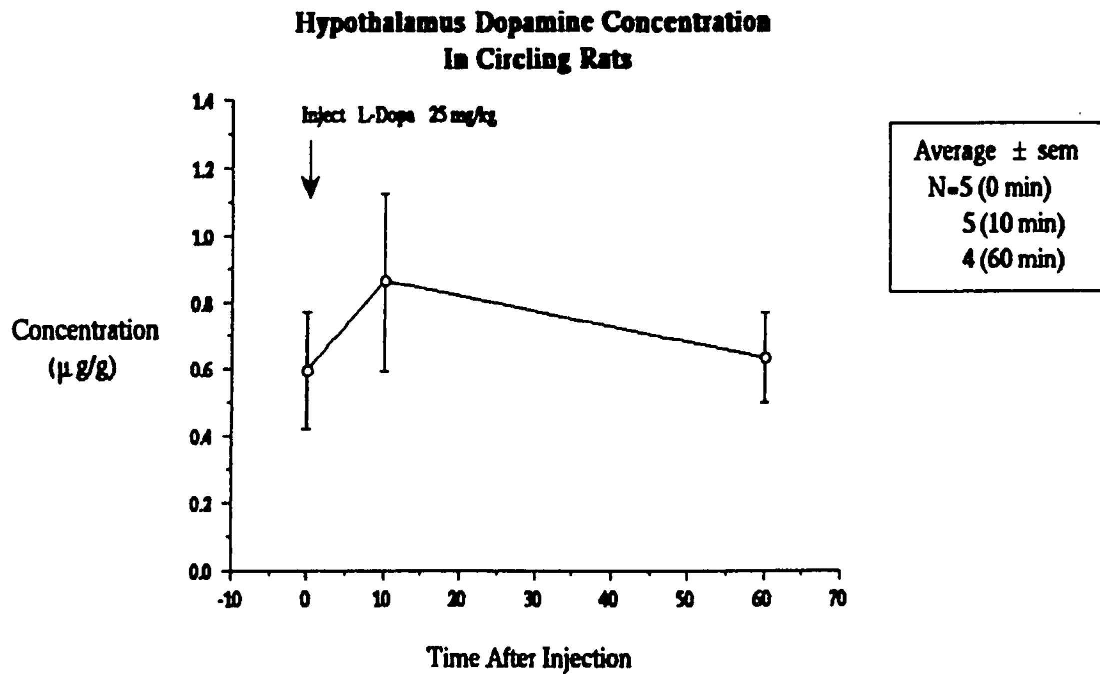
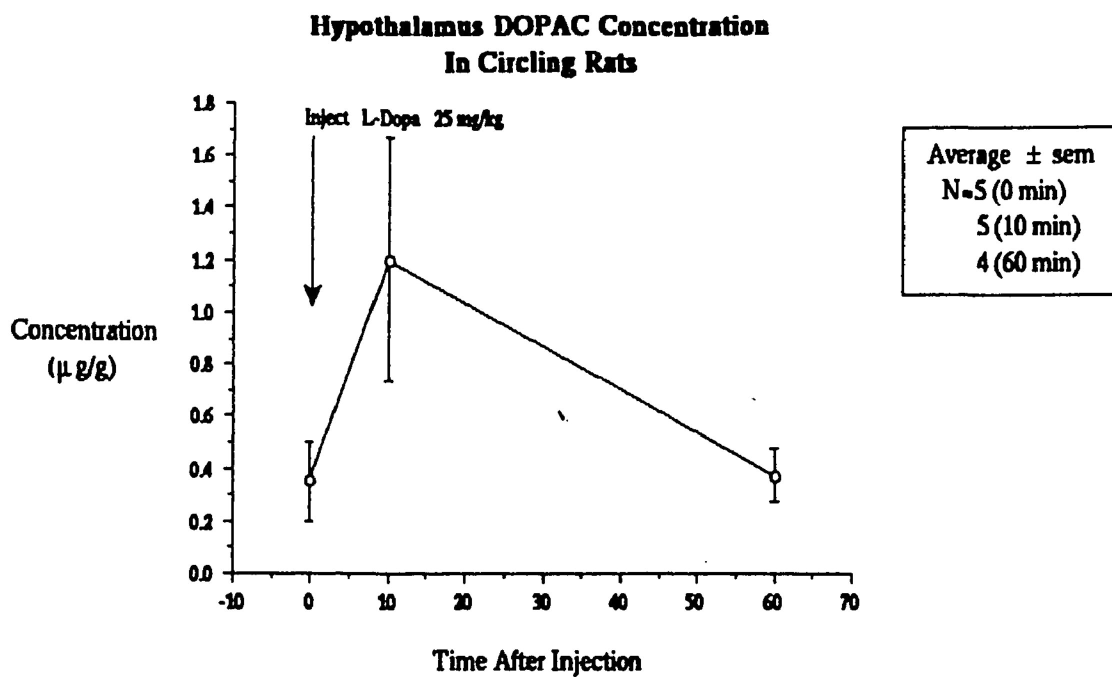


Figure 72



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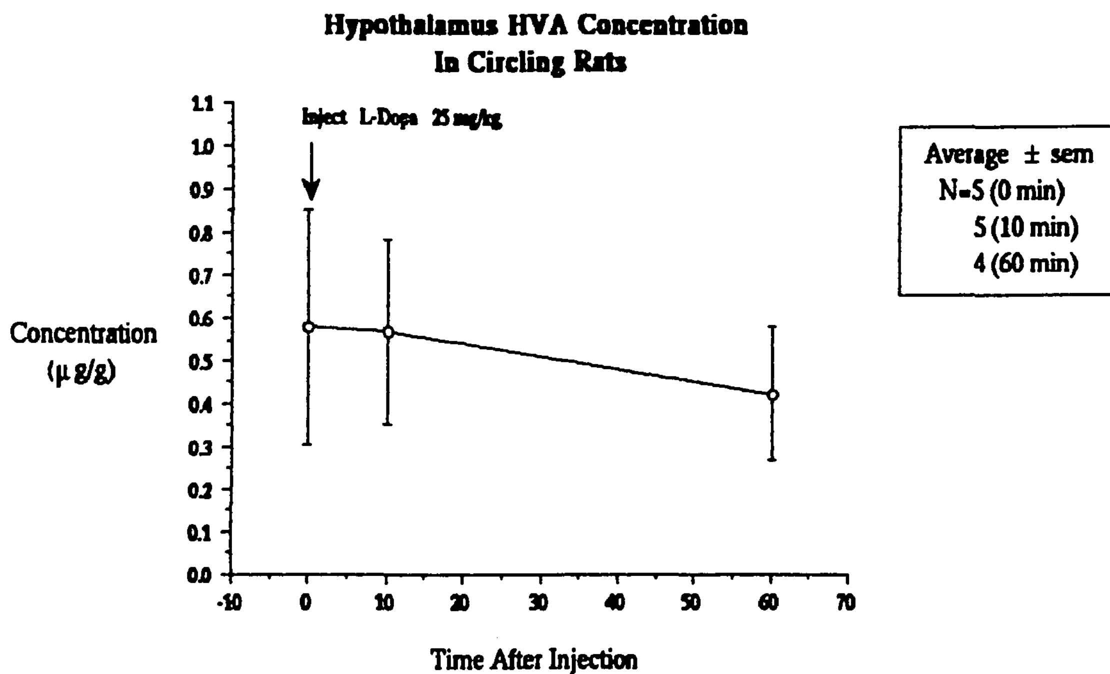

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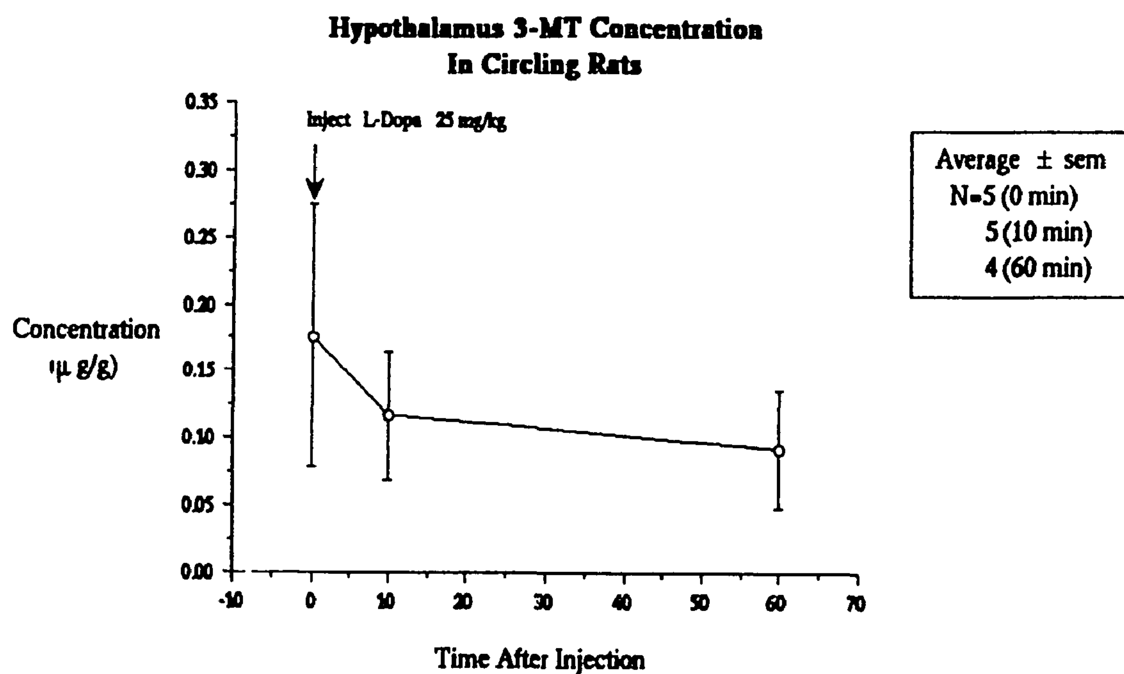

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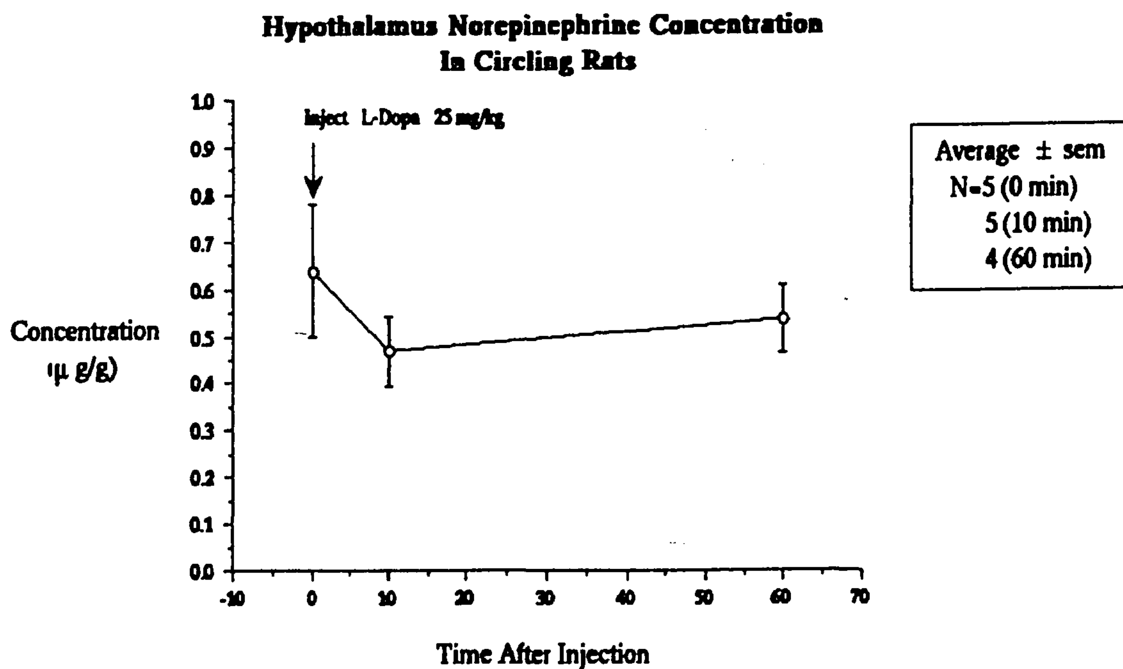

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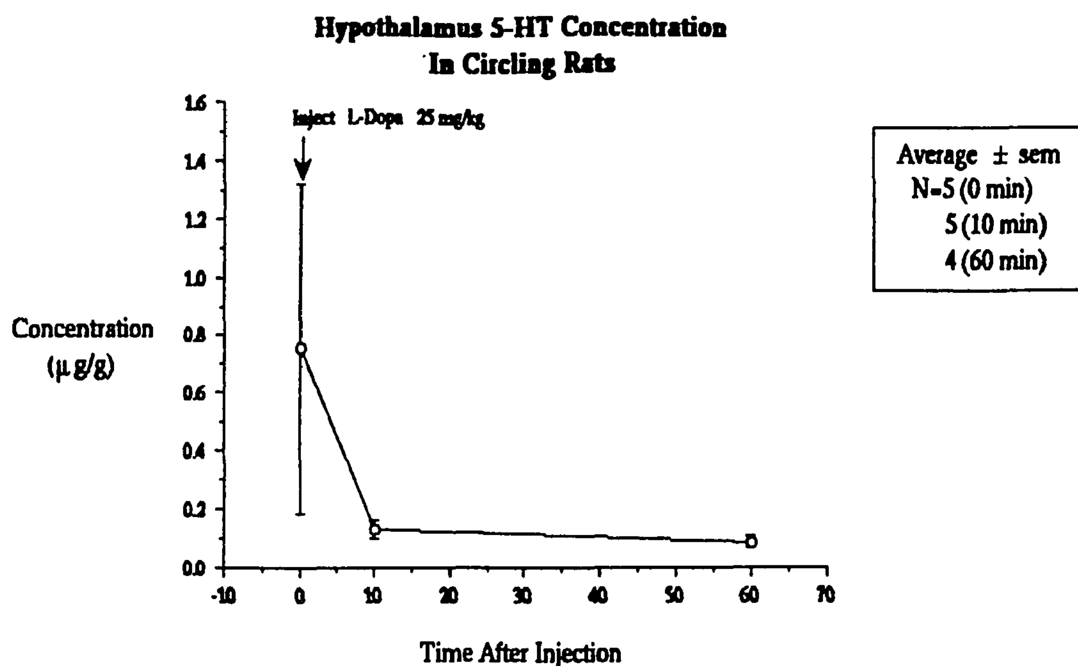

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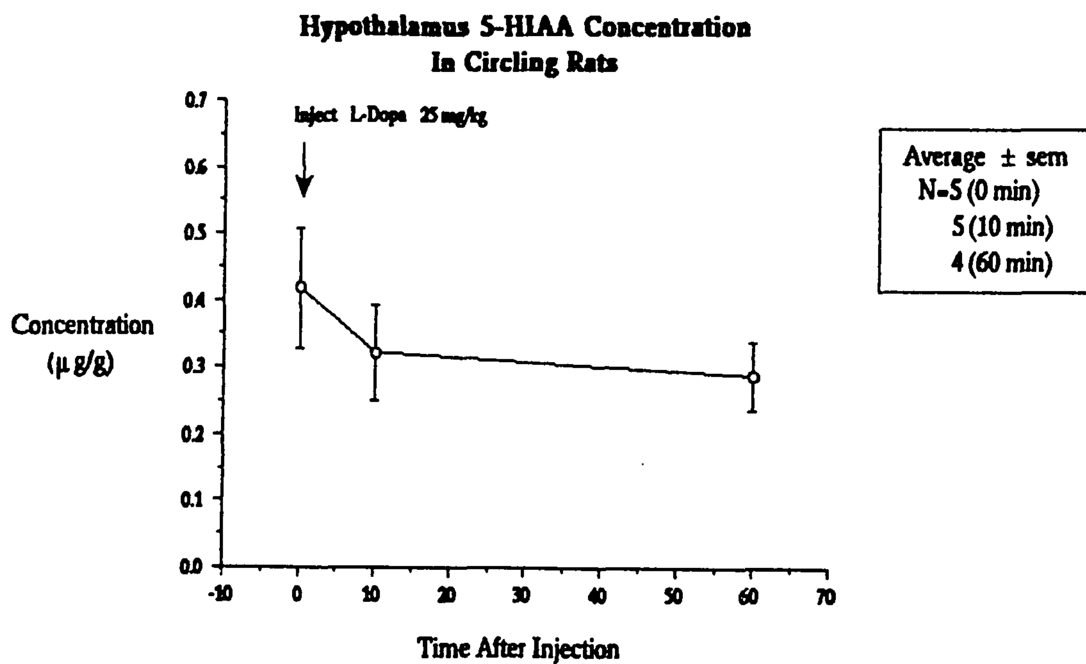

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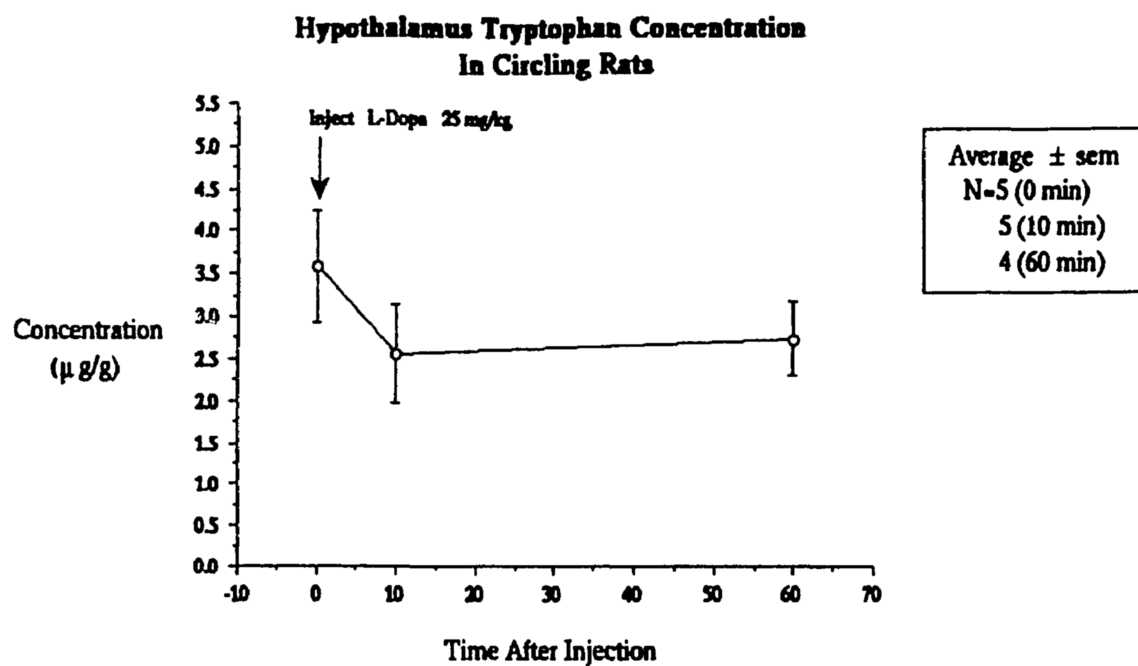

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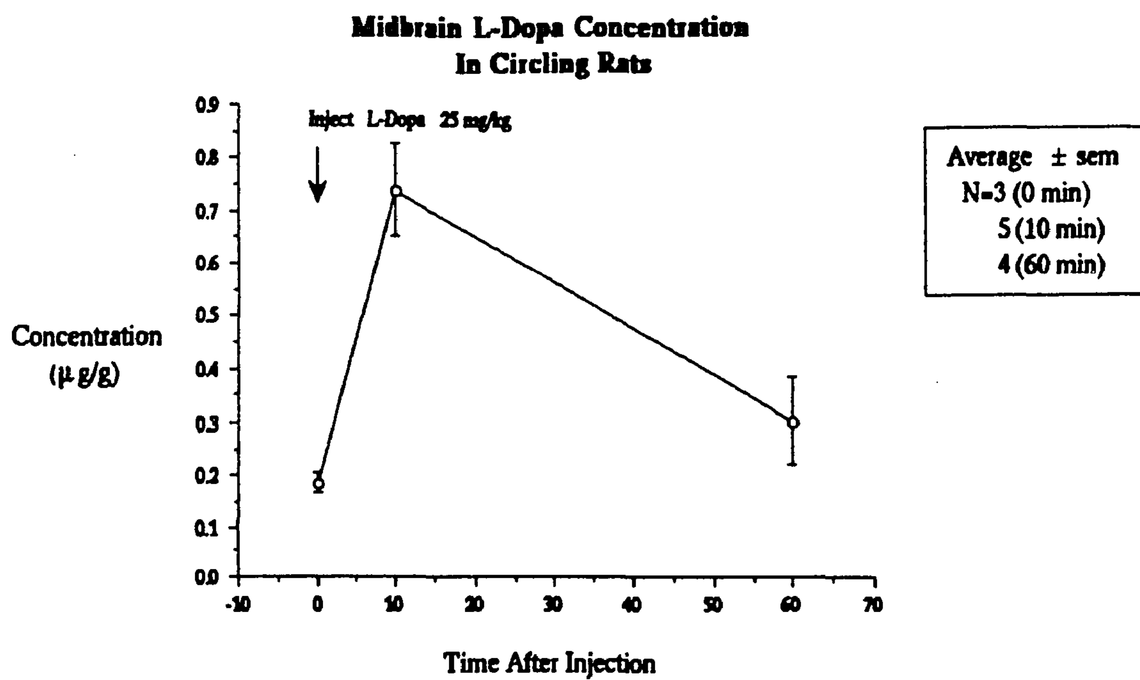

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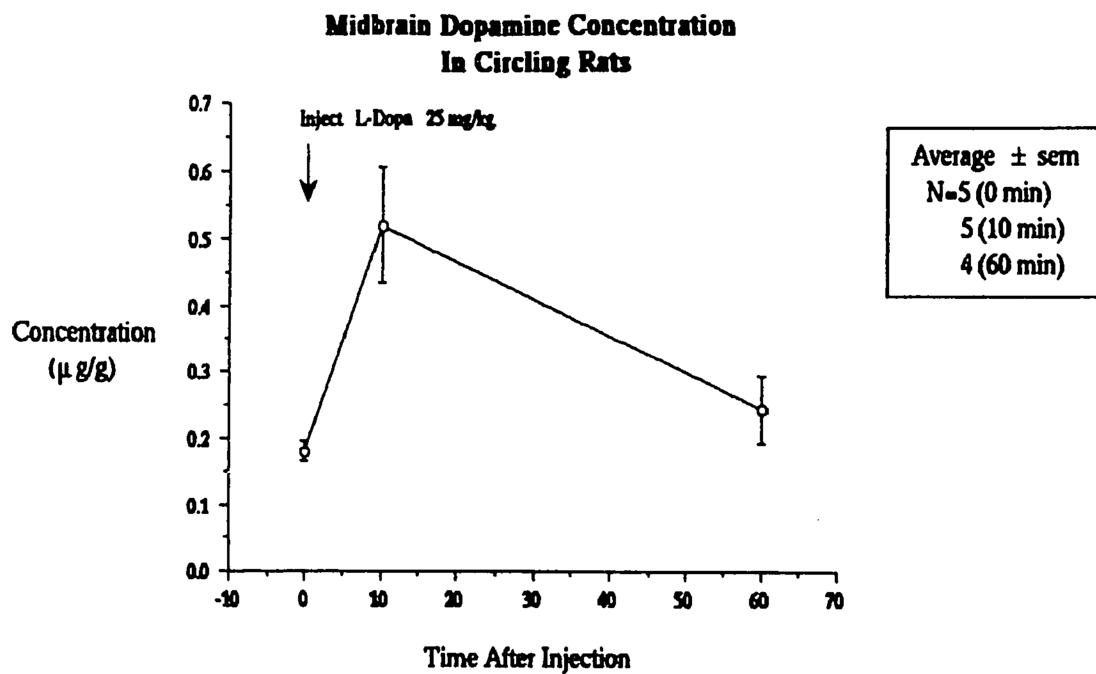

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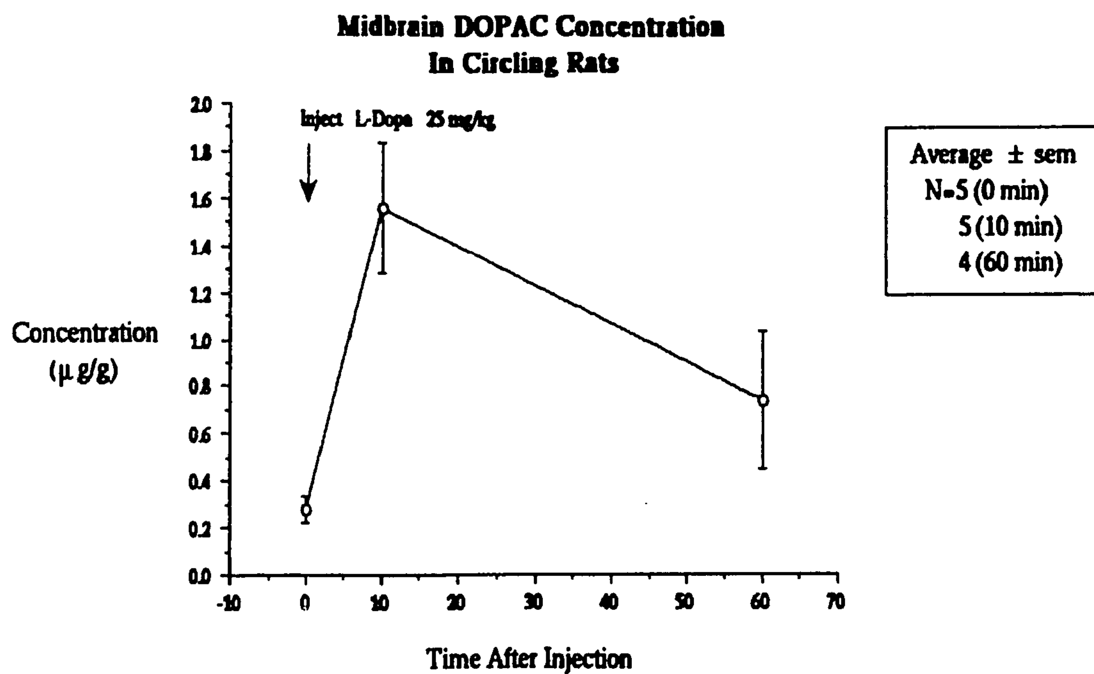

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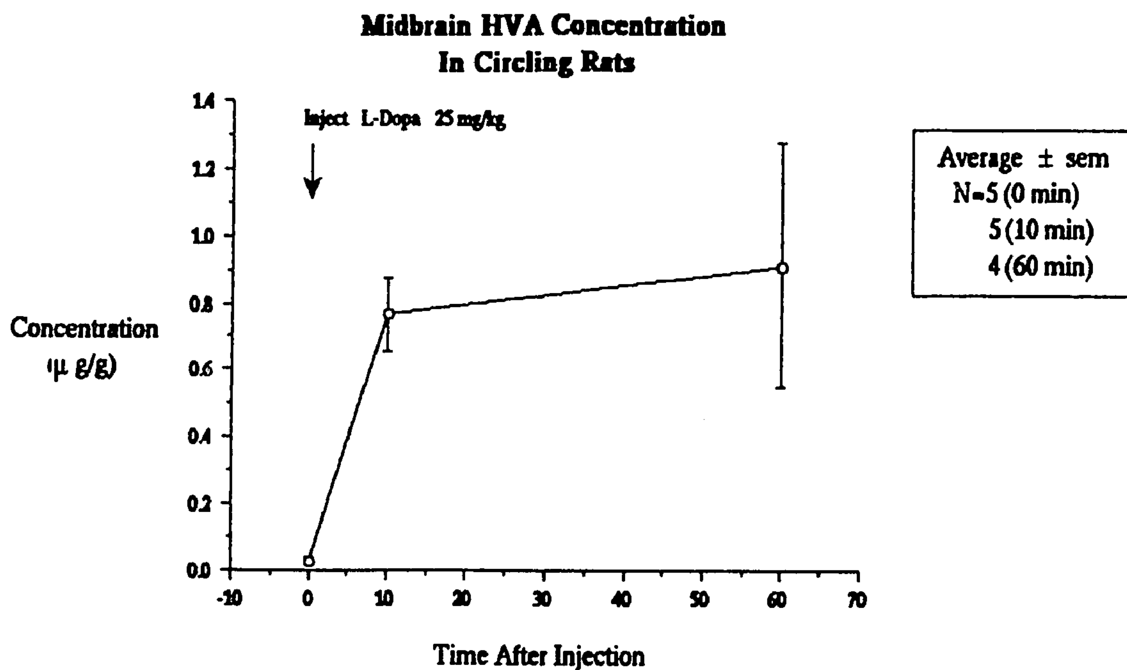

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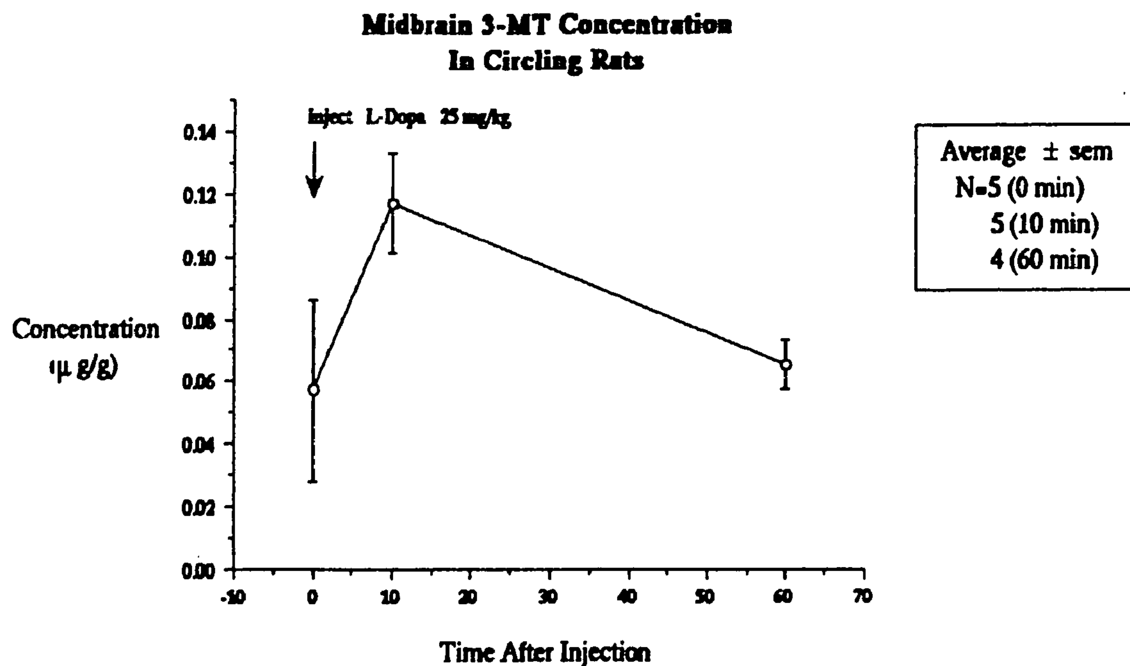

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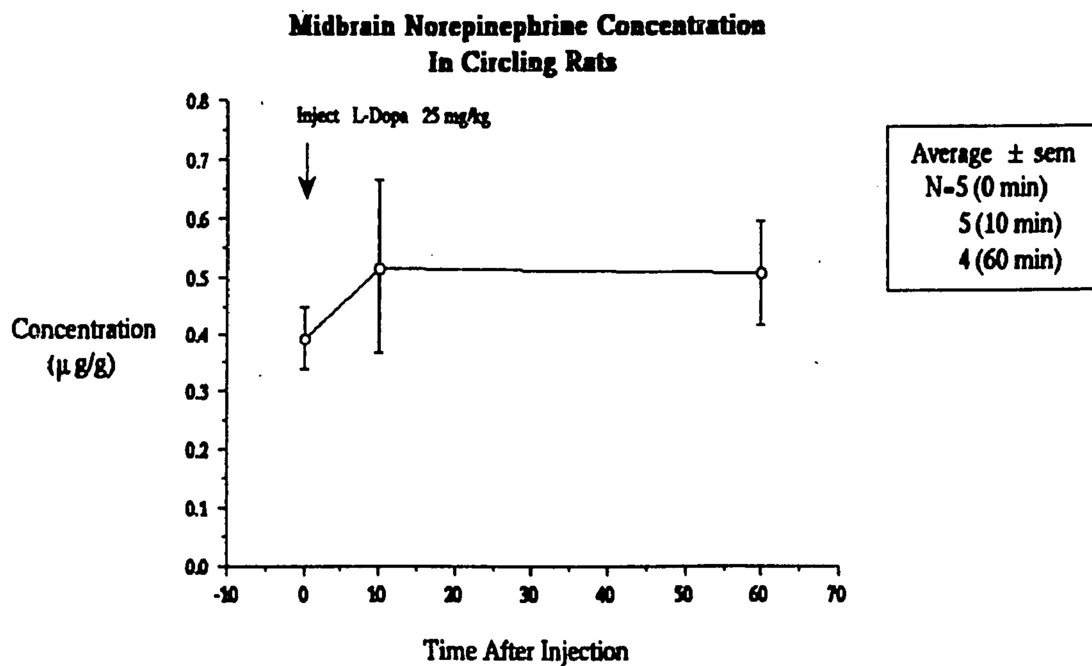

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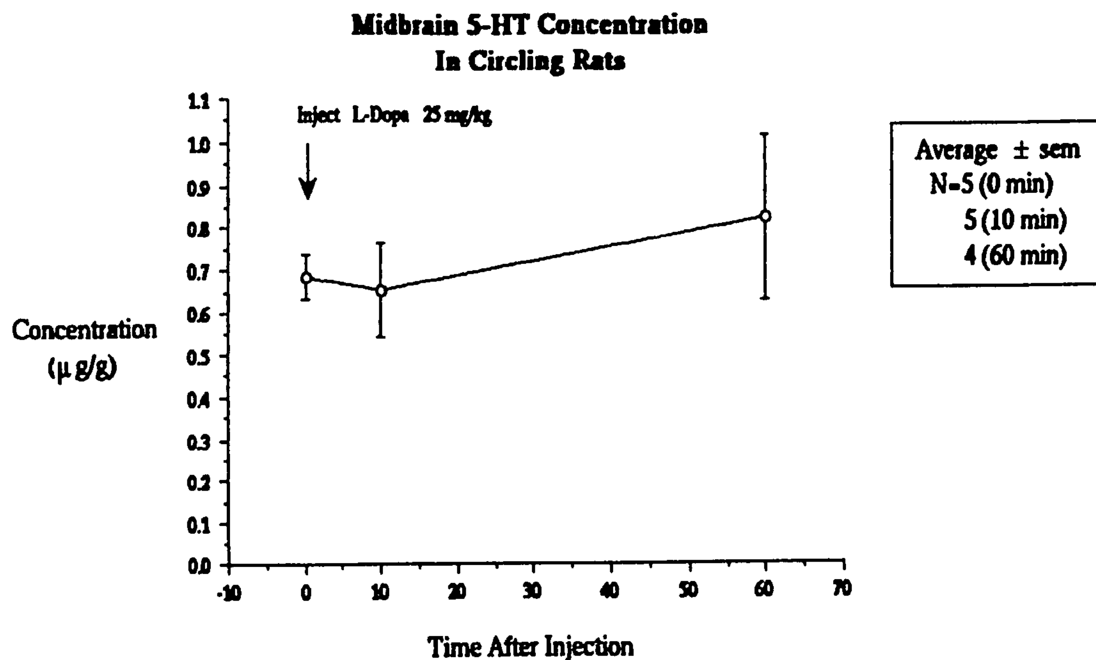

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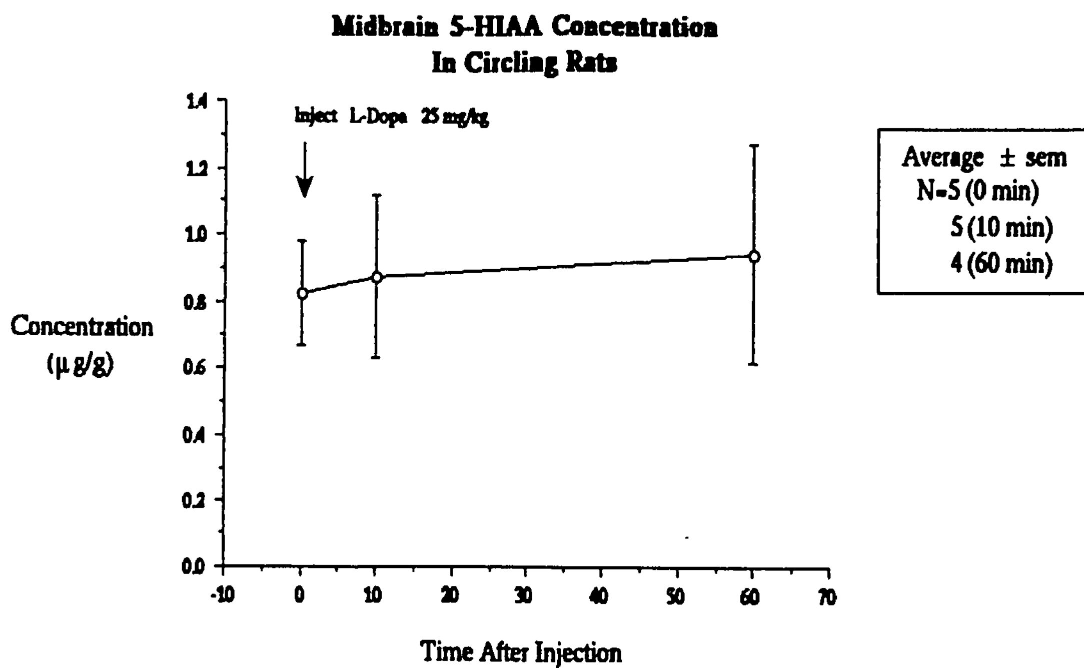

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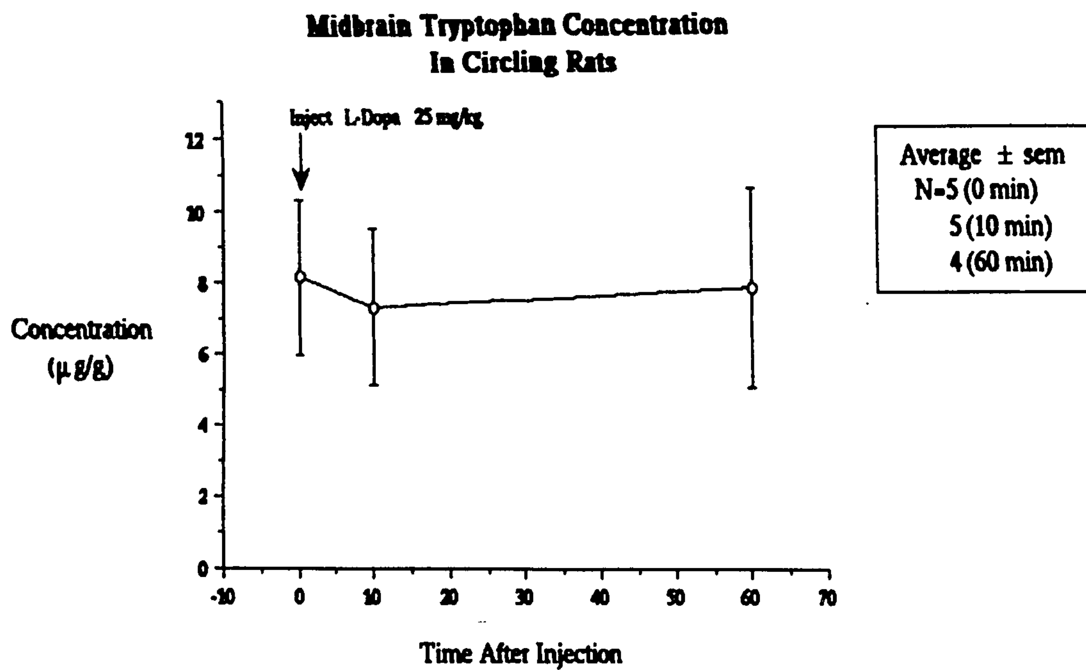

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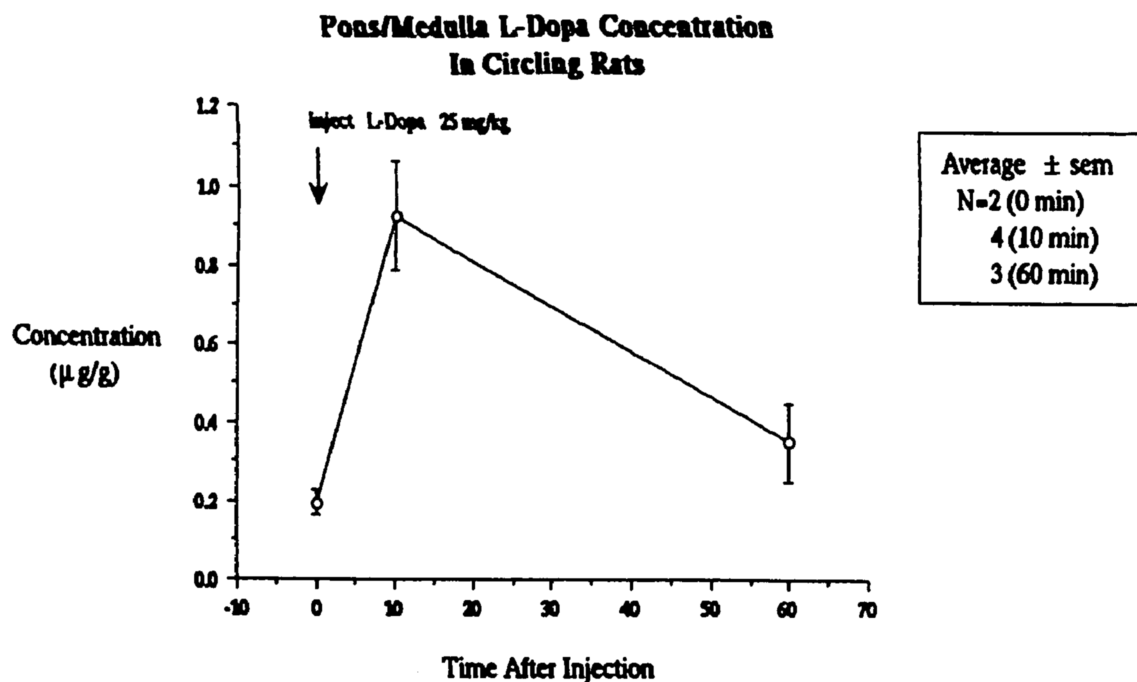

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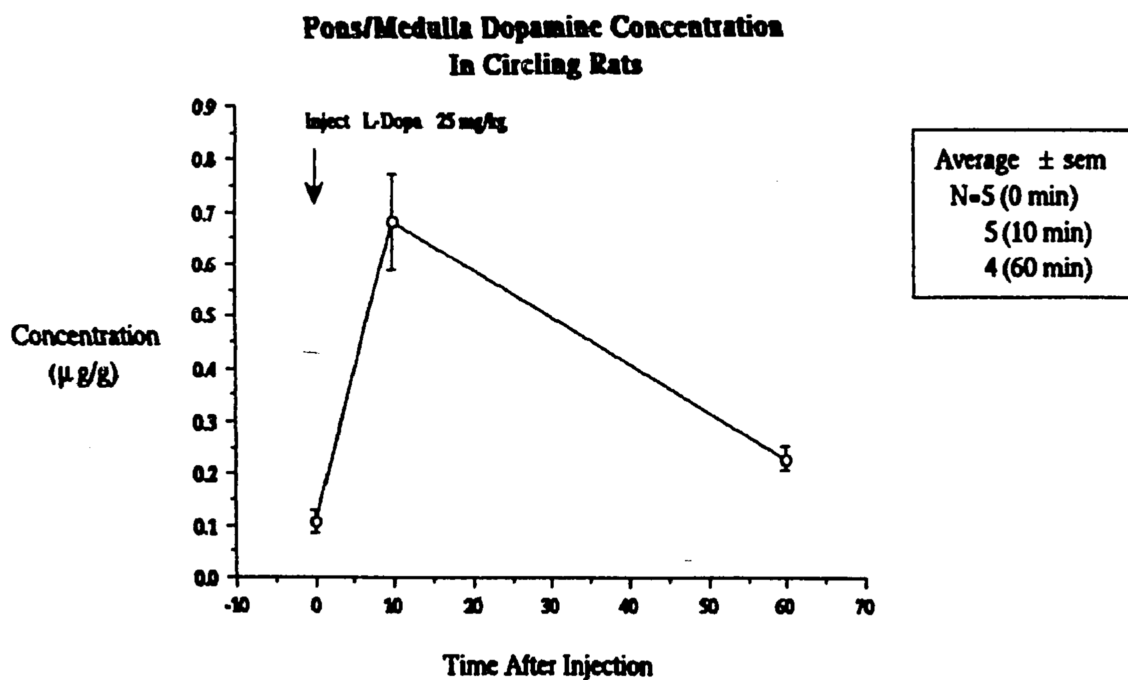
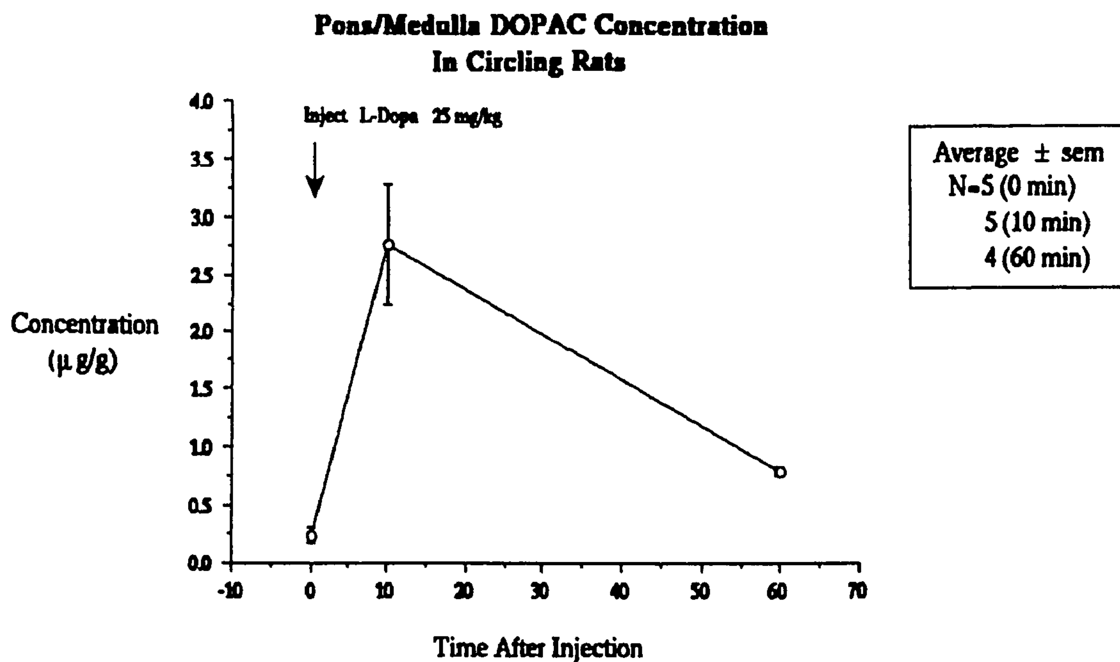


Figure 90



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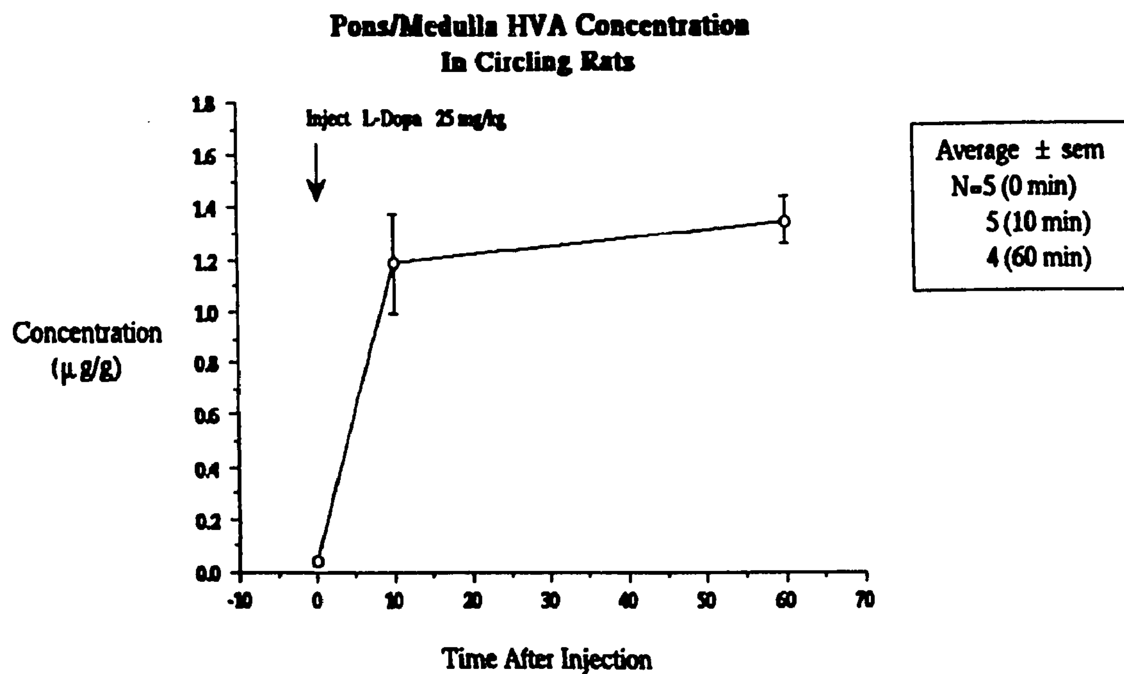

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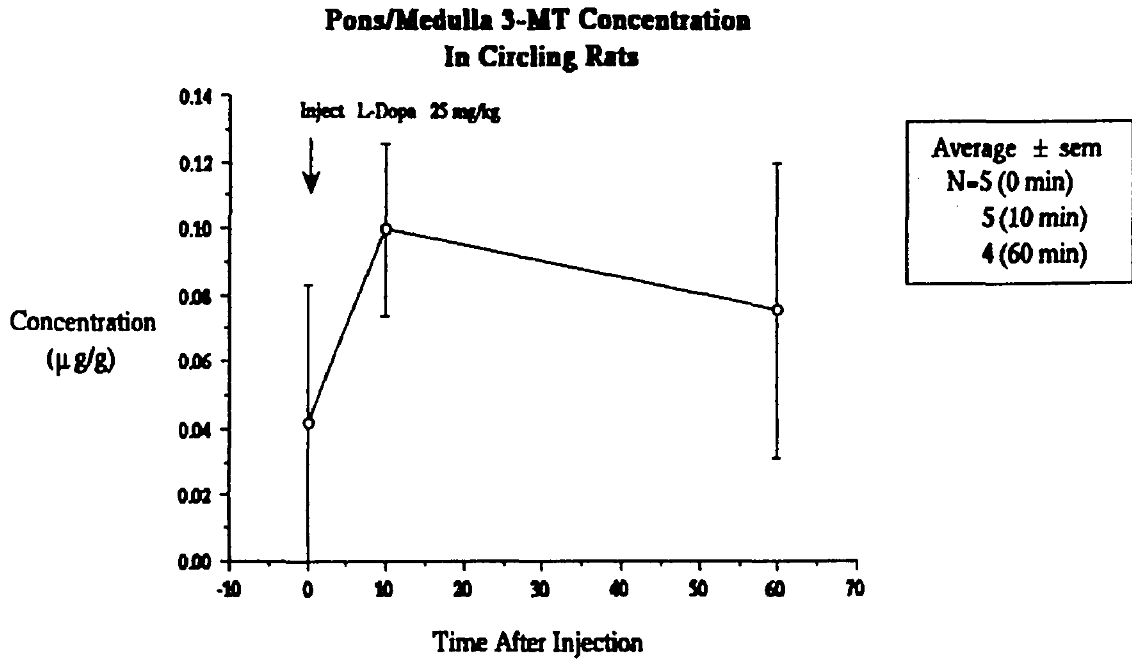

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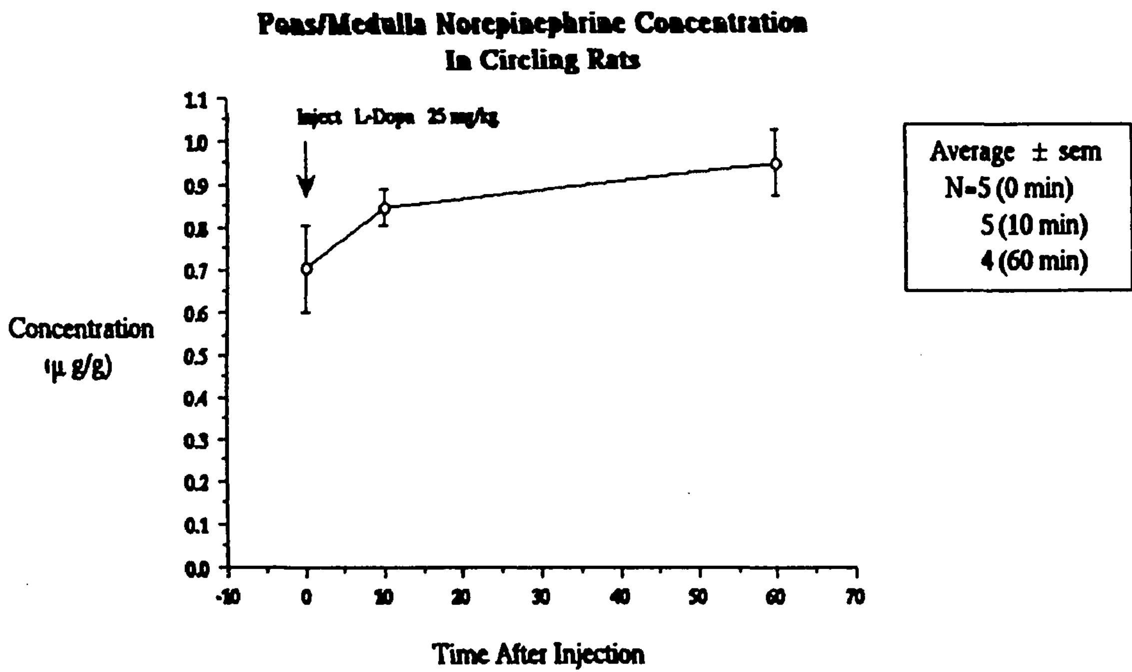

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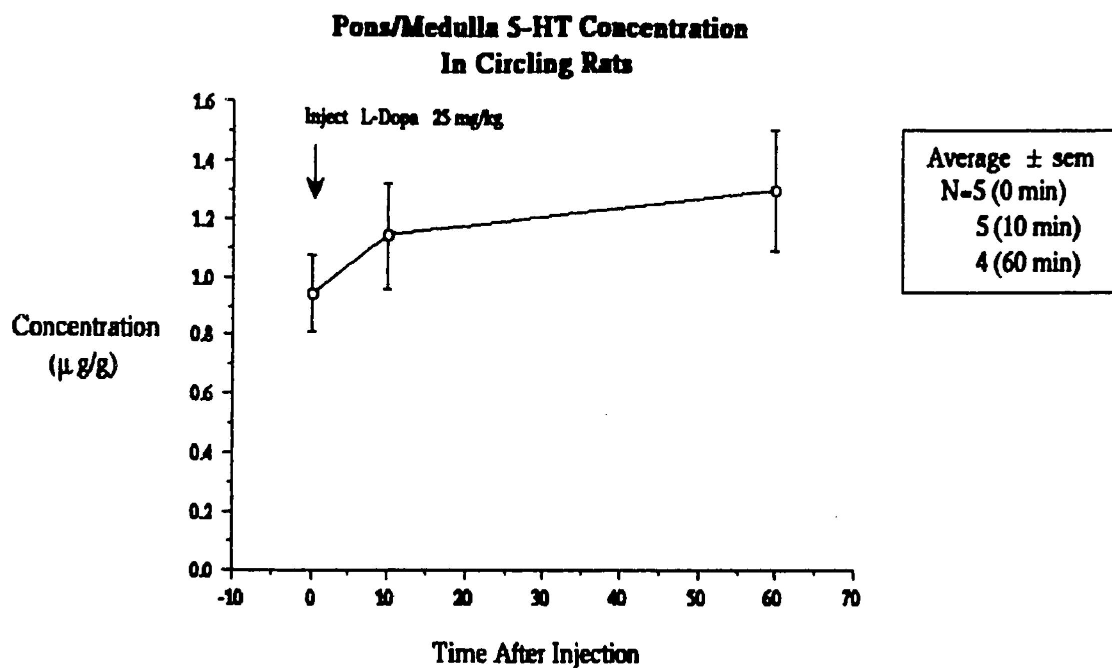

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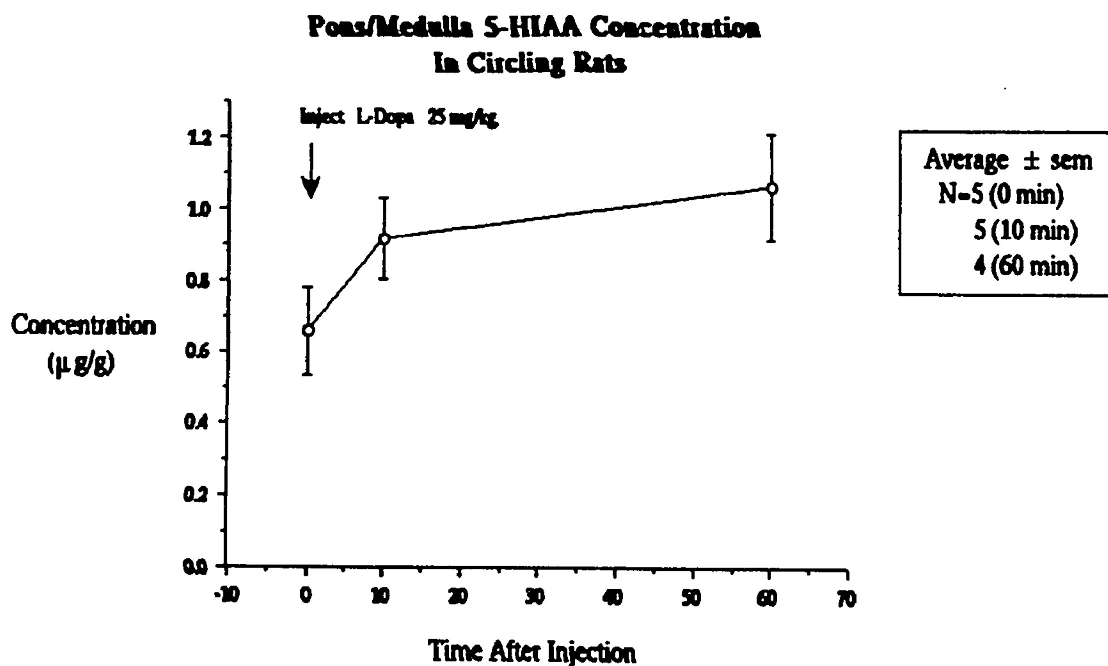

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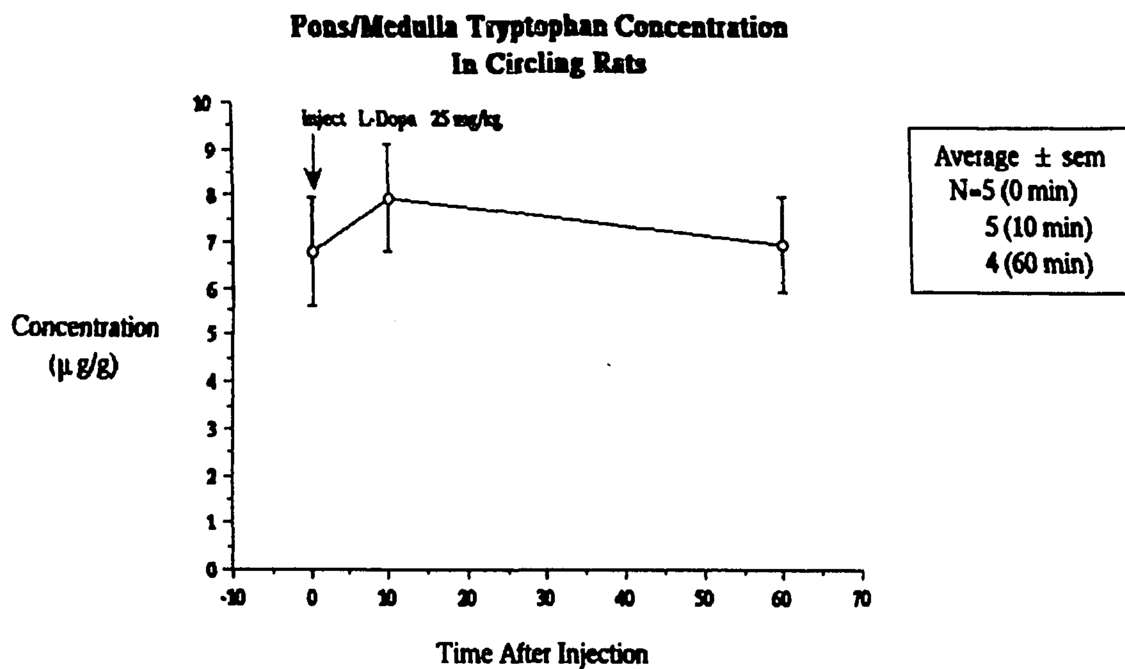

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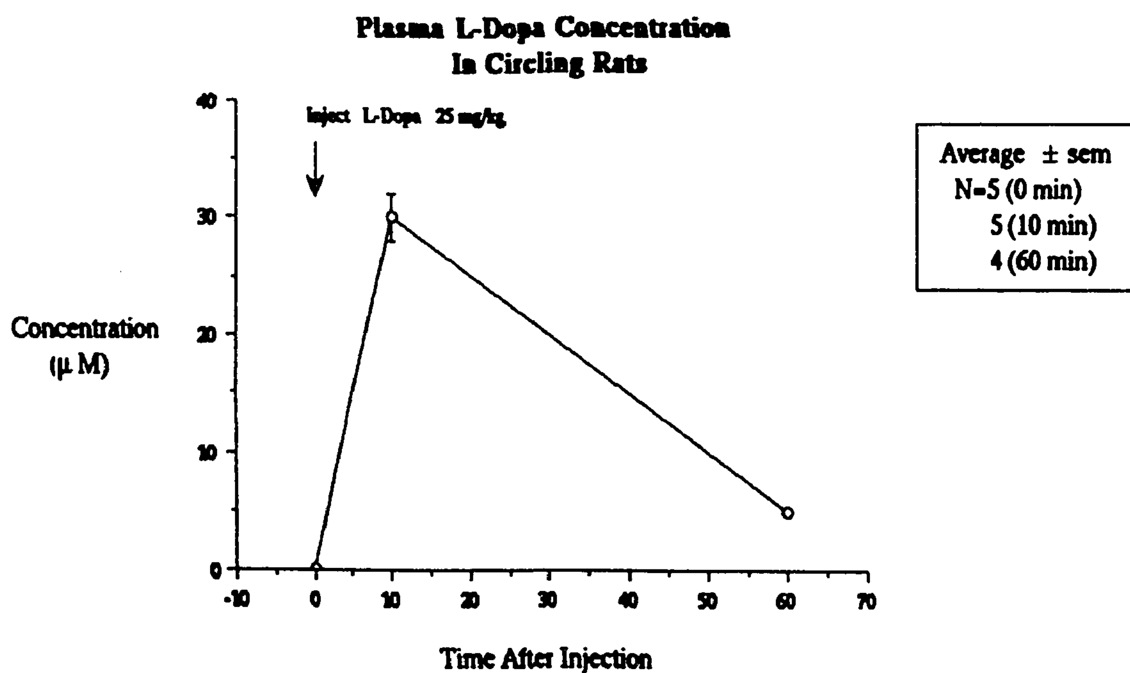

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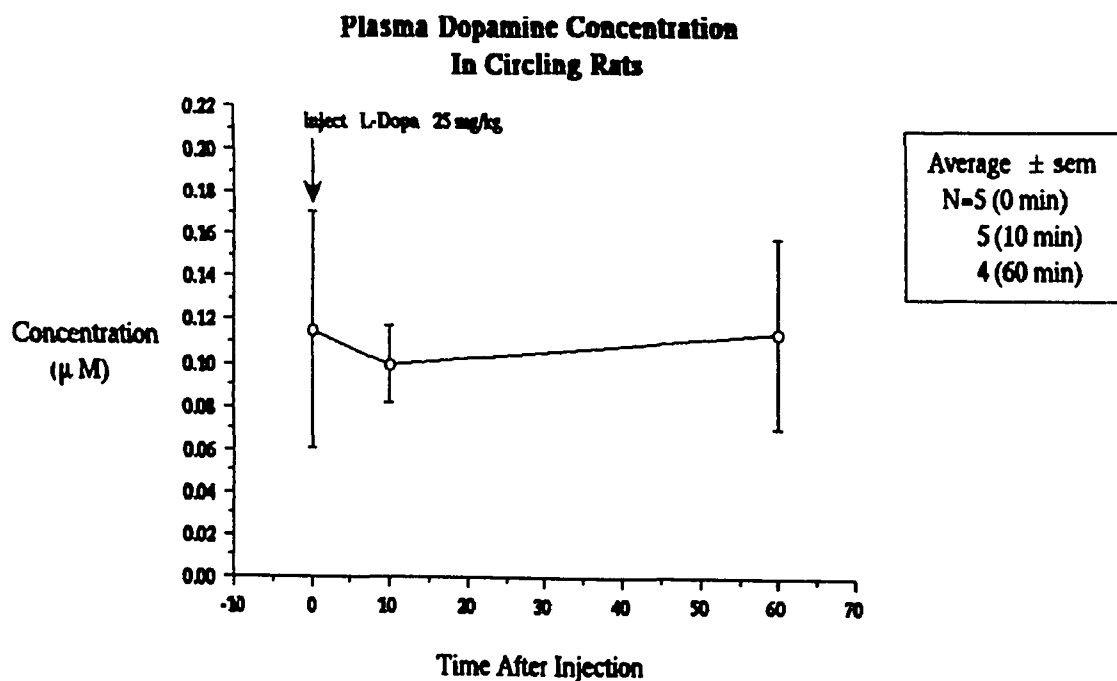

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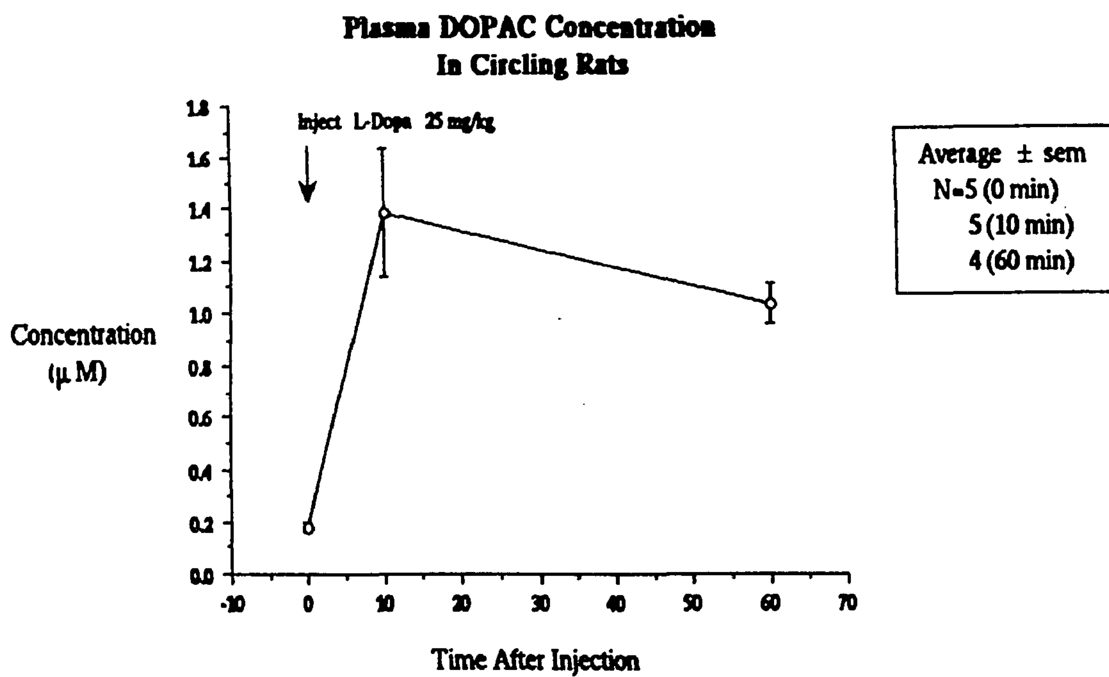

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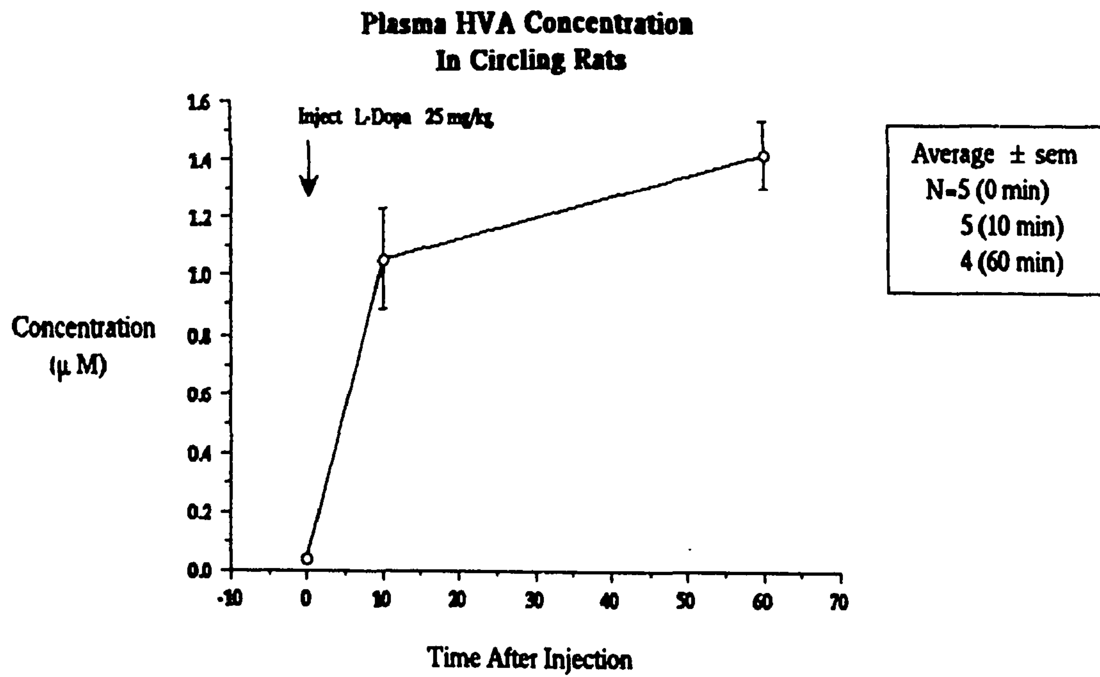

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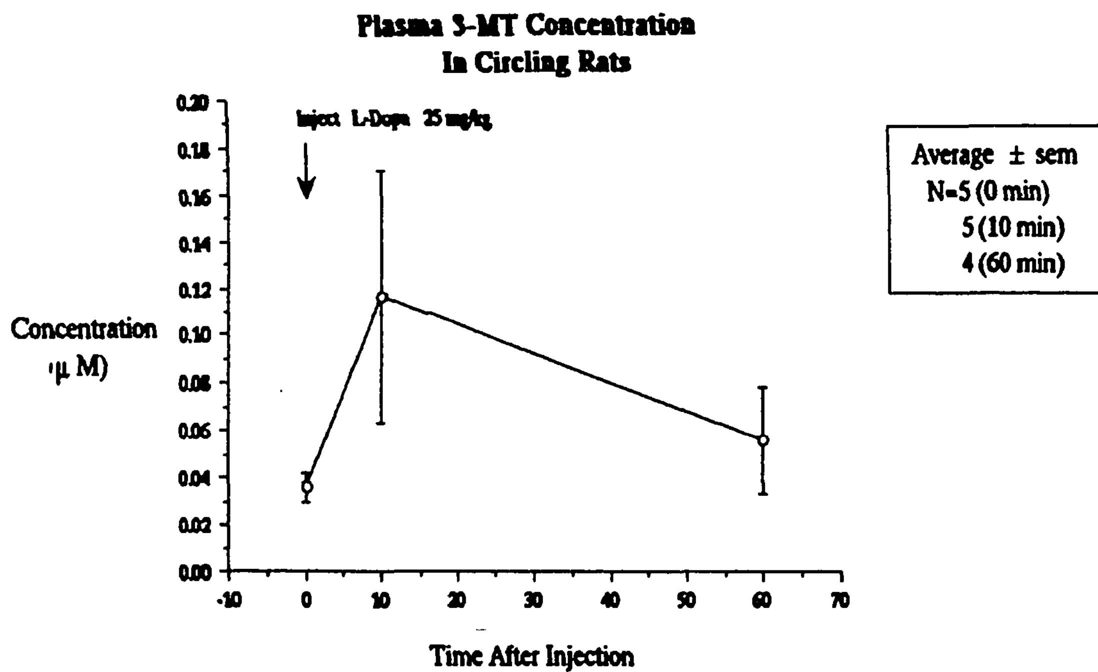

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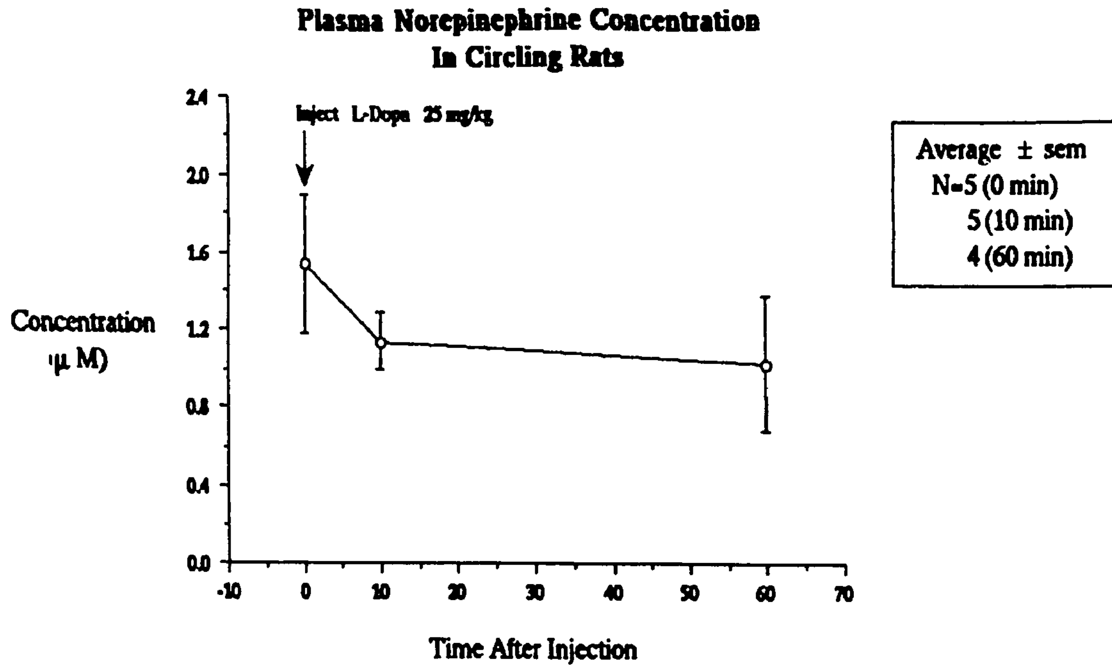

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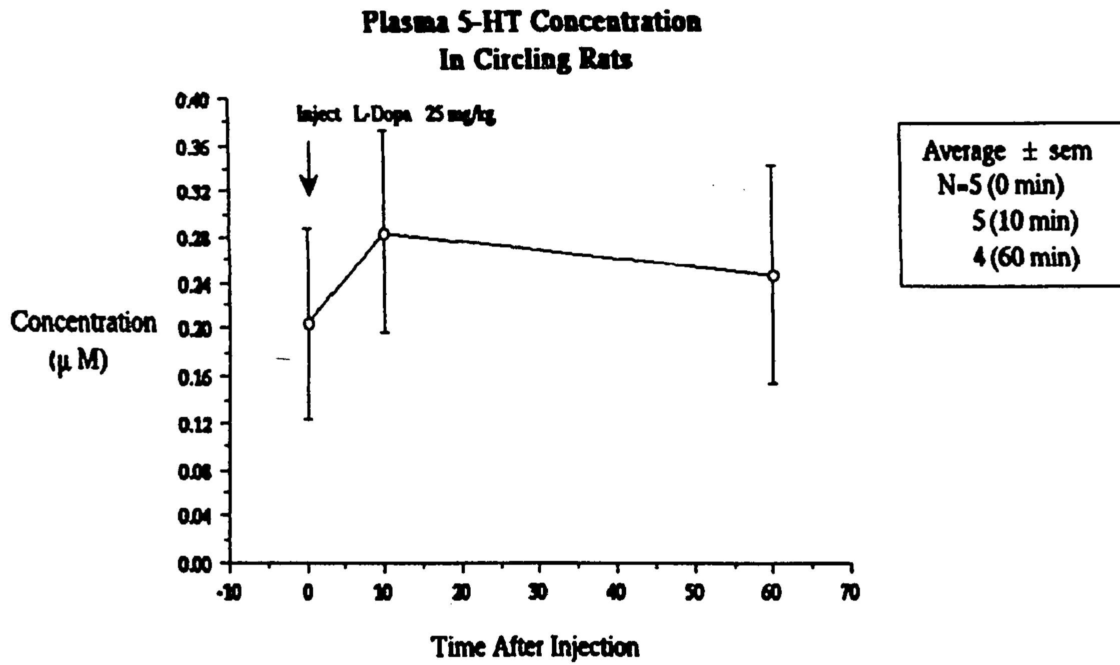

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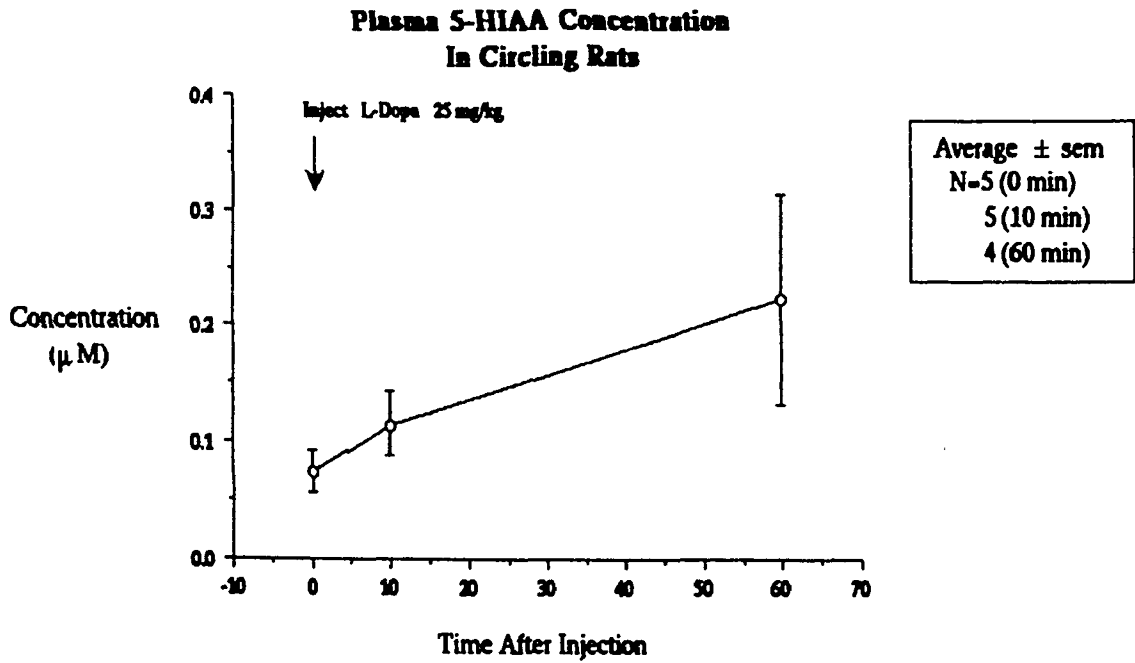

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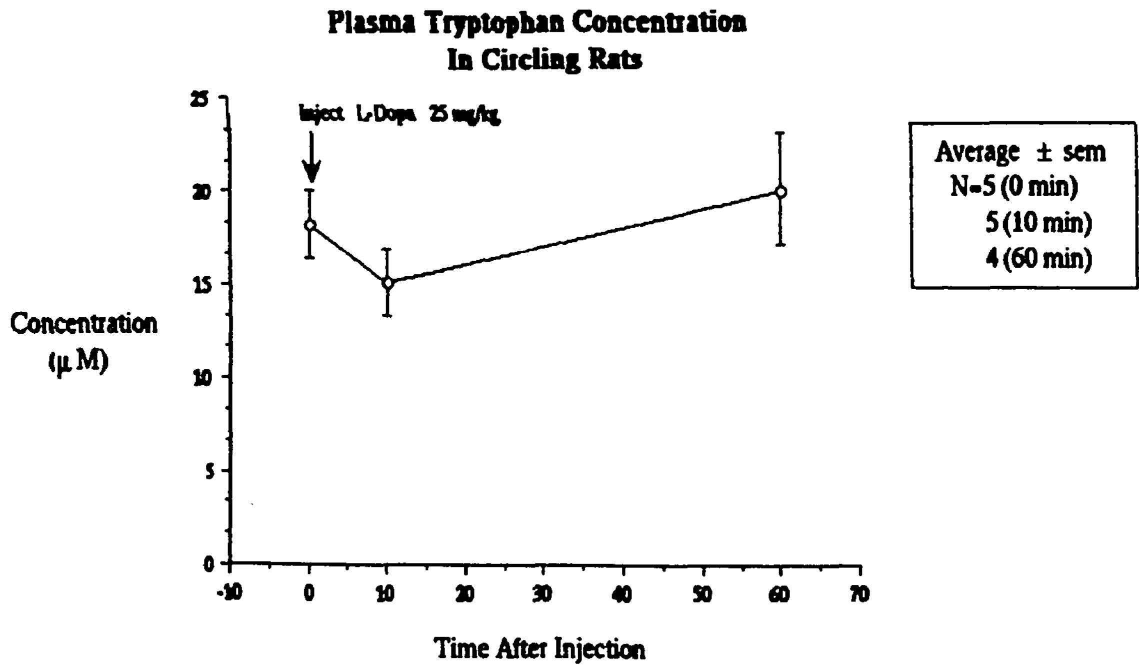

APPENDIX 5: STATISTICAL RESULTS SUMMARIZED

The following tables summarize the statistical results of the studies analyzed in the biochemistry and IVED/microinjection studies. The results of the analysis variance includes the "p" values for the inter-group variable (side: lesioned versus unlesioned), the repeated measures variable (time), and the interaction of time and side. In the biochemistry calculations, there were two levels of the side variable (lesioned versus unlesioned) and three levels of the time variable (times 0, 10, and 60). In all cases, there were two levels of the side variable (lesioned versus unlesioned) and eleven levels of the time variable (sessions 9 to 19).

Table III
SUMMARY OF STATISTICAL CALCULATIONS
FOR BIOCHEMICAL EXPERIMENTS

	Side	Time	S x T
Dopamine	**0.0001**	0.7375	0.2910
DOPAC	**0.0001**	**0.0003**	0.1098
HVA	**0.0001**	**0.0001**	**0.0014**
3-MT	**0.0001**	0.8910	0.2666
Norepinephrine	0.5818	**0.0222**	0.9627
Serotonin	**0.0028**	0.3038	0.6928
5-HIAA	0.1203	0.2704	0.9663
Tryptophan	0.4675	**0.0146**	0.9712

Comparison of the concentrations of neurochemical levels in the denervated and intact caudates of circling rats. The two sides were compared by repeated measures analysis of variance with time as the repeated measure and side as the inter-group factor. The p values are shown. Statistically significant values are shown in bold print.

Table IV
SUMMARY OF STATISTICAL CALCULATIONS FOR
URIC ACID / ADENOSINE EXPERIMENTS

	Peak 2				Peak 1			
Study	Side	Time	S x T	N	Side	Time	S x T	N
S/S/-				9				9
S/S/S				12				12
S/S/D25				6				5
U/U/D25	0.9612	**0.0001**	0.9993	6	0.5420	**0.0001**	0.3961	5
U/S/S	0.0657	0.0949	**0.0427**	5	**0.0019**	**0.0001**	0.1351	5
U/S/D25	**0.0004**	**0.0001**	**0.0002**	5	0.7859	**0.0001**	0.0893	5
A1/S/-	**0.0338**	**0.0001**	0.3899	13	0.9483	**0.0001**	0.4759	13
A10/S/-	0.3947	0.1425	0.5819	2	**0.0232**	**0.0001**	**0.0157**	2
2/S/S	0.4786	0.0790	0.9868	2	0.4760	**0.0172**	0.9444	2
2/S/D25	0.4705	**0.0174**	0.5598	6	0.5204	**0.0001**	0.6873	5
2/S/D100	0.5205	0.2497	0.6731	8	0.8804	0.1871	0.7809	8

Comparison of the heights of Peaks 1 and 2 in the striatum of rats for the experiments described in Section 5 — Uric Acid / Adenosine Findings. The sides are classified as either ipsilateral or contralateral to the microinjected drug, or, in the case of the bilateral uricase microinjections, ipsilateral or contralateral to the denervated striatum. The two sides were compared by two-tailed paired t-test, and the p values are shown. Statistically significant values are shown in bold print.

The studies are listed according to the key of:

Ipsilateral Microinjection / Contralateral Microinjection / Systemic Injection

A1 = Adenosine 1 mM
A10 = Adenosine 10 mM
D25 = L-Dopa 25 mg/kg
D100 = L-Dopa 100 mg/kg i.p.
S = Saline
U = Uricase 0.1 units
2 = 2'-Deoxycoformycin
- = No Injection

Table V
SUMMARY OF STATISTICAL CALCULATIONS
FOR DOPAMINE EXPERIMENTS

	Peak 2				Peak 1			
Study	Side	Time	S x T	N	Side	Time	S x T	N
D/S/S	0.1751	**0.0001**	0.2641	7	0.7569	**0.0001**	0.2054	6
C/S/S	**0.0338**	**0.0001**	0.9998	6	0.3323	**0.0137**	0.6085	6
C/S/D25	0.5477	**0.0052**	0.9987	2	0.7349	0.7341	0.9761	2
R/S/S	0.4886	**0.0043**	0.8364	2	0.5177	0.9397	0.9975	2
R/S/D25	0.8634	**0.0166**	0.1936	3	0.4091	**0.0066**	0.2879	3
H/V/S	0.9212	**0.0001**	0.9697	9	0.8876	**0.0001**	0.9146	9
H/V/D25	0.8501	**0.0053**	0.4974	2	0.4331	**0.0032**	0.3113	2
H/V/D100	0.6144	**0.0001**	0.8672	8	0.4147	0.3019	0.2745	8
Apo/S/-	0.5477	**0.0001**	0.9788	4	0.3635	**0.0001**	0.8963	4

Comparison of the heights of Peaks 1 and 2 in the striatum of rats for the experiments described in Section 6 — Dopamine Findings. The sides are classified as ipsilateral or contralateral to the microinjected drug. The two sides were compared by two-tailed paired t-test, and the p values are shown. Statistically significant values are shown in bold print.

The studies are listed according to the key of:

Ipsilateral Microinjection / Contralateral Microinjection / Systemic Injection

Apo	=	Apomorphine 1 mM
C	=	Carbidopa 1 mM
D	=	Dopamine 1 mM
D25	=	L-Dopa 25 mg/kg
D100	=	L-Dopa 100 mg/kg i.p.
H	=	Haloperidol 1 mM
R	=	Ro4-4602 1 mM
S	=	Saline
V	=	Vehicle
-	=	No Injection

APPENDIX 6: CAFFEINE AND PARKINSON'S DISEASE

Included here is a copy of the report for the project examining the relationship between caffeine and Parkinson's disease.

THE EFFECT OF DIETARY CAFFEINE ON THE COURSE OF PARKINSON'S DISEASE

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ABSTRACT

Caffeine is commonly consumed in beverages such as coffee, tea, and cola drinks. Since animal work has shown it to have an effect on brain dopaminergic activity, the possible role of dietary caffeine was evaluated with regard to the evolution of Parkinson's disease and levodopa therapy. Patients with established Parkinson's disease drawn from the Mount Sinai Parkinson's Disease Center were interviewed regarding their dietary caffeine intake, and their records reviewed as to clinical symptoms, which had been evaluated numerically at each patient examination. Information on drug therapy was also gathered. Caffeine intake was correlated with several of the scores. Caffeine intake showed no interaction with levodopa drug treatment. It also did not affect a patient's overall symptom score, dyskinesia score or disease stage. Chronic dietary caffeine did have a statistically significant correlation with the rate of progression of Parkinson's disease ($r=0.368$, $p=0.02$). This suggests long-term dietary caffeine may accelerate the natural progression of Parkinson's disease. These results, although preliminary, suggest that caffeine restrictions may be advisable for those with this disorder. Possibly caffeine's antagonism of the adenosine receptor enhances dopaminergic activity by blocking adenosinergic inhibition, increasing cell injury by augmenting free radical formation secondary to the increase in dopamine turnover.

Key Words:
Caffeine
Parkinson's Disease
Levodopa
Coffee/Tea

INTRODUCTION

Caffeine is one of the most commonly used (abused?) drugs throughout the world. Millions of Americans consume it daily as part of their normal diet, averaging about 200 mg per person[1]. It is a component of many beverages, most notably coffee, tea, and cola drinks. Because of its widespread use, the fact that caffeine has several drug actions has been often overlooked, although awareness has been more apparent in recent years. In addition to its properties as a central nervous system stimulant, caffeine is noted for its diuretic properties and cardiovascular effects[2].

Because it has pharmacologic properties, caffeine has the potential to interact with disease processes as well as with drug therapies. This has particular importance for disorders of the central nervous system. One neurological disorder that may be affected by caffeine is Parkinson's disease.

James Parkinson first described the disease that bears his name in his Essay on the Shaking Palsy[3]. Parkinson's disease is a neurological movement disorder that has three cardinal characteristic symptoms: resting tremor, muscular rigidity, and bradykinesia. A major increase in our understanding of parkinsonism resulted from the demonstration of an association between the disease and the loss of dopamine from the striatum. A major pathological sign of this disease, perhaps the one responsible for the movement disorders, is the death of the dopaminergic nigrostriatal neurons. (For a historical perspective see 4, with references in 5) The strong association of this pathway with the classic symptoms of Parkinson's disease, rigidity, bradykinesia, and tremor, has recently been underscored by the clinical cases of drug-induced parkinsonism caused by MPTP in humans[6], and subsequent investigations in animals[7,8]. The loss of dopamine suggested that replacement of this neurotransmitter from exogenous sources might prove to be a useful mode of treatment. Indeed, this was shown to be the case, and, in fact, the current treatment of choice in this disorder is administration of levodopa (L-dihydroxyphenylalanine), the biosynthetic precursor of dopamine[4,9]. The proposed

mechanism of action is that within the brain, levodopa is decarboxylated by the enzyme dopa decarboxylase (aromatic L-amino acid decarboxylase), and the dopamine formed compensates for the loss in the striatum. There have been extensive studies examining how and where the levodopa is decarboxylated to dopamine[5,10,11,12].

With time, however, levodopa's effectiveness diminishes. Fluctuating responses in which the patient experiences abrupt shifts between clinical improvement and full blown symptomatology, adventitious movements, and overall less control of parkinsonian symptoms develop. The exact cause of these phenomena are unknown[9,13], and a controversy exists around whether treatment with levodopa contributes to their development or whether this represents the natural progression of the disease[14,15,16].

In 1971, Urban Ungerstedt published a series of papers describing an animal model of Parkinson's disease in which the neurotoxin 6-hydroxydopamine is used to selectively and unilaterally destroy the nigrostriatal system in rats. When appropriately stimulated, the rats would show a marked side preference in which direction they would turn while walking; the rats would circle. When these rats were injected with levodopa, they turned toward the unlesioned side[11]. This was believed to represent the development of receptor supersensitivity in the denervated striatum. This system has become a model for testing antiparkinsonian drugs: administer the drug to a unilaterally lesioned rat and see if it circles. The circling model has been subjected to extensive examination (for review see 17). Although the underlying neuronal mechanism in the circling model was once believed to be simply an asymmetry in dopaminergic activity, a considerable body of research has accumulated suggesting that it is much more complex.

The methylxanthines have an effect on turning behavior in unilaterally lesioned rats[18,19,20,21]. Administration of caffeine or theophylline causes marked rotation away from the lesioned side. The mechanism by which caffeine induces turning is unclear. It is not due to phosphodiesterase inhibition, as other phosphodiesterase inhibitors do not induce turning[18]. It has been proposed that caffeine and theophylline may have a direct effect on dopamine

receptors [18]. Another, perhaps more likely, explanation is that their effects are mediated by their antagonism of the adenosine receptor.

Adenosine has become a major candidate for neurotransmitter status in recent years (for reviews see 22, 23, 24). The existence of several adenosine receptors has been demonstrated in the brain[25,26], and a variety of drugs have been shown to have properties as agonists and antagonists at these receptors[25,27]. Caffeine is well known for its behavioral stimulating properties [2], and adenosine has been shown to have sedative effects[28]. In addition, there is evidence that adenosine may interact with a number of other neuronal systems, including cholinergic, adrenergic, and dopaminergic neurons[29,30,31,32]. Both cholinergic and dopaminergic neurons play important roles is Parkinson's disease.

The interaction of adenosine with dopamine may help explain the effects of methylxanthines on turning behavior. Adenosine is able to presynaptically inhibit the release of dopamine[30,31]. In addition, A1 and A2 adenosine receptors affect adenylate cyclase activity[33,34,35], and so may interact with the effects of dopamine-sensitive adenylate cyclase in the post-synaptic cell[35,36]. Unilateral microinjection of adenosine analogs into the striatum of normal, naïve rats elicits circling upon apomorphine challenge, further suggesting that adenosine plays a significant role in this behavior[37]. In connection with this, it has been shown that adenosine production/content, and its depolarization-dependent release are associated with intrinsic neurons of the striatum (kainic acid-sensitive). The dopaminergic nerve terminals also seem to contain adenosine, since it is decreased after 6-hydroxydopamine lesion. The kainic acid lesions of the striatum also decrease adenosine-sensitive adenylate cyclase 99%[38,39], leading the investigators to suggest that essentially all the A2 receptors in this brain area are located on intrinsic neurons.

Fuxe and Ungerstedt found that caffeine considerably enhanced levodopa-induced rotation[40]. This was later confirmed in a repeat study also done in Ungerstedt's lab[18]. This was interpreted as supporting the hypothesis that endogenous adenosine inhibits rotational behavior, and that caffeine blocks this inhibition.

Strömberg and Waldeck investigated the effects of caffeine and levodopa on locomotor activity in normal mice[41,42]. They found that caffeine greatly potentiated the hyperactivity that was induced by levodopa, both by increasing activity at a single dose of levodopa and by shifting the dose-response curve to the left, so that hyperactivity was seen at doses of levodopa that elicited no response without the addition of caffeine. They speculate that "... caffeine may modify the functional response to L-dopa both by increasing the level of DA [dopamine] in the brain and by sensitization of the DA-receptor."[42,43] In view of their findings, the investigators suggested that "the possibility should thus be considered that caffeine ingested, e.g. in beverages, may alter the therapeutic response to L-dopa."[41].

In another study by Maj, et al., caffeine potentiated the reversal by levodopa of neuroleptic-induced catalepsy in rats[44], lending further support to the notion that caffeine may alter the response to levodopa. The authors speculated that giving caffeine together with antiparkinsonian drugs may permit lower doses to be used and may reduce side-effects.

Overall, these studies indicate that caffeine may have a therapeutic effect in Parkinson's disease. The mechanism of action of caffeine, antagonism of the adenosine receptor, suggests, however, that caffeine alone may not be effective. Because adenosine is an inhibitory neurotransmitter, caffeine's action would be to disinhibit striatal neurons. This does not necessarily mean that striatal activity would increase, but it suggests that other stimulatory activity would be potentiated. In a parkinsonian patient, exogenous stimulatory drugs may be required to compensate for the loss of the intrinsic stimulation from the nigrostriatal pathway. Thus, while caffeine alone may not be efficacious in the treatment of Parkinson's disease, it could be useful as an adjunctive agent to levodopa.

Because of the animal studies described above, some clinical studies have been undertaken to study caffeine's usefulness as a drug therapy. These treatments focused upon giving it in combination with other drugs such as levodopa and direct dopamine receptor agonists like bromocriptine and piribedil.

Shoulson and Chase examined the use of caffeine when given to patients taking levodopa or piribedil[45]. In this double-blind crossover study, they found that caffeine at a variety of dose levels did not contribute to the further alleviation of symptoms. It did, however, increase the duration of periods of dyskinesias reported by patients who had developed this complication from levodopa treatment. This has two possible interpretations. One, which the authors state, is that caffeine has no beneficial effects, but rather exacerbates the unwanted complications of levodopa therapy. The other is that caffeine potentiates the actions of levodopa, so that the addition of caffeine will increase symptom improvement to the same level that the optimal dose of levodopa alone will, but beyond that, it will only contribute to unwanted side-effects the same way that a supra-optimal dose of levodopa would. In this study, patients received the same doses of levodopa (or piribedil) whether on caffeine or placebo, implying that they were always receiving the optimal level of drug treatment. Thus it cannot be determined which of the two interpretations is correct.

A later study, in which one of the same authors was involved, attempted to take this dosage difference into account. An evaluation, similar to that in the first study, was made of the effect of caffeine on the efficacy of drug treatment with bromocriptine[46]. In this case, the level of bromocriptine was reduced from 100 mg to 40 mg prior to starting the patients on caffeine. Again, caffeine failed to improve the symptoms in these patients. In fact, there was a trend toward worsening when a patient's symptom scores when on 40 mg bromocriptine with caffeine were compared to the scores on 100 mg bromocriptine alone. Two factors could be involved. First, when the bromocriptine was reduced from 100 mg to 40 mg, the symptom scores did not show a significant change, which suggests that the dose of bromocriptine was not reduced to a level below maximal effectiveness. This could explain why no additional alleviation of symptoms did not occur with caffeine, since the would be the same problem as before, and the worsening could be a manifestation of a supra-therapeutic overdose. And second, bromocriptine, in the rotational model with unilaterally lesioned rats, shows somewhat different properties from levodopa and apomorphine[20].

A related study by Magnussen, et al., reports that theophylline, another methylxanthine derivative with similar properties to caffeine, does not potentiate the response to levodopa, but, again, the patients were kept on the optimal dose of levodopa throughout the study[47].

And finally, a study by Strang mentioned, in passing, a possible effect caffeine has upon treatment of Parkinson's disease[48]. Two drug therapies were being compared: a) methixene; and b)chlorphenoxamine (an antihistamine with anticholinergic properties) with caffeine. Although no direct comparisons were run between chlorphenoxamine with caffeine and chlorphenoxamine without caffeine, Strang anecdotally mentions in the discussion section that

> One of the most surprising features of the results of the present trial was the clear superiority, compared with those obtained when chlorphenoxamine alone was used [49]. This was evident for both rigidity and depression, although the relief of tremor was also significantly better. The better results were probably due partly to the inclusion of caffeine, and partly to an improved and closer contact with the patients throughout the trial and the energetic efforts made to obtain an "optimal" rather than a "clinically satisfactory" level of therapeutic response. It is also possible that caffeine may exert a slight potentiating effect onchlorphenoxamine.

This conclusion must be viewed somewhat cautiously due to the lack of direct comparisons and the lack of statistical analysis of this result.

Patients, on occasion, report that caffeine may exacerbate some of the symptoms of their Parkinson's disease. Most specifically, they may feel that coffee exacerbates their tremor. A recent study examined the question of whether caffeine administration increases tremor as measured with an accelerometer[50]. The authors found that caffeine did not increase parkinsonian tremor (nor did it alter essential tremor or physiologic tremor in normal individuals). This indicates that despite a patient's subjective assessment of tremor, no objectively measurable increase occurs. The study, however, was biased. Only patients that drank regular (nondecaffeinated) coffee were allowed to participate. This allows examination of the patient population that is most directly affected by caffeine, but it precludes the ability to generalize the effects caffeine may have to populations not chronically taking caffeine.

The present study attempted to determine the effect of caffeine in the diet on the course of Parkinson's disease and on drug treatments. More specifically, caffeine intake was compared with the rate of progression of the disease as measured by the worsening of symptoms, with the dose of levodopa that yielded the optimal response in a group of patients that recently underwent a dose titration, and with several clinical parameters at a specific time point (seven years) after diagnosis.

METHODS

Patients for this study were selected from the registry of the Clinical Center for Parkinson's disease at the Mount Sinai Medical Center. Since this is a research center for Parkinson's disease, the patient population represents those with more difficult therapeutic problems. In addition, a number of patients were participating in a double-blind clinical trial comparing the effectiveness of an alternative, experimental form of Sinemet with standard Sinemet. As part of this study, these patients underwent a standard Sinemet dose titration so that their drug dose could be optimized.

The general sequence of events in gathering the data for this study was: a) interview patients to elicit a beverage consumption history using a standard questionnaire; b) use the beverage history to calculate daily caffeine intake; c) review the patients' Parkinson's disease charts to assemble their clinical scores for various symptoms, side-effects, and drug doses; d) use the clinical scores to calculate a rate of disease progression; and e) correlate the various disease parameters with caffeine intake.

Interview/Questionnaire. The interview was conducted to elicit a history for coffee, tea, and cola consumption history. It spanned the time from ten years prior to diagnosis until the present. A questionnaire was used to standardize the interview, and an opening statement was read to each patient explaining what was being asked of them and assuring them that their rights and privacy were being protected. Questions were organized to determine consumption at the present, at the time of diagnosis, and ten years prior to diagnosis. If any differences were

noted in beverage intake between these times, follow-up questions were asked to determine when the change occurred and/or if it was abrupt or gradual. In order to prevent the patient from focusing too much on caffeine, distracting questions were asked about consumption of other beverages (i.e., milk and juices), and the overall interview was presented as a "beverage survey".

Interview Format. The patients were interviewed at their regular clinic visit. After introductions and a preliminary agreement to participate, the patient was led to a private area, the opening statement was read, and then, assuming the patient agreed to continue, the interview was conducted. If a relative accompanied the patient (generally a spouse), he/she was allowed to be present if he/she so wished. The interview took five to ten minutes.

The patients participating in the titrated dose study were considered to be valuable sources for analysis, so those of them that were not seen in person were contacted on the telephone. An essentially identical format was followed.

Elicitation of Caffeine History. Because the caffeine history was considered to be the most important, follow-up questions were most fully pursued in the areas of coffee, tea, and soft drink consumption. Particular care was taken in determining the type of coffee and tea (especially whether or not it was decaffeinated), and the brand (or brands) of soda. If any decaffeinated beverages were consumed, the patient was asked if previously a non-decaffeinated beverage had been replaced, and, if so, when. Except where other circumstances precluded it, i.e., communication problems or an uncooperative patient, similar thoroughness was used for the other questions. In almost all cases, the answers were elicited directly from the patient. In a few cases, the spouse or other relative helped or answered for the patient, due to communication problems brought on by the disease. Patient responses seemed to be reliable and reasonably accurate, because they generally consisted of specific figures in response to the questions.

In addition to the beverage questions, the patient was asked about when he/she was first diagnosed with Parkinson's disease and when he/she first came to the Center for treatment for

Parkinson's disease. These dates were compared with their charts to assess the overall reliability of their responses. In one case a rather large difference could not be reconciled. The patient was excluded from any further analyses. (Subsequent investigation indicated that dementia was the probable cause of the unreliability of the responses.)

The beverage history was transformed into a caffeine dose history with the use of "The Latest Caffeine Scorecard", a publication from the federal government[51] which reported caffeine levels for various types of coffees and teas, and for different brands of soft drinks. The questionnaire had been tailored to maximize the utility of the information in this publication. If a patient did not or could not specify between two or more of the choices listed in the scorecard, i.e., "Whatever cola is on sale", or "I drink whatever coffee is available, whether it is percolated or drip filtered I do not know", then the relevant scores were averaged to arrive at the beverage serving caffeine content. Caffeine content was totalled in milligrams per day and were recorded for six month intervals.

As a methodological point, it is important to discuss the validity of assessing caffeine intake by determining the consumption of caffeine-containing beverages, i.e., coffee, tea and colas. Briefly, the vast majority of caffeine in the United States is consumed in the form of coffee (75%) and tea (15%). After adding in cola soft drinks, only a negligible amount of caffeine consumption remains[1]. Transformation of a beverage count into a caffeine dose can be accomplished with the aid of one of several tables listing caffeine content[51,52]. Caffeine intake calculated in this way has been found to have a very high correlation with actual caffeine intake[53].

Parameters of Disease Assessment and Progression. A full scoring assessment had been made each time the patient was seen, based upon a modified version of the Unified Rating Scale for Parkinsonism. The information recorded from the chart for analysis included: a number of symptom scores, focusing mainly on the movement manifestations, that were summed as a total symptom score; the disease stage as assessed by the Hoehn and Yahr staging scale; a score for dyskinesias; and the names and doses of drugs being taken by the patient. Excluded from analysis were those visits when the patient was not yet begun on an optimal dose of Sinemet,

or when the patient was specified as being in an "off" period (in order not to confuse disease progression with the changes between "on" periods and "off" periods, and to keep measurements comparable from patient to patient).

The total symptom score was plotted over time and a line was fitted to the points by the method of least squares. (For an example of this, see Figure 1.) The slope of this line was considered to be a measure of the rate of progression of the disease. A slope was determined for each patient. Because of the high degree of variation of the patient's clinical scores, the slope can only be considered to be a crude estimate of disease progression. It is, however, the best estimate available.

Sinemet Titrations. Those patients that had had their optimal Sinemet dose determined by titration in a separate study were treated in essentially the same manner as the other patients. The only additional information gathered from their charts was the dose of Sinemet.

Statistical Tests. Once this information was gathered, three specific statistical tests were run calculating the Pearson's product moment correlation.

1. Correlation of caffeine intake at the time of titration with optimal Sinemet dose in those patients that underwent titration in the other study. This examined the possibility that caffeine consumption affects the optimal dose of levodopa. In other words, it determined whether or not caffeine causes any shift in the dose-response to caffeine.

2. Correlation of caffeine intake averaged over the life of the disease with the disease progression (slope calculated as above). This examined the possibility that caffeine consumption accelerates or slows the normal worsening of the disease.

3. Correlation of caffeine intake at year seven post-diagnosis with the various clinical parameters (disease stage, total symptom score, dyskinesia score, and current levodopa dose) at that point in time. This examined the possibility that caffeine consumption affects the clinical state in "comparable" patients. (Year seven post-diagnosis was chosen because it was the year that had the most patient data available.)

RESULTS

Demographics. The fifty patients interviewed ranged in age from 39 to 87 years old. Fifty percent were in their seventh decade of life, and ninety-four percent were in their sixth to eighth decades. Sixty-six percent of them were male, and thirty-four percent were female. Disease length ranged from one to thirty-one years, and the age at diagnosis ranged from 30 to 77 years old. Most of the patients were diagnosed at an age between the late forties and early sixties. At this time, most patients were classified as Stage 3. Referral patients seem to come here only in the later stages of their disease (usually Stage 3). By Stage 5, the patient is no longer ambulatory. No Stage 5 patients were seen. Patients were generally examined several times a year, although the length of time between exams varied widely.

Caffeine intake. When the caffeine consumption was averaged over the life of the disease (or at least that part of the disease for which clinical scores were available), the daily intake ranged from 0 to 598 mg. Most of the patients had a fairly constant caffeine intake over the life of the disease. Those that had changed their caffeine habits (generally decreasing intake) seemed to have done so either at the time of diagnosis, because they became more attentive to general health habits at that time, or fairly recently (within the past few years), apparently as a result of the greater attention caffeine's health effects have been receiving. Some of the patients expressed a feeling that caffeine in conjunction with their disease made them feel "jittery", but this did not seem to be a major factor in decreasing consumption.

Exclusion of Patients. Before the statistical analyses were carried out, various patients were excluded from the test groups. This was done to insure that the specific variables being examined would not be confounded by other identified factors.

Patients with titrated Sinemet doses were included in the correlation (statistical test 1) only if they were scored as being Stage 3 (moderately advanced Parkinson's disease). Patients with other disease stages may require different amounts of Sinemet due to the varying degree of their illness, as opposed to a possible drug interaction with caffeine.

The correlation of slope with caffeine consumption included only those patients that had been seen for longer than one year. This was done in order to increase the validity of using the computed slope to represent the disease progression. With shorter observation periods, minor variations for any one examination may disproportionately influence the slope of the fitted line.

The patients included in the comparisons at year seven were only those that had had little change in their caffeine history. Because the correlations were made with caffeine intake at year seven, it seemed wise to exclude those patients that had changed their habits within the last year or two.

Titrated Sinemet Correlation (See Figure 2). Eighteen patients were included in the correlation. The correlation coefficient was very low ($r=0.099$), and there was not a significant correlation ($p=0.70$). This indicates that there is no connection between dietary caffeine intake and therapeutic Sinemet dose.

Slope Correlation (See Figure 3). Thirty-seven patients were included in the correlation. A positive correlation was found ($r=0.368$), which was statistically significant ($p=0.02$). This indicated that caffeine may influence Parkinson's disease by increasing the rate of disease progression.

Year Seven Correlations (See Figures 4-7). Thirteen patients were included in the year seven analyses. One patient was not included in the correlation with disease stage, because no disease stage was listed in the chart for this time period. None of these comparisons showed a significant correlation. (Stage: $r=0.097$, $p=0.76$; total symptom score: $r=0.031$, $p=0.88$; dyskinesia score: $r=0.007$, $p=0.93$; levodopa dose: $r=0.162$, $p=0.60$.)

DISCUSSION

Caffeine's effect on central nervous system function and on dopaminergic function is well known in animal studies. In attempting to examine the effects on this drug on Parkinson's disease, this study has shown that dietary caffeine intake may accelerate the gradual worsening of the patient's symptoms normally seen as the disease progresses. However, the data do not

support the hypotheses that dietary caffeine, in the shorter-term, affects the dose of levodopa needed to achieve optimal clinical effectiveness against Parkinson's disease, that it affects the clinical stage or the symptoms of Parkinson's disease, or that it exacerbates the side effects of levodopa treatment.

This study failed to establish an interaction between caffeine and levodopa dose conflicting with the animal studies mentioned above. This result supports the idea that caffeine is not an effective therapeutic agent for Parkinson's disease, reinforcing the other clinical studies, although it is difficult to rule out an interaction completely, especially in light of the evidence from the study cited above in which caffeine exacerbated levodopa-induced dyskinesias[45].

Because different amounts of caffeine consumption has no correlation with either the stage of disease, the level of symptoms, or the amount of dyskinesia, it appears that the subjective reports of the patients do not hold up under objective scrutiny. This is not to say that the patients do not experience any real discomfort, only that it is not manifested in a manner measurable with these methods.

Finally, the one positive finding of this study suggests that dietary caffeine may accelerate the degenerative processes of Parkinson's disease. This finding, however, should be interpreted cautiously, until more data can be gathered. With that precaution, the results suggest that dietary caffeine consumption should be decreased in Parkinson's patients. A mechanism to explain this finding is not apparent, and it does not fit in with the literature discussed above. A clue to understanding what processes may underlie the accelerated degeneration might be found in some of the literature of Lesch-Nyhan syndrome and a proposed animal model of it.

The metabolic basis of Lesch-Nyhan syndrome is a disorder of purine metabolism, which could conceivably affect adenosine neurotransmission. (Nyhan proposed that chronic administration of high doses of caffeine be used as an animal model.) It has been reported that patients with this disorder have a functional loss of dopaminergic nerve terminals[54,55], and a movement disorder is part of the syndrome. Animal models of this disorder suggest involvement of both purinergic and dopaminergic neurotransmission[11,37,56,57,58,59]. It is interesting to

speculate that loss of adenosinergic activity could have a toxic or degenerative effect on nigrostriatal neurons.

One way adenosine is believed to affect dopaminergic transmission is by inhibition of dopaminergic neurotransmitter release presynaptically[30,31]. It is possible that caffeine, by antagonizing adenosine, could enhance dopamine turnover in the nigrostriatal neurons that remain in the parkinsonian patient, and thus could enhance formation of free radicals formed as a result of dopamine metabolism, leading to an increase in the rate of dopaminergic cell injury, thus accelerating the progression of the disease. This study could provide a springboard for future investigation in this area.

The results obtained should be viewed from the perspective that this was a selected patient population. In this regard, several factors are involved. The patients were in a moderately advanced phase of disease, they were difficult therapeutic problems, and they were receiving antiparkinsonian agents other than levodopa – with regard to which the assumption had to be made that the use of nondopaminergic agents was a variable factor throughout the patient population that did not confound these results.

One final factor is caffeine tolerance. Tolerance to the effects of caffeine, and withdrawal when its use is ended, is a well documented phenomenon. It is generally a consideration for subjects taking much larger amounts of caffeine than the bulk of these patients. Its effects here, however, are not known and thus were not taken into account. From animal studies, receptor up-regulation has been shown to occur in some brain regions in response to chronic caffeine[60,61,62], so it is possible that the lack of correlation of caffeine consumption with the symptom score or the dose of levodopa is related to a compensatory mechanism within the brain. An area for future study would be to investigate the effect of acute caffeine administration on the levodopa requirement, utilizing a prospective study, perhaps by alternating regular and caffeine-free diets.

In summary, this study suggests that beverages containing caffeine have no noticeable effect on the clinical picture a Parkinson's disease patient presents, but that in the long term, it

may accelerate the rate at which the disease worsens. This conclusion suggests that it may beneficial to advise Parkinson's disease patients to reduce their intake of caffeine-containing beverages.

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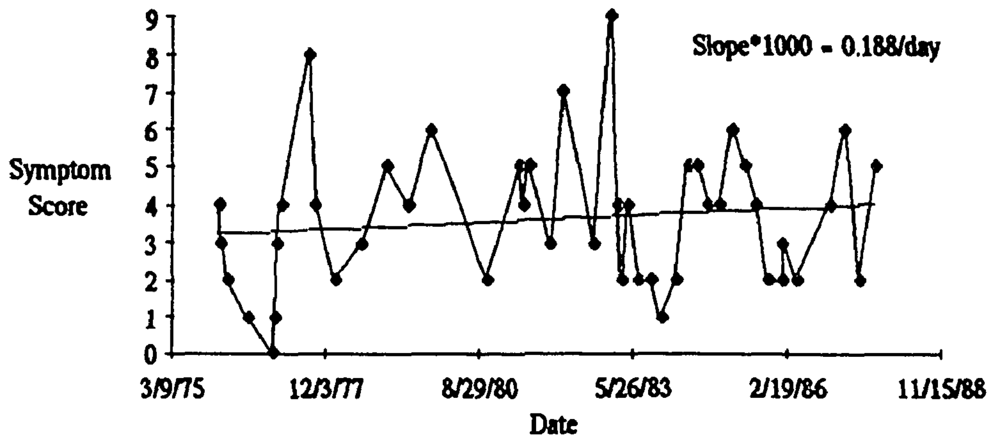
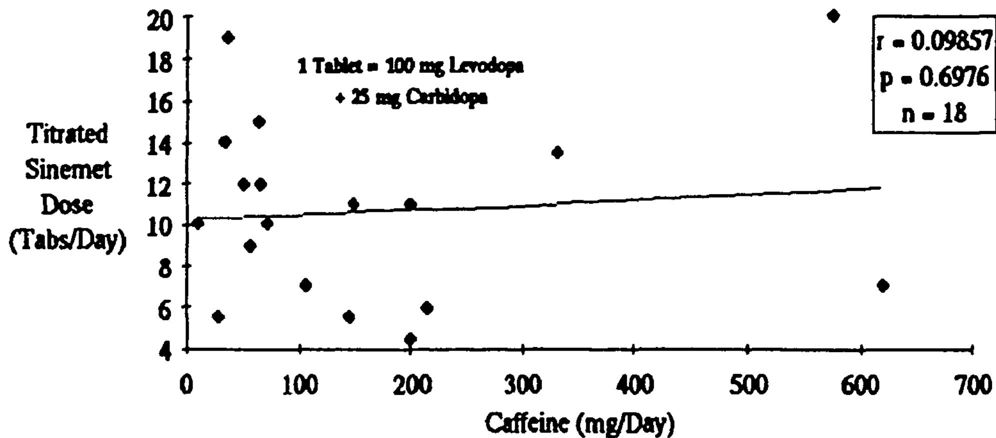
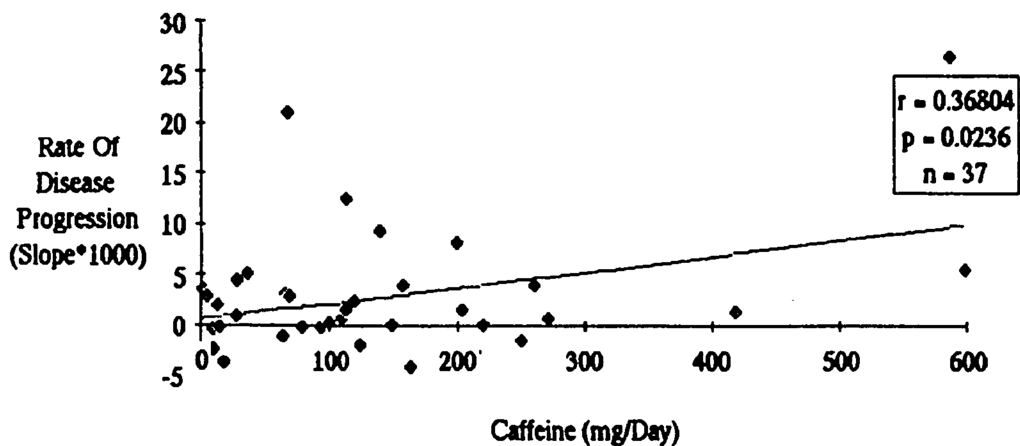
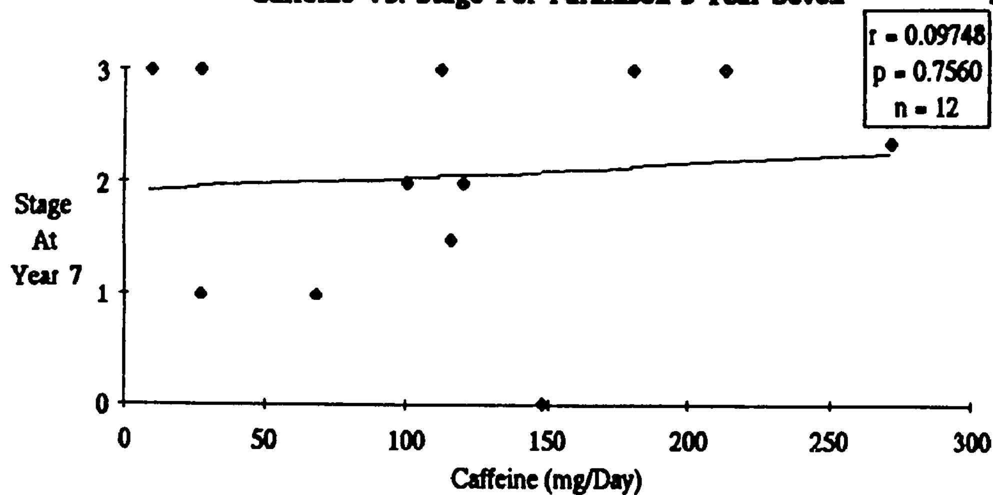

Patient 004 Disease Progression
Figure 1
Slope*1000 = 0.188/day
Symptom Score
0
1
2
3
4
5
6
7
8
9
3/9/75
12/3/77
8/29/80
5/26/83
2/19/86
11/15/88
Date

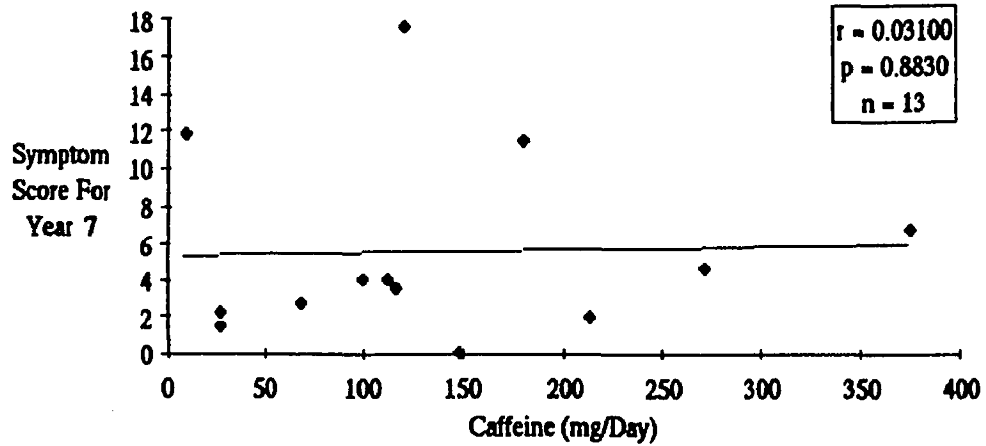

Caffeine Vs. Titrated Sinemet Dose
Figure 2
1 Tablet = 100 mg Levodopa
+ 25 mg Carbidopa
r = 0.09857
p = 0.6976
n = 18
Titrated Sinemet Dose (Tabs/Day)
4
6
8
10
12
14
16
18
20
0
100
200
300
400
500
600
700
Caffeine (mg/Day)

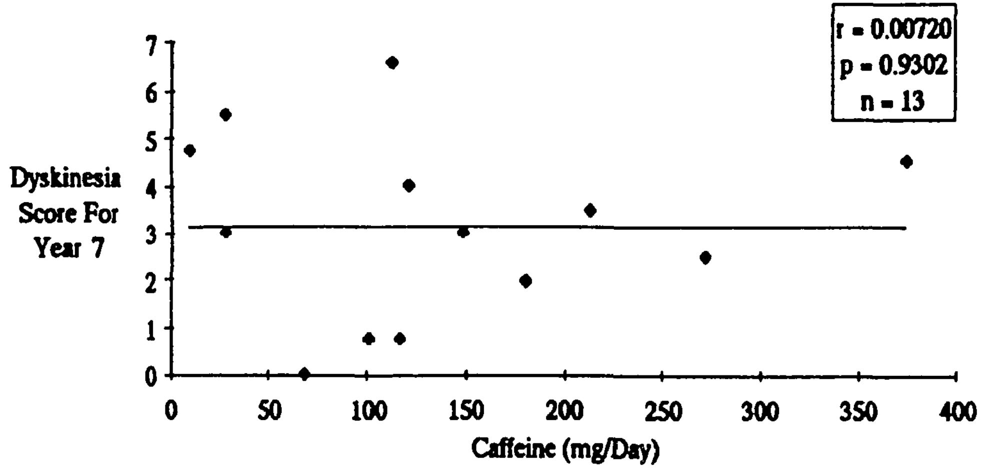
Caffeine Vs. Rate Of Disease Progression Figure 3

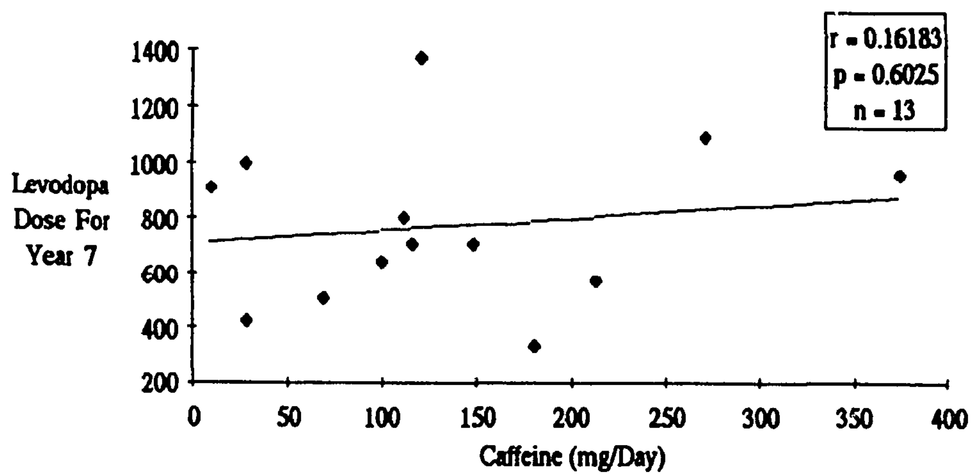


Caffeine Vs. Stage For Parkinson's Year Seven Figure 4




Caffeine Vs. Symptom Score For Parkinson's Year Seven
Figure 5
r = 0.03100
p = 0.8830
n = 13
Symptom Score For Year 7
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
400
Caffeine (mg/Day)


Caffeine Vs. Dyskinesia Score For Parkinson's Year Seven
Figure 6
r = 0.00720
p = 0.9302
n = 13
Dyskinesia Score For Year 7
7
6
5
4
3
2
1
0
0
50
100
150
200
250
300
350
400
Caffeine (mg/Day)


Caffeine Vs. Levodopa Dose For Parkinson's Year Seven
Figure 7
r = 0.16183
p = 0.6025
n = 13
Levodopa Dose For Year 7
1400
1200
1000
800
600
400
200
0
50
100
150
200
250
300
350
400
Caffeine (mg/Day)

APPENDIX 7: STATISTICS AND CALCULATIONS

All graphical and statistical analyses were performed on an Apple Macintosh™ computer. Microsoft Excel™ (Versions 1.0 to 1.5) was used for data entry and transformation; Microsoft Excel™, Microsoft Chart™ (Version 1.00), and Cricket Graph™ (Version 1.2) were used for graphical analyses; STATFAST™ (Version 2.0) by StatSoft and StatView 512+™ (Version 1.0) by BrainPower, Inc. were used for statistical analyses.

APPENDIX 8: ADDITIONAL CORRELATIONS

Later discussions regarding the data from the early stages of this investigation suggested that other ways of correlating the turning data with the peak height data might yield interesting results. The idea was to determine whether the correlations noted previously with the net turns data were dependent upon the lateralization of the turning and of the peak height changes, or whether they held up with absolute changes in the number or turns toward the intact side or with the height of peak two in the lesioned striatum. These comparisons involved correlating the heights of peak 2 on the lesioned side with the number of turns toward the intact side, as well as with net turns. The results from these calculations are presented in Tables VI and VII.

The behavioral data, however, showed a lack of turning toward the lesioned side. The lesions caused L-dopa-induced turning toward the unlesioned side to the extent that essentially no turns were made in the opposite direction. (See Table VIII) Of the rats used in the data analyses, only two of them made more than three turns toward the lesioned side in the ninety minute observation period: Rat 2 (20 turns) and Rat 70 (7 turns). The relative and absolute lack of difference between the number of net turns and the number of turns toward the intact side means that any correlations computed for these two measurements are essentially identical. The calculated correlations confirm this prediction, including the correlations for Rat 2 and for Rat 70, the rats with the greatest differences. (See Tables VI and VII.)

As can be seen in Tables VI and VII, the height of Peak 2 on the lesioned side had the strongest correlation with the number of turns (both net and absolute toward the intact side) from the session preceding the peak height measurement by twenty minutes. This contrasts with the correlations from the "lateralized Peak 2" height measurements that were calculated previously, in which the strongest correlations were seen when the number of turns was correlated with the net peak height from the session preceding the

peak height measurement by ten minutes. The importance of this result is difficult to assess, as it was not examined because of an a priori hypothesis. This line of thought was not originally pursued because the initial hypothesis involved correlating an asymmetry in striatal activity with an asymmetry in circling behavior.

It may be that the increase in activity in an individual striatum directly drives the amount of circling behavior away from that side. This view, however, is not supported by other data from this investigation. As was shown in Figure 14, the height of Peak 2 increases in the unlesioned as well as in the lesioned striatum. This increase is not accompanied by an increase in circling behavior. This is not what would be predicted by the hypothesis that striatal activity directly drives circling, and thus strengthens the possibility that it is the asymmetry in activity that causes circling. Further investigation into this area, however, is warranted.

Table VI
CORRELATION OF PEAK 2 HEIGHTS IN THE LESIONED STRIATUM WITH TURNING TOWARD THE INTACT SIDE

Rat Number	Peak 2 (t) : Turn (t) N=9	Peak 2 (t) : Turn (t-1) N=8	Peak 2 (t) : Turn (t-2) N=7
L2	-0.457	0.453	0.859
L8	-0.907	-0.379	0.526
L11	0.055	0.714	0.663
L63	-0.331	0.628	0.972
L66	0.798	0.885	0.449
L68	0.041	0.702	0.943
L70	-0.180	0.685	0.914
Averages	-0.140	0.527	0.761

Pearson's product moment correlations of Peak 2 height differences with net rotations for the sessions following L-dopa administration. For Peak 2 (t) : Turn (t), the correlation coefficient is based upon 9 points. For Peak 2 (t) : Turn (t-1), the correlation coefficient is based upon 8 points. For Peak 2 (t) : Turn (t-2), the correlation coefficient is based upon 7 points.

Table VII
CORRELATION OF PEAK 2 HEIGHTS IN THE LESIONED STRIATUM WITH NET TURNS

Rat Number	Peak 2 (t) : Net (t) N=9	Peak 2 (t) : Net (t-1) N=8	Peak 2 (t) : Net (t-2) N=7
L2	-0.434	0.488	0.873
L8	-0.905	-0.386	0.499
L11	0.049	0.713	0.666
L63	-0.214	0.681	0.924
L66	0.798	0.885	0.449
L68	0.057	0.709	0.938
L70	-0.220	0.651	0.893
Averages	-0.124	0.534	0.749

Pearson's product moment correlations of Peak 2 height differences with net rotations for the sessions following L-dopa administration. For Peak 2 (t) : Net (t), the correlation coefficient is based upon 9 points. For Peak 2 (t) : Net (t-1), the correlation coefficient is based upon 8 points. For Peak 2 (t) : Net (t-2), the correlation coefficient is based upon 7 points.

Table VIII
COMPARISON OF TURNS AND NET TURNS

Rat Number	Turns From Lesion	Turns To Lesion	Net Turns
L2	184	20	164
L8	22	3	19
L11	52	1	51
L63	7	1	6
L66	105	0	105
L68	178	2	176
L70	113	7	106

Comparisons of the number of the total number of turns made in each individual direction with the number of net turns. "Turns From Lesion" is the number of turns that the rat made in the direction away from the side of the striatum that was lesioned. "Turns To Lesion" refers to the number of turns that the rat made in the direction toward the side of the striatum that was lesioned. Net turns = Turns From Lesion - Turns To Lesion. All figures are expressed as the sum of the number of turns made in nine ten-minute sessions following L-dopa administration.

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