Estrogen Modulation of MPP^+-Induced Dopamine Secretion in the Corpus Striatum and Nucleus Accumbens of the Rat Brain

by

Michael Arvin Jr.

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ABSTRACT

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Michael Arvin Jr.

Master of Science

Youngstown State University

Parkinson's disease, first described in 1817, is a progressive disorder affecting men and women of middle age and older. The disease results in a disruption of normal motor function. Classic features include muscle rigidity, resting tremor, and the inability to initiate normal muscle movement. These symptoms are the result of the destruction of dopamine producing neurons in an area of the brain known as the Substantia Nigra and the subsequent depletion of dopamine in the striatum.

The cause of the disorder is still unknown. Possible contributing factors proposed include environmentally acquired toxins, and/or endogenous toxic

metabolic byproducts. There is also the possibility of a genetic component predisposing certain individuals to the disorder.

This study was designed to investigate the modulatory and possible neuroprotective effects of the steroid hormone estrogen upon dopamine release and clearance from neurons of the nigrostriatal system.

An *in vivo* animal model, along with the technique of *in vivo* electrochemistry was used to demonstrate, in real time, the characteristics of dopamine release and clearance. This is accomplished through the use of a stereotaxic instrument which allows for the placement of an electrode and micropipette assembly into specific brain regions. A neurotoxin, MPP^+, which simulates the biochemical events seen in Parkinson's disease was used to stimulate the release of dopamine from the nigrostriatal neurons.

Female ovarectomized rats were divided into two treatment groups, MPP^+ alone, and MPP^+ with estrogen. These treatments were applied to the rat forebrain and measurements of the ensuing dopamine release were monitored using the IVEC-10 system and software capable of measuring neurochemical substances such as dopamine.

Results of this study demonstrate a modulatory and/or neuroprotective effect of estrogen upon neurons of the nigrostriatal pathway by decreasing the effectiveness of MPP^+ to elicit the release of dopamine from these neurons. The results of this study also reveal differences, in some release and clearance parameters, between the corpus striatum and nucleus accumbens.

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CHAPTER I

INTRODUCTION

BACKGROUND / HISTORY

The symptoms and resulting debilitation of Parkinson's Disease (PD) was first described by James Parkinson in 1817 in a communication entitled "An Essay on the Shaking Palsy." At that time he had no pathological or histological evidence to identify the site of the disease. It was not until much later in 1895 when Brissaud reported three cases where patients with Parkinsonism presented with tuberculomas in the Substantia Nigra (SN). It was at this time that the SN became implicated. Then in 1960 Ehringer and Hornykiewicz detected a deficiency of dopamine in the striatum and the nigrostriatal dopaminergic pathway. These findings led to a suspicion of nerve cell damage in the substantia nigra as a critical part of Parkinson's Disease (Forano, 1995). Other researchers in the 1960's, Tretiakoff, Hassler, Greenfield, and Besanquet, without knowing why,

suspected the involvement of the SN in the pathology of Parkinson's Disease. Cotzias provided further support for the idea of dopamine deficiency by successfully reversing Parkinsonian symptoms in patients with large doses of D, L-dihydroxyphenylalanine (dopa), a biochemical precursor to dopamine (Cotzias et al., 1967).

It was not until 1973 that a correlation was established between nerve cell degeneration in the Zona compacta of the SN and the decrease observed in dopamine content. Bernheimer et al., (1973) gathered information from a relatively large group of Parkinsonian patients, including those having idiopathic Parkinson's Disease. From this information it was determined that the decreases in dopamine observed in the striatum correlated positively with the degree of nerve cell loss in the SN. It was also determined that the decreases in dopamine content, seen in idiopathic cases, was most severe in the putanem.

Since 1817, when James Parkinson identified the major symptoms related to Parkinson's Disease, considerable advancements have been made towards the characterization of the symptomatology of this disorder. The clinical definition of the disease is one of a progressive disorder, usually striking persons of middle age and older, resulting in a disruption of normal motor function. Classic features of the disease include rigidity in both flexors and extensors, resting tremor, often involving the thumb and forefinger (Pahwa and Koller, 1995). Bradykinesia, which is the inability to initiate or perform common motor movements with normal speed (Pahwa and Koller, 1995), is quite often the most disruptive of the symptoms. Many patients exhibit a postural instability forcing the patients center of gravity

forward and causing a loss of balance. There is often involvement of the arms, legs and head and as the disease progresses there is a loss of postural righting reflexes (Yahr, 1977), causing further balance instability and subsequent falls.

Years before the manifestation of motor disturbances many patients experience milder, less obvious symptoms, such as slowness of movement, depression, loss of smell, and decrease in dexterity. Nearly one half of all patients diagnosed complain of pain, numbness, and/or tingling in the affected areas.

The presence of dementia as a part of the disease process associated with Parkinson's Disease, is still a matter of some debate. While once thought not to be a major consideration in the disease process (Lieberman et al., 1979, Polloc and Hornabrock, 1966), more recent investigations have discovered a higher incidence of associated dementia than was once thought. Early estimates of the occurrence of dementia with PD ranged from 5.7 to 15%. In a population based study, more recent work reported by Mayeux et al., (1992), demonstrated a frequency of 41.3% for dementia associated with Parkinson's Disease. There also seems to be an age factor associated with dementia, in that, as the age of onset of motor symptoms increases, so too does the frequency of dementia (Mayeux et al., 1992). This more recent study also supports previous work, suggesting that PD effects men more than women and Caucasians more than non-caucasians. Interestingly, even though the disease effects men more frequently, mortality rates for women have been reported to be higher by as much as 98% (Diamond et al., 1989), while the mean age of death is about the same for both men and women, at

73.4 and 73.1 respectively (Diamond et al., 1989). Evidence for dementia as a shared risk factor among first degree relatives of PD patients has been suggested (Marder et al., 1990). This is interesting and suggests a possible genetic component in the sequella of Parkinson's Disease. Determining the onset of idiopathic PD remains difficult at best. Evidence suggests that there is a long presymptomatic phase associated with the disorder (Koller et al., 1992, Koller, 1991). Duration of the presymptomatic phase varies from several months, for such symptoms as primary sensory deficits (Koller, 1984, Snider, 1976), to as long as 10-20 years for complaints of loss of olfaction or depression (Santamaria et al., 1986). While diagnosis of the onset of PD is difficult, determining the extent of progression is simpler and is accomplished through clinical observation. Progress is based on disability and a system of 5 stages can be utilized for assessment (Pahwa and Koller, 1995). Recently, a Unified Parkinson's Disease Rating Scale (UPDRS) has been developed to determine the extent of the disease.

The term Parkinsonism is used to represent a variety of disorders which also effect the nigrostriatal pathway, and manifest themselves with symptoms similar to those of Parkinson's Disease. While these disorders are numerous and quite varied in their etiology, there is a commonality of tremor, rigidity, and bradykinesia seen throughout (Stern and Koller, 1993). Hereditary disease, toxins, metabolic disorders, infections, multiple system atrophies, and other CNS diseases with degenerative components, are a few of the categories included in Parkinsonism.

Distinguishing between PD and other neurodegeneritive disorders is an imperative and relevant determination. While therapeutic regimes may be

similar, atypical Parkinsonism does not respond well to current pharmacological treatment. An equally important benefit to differential diagnosis is the positive impact it imparts to areas of current research.

CAUSES OF PARKINSON'S DISEASE

While still unknown, the causes of idiopathic Parkinson's Disease has been the focus of intense investigation over the last 25 years. Since 1817 there has been a variety of suggestions as to the cause of this disease. Recently most hypotheses center around exposure to some exogenous or endogenous toxic agent. Also, since there is obvious variability of persons in their susceptibility to the disease, the issue of genetic predisposition must also be considered. Current research suggests a multicausational pathogenesis for PD rather than a single metabolic, neurotoxic, or genetic defect. The validity of a "Global Hypothesis" as described by Barbeau (1986) seems a plausible explanation for the cause of Parkinson's Disease. Barbeau's theory suggests that PD is the result of a number of factors, including general cell aging, accentuated by a genetically predetermined susceptibility of the cells involved. This, in conjunction with other factors, results in the pattern of increased catecholamine turnover contributing to free radical and neuromelanin accumulation in the affected neurons. The result of this is an exhaustion of cellular compensatory mechanisms leading to membrane or organelle damage. Later, the formation of Lewy Bodies and cell death is observed. The formation of Lewy Bodies, while not a phenomenon reserved strictly to PD (Gibb, 1988), is clearly an important

aspect of the pathology of PD (Forno and Langston, 1993). Lewy Body formation seems closely associated with nerve cell destruction (Forno, 1986, Gibb et al., 1985) and certainly is an important factor in the total presentation of PD. Other than the "global hypothesis", the remaining theories as to the cause of Parkinson's Disease have a much narrower focus, centering instead on one specific biochemical or neurophysiological process. These theories include, aging, metabolic factors such as those that involve neuromelanin, free radical damage, mitochondrial disruption, catecholamine metabolites, and exogenous environmental toxins, such as heavy metals or pesticides. All of these etiological theories, as to the cause of Parkinson's Disease, have support and will be discussed in some detail.

Aging Affects. It is quite clear that Parkinson's Disease primarily effects people of middle age and older. While there have been documented cases of patients forty years of age and younger, these are rare and account for only a small percentage of patients with the disorder. Onset of symptoms usually occurs between age fifty and seventy nine, with an average nearer to sixty (Forno, 1995). Several studies have been done with aging as the central theme (Thiessen et al., 1990, Scherman et al., 1989, Caline and Langston, 1983, Riederer et al., 1976,). These studies consider aging and it's effects, direct or indirect, as the cause of nerve cell loss in specific areas of the brain, such as the substantia nigra. It is still unclear as to the definitive effect of aging and it is well documented that some nerve cell loss in the SN accompanies the normal process of aging (Theses et al., 1990). Whether or not this is overtly responsible for the disease itself, or rather acts as a contributing component to the deterioration of the nigrostriatal system,

is still a matter of debate. Some researchers (Fearnley and Lees, 1991) have examined aging and cell loss in the SN as possible unrelated events. In fact, the nerve cells most susceptible to PD have been shown to be somewhat resistant to the effects of aging.

Endogenous Metabolic Toxins. It has been well established that the loss of dopamine in the nigrostriatal system is the primary neurochemical disruption present in Parkinson's Disease. While this is undoubtedly true, many scientists would argue that other biochemical disturbances may play a role in the etiology of the disease. There is currently great interest in the prospect of one or more possible toxic agents as the cause of, or a contributing factor to the disease process.

There have literally been dozens of compounds implicated as the possible causative agent involved. Some of the most likely candidates are considered here.

Dopamine. The fact that dopamine is found in much higher concentrations in its neurons than other catecholamines, has led some to suspect it as the causative factor in Parkinson's Disease. This is due to several biochemical conditions which occur as a result of its metabolism or interaction with other compounds.

During normal catabolism of dopamine a number of oxidatively active products are generated. Monoamine oxidase (MAO) initially degrades dopamine, and following an intermediate step, results in the production of one of the major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC). This process utilizes molecular oxygen, and thereby generates one molecule of hydrogen peroxide for every molecule of dopamine catabolized. These

increasing levels of hydrogen peroxide can be damaging to the dopaminergic neurons themselves, eventually resulting in cell death. The result of such a process would be a compensatory response from the remaining neurons to produce more dopamine, which would be metabolized as well. This cycle of cell damage, followed by increased dopamine release and subsequent cell damage, could create a downward spiral leading to the demise of the nigrostriatal system.

Other dopamine derived compounds may also have a toxic affect on the nigrostriatal system. Again, in the presence of oxygen, dopamine can auto-oxidize and be converted through an intermediate to an orthoquinone derivative (DOQ). In this process, oxygen is converted to either a superoxide or a peroxide (Irwin and Langston, 1995). The intermediate compound, dopamine semiquinone (DSQ), as well as DOQ, can act as cytotoxic compounds. Their cytotoxicity stems from their ability to generate peroxides and superoxides (Graham, 1978a, Graham et al., 1978b). This process is enhanced in the presence of certain metal cations such as iron, magnesium and calcium. Covalent bonding of these compounds with nucleophilies, such as glutathione or cysteine, would lesson the availability of these important protective compounds.

However, there is still no direct evidence identifying DOQ or DSQ as the endogenous toxic compound of PD. If autoxidation of dopamine leads to damage of dopamine cells in the nigrostriatal system, then treatment with MAO inhibitors should result in an acceleration of the damaging effects. In contrast results have demonstrated quite the opposite (Irwin and Langston, 1995a).

From DOQ it is possible to get an interaction with amino nitrogens, resulting in cyclization and the generation of leukochromes, and then via oxidation, aminochromes. Aminochromes could then undergo cycling, greatly enhancing the potential to generate oxygen radicals (Pileblad et al., 1988). Again, there is no direct evidence that this biochemical process is linked to PD.

6 - hydroxydopamine (6-OHDA) has been considered one of the leading contenders to the title of the endogenous toxic agent of PD. This is primarily due to its strong selectivity for catecholaminergic neurons. 6-OHDA does not cross the blood brain barrier and therefore must be administered directly to cause its toxic affects. Whether or not 6-OHDA is produced endogenously is also a matter of some debate. 6-OHDA could be generated endogenously by way of several metabolic pathways. DOQ can react with H_2O or with hydroxide radicals and Fe^{+2}. In these situations 6-OHDA can be generated at a physiological pH (Slivka and Cohen, 1985). While these metabolic processes produce 6-OHDA at a relatively slow rate, and in limited quantities, the severe toxicity of 6-OHDA is such that only small quantities would be necessary to result in cell destruction. There is evidence that supports the endogenous production of 6-OHDA. The presence of 6-OHDA in the caudate nucleus following methamphetamine administration has been demonstrated in the rat (Seiden and Vosmer, 1984). In addition, pretreatment with MAO inhibitors results in an increase of 6-OHDA following methamphetamine treatment (Marek et al., 1990a, Marek et al., 1990b).

Neuromelanin . Neuromelanin is a black pigment deposited in nerve cells secondary to catecholamine activity and metabolism. Evidence suggests that spontaneous oxidation of dopamine is responsible for this substance being deposited in cells of the SN (Forano, 1995, Carstom et al., 1991, Graham, 1978), and that accumulation of the pigment in this area may be age related (Shoffner et al., 1991). While some studies have reported a protective role for neuromelanin, others have attributed a damaging role to neuromelanin in the etiology of PD. A protective role for neuromelanin has been suggested from experiments that have demonstrated that melanin can bind reducible active metal ions. This binding would limit the ability of these ions to participate in free radical generation. Melanin can also act as a storehouse for potentially toxic substances, thereby sequestering them from other parts of the cell where they could potentially have damaging effects. Finally, melanin acts as a scavenger entering into reactions with superoxides, hydroxyls, and peroxide which would removes these toxic compounds from inside the cell (Swartz et al., 1992, Lindequest et al., 1987). These seemingly protective features of neuromelanin have led some researchers to speculate that the loss of pigmented cells seen in PD reflects a disruption of normal neuromelanin function, which may be related to nerve cell death (Gan et al., 1976).

In contrast to the potential benefits, there is considerable evidence that neuromelanin accumulation may actually compromise nerve cell function. Neuromelanin accumulation has been associated with a reduction in the Golgi network and cytoplasmic RNA, either of which could lead to nerve cell destruction. Negative effects, secondary to neuromelanin accumulation

are supported by the biochemical properties of the compound. These include the ability to accumulate toxic substances which could later be released in the cell (D'Amato et al., 1986, 1987, Irwin and Langston, 1985), as well as the interaction with reducible metal ions. Although the interaction with reducible metal ions was suggested as having potential benefits, this interaction could generate damaging superoxide, peroxide, and hydroxyl free radicals. There is also evidence that neuromelanin may alter the oxidative state of metal ions favoring the formation of these same damaging compounds (Kurytowski et al., 1985).

Heavy Metals. The presence and subsequent role of heavy metals, in particular iron, and to a lesser extent aluminum, in the pathology of PD is another area of considerable controversy. A number of studies demonstrated the presence of iron in the substantia nigra of patients with PD (Sofic et al., 1991, Hirsch et al., 1991), while others did not (Uitti et al., 1989). It is unclear if the level of ferritin, an iron binding protein sometimes seen in niagral cells, plays a role in the pathology of PD. Some studies have shown ferritin levels to be increased in idiopathic PD (Jellinger et al., 1993), while others have failed to demonstrate any difference in ferritin levels compared with those of controls (Dexter et al., 1993).

Also unclear is the exact neural site of action of iron and other heavy metals in association with PD. Suggested locations include astrocytes of the SN, macrophages, as well as some non-pigmented neurons, and even Lewy bodies (Jellinger et al., 1990, Hirsch et al., 1991). However, Jellinger et al., (1993) demonstrated that iron tends to deposit into neuromelanin, but not into the Lewy bodies of Parkinson's patients.

In addition to iron, manganese and copper have also been suggested as possible transitional metals with the capability of causing neurotoxicity. In Wilson's Disease, a similar neurological disorder, copper is found in elevated levels in the basil ganglia and manganese poisoning results in symptoms similar to PD, in particular, dystonia (Irwin and Langston, 1995). However, the symptomatology produced by toxic exposure with these metals, differs substantially from what is classically seen in idiopathic PD.

Even in the light of current findings one should approach the role of iron and other heavy metals, in the pathology of PD, with guarded skepticism.

The search for an endogenous toxic substance, which can be definitively linked to PD, has been the subject of intensive investigation for many years and will likely continue for some time to come. The limited knowledge available concerning a number of these metabolic compounds only leads to more questions about their potential roles in neurological disorders, including PD.

Other Metabolic Agents. A number of metabolic agents have been proposed as possible endogenous neurotoxic substances that contribute to Parkinson's Disease. Most of the information available concerning these metabolic agents is focused on the Isoquinolines and Beta-Carbolines. Isoquinolines are derived from phenethylamines and occur both environmentally and endogenously (Irwin and Langston, 1995). The interest in this group of substances as possible endogenous toxic substances is due to several factors. First, isoquinolines are highly oxidizable substances with a number of double bonds and different states of saturation. This allows them to be good candidates for substitution reactions on their nitrogenous

rings. It has also been determined that these substances occur in a number of common food types, such as wine, cheese, and cocoa (Niwa et al., 1990), as well as in various mammalian tissues including the brain (Niwa et al., 1990, Niwa et al, 1987).

Beta-carbolines are similar to isoquinolines in that they also occur in a number of oxidative states. Beta-carbolines can also be found in several human tissues including neural tissue, these compounds are an alkaloid derivative and can be found in somewhat higher quantities than isoquinolines. There is a rather large body of information regarding these compounds, partly due to their involvement in other medically related areas such as benzodiazepine receptor activity and alcoholism (Irwin and Langston, 1995). The consideration of these substances in the etiology of PD arises from their structural similarities to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and MPP^+. Two such compounds are 1,2,3,4-tetrahydro-beta-carboline (MTBC) and 2-methyl-beta-carbolinium ion (MBC^+). MBC^+ has been shown to inhibit mitochondrial respiration, similar to MPP^+,(Albores et al., 1990, Hoppel et al., 1987) and cause dopamine depletion in the striatum with suspicious neural lesions (Drucker et al., 1990, Neafsey et al., 1989).

Exogenous Metabolic Toxins. An exogenous, or environmental agent as the causative factor in PD, might at first seem an unrealistic consideration, since it is clear that PD is a progressive disorder with symptoms becoming worse as time passes. In contrast, most environmentally related disorders either stabilize or improve when the affected individual is removed from the toxic environment. Recently, this line of thinking has been shown to be

somewhat narrow. For example, in the western pacific region there are cases of amyotrophic lateral sclerosis (ALS) and Parkinsonism with dementia (P-D) that have been shown to lack any hereditary connections (Spencer and Butterfield, 1995). These same studies show a decline of these disorders in areas where substantial environmental changes have been made. The most striking consideration is the pattern of irreversible striatal damage and neuronal cell death in humans and primates following exposure to MPTP. These occurrences, when considered together, are suggestive of an environmental toxin as a plausible explanation to the cause of PD.

The search for an exogenous agent in relation to PD has led researchers in a number of directions. While substances investigated have come from a variety of categories, the focus of work in this area seems to center around herbicides and insecticides and their possible involvement. When considering an environmental agent's toxic potential, it is normal to classify the substance as an agent that will result in either regional or global brain dysfunction. Regional brain dysfunction is usually associated with some aspect of mitochondrial disruption. Globally active neurological agents cause much more widespread neuropathological changes, like those seen secondary to hypoxia.

Of the compounds considered, most have striking similarities to the toxic metabolite of MPTP, 1-methyl-4-phenylpyridinium ion (MPP^+). Isoquinolines and beta-carbolines are two such compounds. While previously mentioned as possible endogenous metabolites, these compounds do occur naturally in some plants and animal tissues as well (Spencer and Butterfield, 1995). Another chemical with regional affects is the 1,1-

dimethyl-4,4-bipyridinium ion (paraquat). Paraquat is a commercial herbicide that is widely used. Again, interest in this compound is due to its structural similarity to MPP^+. Paraquat is acutely toxic, normally accumulating in the lungs of various species, including humans, where it contributes to free radical cell damage. To date there is no direct evidence linking these compounds to neural damage similar to that seen in PD.

Other chemical compounds of some interest contribute to a broader, more global cerebral energy disruption. Substances such as carbon monoxide, manganese compounds, organic mercurials, and carbon disulfides are some of the chemicals which can produce varying degrees of neural toxicity. Several organic compounds, such as n-hexane and hexachlorobenzene, have also been implicated as possible neurotoxins in the development of PD. While the production and use of both of these organic compounds has been drastically reduced, there are still large quantities encountered by individuals in several industrial situations.

In addition to specific chemical agents, certain environmental situations have been considered as having a potential connection to PD. Examples of these, include farming, rural living, rural water supplies, and sawmill or paper processing. Various epidemiological studies have examined all of these situational conditions in an attempt to make a connection to idiopathic PD. Studies examining rural residency have found a higher incidence of the disease in those individuals maintaining such residency compared to those that have an urban lifestyle. Patients with an early age of onset seem particularly over represented in rural populations (Rajput et al., 1986, Tanner, 1986). Numbers of patients affected in rural versus urban

communities become more similar as the age of onset of the disorder increases and are nearly equal at age 75 (Rajput et al., 1988).
Studies investigating paper processing, sawmills or wood related industries have produced somewhat conflicting results. Persons involved in these types of industries are regularly exposed to a wide variety of chemical agents, which could prove to have neurotoxic affects. One study in Quebec compared mortality rates, along with L-dopa use, across several regions. This study identified fifteen towns with incident rates for PD three times greater than the normal level. In ten of these towns a paper or pulp processing plant was in place, while the other five towns were located directly downstream of such an industrial complex (Barbeau et al., 1987). In a another study Sethi et al., (1989), using some of the same criteria as Barbeau et al., (1987) was unable to demonstrate a higher incidence of PD in similar wood processing areas in Georgia.

Pesticides, herbicides and fungicides have received a considerable amount of attention as the possible source of the environmental toxin or toxins involved in the etiology of Parkinson's disease. Prior to 1940 pesticides were the predominant types of agents used, while more recently the increase in sales of herbicidal agents has raised this category of agents into the number one spot. Herbicides contain primarily chlorophenoxy compounds like 2,4-D(2,4-dichlorphenoxy acetic acid) or 2,4,5-T(2,4,5-trichlorophenoxy acetic acid) (Spencer and Butterfield, 1995). Exposure to these and other similar compounds has produced various neurological abnormalities. However, to date no particular agent has been identified that produces a pattern of symptomatology identical to that of idiopathic PD.

Whether PD is due to a single entity or to a compilation of endogenous, exogenous and genetic factors, remains a very important unanswered question. With the exception of MPTP, no agent has shown the selectivity for the dopaminergic neurons in question that would be required of a toxin to cause the nerve cell damage seen with PD.

Mitochondrial toxins. Endogenous and or exogenous toxic substances, which effect mitochondrial respiration, have been given serious consideration as the possible causative agents of PD. While the subject of cell death will be discussed in more detail later, it is important to mention the possible influence of these agents as a causative factor for the disorder. Clearly, there is some correlation between mitochondrial disruption and the previously cited possible causes of PD. Decreases in complex I, and minor changes in complex II, have been demonstrated in PD patients through immunocytochemical techniques (Mizuno et al., 1989, Hattori et al., 1991).

Much of the information available concerning the mitochondrial respiratory chain and the effects of toxins is a result of studies done with MPP^+. This is the suspected toxic metabolite of MPTP. Studies have shown MPP^+ to accumulate in mitochondria where it is thought to have disruptive effects on the respiratory chain in the area of complex I (NADH dehydrogenase) (Ramsey et al., 1986, 1987, Vyas et al., 1986). MAO-B has been shown to be the important element mediating the formation of MPP^+ from its substrate MPTP. This is interesting due to the presence of MAO-B in astrocytes found in the areas of cell destruction associated with PD.

Lewy bodies. Lewy bodies are a structural inclusion located most often in the SN and Locus ceruleus (LC), and are associated with neurodegenerative disorders such as PD, Alzheimer's Disease, Neuroaxonal Dystrophy, and others (Forno, 1995). While not seen as a direct cause of PD, the close association of Lewy bodies with nerve cell degeneration deserves special consideration. These somewhat curious structures are composed of fibrous material most likely of cytoskeletal origin. They have a dense core with fibers radiating outward and are often seen surrounded by neuromelanin. Lewy body formation is usually seen in people over sixty, and very rarely in younger individuals. Therefore their presence, as it relates to age, is of great interest. In a study by Forno and Langston (1993), Lewy bodies were often found in brain tissue of non-parkinson patients. These discoveries would suggest a direct relationship between age and Lewy body formation. In their study, Lewy bodies were present in 8% of the population older than fifty (Forno and Langston, 1993). This percentage was increased to 12.8% for people older than seventy and to 15.9% in those examined over eighty years of age (Forno and Langston, 1993). Appearance of these structures in areas of the cerebral cortex, in particular the limbic area, has been referred to as diffuse Lewy body disease (Kosaka et al., 1984). Findings such as this located outside the SN and LC, has brought into question the role of Lewy bodies in PD. Similar research has raised questions of a possible closer relationship between PD and Alzheimer's Disease than was once thought to exist (Forno, 1992). Lewy bodies interact quite readily with antibodies directed against Ubiquitin, which is a protein involved in ATP-dependent proteolysis (Gibb et al., 1991).

Research concerning the role of Lewy bodies in neurostriatal degenerative disorders, particularly PD, continues. Studies by Gibb and Lees (1988) allude to the possibility that Lewy body formation may be a pre-disposing factor of PD.

Currently there are no acceptable animal models suitable for the study of Lewy bodies. However, experiments have used MPTP treated animals, and while successful in developing neural inclusions, they lacked some of the features of naturally occurring Lewy bodies.

Infectious Agents. It was not long after the initial description of PD that a number of infectious agents were being considered as the possible etiological agents of the disease. This was due primarily to the appearance in the early 1900's of the condition known as encephalitic lethargica. This condition was first described in patients located in France and Austria. The symptomatology began with recurrent headaches, fever, lethargy, and some paralysis of the extremities (Zack and Tanner, 1995). Thirty percent of those infected died, 2 to 20 % of the survivors developed Parkinsonian like symptoms within the next 5 to 10 years (Ziegler L, 1928). While similar to PD in many respects, some of the symptoms exhibited by these patients were quite atypical. The most notable of these differences was the age of onset of symptoms. In most cases the age of onset was much earlier than typically seen in PD. Occurrences appeared most frequently during the winter months and seemed to be spread via airborne mechanisms. By 1919 this disorder had spread across Europe and to North America, Japan, Africa, and India. Post-mortum histological studies verified localized neurological damage to the mid-brain and in particular the substantia nigra (Hohman L,

1925). While the causative agent was never identified, it was speculated that a possible viral agent of the herpes type, or the same unidentified infectious agent responsible for the influenza pandemic of the same period, was to blame. The influenza agent seemed a likely candidate, because of its epidemiological factors rather than its clinical ones. Some of the information which led investigators in this direction was the fact that the peak in occurrence of encephalitis lethargica accompanied peaks in influenza and lessened with decreases in influenza. Also, the disease was virtually nonexistent in areas that were devoid of influenza outbreaks altogether (Jordan E, 1918).

Following this epidemic outbreak of encephalitis lethargica in the 1920's, a number of researchers became proponents of a theory, known as the "Cohort Hypothesis", which attributed all cases of PD to this unidentified infectious agent responsible for the influenza outbreak. This theory had substantial support despite a number of glaring symptomatic differences between idiopathic PD and encephalitis induced parkinsonianism. For example oculogyric crises and more extensive CNS injury were more commonly associated with encephalitis induced parkinsonianism. Researchers of the period, observing no further outbreaks of influenza, suggested that since exposure to this infectious agent would most likely have occurred between 1910-1920, all present cases of PD, as well as any new cases should decrease in number and no longer be a major medical problem by the year 1980 (Puskanze and Schwab, 1963). Obviously this has not been the case, as the predicted decline in the incidence of the disease through the 1970's and 80's has not occurred.

Other viral, bacterial and fungal organisms have been reported to cause CNS disease. While some of them cause disorders with Parkinson-like symptoms they are clinically different in many ways. With these types of agents, the occurrence of symptomatology is acutely linked to the infection and improves as the infection wanes, or at least stabilizes and does not demonstrate a progressive nature. Also, there is typically a more widespread neurological destruction than that associated with idiopathic PD (Zack and Tanner, 1995). Therefore, with the evidence available, there seems to be no direct correlation between any of these infectious agents and idiopathic PD.

Other infectious mechanisms have been investigated and include latent, slow viral infections, like those suggested for Kuru and Creutzfeld-Jakob diseases. Interest in these types of infectious mechanisms arises due to data which suggests there are Ribonucleic acid (RNA) base changes seen in the globus pallidus of Parkinson's patients (Gomirato and Hyden, 1963). Isolation of such an infectious agent present in Parkinson's patients has not yet been demonstrated.

More recently two other rather unusual infectious agents have been implicated in the pathology of PD. Enzyme-linked immunoabsorbent assay (ELIZA) has demonstrated an increased antibody level to coronavirus in the spinal fluid of Parkinson's patients (Fazzini et al., 1992). In addition, a murine hepatitis virus, a variant of the coronavirus, has been shown to localize in the substantia nigra (Fishman et al., 1985). Another infectious agent of interest is the common soil pathogen, *Nocardia asteroides*. While this soil pathogen is thought not to cause infection in humans, it is capable of

causing neurological symptoms and a persistent movement disorder in mice. Pathological studies in mice have revealed cell damage and inclusion bodies, similar to the Lewy bodies of idiopathic PD in the SN of mice (Kohbata and Beaman, 1991).

Lastly it is possible that exposure to an infectious agent might result in increased susceptibility to whatever the events are that lead to Parkinson's disease. Niagural cell neurons could be damaged leaving fewer neurons to combat an insult by PD. Alterations in genetic material at the time of the infection might not be detected or exert their effects until a much later date. Failure to identify such an infectious agent may simply be due to the lack of investigation into that particular organism, or of a lower sensitivity for its detection by currently available techniques. Finally, an infectious agent may represent only one entity in a multiple component mechanism, leading to the development of Parkinson's disease.

GENETIC CONSIDERATIONS

For many years the search for the cause of PD centered primarily around the idea of some inheritable factor which resulted in the development of the disorder. In recent years the development of the "environmental hypothesis", focusing on a yet unidentified exogenous toxin as the cause of PD, has garnered much of the attention of current researchers. This, along with the inability to identify a single genetic common denominator among PD patients, has led many investigators to all but abandon the idea of a common genetic factor. However, recent developments have again

stimulated interest in the possibility that some intrinsic inheritable factor, may at least in part, play a role in the etiology of PD.

Until about 1937 investigations into the genetic cause of PD included primarily anecdotal accounts concerning the disease. With examination, these early reports demonstrate a pattern that is suggestive of an autosomal dominant factor. However, none of this data was confirmed by clinical or pathological evidence (Golbe, 1995). Reports never alluded to an autosomal recessive or X-linked factor associated with the development of PD. Early kindred studies provided little detail concerning individual cases and descriptions were not precise enough to separate PD from other neurodegenerative disorders.

Only more recently have hereditary studies been done that include at least some post-mortum information. One study involved a family in Salerno, that as of 1993 had 45 members affected with PD (Globe et al., 1990). The effected family members span four generations, and despite some minor abnormalities, like a slightly early age of onset, (46.5) years, they present a typical pattern for PD, including Lewy body formation (Golbe et al., 1990). Another study included only three patients of which two are still living. Pathological examination of the one deceased member revealed a pattern of cell loss and lesions that are typical of PD (Mark and Dickson, 1992).

A number of other studies have been done concerning PD kindred's, and in many of these, either the clinical or pathological presentation is atypical for PD. Therefore their contribution with respect to a genetic link in PD is questionable (Golbe, 1995).

To date, four studies involving twins have been conducted. This includes an ongoing study based in the US using 8000 white male military veteran pairs. In each of these, the reported concordance rates for monozygos twins is much lower than what would be expected in a genetic illness (Golbe 1995). While interesting, all the studies involving twins must be considered carefully due to possible bias in the study design or the way participating members were recruited.

A number of studies have attempted to determine the hereditary mechanism at work in PD. While the largest body of work points toward an autosomal dominant mechanism, a small number of studies suggest a polygenic cause may be at work.

Another area of genetic research concerning sporadic PD centers around two mechanisms, somatic mutation and mitochrondrial genetic defects. Either of these could result in genetic alterations and subsequent development of PD. In one study a dominantly inherited mutation, resulting in the latent onset of neuronal cell death was isolated (Chalfie and Wolinsky, 1990). In this study the gene sequences, which coded for a previously unidentified protein, were located in the genome of the nematode *Caenorhabditis elegans.* This protein seems to alter neural membrane function resulting in nerve cell death, in a manner uncharacteristic of the normal aging process.

Other possible genetic models exist, which could explain some of the unexpected findings reported in family and twin studies. One such mechanism involves DNA rearrangement during development that would cause an increased susceptibility to PD (Johnson, 1991). Another possibility

involves the phenomenon of post conceptual trinucleotide repeat expansion. This phenomenon involves the inclusion of additional nucleotides into a C-G rich repeat sequence during different developmental stages, for example, early in mitosis. The length of the expansion is inversely linked to the age of onset of neurodegenerative disorders, which exhibit this type of genetic expansion. Other neurodegenerative diseases, such as Huntington's disease, Kennedy's syndrome, Myotonic dystrophy, and Fragile X syndrome, exhibit this type of abnormal nucleotide expansion (Golbe, 1995).

There is also the possibility that a mitochondrial genetic deletion exists in PD patients. Mizuno et al., (1989) was able to demonstrate a deficiency in the genome of some PD patients which codes for complex I subunits. This is interesting since it has been shown that complex I activity, which is involved in the respiratory chain, is decreased in PD patients (Parker et al., 1989, Schapira et al., 1990). An inherited disease linked to a mitochondrial mechanism would only be passed maternally since only the ovum contains mitochondria. To date, no such maternally passed pattern seems to exist in any of the kindred studies of PD (Golbe, 1995).

It seems clear that PD, is at least in part the result of hereditary factors, the extent of which is yet to be determined. The idea of a genetic predisposition, in conjunction with activation by some endogenous or exogenous toxic substance is not an unrealistic concept.

NIGROSTRIATAL SYSTEM

The primary dopamine containing systems of the brain have been revealed to be more complex than non-adrenergic systems. The dopaminergic systems contain fifteen to twenty thousand dopamine containing cells per hemisphere, compared to five thousand cells per hemisphere for non-adrenergic neurons (Cooper et al., 1991, Kandel et al., 1991). Based on studies of the anatomy of dopaminergic systems, they can be categorized into three different types. First is the ultrashort system like the one containing the periglomerular dopamine cells. The intermediate length systems include the tuberohypophysial dopamine cells in the arcuate and periventricular nuclei, as well as the incertohypothalamic neurons and the medullary periventricular cells. Long length systems link the ventral tegmental and substantia nigra dopaminergic cells with the caudate nucleus and the putamen, along with the limbic cortex and other limbic structures (Cooper et al., 1991, Kandel et al, 1991). It is the long length systems, which originate in the SN, that are of special interest in the physiology of PD. The nigrostriatal dopaminergic system is located in the mid-brain. This group of neurons is responsible for 70% of all the dopamine present in the brain (Thompson, 1985). Cell bodies in this system project from an area of the lower mid-brain, known as the substantia nigra (SN), with its two divisions, the pars compacta (PC) and the pars reticulata (PR). The cell bodies located in these areas send projections into the corpus striatum, which consists of the putamen, caudate nucleus, and the globus pallidus. These three areas are collectively referred to as the basal ganglia. It is cell

loss in the area of the SN that is the key factor in the development of Parkinson's symptoms, secondary to idiopathic PD and MPTP induced Parkinsonism (German et al., 1992). There is of course no cellular regeneration and little or no compensation by the remaining dopaminergic neurons.

Substantia nigra. The areas of the SN, the pars compacta and pars reticulata, have been recently reconsidered and a more precise means of dividing this area anatomically has been developed. The results of this work have reorganized the area into three nuclei, A8, A9 and A10. A8 includes part of the pars reticulata, A9 includes the former pars compacta, while A10 is known as the ventral tegmental area. All of these areas send neural projections into the striatum (German et al., 1989). Studies of PD patients have shown a pattern of cell loss in all three areas, with the greatest loss occurring in the A9 nucleus.

Many experiments have focused on nerve cell loss in the SN as the main target of PD, as well as MPTP induced Parkinsonism. There is a large body of evidence that has implicated nerve terminals of the nigrostriatum as the area damaged by PD (Hornykiewicz, 1992). If this is true, then cell death most likely occurs via some means of retrograde degeneration or transport of a neurotoxic substance. This is interesting since the neurodegenerative effects of MPTP are first thought to begin in nerve terminals (Herkenhan et al., 1991). This adds further credence to MPTP as a good model for neuronal cell death in the SN in PD patients.

The exact makeup and function of the dopamine terminal in the striatum is uncertain. Once thought to exert a non specific modulatory effect on

striatal neurons, it now seems evident that the median spiny output neurons of the striatum receive symmetrical input from dopamine terminals as well as excitatory input originating in the cortex (Smith and Bolam, 1990). This would suggest an interaction between these two afferent systems, which could result in excitatory signals from the cortex being exaggerated when inhibitory actions are lost due to dopamine depletion. Consequently, this could cause some of the motor symptoms observed in PD such as resting tremor or slowness of movement.

The organization of the circuits in the basil ganglia cannot be easily explained. Recent evidence suggest that multiple, functionally segregated, or parallel circuits do not explain the processing of information or the control of movement by the basal ganglia (Parent and Hazrati., 1993).

Corpus Striatum. The corpus striatum (CPU) receives projections from nerve cell bodies located in the SN. The CPU contains the caudate nucleus and the putamen, and receive incoming stimuli from all areas of the cerebral cortex. Output from striatal neurons is through the thalamus and back to the motor cortex.

Damage to these areas of the striatum results in the different symptoms seen in PD. The caudate nucleus is the site of the greatest striatal dopamine loss (Fearnley and Lees., 1990). Damage in this area results in the disruption of motor signals back to the cortex. The organization of the putamen is such that specific areas of the putamen are linked to specific areas of the motor cortex. Fearnley and Lees (1991) were able to detect a high level of cell loss in the intermediate zone of the caudate nucleus. It is the intermediate zone which is associated with the arm, and symptomatology

of PD most often begins in this extremity. In contrast to this, Moratalla et al., (1992) found a greater loss of uptake sites in the putamen, than in the caudate nucleus. This implies that the putamen is more severely affected by dopamine deficiency than the caudate nucleus. The globus pallidus also receives innervation from the nigrostriatal system, and functionally is responsible for a considerable amount of the output from the basal ganglia. Parkinson's Disease has been shown to have effects in this area as well, resulting in nerve cell disruption and a decrease in neuronal cell numbers (Fearnley and Lees, 1990).

In addition to the dopaminergic projections of the nigrostriatal system, there is also considerable input to parts of the limbic system. The mesolimbic dopaminergic system originates in the ventral tegmental area and sends projections to various areas of the limbic system, parts of the amygdala and hippocampus, and to the nucleus accumbens. The role of the mesolimbic system, and in particular the nucleus accumbens, in emotions, memory and schizophrenia is thought to be considerable. The akinesia, along with some of the cognitive deficits seen in Parkinson's patients may be due, in part to dopamine depletion in the nucleus accumbens (Kandel et al., 1991).

DOPAMINE AND CATECHOLAMINES

To better understand the mechanisms at work in PD, it is necessary to be familiar with the biochemical aspects of neurotransmitters. In general, neurotransmitters are those substances synthesized by neurons that function

to influence other tissues. Neurotransmitters can act upon neurons, glandular, or muscle tissue, and can produce profound effects in these target tissues. Catecholamines, of which dopamine is an example, are a specific class of neurotransmitter.

Catecholamines contain what is known as a catechol nucleus, which has at its center a benzene ring with two adjacent hydroxyl groups, along with an amine group. Catecholamines are synthesized in a number of sites. The amino acid tyrosine serves as the precursor and can be found in the brain, sympathetic nerves, chromaffin cells, and the sympathetic ganglia (Cooper et al., 1986). The synthesis of catecholamines requires a sequence of enzymatic reactions, which was first postulated in 1939 and then confirmed in 1964. Tyrosine is first converted into 3,4-dihydroxy-L-phenylalanine (L-Dopa) through the actions of tyrosine hydroxylase (TH), which uses molecular oxygen to catalyze the addition of a hydroxyl group in the meta position on tyrosine (Siegel et al., 1989). L-Dopa is then converted to dopamine (DA) through the actions of Dopa decarboxylase, more accurately referred to as L-aromatic amino acid decarboxylase since it will act upon all aromatic L-amino acids. Once formed, dopamine is taken up and sequestered in storage vesicles. Further modification of the catecholamine can occur if the proper enzymatic substrates are present. Dopamine can be converted to norepinephrine by dopamine beta-hydroxylase, and finally to epinephrine, if phenylethanolamine N-methyltransferase (PNMT) is present. The pace at which this biosynthetic process takes place is determined by the availability of tyrosine hydroxylase, and therefore constitutes the rate limiting step.

Once synthesized dopamine can be concentrated in nerve terminals through a process of active transport which utilizes an ATP dependent pump (Siegal et al., 1989). These vesiculated catecholamines exist in complexes with adenosine triphosphate (ATP) and chromogranins, which are acid proteins. Once stored, these vesicles act as a ready releasable supply of the neurotransmitter.

Release of dopamine occurs through a calcium dependent mechanism of exocytosis. Once stimulated by an action potential propagating down the nerve, calcium channels in the nerve terminal open, allowing the influx of this positively charged ion into the nerve terminal. This increase in cytosolic calcium levels provides the stimulus for the fusion of dopamine containing vesicles with the plasma membrane in the area of the nerve terminal. Dopamine in the vesicles is then released into the synaptic cleft where it can combine with its receptors on the post-synaptic membrane (See Figure 1).

Deactivation of dopamine occurs by way of several processes. Dopamine can be catabolized by the enzymatic actions of two different enzyme substrates. Monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT) are the enzymes responsible for the biochemical deactivation of dopamine. MAO contains flavin and is located on the mitochondrial outer membrane of both pre and post synaptic cells. Deactivation occurs through a process of deamination, resulting in the generation of an aldehyde (Siegal et al., 1989). Two forms of MAO have been identified, MAO-A which deaminates norepinephrine and serotonin preferentially, and MAO-B which acts on a wide range of phenylethylamines, including dopamine. COMT is found in nearly all cell

types and can be seen in association with the post synaptic cell membrane in neural systems.

Catecholamine metabolism results in products, that when measured, can provide insight into the turnover of these products in the brain. In the case of dopamine, the major metabolic products are 4-hydroxy-3-methoxy-phenylacetic acid, more commonly known as homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC), and a small amount of 3-methoxytyramine. Accumulations of HVA in the brain and spinal fluid, has quite often been used as an index to determine the activity of dopaminergic neurons. In the rat brain DOPAC levels in the striatum have been shown to provide an accurate picture of the function of dopaminergic neurons in the nigrostriatal pathway (Cooper et al., 1991).

The most striking biochemical changes associated with PD are the decreases seen in dopamine content and its metabolites. Along with decreased levels of dopamine itself, other biochemical changes associated with dopamine synthesis can be seen with PD. Tyrosine hydroxylase, the enzyme responsible for the synthesis of dopamine from L-Dopa, is also decreased. There are four types of tyrosine hydroxylase found in the human substantia nigra and decreases in the messenger RNA for all four have been demonstrated in the brains of Parkinson's patients (Nagatsu and Ichinose, 1996). Lower amounts of tetrahydrobiopterin (BPH), a cofactor of tyrosine hydroxylase, and GTP cycolhydrolase (GCH-1), the enzyme necessary for its synthesis, are also reduced in Parkinson's patients (Nagatso and Ichinase, 1996).

The severity of dopamine loss in the brains of Parkinson's patients varies from region to region, with some of the largest decreases seen in the putamen, caudate nucleus, and both the pars compacta and the pars reticulata of the SN. Decreases of dopamine content in the putamen to 3%, in the caudate to 15%, and to 21% and 13% respectively in the pars compacta and pars reticulata when compared to control values have been reported (Siegal et al., 1989).

Catecholamines, not inactivated by MAO or COMT, can also be removed from the synaptic cleft through a powerful active reuptake mechanism located in the presynaptic nerve terminal. This process is mediated by a specific carrier molecule for the particular catecholamine in question. This transporter is an energy dependent mechanism. Actions of these carriers are concentration dependent as well, obeying Michaelis-Menten kinetics. The reuptake process is linked to the sodium gradient across the membrane. This is important because during depolarization and release of catecholamines, the reuptake process is suspended. In recent years amine transporters have been reorganized as part of a new gene family known as biogenic amines. Cloning of these biogenic amine transporters is now possible. The first sequencing information was determined from studies with GABA, and noradrenaline transporters followed by isolation of copy DNA (cDNA) for the dopamine transporter (DAT). All of the transporters isolated to date seem to share significant amino acid identities ranging from 30% to 78% (Pifl et al., 1996). Analysis of the transporter reveals an arrangement of twelve hydrophobic amino acid sequences representing the membrane spanning domains (Pifl et al., 1996). Studies

using chimeric proteins from related protein families allow for exchange of protein segments followed by observation for functional activity. Results from such studies, suggests that the major determinants common to all transporter function exist in an area from the N-terminis to the fifth transmembrane domain. The region from the fifth through the eighth domain determines interactions with tricyclic antidepressants. The region of the C-terminis, including parts of the eighth and twelfth transmembrane segments, confers substrate specificity and stereoselectivity (Pifl et al., 1996).

Due to the specificity of these transporters, they can be selectively inhibited, through the use of certain pharmacological agents. In addition to inhibition, a variety of substrates, such as amphetamines and cocaine, as well as certain toxins, can bind to the transporter and compete with the normal catecholamine substrate. Such competition could effectively displace the original substrate, thereby affecting the functional levels of catecholamines in the presynaptic nerve terminal. It is thought that this is the mechanism, by which the neurotoxin MPP^+ displaces dopamine from nerve terminals, triggering the release of this transmitter and inhibiting its reuptake into the presynaptic neuron.

NERVE CELL DEATH

Parkinson's Disease is a progressive disorder resulting from neurodegeneration and neuronal cell death at the heart of the disorder. Neurons die via a number of different methods secondary to a variety of metabolic disturbances and some of these mechanisms are implicated in PD.

Oxidative stress. Since oxygen is necessary for all higher forms of cellular life, the reduction of oxidative species is an integral part of cellular survival and energy production. Reduced oxidative species can be produced through a number of chemical reactions and are necessary intermediates in the production of cellular energy (Irwin and Langston, 1995). When these same reduced species are found in too high a concentration they can become detrimental to cellular survival, resulting in a condition known as "oxidative stress", with subsequent cell damage occurring. Cellular destruction is a result of oxidative species interacting with lipid membranes, protein sulfhydryls, and similar groups, or by disrupting DNA (Halliwell and Glutteridge, 1985).

Oxygen free radicals are formed by the transfer of electrons to molecular oxygen, culminating in the generation of several possible oxygen free radical species (Irwin and Langston, 1995). A stepwise reduction of oxygen gives rise to several reactive species, including O_2^- (superoxide anion radical), PO_2^{-2} (peroxide ion), and OH (hydroxyl radical). Interactions between several of these species, such as the hydroxyl radical and hydrogen peroxide, can be facilitated in the presence of an oxidizable transitional metal ion. Some researchers have suggested that hydroxyl radical generation may be the critical component to nerve cell cytotoxicity. Hydroxyl free radicals are extremely reactive and enter into chain reactions, which produce other radical species (Irwin and Langston, 1995). In most situations, the production of hydroxyl radicals, by the Haber-Weiss reaction, is very slow. The addition of even small amounts of transitional metals, such as iron or

copper, acting as a catalyst, substantially increases the production of hydroxyl free radicals.

Some oxygen free radicals are produced in the normal course of cellular metabolism. Therefore, there are mechanisms in place to deal with the detoxification of such species. Superoxide dismutase (SOD) is an enzyme that reduces superoxides to hydrogen peroxide and oxygen. Hydrogen peroxide reacts with catalase and is converted to oxygen and water in the cell, and by glutathione peroxidase in the mitochondria. Small molecules such as vitamin C and E can also provide some defense against reactive oxygen species by acting as free radical scavengers. These small molecules enter into covalent bonds with the reactive species and deactivate them.

In PD there are some biochemical changes, or conditions, which could lead to situations of accelerated generation of reactive oxygen species, resulting in oxidative stress. In the substantia nigra, the levels of superoxide dismutase activity is as much as 50% higher than in other areas of the brain (Marttila et al., 1988). This suggests higher amounts of superoxide in the area of the SN. As a result of increased levels of superoxide activities, we would expect to see increased formation of peroxide. This sets up a dangerous situation, since studies suggest catalase activity and glutathione peroxidase levels are not substantially increased in the SN. Consequently, the increased peroxide levels in the SN would not be compensated for by elevated levels of the substrates necessary for peroxide detoxification (Marttila et al., 1988, Kish et al., 1985). Studies done with PD patients have shown decreased levels of catalase (Marttila et al., 1988), and

glutathione peroxidase activity (Kish et al., 1985) when compared to non Parkinson's diseased patients (Perry et al., 1982, 1986).

Therefore there is a reasonable body of evidence in support of oxidative stress as a possible component to the nerve cell destruction associated with PD.

Calcium. Normally, free calcium is maintained in relatively low levels in the cytosol when compared to extracellular calcium levels. This is accomplished by an active means of transport of calcium out of the cytosol and through the sequestering of intracellular calcium within the mitochondria or endoplasmic reticulum. When these closely regulated calcium levels are disturbed, and there is an increase in cytosolic free calcium, the resulting conditions can negatively impact cellular membrane integrity, and consequently cell survival. This negative impact is a consequence of calcium's activation of certain protease's and phospholipases that can act upon cell membranes, membrane proteins, and other cytosolic structures, resulting in compromised cellular integrity.

Several endogenous or exogenous toxins are believed to be activated though calcium dependent mechanisms. Once activated, these toxins could affect cellular metabolic processes, thereby causing cellular ischemia and eventual cell death (Choi, 1988). The best example of this is calcium's known role in activating amino acid toxicity through N-methyl-d-aspartate (NMDA) receptors. Further examples of toxins requiring elevated cytosolic calcium levels for activation, include, diquate, hydroperoxides, and toxic quinones (Irwin and Langston, 1995).

As of yet there is little evidence to suggest altered calcium homeostasis as a component of PD. At this time, little research is being done in this area. Interestingly, there is some evidence that suggests that neurons, which contain the calcium binding protein calbindin, are spared in PD (Yamada et al., 1990) and MPTP induced Parkinsonism (German et al., 1992). Such discoveries may lead to a new direction in the investigation of neuronal cell death secondary to PD.

ATP Synthesis Disruption: Energy Depletion. Probably the most recently investigated mechanism of cellular death, in relation to PD, has been that of energy depletion. We know that adenosine triphosphate (ATP) is the energy rich compound utilized during many basic metabolic cell processes, for example, the operation of transport pumps, the maintenance of cytoskeletal elements, and of course, the synthesis of DNA and proteins.

ATP is produced in the mitochondria through a complex stepwise operation. This entire process is referred to as the mitochondrial respiratory chain. There are four main steps involved in this synthetic process. Each step is controlled by a specific enzyme complex. These complexes catalyze specific reactions at each step, resulting in a product which can then enter into the next phase of the overall reaction. There is evidence that a number of toxic substances, such as Rotenone, Piericidin, and MPP^+, are shown to exert their affects at some point along this mitochondrial respiratory chain (Tipton and Singer, 1993, Ramsay et al., 1991, 1989, Gerlach et al., 1991, Singer et al., 1987,).

There are several ways that such disturbances of the respiratory chain can be accounted for in PD. First, some genetic abnormality could be

present in the mitochondrial DNA which codes for the complex I subunits, resulting in the faulty synthesis or operation of these complexes. In the past, studies have been unable to identify with any consistency, where in the mitochondrial DNA of Parkinson's patients, such a site might be located (Ikebe et al., 1990, Lestienne et al., 1990, Schapira et al., 1990a, 1990b, 1990c). There is also the possibility that mitochondrial defects may be the result of some mitochondrial toxin. However, it is now clear that disruption of ATP synthesis is a component of idiopathic PD. Studies have clearly shown a reduction in complex I activity in several tissues, including the striatum and the SN (Shoffner et al., 1992, 1991, Yoshimo et al., 1992, Hattori et al., 1991, Schapria et al., 1990, 1989,). Deficits in complex I have also been documented with MPTP induced parkinsonism (Swerdlow et al., 1996, Cleeter et al., 1992). While complex I seems to be the primary site affected by these toxic agents, there has been some information that suggests the possible involvement of other areas in the respiratory chain (Shoffner et al., 1991). If disruption of these mitochondrial mechanisms can be definitively linked to neural cell death in PD, it would be persuasive evidence in support of energy depletion as the primary cause of cell loss in neurodegenerative diseases.

Apoptosis. Apoptosis is by definition the process of programmed cell death. Here we see cells die secondary to a genetically encoded message that is turned on at a specific time in response to some particular stimuli. This process has been shown to occur naturally in specific cell types, for example, neutrophils. Some of the stimuli thought to activate this process of cellular self-destruction include chemical compounds such as tributyltin and

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), (Irwin and Langston, 1995). Other contributing factors might be the withdrawal of necessary growth promoting factors, or secondary effects due to increased levels of cytosolic calcium. Calcium levels and denovo protein synthesis are two areas of current interest concerning apoptosis. It seems calcium chelators and protein synthesis inhibitors may block the process of apoptosis in some cell types. Taken together this would suggest a process linking cytosolic calcium levels and regulatory protein control.

At this time, there is no direct evidence to connect the cell loss seen in PD to a process of apoptosis. However, there have been some interesting findings in relation to apoptosis in the use of MPTP. Dipasquale (1991) observed structures associated with apoptosis in cerebral cell cultures after treatment with MPP^+. The previously discussed mechanisms of cell death, while limited in number, are not necessarily unrelated events. The effects we see associated with disturbances in mitochondrial respiration can easily be influenced by properties present in the other mechanisms. Decreases in ATP synthesis and subsequent depletion of ATP stores could create a more susceptible environment for oxidative stress to occur with the generation of reactive oxygen species. Reduction of ATP could also contribute to rising cytosolic calcium levels by shutting down mechanisms responsible for sequestering calcium in the cell, resulting in disruptions of the calcium gradient. When viewed in this manner it is not unrealistic to see the interrelation of multiple factors culminating in the neurodegenerative process of PD.

MPTP

MPTP is a contaminate in the biosynthesis of synthetic heroin. This compound first came into notoriety when a number of young I.V. drug users in northern California accidentally injected this contaminant, along with the desired drug, synthetic pethidine. Initially upon injection, the subjects experienced a number of symptoms, including hallucinations, visual changes, a metallic taste, and irregular uncoordinated movements of the extremities (Ballard et al., 1985, Langston et al., 1983). Following repeated use of the substance individuals developed numerous motor disorders which increased in severity with use, eventually becoming chronic. Symptoms expressed included muscle stiffness, rigidity, delayed movement, resting tremor, and micrographia (a decrease in handwriting size). Such motor symptoms are virtually identical to those seen in Parkinson's Disease (Langston, 1995). Consequently, examination of these patients revealed striking similarities to idiopathic PD, including alleviation of symptoms with classic treatment regimes for PD. Just as in PD, these patients experienced the same decrease in effectiveness that is seen with long term treatment, including fluctuations in effectiveness (the so called on-off effect), dyskinesias, hallucinations and a narrowing of the therapeutic window (Langston, 1995).

While most of these original subjects are still living, there was a case of one young man who committed suicide, making part of his brain available for pathological study. Examination revealed a striking similarity to the histological studies of the brains of PD patients. While no Lewy bodies

were seen, there was a substantial loss of nerve cells in the substantia nigra, suggesting a specificity of nerve cell degeneration never seen before except in PD.

Animal models. Animal models intended to mimic disease processes are many times limited, in that we are never actually sure if the anticipated effects would actually be seen in humans. Unfortunately, in the cases of accidental MPTP use by the I.V. drug users, we have been able to see the disastrous effects of this drug in human subjects. Since its discovery, MPTP has been administered to a variety of animals from invertebrates to non-human primates. While various animal trials have been attempted, the most widely utilized models involve primates or rodents. In rodent models, both rats and mice have been used. While rats seem insensitive to systemic MPTP exposure, (Boyce et al., 1984) toxic effects can be observed with intranigral injection of MPP^+, the primary metabolite of MPTP (Chiueh et al., 1984). Mouse studies provide a good model for neural plasticity or regeneration, since there is considerable recovery from the effects of MPTP in this species. The primate model seems best suited to studies of neurotoxicity or behavioral features (Langston, 1995).

The extent to which MPP^+ exerts its chronic neurotoxic effect appears linked to the age of the animal (Forno et al., 1988), (Gupta et al., 1986). Aged C57 black mice, at twelve months, were more severely effected by MPTP than were young mice of the same strain. Recovery in aged animals was also effected with only minimal improvement being reported for the aged mice (Date et al., 1990).

Bioactivation of MPTP. Evidence that MPTP must first be activated to exert its effects was provided by Chiba et al., (1984). It was demonstrated that rat brain samples metabolized MPTP to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and MPP^+. Simultaneously, it was shown that monoamine oxidase (MAO) inhibitors, in particular those of the B form, could effectively block this bioactivation process (Chiba et al., 1984). Both forms of MAO have been shown to oxidize MPTP *in vitro* with the rate of MAO-B activity being some fourteen times greater than that of MAO-A (Salach et al., 1984). When MAO-B activity is blocked the small amounts of MPP^+, formed through activation by MAO-A, has been shown to be insufficient to cause neurotoxicity (Buckman, 1991). MPTP is initially oxidized to the $MPDP^+$ ion, which is then further oxidized to MPP^+. The reaction is self-limiting, in that the amount of MPP^+ formed depends on the amount of MAO-B present. This could be a means to explain some of the species specificity seen in response to this neurotoxin, since the levels of MAO-B vary from species to species.

MPP^+ is not effective when administered peripherally since, due to charge, it does not cross the blood brain barrier. Furthermore, MPP^+ is not effected by MAO inhibitors, which suggests the role of MAO is limited to the oxidation of MPTP (Tipton and Singer, 1993).

Sites of MPTP Activation. The neurotoxic effect of MPTP or MPP^+ can be prevented by pretreatment with a presynaptic dopamine uptake inhibitor (Javitch et al., 1985). This suggests that MPTP's selective toxicity for dopaminergic neurons, may be linked to the specific energy dependent uptake of the metabolite MPP^+ by pre-synaptic terminals. This evidence

points to the activation of MPP^+ outside the nerve terminal. Presently, it is generally accepted that glial cells are the sites of MPP^+ formation. This has been demonstrated in cultured astrocytes where MPTP is taken up and then metabolized to MPP^+ (Marin et al., 1992, Ramson et al., 1987, Javitch and Snyder, 1984). MPP^+ is then readily released from these astroglial cells. Since astroglial cells surround the dopaminergic neurons, the close proximity would permit easy access of MPP^+ to these dopaminergic neurons.

There is a relatively large body of evidence, which points to MPP^+ as the primary neurotoxic agent in MPTP models of PD. The most convincing of this evidence includes:

1. MPP^+ can accumulate in the dopamine nerve cells of the substantia nigra following systemic administration of MPTP (Irwin and Langston, 1985).
2. MPP^+ is toxic to dopaminergic neurons raised in culture (Mytilineau et al., 1985).
3. MPP^+ infused directly into the striatum and substantia nigra of rats produces toxic effects (Heikila et al., 1985a).
4. MPP^+ toxicity can be prevented through the use of re-uptake inhibitors (Javitch et al., 1985).
5. MPP^+ inhibits the synthesis of dopamine via enzyme inhibition (Ozaki, 1988).

Mechanisms of MPP^+ Neurotoxicity/ Free Radical Generation.

One hypothesis, concerning the events leading to the destruction of the dopaminergic neurons, is the generation of a toxic free radical or superoxide

species during the reduction and re-oxidation of MPP^+. It has been suggested that when such compounds are generated in sufficient quantities, they could overwhelm the cell's capacity to neutralize them. This type of action would be similar to the mechanism of the herbicide, paraquat, a compound similar in structure to MPTP (Kopin, 1986, Mytilineou et al., 1985). It has also been suggested that this free radical generation may be enhanced in the presence of certain heavy metal ions (Mytilineou et al., 1985). Electron spin resonance studies have provided some support for this hypothesis. Such studies have shown an accelerated accumulation of lipofusion in, and peroxidation of, lipid membranes (Rios et al., 1987). Some studies also point to MPTP and MPP^+ enhancing the auto-oxidation of dopamine. If this occurs it could lead to free radical generation as well (Poirier et al., 1985). However, there is some evidence against this type of process. Treatment with superoxide inhibitors should, in theory, result in an improvement. This does not seem to be the case. Studies using a superoxide dismutase inhibitor and a copper chelator (diethyldithiocarbonate) have resulted in more sever MPTP neurotoxicity (Corsini et al., 1985). This is not suggestive of an oxygen free radical formation problem. Oxygen free radical scavengers, like metal chelators, should have a protective effect but results from DiMonte et al., (1989) have not supported this idea. Because of conflicting results with respect to free radical generation, this process can not be considered the definitive cause of MPTP induced neurotoxicity.

Mitochondrial Effects of MPP^+. In recent years, there has been an increase in experimental evidence to support the hypothesis that MPP^+

expresses its neurotoxic effects through a mechanism, which ultimately causes mitochrondrial damage. It is thought that this damage stems from interruption of the respiratory chain in the region of complex I. It has been suggested that MPP^+ blocks the reoxidation of NADH dehydrogenase by ubiquinone (Vyas et al., 1986). The events, which lead to the disruption of the mitochrondrial respiratory chain, have been clarified to some extent. First, MPP^+ must be concentrated in the mitochondria. This is accomplished through a pumping action of the inner membrane, and is energized by the electrical gradient present. The evidence to support this stems from studies requiring much higher concentrations of MPP^+ to block NADH oxidation in disrupted mitochrondrial particles, than in intact mitochondria (Ramsay and Singer, 1986). While this suggests the presents of a specific carrier molecule, other studies using tetraphenylboron ion (TPB^-) demonstrated a more passive means of transport. TPB^- is known to facilitate the entry of positively charged particles into the mitochondria (Heikkila et al., 1990). Studies by Davey et al (1992), using an ion selective electrode, indicated a rate of transport, which was non-saturable. These results suggest the actions of a non-carrier mediated transport mechanism. The inhibition of the respiratory chain seems to involve two sequential events: Concentration against a gradient into the mitochrondrial matrix followed by a slower penetration into a portion of the NADH dehydrogenase molecule (See Insert Figure 1). The actual cessation of electron transport from NADH dehydrogenase to ubiquinone, occurs by some as yet unexplained method. The location of complex I inhibition, being at the Fe-S cluster in NADH dehydrogenase, is supported by the finding that MPP^+ does not inhibit

succinate respiration (Nicklas et al., 1985), nor does it interfere with ferricyanide reduction by complex I (Ramsay et al., 1987). Interruption of the respiratory chain would then be followed by the rapid depletion of cellular stores of ATP, making it impossible for cell survival. Such ATP depletion and cell death is seen in studies using hepatocytes treated with MPTP and MPP^+ (Di Monte et al., 1986), neuroblastoma cells (Kutty et al., 1991), PC 12 cells (Denton et al., 1987) and in mouse brain synaptosomes (Scotcher et al., 1990). There is further support for this mechanism as the cause of cellular death secondary to MPP^+ administration. Studies using rotenone, a known complex I inhibitor, results in the same pattern of damage to striatal neurons as that seen with MPP^+, when administered to the rat median forebrain (Heikkila et al., 1985b).

Disruption of Calcium Homeostasis. Some studies have suggested that the critical factor leading to cell death is a depletion of mitochrondrial Ca^{+2}, followed by an increase in cytosolic calcium levels (Kass et al., 1988). While there is little evidence to connect cell death and calcium overload, it could serve as a link between the otherwise different MPTP and 6-hydroxydopamine generated neurotoxicities (Turski et al., 1991).

Dopamine Oxidation. Both MPP^+ and $MPDP^+$ have been shown to cause a dopamine release in the striatum. It has been suggested that the oxidation of this dopamine is directly responsible for hydroxyl radical formation (Chiueh et al., 1992). When dopamine auto-oxidizes we see the formation of semiquinone radicals and melanin. It is thought that this melanin is deposited in the neurons of the substantia nigra, as a result of oxidative stress (Chiueh et al., 1993).

Nigrostriatal Selectively of MPTP. While there has been no complete explanation as to how MPTP exerts such a strong selectivity for neurons of the substantia nigra, a number of factors have begun to point in the same direction. If we assume that the hypothesis of mitochrondrial respiratory chain disruption, followed by ATP depletion is correct, we can then begin to draw some basic conclusions. For MPTP to be toxic it must first be converted to MPP^+. Then MPP^+ acts as a substrate for a membrane carrier system where, once inside the cell, it has to remain long enough to gain entrance into the mitochrondrial matrix. Here it can exert its effects on the respiratory chain. The studies demonstrating the involvement of the high affinity dopamine uptake system, as the method of entry into dopaminergic neurons for MPP^+, help explain the selectivity of this toxin (Chiba et al., 1985, Javitch et al., 1985). The fact that other tissues and organs do not have such a concentrating mechanism for MPP^+ could explain its selectivity for the CNS and the neural areas previously mentioned. Enhanced striatal cell sensitivity, as compared to other dopaminergic cells, could be linked to the sequestering effects of neuromelanin. Neuromelanin has been shown to bind to MPP^+ (D'Amato et al., 1986). This would provide a means by which the cells could retain the MPP^+ until transported into the mitochondria. Other organs and tissues may contain various enzymatic mechanisms, which would metabolize MPP^+, via a different route, to some non-toxic compound (Singer et al., 1987). Other factors, such as those that allow for the removal of toxic metabolites, or those that involve the regenerative capacity of certain cell types, may also be of importance.

Mitochrondrial Function as it Relates to Idiopathic PD. The disruption of mitochrondrial function that is seen with MPTP treatment appears to parallel that which is seen in idiopathic PD. Studies have shown that levels of NADH-COQ reductase activity are significantly reduced in the SN and in the putamen of Parkinson's patients (Schapira et al., 1990, Mizuno et al., 1990). The cause of this decreased activity is not clear. Such effects could be due to some toxic process like that observed with MPTP, or there could be some genetically predisposing factor. While current evidence does not seem to support the latter (Marsden, 1987), there may be some inherited characteristic through an autosomal dominant process (Golbe et al., 1990).

Aging and MPTP. Researchers have demonstrated a decrease in mitochondrial activity with aging (Victoria et al., 1986). Species sensitivity to MPTP has also been shown to increase with age. For example, Irwin et al. (1992) and Lange et al. (1990) were able to demonstrate such increases in sensitivity in rodents. Similar increases have been seen in primate models (Forno et al., 1988). This reported age related increase in sensitivity to MPTP may be due to several factors. With aging, there may be a decline in the nigrostriatal neuron number with a subsequent increase in glial cells, and/or a decrease in dopamine levels. This would result in a compromised nigrostriatal neuronal system that is more susceptible to the actions of MPTP.

Age is also seen as a variable in the types of neural lesions that are present. Inclusions, similar to Lewy bodies, have been identified in aged chronically treated MPTP animals versus younger specimens (Forno et al., 1986). In certain species such as rats, mice, cats and dogs it has been

possible to demonstrate a time dependent recovery from the toxic effects. However, aged animals were much less able to recover from these same toxic effects (Rose et al., 1989). Lastly, melanin levels seem to increase with age, and melanized neurons show a greater sensitivity to the degenerative effects of MPTP and PD (Hirsch et al., 1988).

Relevance of MPTP and MPP^+ to Idiopathic Parkinson's Disease. To date, the MPTP model, even with it's limitations, is the best and certainly the most effectively investigated animal model for PD. Investigations into MPTP's mechanisms of neurotoxicity have provided considerable information as to the methods surrounding nerve cell death. Reports of similarities of mitochondrial dysfunction and ATP depletion, in both MPTP animal models and idiopathic PD, is very encouraging. The differences that do exist, between PD and MPTP induced neurotoxicity, may be due in part, to inherent differences, which may exist when comparing an acute toxic effect as seen with MPP^+, to a chronic progressive degenerative process as with PD (Tipton and Singer, 1993).

It is clear that there are still a number of questions to be resolved concerning the activation and toxic effects of MPTP as it relates to idiopathic PD. Despite these limitations, MPTP and it's metabolite MPP^+, provide us with a model that is most indicative of the neurodegenerative effects seen in PD. In this study, this neurotoxin, will serve as an excellent tool for an investigation into the dynamics of dopamine release in the corpus striatum of the rat brain.

ESTROGEN \ 17-BETA ESTRADIOL

Estrogen is a gonadal steroid hormone produced primarily by the female ovaries. The amounts of estrogen, like other female gonadal steroid hormones, fluctuate throughout the menstrual cycle. Estradiol is the major steroid hormone during the follicular phase of the cycle. The follicular phase is the period commencing with day 0 up to day 14, of a normal 28 day cycle. As estrogen predominates during this time, the follicle begins developing. Estrogen levels continue to increase, due to the maturing follicle, resulting in a negative feedback effect on follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary.

Along with its stimulatory role during the early stage of follicle development, estrogen is also necessary for the adequate development of the female secondary sex characteristics. These include development of pubic hair, additional deposits of adipose tissue, and mammary gland development (Hadley, 1996).

Estrogen synthesis is believed to begin in thecal cells of the ovary under the influence of luteinizing hormone. Once the C-19 form is synthesized it is transferred to the granulosa cells where it is modified to the C-18 estrogen (Hadley, 1996). As an alternate means of biosynthesis it has been suggested that luteinizing hormone stimulates the C-19 androgen synthesis in the thecal cells, where it is then aromatized, as well as being sent to granulosa cells, where aromatization can also occur. In this model, estrogen from the thecal cells provides the source for circulating estrogen, where as estrogen from

the granulosa cells acts in a local role during follicle development (Hadley, 1996).

Estrogen is a steroid hormone that is derived from cholesterol. This biosynthetic process is a cyclic AMP (cAMP) dependent process that may proceed via several different pathways. The primary route of biosynthesis begins with the conversion of cholesterol to pregnenolone, then to 17 alpha-hydroxypregnenolone and on to dehydroepiandrosterone. From this point, the conversion continues to androstenedione then to testosterone, which is acted on by aromatase and is converted to estradiol (Hadley, 1996). Estradiol is oxidized by the liver into estrone and estriol, which can then be excreted in the urine.

The ovarian follicle is not the only source of estrogens. The placenta, the adrenals, as well as some peripheral tissues, can produce substantial amounts of estrogens from steroid precursor molecules. For example, testosterone and androstenedione can be aromatized by certain tissues, and serve as the major source of estrogens in men and post-menopausal women (Hadley, 1996).

Estrogen as it Relates to Dopamine. Estrogen's effects on striatal dopamine release were first illustrated in 1980. Findings at that time suggested that gonadal hormones had a modulatory effect on striatal dopamine release (Becker and Ramirez, 1980). Researchers found increases in dopamine release and decreases in dopamine tissue content in both young and aged female rats (McDermott, 1993). Discharge rates and autoreceptor sensitivity are also effected (Chiodo and Caggiula, 1983). Changes can be seen in non-reproductive dopamine mediated behavior

(Becker, 1990), and sensory motor task performance (McDermott et al., 1994). Striatal dopamine receptors are of two types, D_1 and D_2. In the rat striatum, increases in the density of both types have been seen in response to estrogen treatments, with increases of 48% and 21% for D_1 and D_2 receptors respectively (Hurska and Nowak, 1988). Prolonged estrogen treatment may result in a shift of the D_2 receptor affinity from high to low (Gomez and Bedard, 1992). Following overectomy (OVX), and in response to acute estrogen administration, female rats demonstrated an increase in dopamine uptake sites. Treatment with progesterone did not have the same effects. The affinity of these uptake sites for their substrate remained unaffected by treatment with either hormone (Morissette et al., 1990).

Dopamine appears to exist in at least two storage sites in the nerve terminal. These are, the readily-releasable and the vesicular storage pools (Justice et al., 1988). While blocking reuptake, amphetamines appear to stimulate the release of dopamine from the readily-releasable pool, while potassium (K^+) effects the release from the vesicular pools. Currently it is thought that estrogen's effects are associated with this readily-releasable dopamine pool in older animals, and the vesicular pool in younger animals (McDermott, 1993).

While evidence seems to substantiate earlier theories concerning estrogen as a modifier of striatal dopamine release, the exact mechanism by which this occurs, remains unknown (Becker, 1990). The concept of nongenomic membrane effects by steroid hormones in the brain is not a new idea. However, supporting biochemical evidence for such membrane effects remains inconclusive. These membrane effects could be the result of

binding to specific extracellular membrane receptors, or, since steroid hormones are lipophillic, through their incorporation into the neural plasma membranes, where they could modify there structure or action, or the action of other membrane proteins.

Data representing the effects of estrogen on striatal dopamine function remains somewhat contradictory. Increased striatal dopamine availability may be beneficial for some dyskinetic symptoms like those seen in early PD. In contrast, prolonged effects may lead to further dopamine depletion and to an increase in the severity of PD and it's associated symptoms (Session et al., 1994, McDermott, 1993).

The role of estrogen as a neuroprotectant has been hypothesized (Bedard et al., 1977) and *in vitro* studies have suggested a decrease in the effectiveness of MPTP to stimulate dopamine release in the presence of estrogen (Dluzen et al., 1996). This protective capacity of estrogen may be linked to its effects on the dopamine transporter. It has previously been shown to increase transporter density without effecting transporter affinity (Morissette et al., 1993, Morissette et al., 1990, Ramirez, 1983). Furthermore, potential nongenomic acute membrane effects of this steroid hormone in modulating DA release and reuptake were investigated in this study.

NEW PHARMACOLOGICAL TREATMENTS

To be effective, treatment of any disorder, including PD, requires a clear understanding of the nature of the disease process and its progression.

Unfortunately, the complete etiology of PD remains unknown, and the pathophysiology behind the disease process is still poorly understood. With this in mind, it is understandable why new treatment regimes have developed so slowly.

Effective management of the disease requires dealing with the side effects of long-term L-DOPA treatment. Some of the resulting problems are dyskinesias, unstable mobility, and increased mental confusion. These symptoms are due at least in part, to L-dopa's down regulation of both D_1 and D_2 dopamine receptors.

Current development of therapeutic agents for the treatment of PD generally fall into one of four categories; catechol-O-methyl transferase (COMT) inhibitors, monoamine oxidase B inhibitors (MAO-B), glutamate receptor antagonists or partial dopamine agonists.

Normally only between 5 and 10% of each dose of L-DOPA reaches the brain. The remainder is acted upon by COMT and is converted into the inactive metabolite 3-O-methyldopa (OMD) (Bracco, 1996). Treatment with COMT inhibitors, which are non-toxic, could increase the availability of L-DOPA by increasing its serum half life. Examples of such drugs currently under investigation are entacapone (OR-611) or tolcapone (RO-40-7592).

MAO-B inhibitors have been widely investigated in recent years, due to their potential neuroprotective effects. Drugs of this type include selegiline (l-deprenyl) and others. MAO-B inhibitors may provide beneficial effects by, increasing dopamine transmission or, by protecting against the generation of free radicals. Other MAO-B inhibitors being developed, such

as lazabemide (RO-196322) are shorter acting and are more reversible than earlier compounds (Bracco, 1996).

Interest in the development of glutathione antagonists has also increased, due to evidence that this excitatory neurotransmitter may be involved in PD. It has been suggested that excessive activity of glutathione receptors, are at least in part, responsible for mediating neural cell damage and death (Rupnik et al., 1992). Glutathione antagonists could decrease the effects of this compound, or protect nerve cell populations from damage.

There are two classes of glutathione receptors: ionotropic receptors, that are linked to membrane ion channels, and metabotropic receptors, which are associated with G-proteins and are believed to modulate second messenger systems. Compounds selective for these receptor sub-types have been less than successful. MK801 and NBQX were at first observed to relieve symptoms and improve motor function in PD patients. More recently, results have not been clearly repeatable, and unwanted side effects may be a problem (Rupnik et al., 1992).

Partial dopamine agonists could possibly achieve a more desirable level of receptor activity than full agonists have been able to accomplish. Several compounds are currently under going clinical trials, the most promising of which are terguride a 9,10-trans-dihydrogenated derivative of lisurside, as well as preclanol, an enantiomer of 3-hydrox-phenyl-N-n-propylipiperidine (3-PPP). Preclanol is thought to have more selective dopamine auto-receptor and post-synaptic effects (Bracco, 1996).

In the light of the current development of anti-parkinsonian therapeutic agents, it seems clear that any gains in this area will most likely be of a modest nature, with no real breakthrough expected in the near future.

SPECIFIC AIMS

This study was designed to investigate the modulatory effects of the steroid hormone estrogen upon the nigrostriatal dopaminergic system, as it relates to the release and clearance of dopamine in the rat corpus striatum and nucleus accumbens. The neurotoxic metabolite of MPTP, MPP^+, was used to stimulate the release of dopamine from the nigrostriatal neurons. Release characteristics, including amplitude, rise time, clearance and secretion rates, T½ and other signal decay parameters were examined, in an attempt to better understand the roles of dopamine, the toxin MPP^+, and the steroid hormone estrogen, as they relate to the Parkinson's disease process. In particular, we examined the effects of MPP^+ stimulated dopamine release from the nigrostriatal neurons, and the possible neuroprotective effects of estrogen.

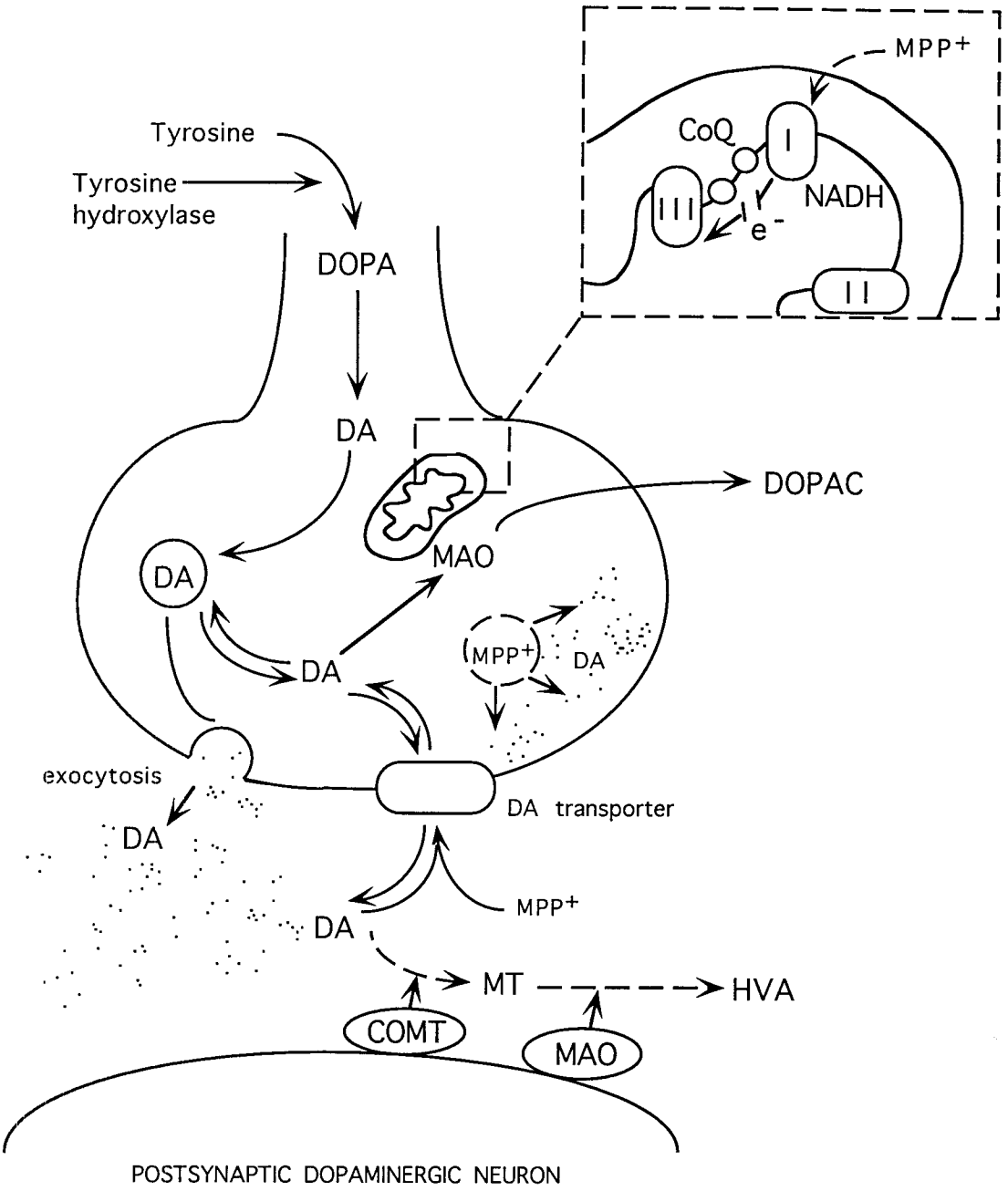
An *in vivo* animal model, along with the technique of *in vivo* electrochemistry, was utilized to measure and record, in real time, the dynamic characteristics of dopamine release and clearance. Animals were separated into two treatment groups, one exposed to MPP^+ alone, and the other to a mixture of MPP^+ with estrogen. The specific characteristics of the release and clearance of dopamine were compared between the two groups. Additionally, the release and clearance characteristics of dopamine were

compared between the corpus striatum and the nucleus accumbens, to determine if MPP^+ stimulated dopamine release, or the effects of estrogen differed between these two important neural regions.

Figure 1. Dopaminergic Terminal.

This figure illustrates a typical dopaminergic nerve terminal. Both pre and post-synaptic neurons are included. Many of the important structural and biochemical components are included. Both normal Ca^{++} dependent exocytosis and MPP^{+} induced dopamine secretion are illustrated in the pre-synaptic terminal. Figure 1 (insert) is of the mitochrondrial inner and outer membranes and is representative of the most widely accepted mechanism of how MPP^{+} shuts down the respiratory chain resulting in death of the dopaminergic neuron.

PRESYNAPTIC DOPAMINERGIC NERVE TERMINAL



CHAPTER II

MATERIALS AND METHODS

ANIMALS

Nineteen female Long-Evans rats, weighing an average of 330g, with an average age of six months were used during this experiment. The animals were housed in groups of two to three, with a reversed twelve hour light-dark cycle, with lights off from 1000 to 2200 hours. Food consisting of standard rat chow, Pro Labs 3000 (PMI Feeds Inc., St. Louis, MO.) and water were available ad lib. All animals were bilaterally ovariectomized (OVX) and allowed to recover for two to four weeks prior to experimentation.

SURGERY

Ovariectomy. All animals were anesthetized prior to surgery, with a dual anesthetic of Ketamine (50 mg/kg) and Xylazine (8 mg/kg). Anesthetics were administered by intramuscular injection. Surgical procedures began 20 to 30 minutes following anesthetic administration, when a suitable level of anesthesia had been obtained. Following anesthesia administration, the skin was prepared by shaving the hair over the flank area, followed by cleansing with alcohol and then a disinfectant solution of Zephrian Chloride (Winthrop Pharmaceuticals, Sterling Drug Inc., New York, NY.)

A small (1 cm) incision along the long axis of the animal was made in the center of the area extending from the last rib, to the proximal margin of the hind leg, and spaced medially, between the dorsal and ventral aspects of the flank. Skin, subcutaneous tissue, and abdominal musculature was cut in this area to expose the uterine horn, which terminates at the ovary. The distal portion of the uterine horn with ovary were ligated with 3-0 surgical silk suture material, and excised distally to the ligation. The area is then examined for any excessive hemorrhage. The abdominal musculature is closed with 4-0 surgical silk, followed by skin closure with two to three stainless steel 18\8 Michael clips (Harvard Apparatus, Holliston, MA.). All animals were monitored post surgically for any complications.

Stereotaxic Surgery. On the morning of the experiment, the animals were deeply anesthetized with a series of three intraperitoneal (ip) injections of Urethane (Sigma Chemical Co., St. Louis, MO). A total dose of 1.5g/kg was divided into three equal amounts, and was administered at twenty

minute intervals. The rat's level of anesthesia was continuously monitored. Withdrawal reflexes were tested intermittently and anesthetic dosage was adjusted accordingly to maintain a surgical level of anesthesia. Core body temperature was monitored by rectal probe and maintained at 37° Celsius with the use of a heating pad placed under the animal. The animal was then placed in the stereotaxic apparatus (Kopf®, Tujunja, CA.) and securely fastened. The hair over the dorsal aspect of the head was shaved, exposing the skin from just behind the ears, to the rostral area near the eyes. The tissue in this area was cut with a mid-line incision, beginning cephallicly just behind the eyes and extending caudally, terminating at a point between the ears over the lambdoidal ridge. The frontal and parietal regions of the skull were exposed. Bregma, (See Figure 3) the junction of the coronal and sagittal sutures, was located and marked for reference. Bregma is an important anatomical landmark and is used as the rostral-caudal zero reference point. An area of each frontal bone was removed, creating windows over the rat forebrain (See Figure 4). Each window extended from the sagittal suture laterally to the parasagittal ridge, and from the coronal suture rostrally to a point just behind the eyes. All bony material was removed, exposing the meninges. Windows were covered with gauze, moistened with 0.9% saline solution, to avoid evaporation from the surface of the brain, until time for probe implantation. Two additional holes were drilled in the right posterior parietal bone (See Figure 4). One hole was used to place two silver/silver chloride (Ag/AgCl) reference electrodes, and a small one eighth inch machine screw was inserted into the other. This area

was then covered with a dental acrylic (Lang Dental M.F.G. Co., Inc., Chicago, IL.) to anchor the reference electrodes and allowed to dry.

Probe placement was determined according to the stereotaxic atlas of Paxinos and Watson (1998). A system of three sets of coordinates, including anterior-posterior (AP), medial-lateral (ML), and vertical (V) was used to determine the exact anatomical location of the probe tip during each stereotaxic pass (See Figure 2). While the stereotaxic coordinates varied for each individual release, those included in the results were obtained within the following locations: AP (1.0-2.0mm), ML (1.5-2.5mm), and V (3.0-7.5mm). These coordinates include Both the CPu and the AcbC and were determined using a stereotaxic atlas (Paxinos and Watson, 1998). Adjustments were made based on specimen breed, sex, and an average weight of 330 grams.

Multiple passes were made bilaterally at 1.0, 1.5, and 2.0 AP and 1.5 or 2.0 ML. Data were gathered using these coordinates at varying depths of 0.5mm between 3.0 and 7.5mm from the surface of the brain in the area of the corpus striatum (Cpu), and nucleus accumbens (AcbC) (See Figure 2).

IN VIVO ELECTROCHEMISTRY

Principles. The electrochemical detection of amine neurotransmitters, in conjunction with high performance liquid chromatography (HPLC), has been the most successful and accurate technique for assaying trace amounts of amine substances in brain tissue since it was first demonstrated in the early

seventies. *In vivo* voltammetry is, in essence, the process of implanting a miniaturized version of the HPLC electrochemical detector.

This process is possible, due to the tendency of catechol based substances, such as dopamine, to be oxidized, and thereby give up electrons. In voltammetry, the oxidizing agent is a carbon fiber electrode (See Figure 5). When a positive potential is applied to such an electrode, it is possible to remove electrons from an oxidizable substance. This sets up a flow of electrons which can then be measured as current, and is directly proportional to the amount of material oxidized (Maidment et al., 1990). Dopamine oxidizes relatively easily due in large part to its basic structure. Surrounding dopamine's catechol nucleus are two hydroxyl groups. It is from these hydroxyl groups that hydrogen atoms, and therefore electrons, are removed.

While there are many chemicals present in neural tissue, only a few are present in sufficient quantities and have oxidation potentials within the detectable range. This range covers an area from -0.2 to +0.8 volts, when compared to a Ag/AgCl reference electrode. Oxygen is reduced below -0.2V, and water at just above +0.8V.

The appropriate oxidation potential for the neurotransmitter dopamine is between +0.15V and +0.25V. The only other substance in sufficient quantity, and with a slightly overlapping potential range, is ascorbic acid whose oxidation potential is -0.2V to +0.2V (Maidment et al., 1990). Resolution of individual amine species in neural tissue depends primarily on two factors, the type of electrode being used and the technique used to make the measurements.

Throughout this experiment, the measurement technique used was high-speed chronoamperometry. This is the simplest means of applying an electrical potential to the working electrode. This is accomplished by the immediate switching of the electrode from an open circuit configuration, to one where the desired potential is immediately applied (Maidment et al., 1990). This potential is held for a predetermined period of time, and then switched back to an open circuit. Current is measured over a particular period within this time frame, creating a current verses time gradient, whereby the oxidizable species is being depleted around the electrode more quickly than it is being replaced through diffusion (Maidment et al., 1990). When concentration, or changes in concentration of the oxidizable species is the variable of interest, then this current time relationship is ignored as long as the electrical potential, once applied, is held constant throughout the experiment (Maidment et al., 1990). In our experiment, measurements using this type of voltametric technique were taken at 5 Hz, and then averaged over one second. This results in the enhancement of the recording as to the signal-to-noise parameter.

Due to the ease at which it can be automated and adapted to software applications, this, or similar techniques, have been used in many laboratories doing *in vivo* neurochemical studies.

Working Electrodes. Single carbon fiber electrodes, with a tip diameter of 30μM, were prepared for each experiment (See Figure 5). Electrodes utilized were obtained from two sources. During the early stages of the experiment, commercial electrodes were obtained from Quanteon (Rocky Mountain Center for Sensor Technology, Denver, CO.). More recently,

construction of working electrodes was done in house at Youngstown State University

Electrode Construction. Single carbon fiber electrodes were constructed through the following procedure. Carbon fibers 30μM in diameter (Textron Specialty Materials, Lowell, MA.) were cut to approximately 5cm in length. Fibers were then soaked in ETOH 95% for 15 minutes to insure that the surface area was as clean as possible. Glass capillary tubes 1.00mm X 0.58mm (A-M Systems Inc., Everett, WA.) were pulled into micropipettes using a Flaming Brown model P-80/PC micropipette puller (Sutter Instrument Co. San Rafael, CA.). The pipette tips were then bumped back to an opening of 50μM, using a ground glass instrument and a microscope fitted with an ocular micrometer. Pipettes were then cut to a final length of 12mm. Carbon fibers were then placed inside the pulled glass capillary tubes such that approximately 1cm extended beyond the glass tips. Fibers were then sealed in place with a very small drop of Epoxylite high temperature baking varnish (Epoxylite Corp., Irvine, CA.). This was accomplished by inserting a 30 gauge stainless steel needle, (Hamilton Company, Reno, NV.), into the back of the pipette and injecting the Epoxylite near the fiber-glass interface at the pipette tip. Electrodes were then heat cured in a constant temperature oven at 120° C for 10-12 hours. Next, the space inside the glass capillary was filled with a conductive graphite epoxy paste (Dylon Industries Inc., Cleveland, OH.). At this point, a 10 cm length of 28 gauge lacquer coated copper wire (Vector Electronic Co., N. Hollywood, CA.) was inserted into the back of the capillary tube containing the graphite paste. About 5mm of lacquer insulation was

removed prior to insertion of the wire. The electrode, with graphite paste and wire, was again heat cured in the oven at 120° C for 10-12 hours. Next, the copper wire-graphite paste interface was back sealed with a drop of Epoxylite and once again, heat cured at 120° C for 10-12 hours. Lastly, the carbon fiber was trimmed to a final working length of 150μM with microscissors and a dissecting scope equipped with an ocular micrometer. Electrodes were then stored in a low humidity environment until use.

Calibration of each electrode was performed separately, *in vitro*. Parameters concerning the calibration were analyzed by the IVEC 10 system, a computer-based neurotransmitter analyzer (Medical Systems Corp., Greenvale, NY. The IVEC-10 system monitors numerous parameters associated with each release. Several of these parameters are shown to the right in figures 9 and 10. Amplitude is defined as the maximal positive deflection of the oxidation signal from baseline. Values were expressed in μM concentrations of dopamine. The signal-to-noise (S/N) ratio is calculated and is considered to be the smallest signal that can be detected when compared to the level of noise in the system and the electrode. The oxidation-reduction current ratio (RED/OX), is given and is defined as the ratio of the reduction current to the oxidation current when the oxidation current is at maximal value. Hysteresis is the point in time when the oxidation current is intersected by the reduction current. Rise time is reported and is considered that period of time from the injection of the test substance, which coincides with the (TTL marker *), to the point where the oxidation signal reaches it's maximum amplitude. The T½ time is reported as the period of time necessary for the signal to decay by 50% from it's

maximal value. Time course is a temporal representation of the entire event, beginning at the infusion of the test substance and ending when the oxidation current returns to baseline. Other release characteristics not given in figures 9 and 10 were evaluated as well, and include T 20-60 and T 40-80. T 20-60 begins when the signal has decayed by 20% of it's maximal value and ends when the signal has decayed by 60% of it's maximal value and includes that period of time in-between. T 40-80 is calculated in a similar manner. The secretion rate of dopamine was calculated for each response and represents the amount of dopamine being released per second from the neural tissue stimulated. Secretion rates are reported in nM concentrations of dopamine per second. Clearance rates were also recorded and are representative of how quickly the dopamine signal is being removed from the recording area. Clearance rates are also reported in nM concentrations of dopamine per second.

Just prior to calibration, 5mm of lacquer insulation was removed from the exposed end of the wire and .040" male gold-plated patch cord pin connectors (Newark Electronics, Chicago, IL.) were soldered to the exposed copper wire. Electrodes were coated with several layers of Nafion®, (Aldrich Chemical Co. Inc., Milwaukee, WI.) which is an electrochemical perfluorosulfonated derivative of Teflon®. Nafion® is applied in multiple coats ranging from 3-15 depending on individual electrode characteristics and environmental conditions. This highly negatively charged substance provides for increased selectivity to monoamines, such as dopamine, rather than ascorbic acid (Maidment et al., 1990). Between each Nafion® coat,

electrodes were heat cured in a constant temperature oven at 160° C for 4-5 minutes.

During calibration, a potential of +0.55V verses a commercially available Ag/AgCl reference electrode (Quanteon, Rocky Mountain Center for Sensor Technology, Denver, CO.) was applied for a period of 100 msec, and then removed, allowing the electrode to return to a resting potential of 0.0V. In this way, both the oxidation and reduction currents produced, can be recorded and analyzed (Friedemann and Gerhardt, 1992). The calibration process takes place *in vitro.* The working and reference electrodes were placed in a beaker containing 40 ml of 0.1M phosphate buffered saline solution and connected to the IVEC 10 headstage which is in turn connected to the IVEC 10 system. The calibration mode of the software was selected and an electrical potential was applied to the working electrode. Oxidation and reduction waveforms were monitored on the computer screen and settings for the gain multiplier and headstage were manipulated until waveforms approximate, as closely as possible, the optimum calibration template.

During *in vitro* calibration, electrodes were tested against 250μM (500μl of 20mM) ascorbic acid (Aldrich Chemical Co. Inc., Milwaukee, WI.) and then challenged with multiple 2mM incremental increases (40μl doses of 2mM) dopamine (Sigma Chemical Co., St. Louis, MO.). Oxidation and reduction current numbers were then recorded and a standard curve was generated (See Figure 6). Parameters such as selectively, sensitivity, oxidation/reduction ratios, and signal to noise ratios were calculated by the software based on values obtained during the calibration process. The OX

slope determines the sensitivity of the electrode to the test substance. RED/OX ratios help identify the identity of the chemical being measured. Selectivity is a measurement of how selective the electrode is for dopamine over ascorbic acid. Signal-to-noise represents how small a signal can be measured compared to the noise in the system and the electrode. Values for the above parameters were stored in the program for each electrode calibrated and measurements made during *in vivo* data acquisition were then compared to the stored values to quantify the dopamine-like signal *in vivo*. This DA-like signal is referred to as dopamine release throughout this thesis. Following calibration, electrodes were stored with their tips immersed in 0.1M phosphate buffered saline solution.

Reference Electrodes. Silver/Silver Chloride (Ag/AgCl) reference electrodes were prepared prior to each experiment. This was accomplished by plating .008" Teflon® coated silver wire (A-M Systems Inc., Everett, WA.) in a solution of 1M HCL saturated with NaCl (40g/100ml distilled water). Electrodes were plated for a period of twenty minutes with a twelve volt power supply producing a constant current at 2.0 ampere. This results in a thin layer of chloride being deposited on the silver wire due to the following reversible reaction, [$Ag + Cl^- \leftrightarrow AgCl + e^-$]. Reference electrodes were then compared to a commercially available reference with a digital multimeter in a 3M NaCl solution. A potential difference of 15.0 millivolts or less between the two was indicative of usable reference electrodes.

Micropipette Construction. Micropipettes were constructed from 1.0mm OD X 0.58mm ID glass capillary tubes (A-M Systems Inc., Everett, WA.).

Capillary tubes were placed in a model P80-/PC Flaming Brown micropipette puller. Tips were pulled to a sub-micron opening using a 2.5mm box type filament and the following settings: Heat = 716, Pull = 150, Velocity = 70, Time = 80. Pulled pipettes were then placed under the light microscope and observed at 10X. Tips were bumped back with a ground glass instrument to an internal opening of 15 microns. Micropipettes were finished to an overall length of 10cm.

Probe Apparatus. Once suitable working and reference electrodes were obtained, the probe apparatus, consisting of the working electrode and a micropipette, was constructed (See Figure 7). The working electrode and micropipette were bonded with a non-conducting adhesive wax (Sticky Wax, Kerr USA, Romulus, MI.) in a manner in which the tips of both were exactly even and spaced in the range of 280 to 320 micrometers (See Figure 7). Distances were verified using a microscope equipped with an ocular micrometer.

Micropipette Filling. Just prior to probe insertion, the micropipette portion of the probe assembly was back filled with either a 10^{-3}M solution of MPP^+ alone (Research Biochemicals International, Natick, MA.), or MPP^+ with estrogen (Sigma Chemical Co., St. Louis MO.) in a concentration of 10^{-5} g/ml of estrogen. MPP^+ and estrogen, when combined, were mixed in equal volumes and administered in cocktail form. Test solutions were drawn up in a 1cc syringe, and pipettes were filled with a 31 gauge micropipette filling needle (World Precision Instruments Inc., Sarasota, FL.). Solutions were passed through a 0.45 micron syringe filter (Micron Separations Inc., Westboro, Ma) to remove any particulate contaminants.

The pipette filling needle was inserted into the micropipette and then back filled with the test solution. Care was taken to insure no air remained trapped in the pipette and a visual inspection of each was performed before experimentation.

Pharmacological solutions were infused through the use of a Picospritzer II (General Valve Corp., Fairfield, NJ.). Volumes were regulated by adjusting the applied pressure and controlling the duration of the pulse. Pressures between 10-30 psi were created with medical grade bottled nitrogen (N_2). Pressure and duration of the pulses was adjusted to maintain infused volumes in the range of 150 to 175nl. Measurements of infused volumes were accomplished by means of observation. A dissecting microscope, equipped with an ocular micrometer, was positioned to observe the meniscus of the solution within the micropipette. The dissecting scope was calibrated against a factory standard micrometer to determine proper magnification. Volumes are calculated by observing the movement of the meniscus with each pulse and calculating the volume infused by applying the conversion factor of 25 µl/gradation of the dissecting scope micrometer.

EXPERIMENTAL DESIGN

This experiment was designed to investigate the effects of MPP^+ alone or the combination of MPP^+ with estrogen on the dynamics of dopamine release and clearance from neurons of the nigrostriatal system. The technique of *in vivo* electrochemistry (*in vivo* chronoamperometry) was used

to record changes in the release and clearance characteristics of dopamine from regions of the corpus striatum and nucleus accumbens (See Figure 2).

Solutions of MPP^+ or MPP^+ with estrogen were injected via the probe assembly (as described above), into the corpus striatum and nucleus accumbens. The characteristics of the MPP^+ stimulated dopamine release were monitored and measured using the IVEC-10 system, and Fast 12 software (Medical Systems Corp., Denver, CO.) installed on a compudyne computer.

Data were collected from sixteen animals. MPP^+ alone was used to stimulate dopamine release, and a total of 176 responses were recorded in eight rats. Of these 176 responses 142 were obtained from areas of the CPu, and 34 were recorded from the AcbC. In the remaining eight animals MPP^+ with E_2 was used to induce dopamine release resulting in a total of 180 responses. Of these 180 responses, 139 were from the CPu and 41 from the AcbC. While the total number of responses recorded for each animal varied throughout the experiments, an average of 22 responses was recorded from each animal.

DRUGS AND SOLUTIONS

Phosphate Buffered Saline. A 0.1M phosphate buffered saline solution is used for calibration and short-term storage of calibrated electrodes. This solution is made by combining the following: Sodium phosphate dibasic anhydrous ($Na_2HPO_4 \cdot 7H_2O$) (10.80 g/1L); Sodium phosphate monobasic

anhydrous ($NaH_2PO_4 \cdot H_2O$) (2.16 g/1L); Sodium Chloride NaCl (9.0 g/1L). The solution is then adjusted to a pH of 7.2 to 7.4.

Ascorbic Acid. A 20 mM ascorbic acid solution was created by combining 0.325g ascorbate with 100ml distilled water. Ascorbic acid is used during the working electrode calibration process.

Dopamine HCL. A 2mM solution of dopamine is made by combining 0.038g dopamine HCL with 100ml of a 0.1 mM perchloric acid solution. Perchloric acid is used as a preservative to stabilize the dopamine and hinder deterioration.

Plating Solution. A plating bath is created by saturating a 1 M HCL solution with NaCl. The ratio is 40g NaCl per 100ml 1 M HCL.

Sodium Chloride. A 3 M NaCl is used to store the commercial reference electrodes used in the plating and calibration processes. A 0.9% NaCl solution is utilized during the post-experiment tissue perfusion process, in preparation for histology.

Formalin. A 10% formaldehyde solution diluted 1:4 in distilled water was used during the post-experiment tissue perfusion process as a tissue fixative prior to staining.

MPP^+. A 10^{-3} M solution of MPP^+ was made by mixing 2.97mg of MPP^+ with iodine in 9.9ml of phosphate buffer solution and .1ml of 95% ETOH.

17-Beta Estradiol. A 10^{-5} g/ml solution of estrogen is made by combining 2.0mg of 17-Beta Estradiol with 2ml of 95% ETOH. This yields a 10^{-3} g/ml concentration, which is then diluted to 10^{-5} g/ml by combining 100µl of the 10^{-3} concentration with 9.9ml of phosphate buffered saline.

MPP^+ & Estrogen. The MPP^+ and estrogen cocktail was made by combining equal amounts of the 10^{-3} M MPP^+ solution and the 10^{-5} g/ml estrogen solution.

HISTOLOGY

Following each experiment rats were perfused, under deep anesthesia, with a 10% formaldehyde solution (Fisher Scientific, Fairlawn, NJ.) diluted 1:4 with distilled water. The thoracic and abdominal cavities were opened and a blunt 18 gauge needle was inserted into the left ventricle of the heart and advanced until the tip was inside the proximal portion of the aorta. Prior to fixation, the animal's circulatory system was flushed with 200 to 250 cc of 0.9% NaCl solution through the same needle. During this process, a small incision was made in the right atrium to allow the efflux of blood and saline solution. Flushing continues until the fluid runs clear from the right atrium. Approximately 150 cc of the 10% formaldehyde solution was then infused in the same manner. This resulted in a good fixation of all animal tissues, including neural tissue.

Following perfusion, animals were decapitated using a small animal decapitation device (Harvard Apparatus, Holliston, MA). Skin and soft tissues covering the skull were then removed. Rongeurs were used to remove bony material and the entire brain was exposed. The brain was then removed from the cranium and stored in the 10% formaldehyde solution until cross-sectional slides of the forebrain were made.

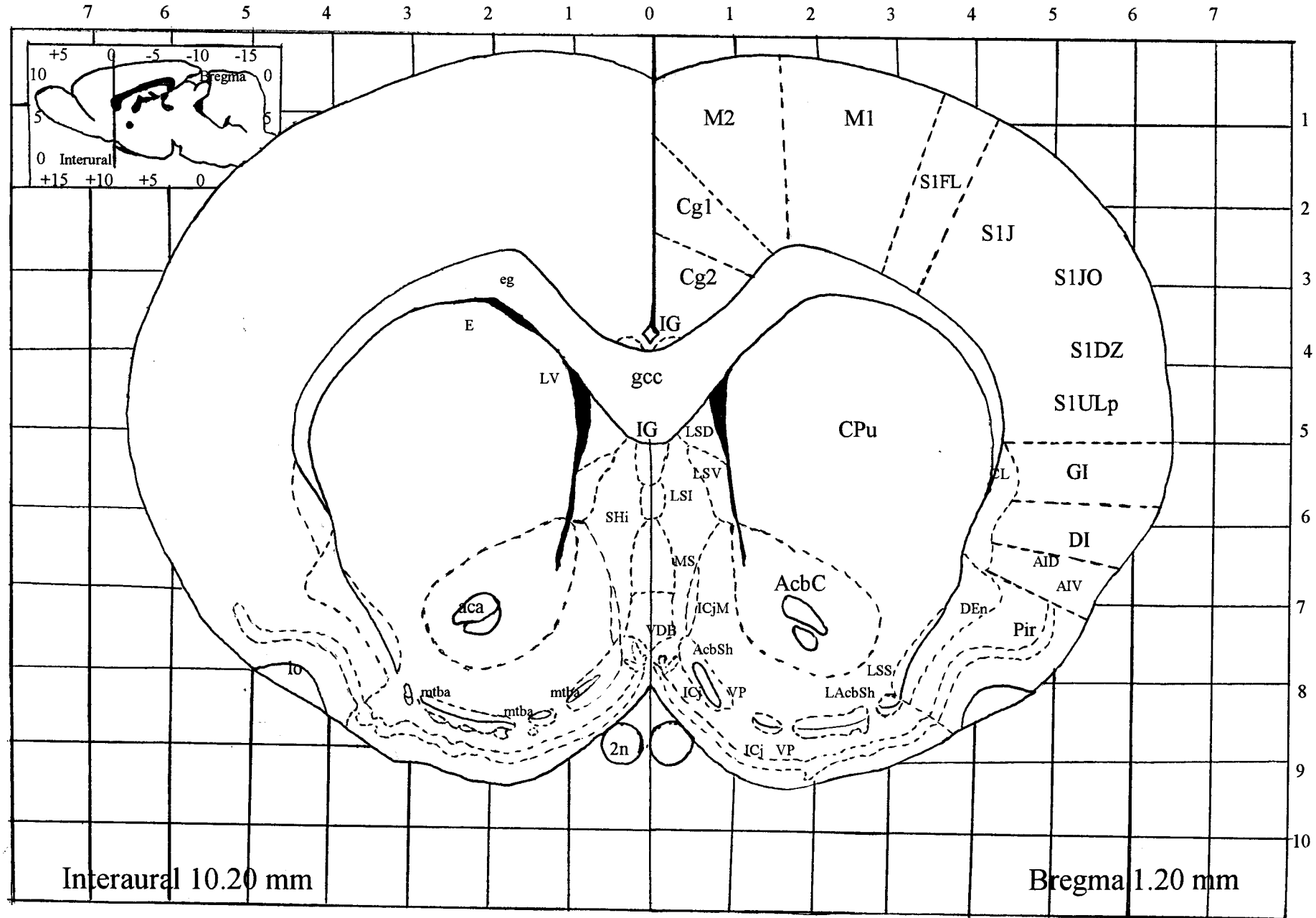
Plain glass microslides 3" X 1" (Corning Scientific Glassware Dept., Corning, NY.) were prepared by coating with 20% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO.) and then dried for 10 minutes on a slide warmer set at 40° C (Fisher Scientific, Fairlawn, NJ.). Whole rat brains were placed on the stage of the cold microtome (Cryo-Histomat, Hacker Instruments Inc., Fairfield, NJ.) and frozen. Thirty micron serial sections of the rat forebrain were made. Individual sections were placed in numbered trays containing 20% ETOH and then floated onto the BSA coated slides. Sections were placed three to a slide and subjected to Basic Fuchsin staining by the following procedure:

1.) 20% ETOH for 3 min.
2.) Distilled water for 3 min.
3.) Distilled water for 3 min.
4.) Distilled water for 3 min.
5.) Basic Fuchsin for 1.5 min.
6.) Distilled water for 5 sec.
7.) Distilled water for 3 min.
8.) Acid Formalin for 5 sec.
9.) Distilled water for 5 sec.
10.) Distilled water for 3 min.
11.) 70% ETOH for 3 min.
12.) 95% ETOH for 3 min.
13.) 100% ETOH for 3 min.
14.) 100% ETOH for 3 min.
15.) Xylene for 5 min.
16.) Xylene for 5 min.

Lastly, sections were covered with 50mm cover slips and sealed with Permount (Fisher Chemical, Fairlawn, NJ.). Slides were then viewed under light microscopy to verify probe placement in the corpus striatum and nucleus accumbens (See Figure 8).

STATISTICAL ANALYSIS

Data was analyzed for variations between groups in amplitudes, rise times, secretion and clearance rates, T50, T20-60, T40-80 signal decay times, as well as total time course with the Sigma Stats program version 1.0 (Jandel Scientific, Corp., Corte Madera, California). Unpaired T-tests were constructed to examine response values for each parameter between groups. Data was assigned a minimum P value of ($p < 0.05$) to determine statistical significance. Graphical representations were constructed using Sigma Plot version 1.02 (Jandel Scientific, Corte Madera, CA.).


+5 0 -5 -10 -15
10
Bregma
0
5
5
0 Interural
+15 +10 +5 0
M2
M1
S1FL
S1J
S1JO
S1DZ
S1ULp
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AID
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DEn
Pir
Cg1
Cg2
eg
E
IG
LV
gcc
IG
LSD
LSV
LSI
SHi
MS
CPu
CL
AcbC
ICjM
VDB
AcbSh
ICj
VP
LAcbSh
LSS
ICj VP
2n
aca
lo
mtba
mtba
mtba
Interaural 10.20 mm
Bregma 1.20 mm

Figure 3. Bregma.

This figure includes both anterior and lateral views of the rat skull and illustrates the location of bregma and the interaural line in relation to other features of the rat skull. The lateral view shows the position of the incisor bar when the specimen is in the stereotaxic device. (Redrawn from The Rat Brain in Stereotaxic Coordinates, 4th ed. by George Paxinos and Charles Watson).

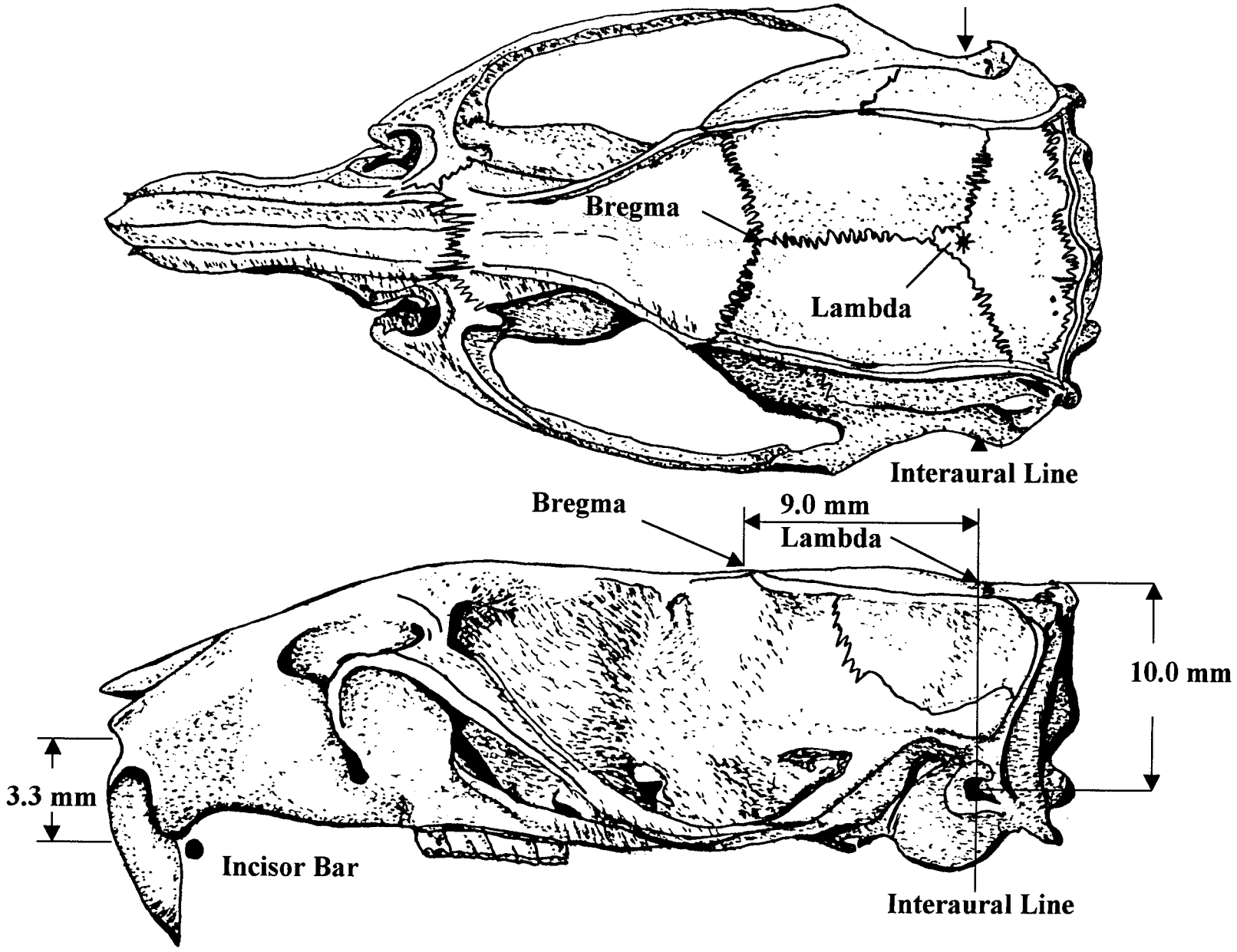

Bregma
Lambda
Interaural Line
Bregma
9.0 mm
Lambda
10.0 mm
3.3 mm
Incisor Bar
Interaural Line

Figure 4. Surgical Windows.

This photo illustrates the location of the stereotaxic windows created in the skull over areas of the rat fore-brain. Bregma is highlighted by a black dot.

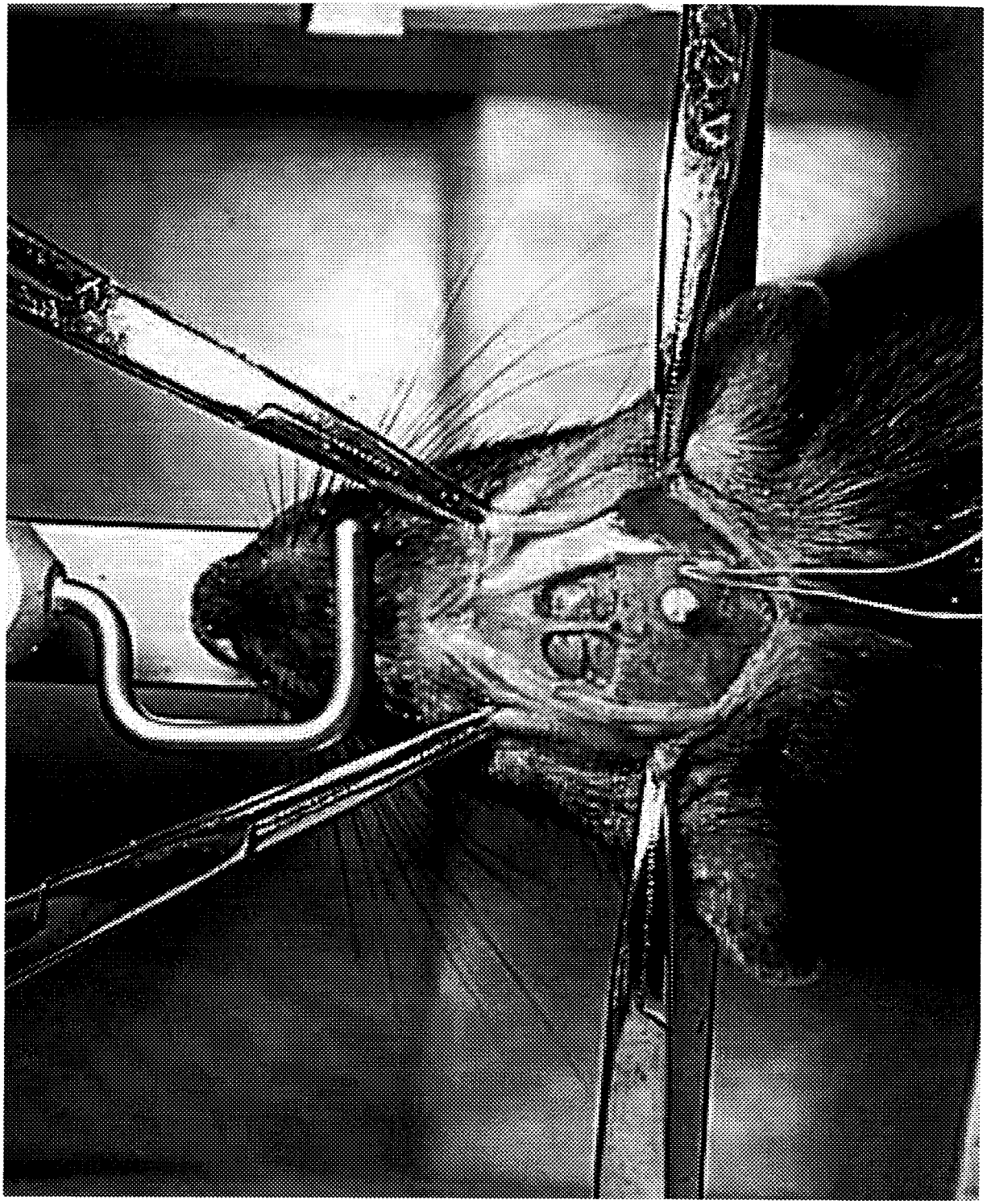


Figure 5. Carbon Fiber Electrode.

This drawing illustrates the components of a 30μM single carbon fiber working electrode.

SINGLE-FIBER CARBON ELECTRODE

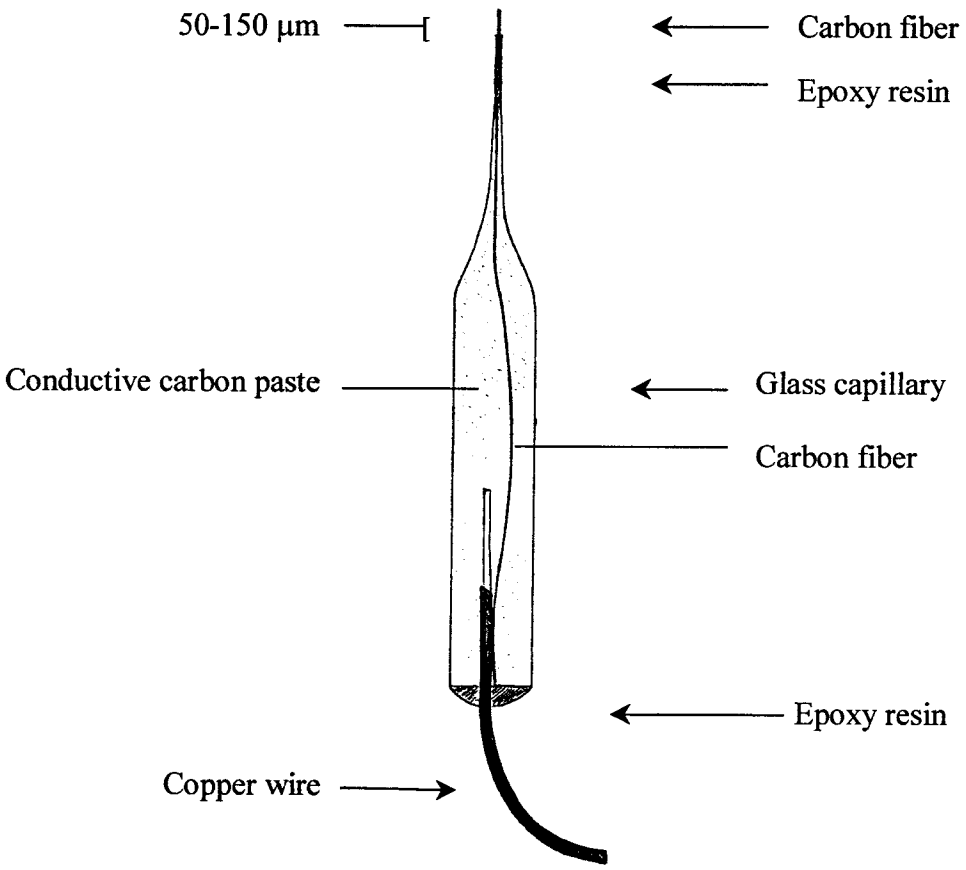
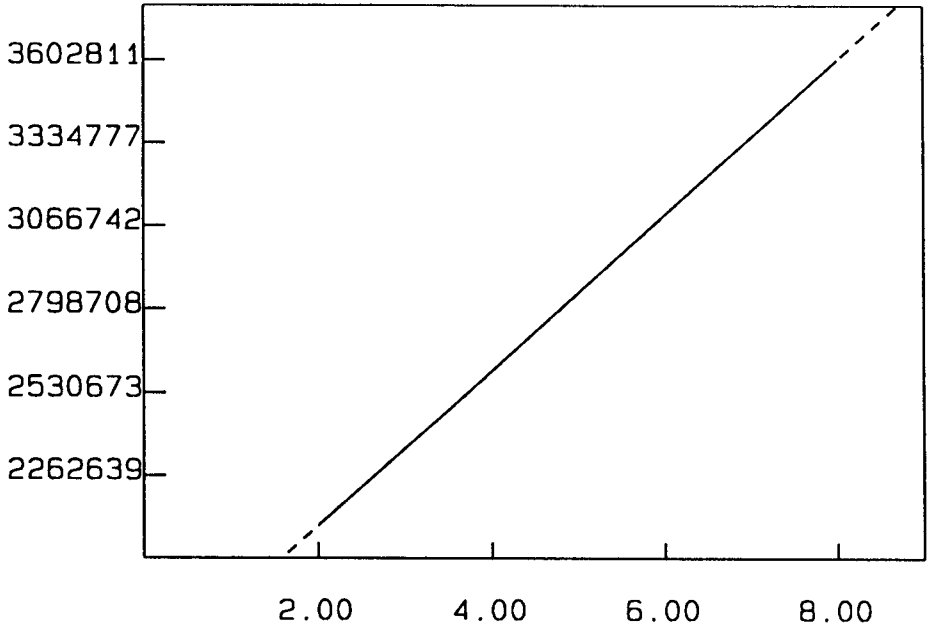
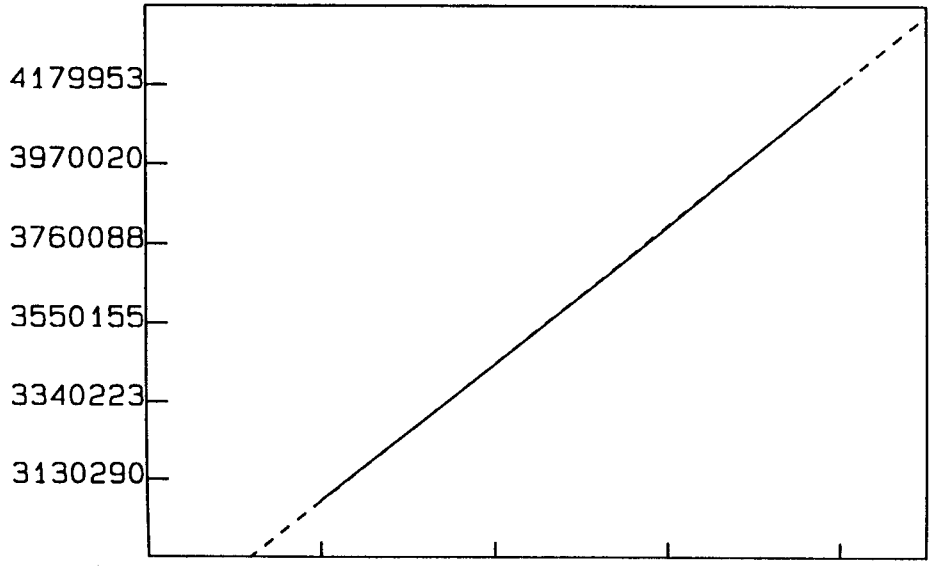


Figure 6. Regression Curve.

This figure illustrates an example of a typical in vitro calibration curve. The oxidation currents (y-axis) top, the reduction currents (y-axis) bottom, and the dopamine concentrations (x-axis) are illustrated. Parameters such as slope, intercept, and correlation coefficients (R^2) are given for both oxidation and reduction currents. Values for the RED/OX ratio, selectively and the signal-to-noise ratio are given as well.





Mon Jan 12 21: 42: 31 1998

OXIDATION

SLOPE	250531.16
INTERCEPT	1599255.50
R^2	1.0000

ELECTRODE # 9

TRANSMITTER	Dopamine
RED/OX RATIO	0.74
SELECTIVITY	-1304849: 1
SIGNAL/NOISE	0.04279
FREQUENCY	5 Hz

REDUCTION

SLOPE	184178.80
INTERCEPT	2703964.00
R^2	1.0000

GAIN SETTING _____

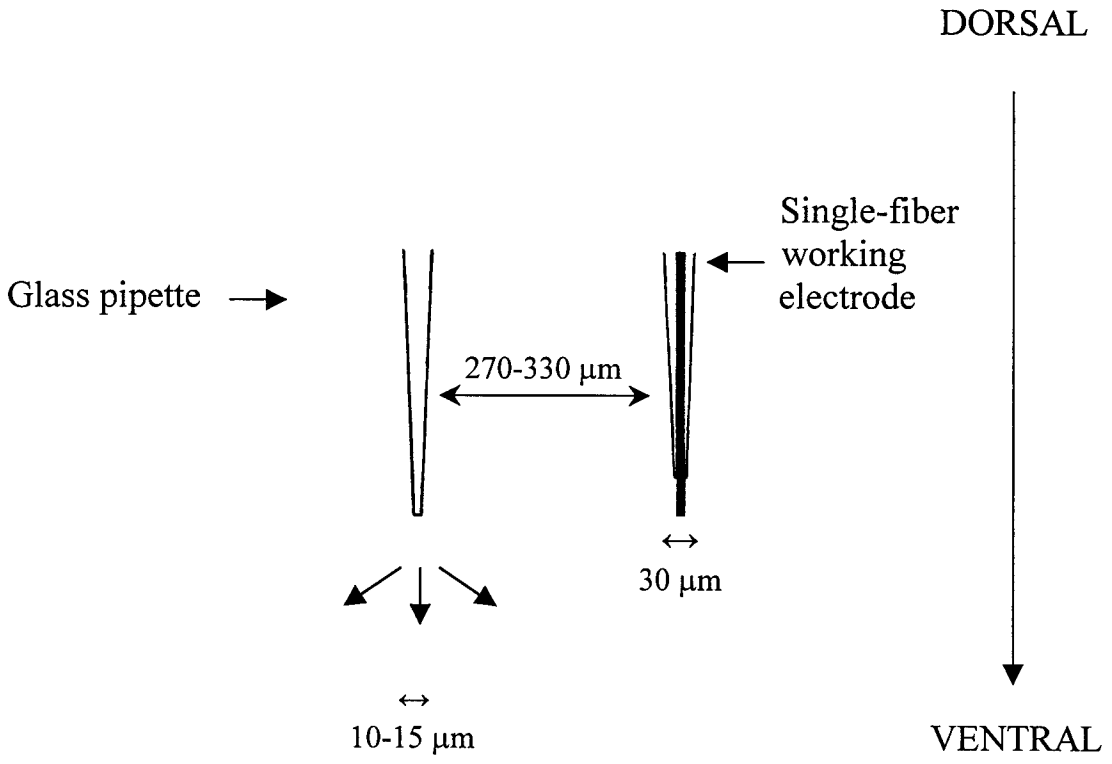
FILTER SETTINGS _____

NAME _____

Figure 7. Probe Assembly.

This drawing illustrates the dimensions necessary for constructing a working probe assembly. The assembly consists of a single barreled micropipette with a tip diameter of 10-15 microns bonded to a 30 micron single carbon fiber recording electrode.

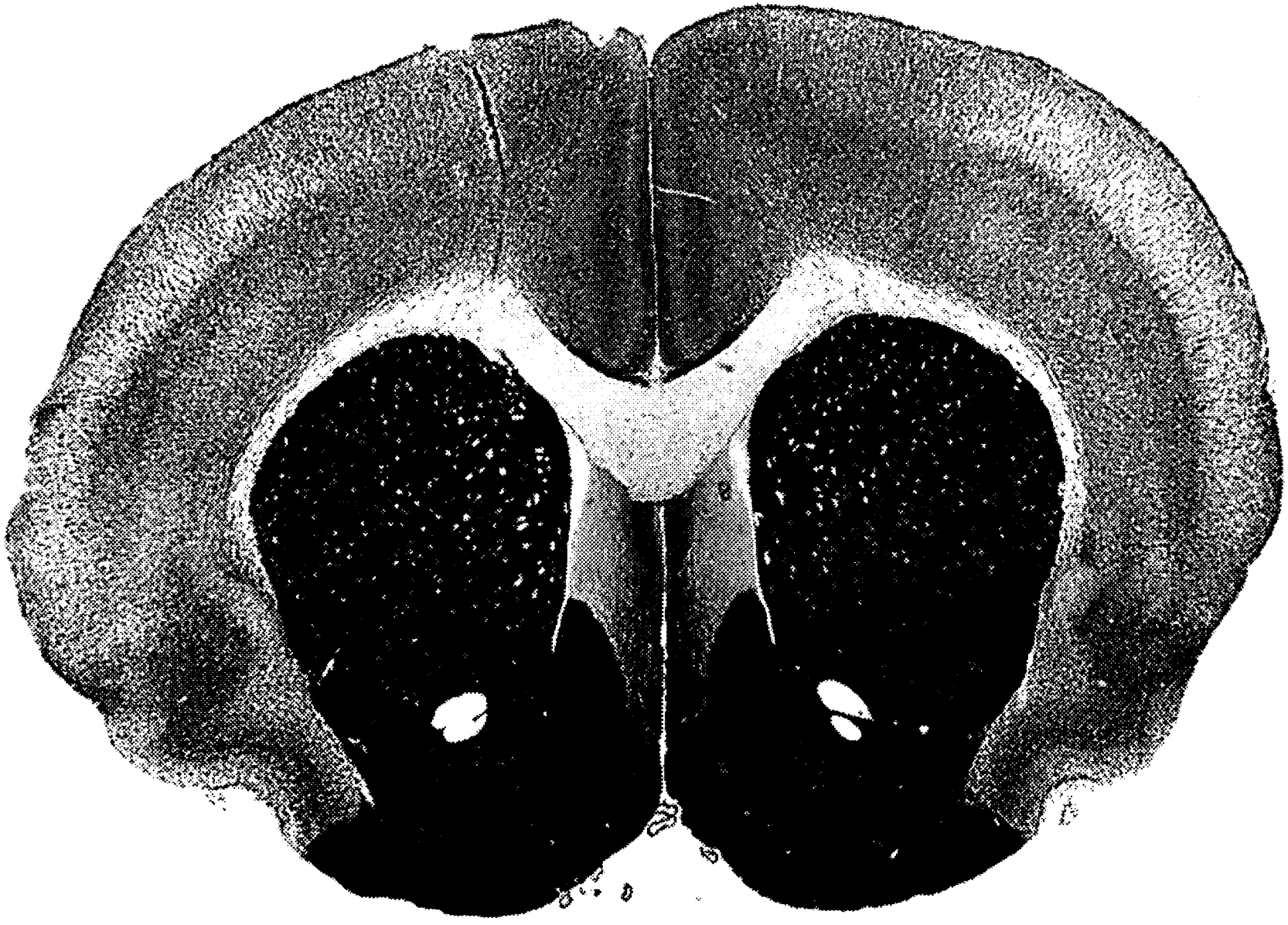
RECORDING ASSEMBLY UNIT



CORPUS STRIATAL TISSUE

Figure 8. Stained Histology Section.

This photo depicts a stained cross-section of the rat fore-brain. The CPu is evident bilaterally.



CHAPTER III

RESULTS

Data was collected from sixteen animals during the completion of this experiment. Results included are based on a total of 176 MPP^+, and 180 MPP^+ with E_2 stimulated responses. Of the 176 MPP^+-stimulated responses 142 were recorded from the CPu and 34 in the AcbC. Of the 180 MPP^+ with estrogen stimulated dopamine responses 139 were recorded in the CPu and 41 in the AcbC. Individual responses were recorded from various areas of the corpus striatum and nucleus accumbens within the rat fore-brain. A typical electrochemical signal detected after local application of MPP^+ can be seen in figure 9. Figure 10 depicts a typical signal as a result of local application of MPP^+ combined with estrogen. For ease of comparison figure 11 is included as a comparison of MPP^+-stimulated (top) and MPP^+ with estrogen stimulated (bottom) dopamine releases. Oxidation currents are represented as a solid line while reduction currents appear as a dashed line. Asterisk's, represent the event time marker which signifies the time point at which test substances were injected.

Figures 12-19 are comparisons of the differences between brain areas and treatment groups with respect to individual parameters of the release events. For each group the following parameters were considered: Amplitude, Rise Time, Secretion Rate, T 50, T 20-60, T 40-80, Clearance Rate, and Total Time Course.

Amplitude. The mean value for MPP^+ stimulated DA responses in the CPu was 1.198 +/- 0.071 μM DA and 0.121 +/- 0.0072μM DA for MPP^+ with estrogen stimulated DA responses ($p = < 0.0001$). This is a 10 fold reduction in the amplitude of the MPP^+-stimulated dopamine signal when estrogen was applied locally along with the MPP^+ (See figure 12). The mean value for MPP^+-stimulated DA responses in the AcbC was 0.536 +/- 0.0435 μM DA and 0.0680 +/- 0.0086 μM DA for MPP^+ with estrogen stimulated DA responses ($p = < 0.0001$). These figures represent an 87.3% reduction in the amplitude of the DA signal when estrogen was applied along with MPP^+ (See figure 12). When comparisons were made between the CPu and the AcbC a reduction in amplitude of 55.3% for MPP^+ ($p = < 0.0001$) and 56.2% for MPP^+ with estrogen ($p = 0.0002$) was observed in the AcbC, when compared to the same treatments in the CPu (See figure 12).

Rise Time. MPP^+-stimulated responses in the CPu had a mean value of 28.3 +/- 2.428 seconds whereas MPP^+ with estrogen responses averaged 22.5 +/- 0.814 seconds, ($p = 0.0251$). This represents a 20.5% reduction in the MPP^+ with E_2 treatment group (See figure 13). In the AcbC the mean for MPP^+-stimulated responses was 28.0 +/- 3.10 seconds and 19.1 +/- 1.40 seconds for MPP^+ with E_2, ($p = 0.0074$). This represents a 31.8% reduction

in the MPP^+ with E_2 treatment group (See figure 13). When comparisons were made between the CPu and the AcbC, no significant differences in rise time were noted between the MPP^+ treatment groups. A slight reduction in rise time of 15.1% was observed in the MPP^+ with E_2 group for the AcbC ($p = 0.0470$), (See figure 13).

Secretion Rate. The secretion rate for MPP^+-stimulated dopamine signals in the CPu was 59.12 +/- 4.816 nM/sec of DA, verses 6.11 +/- 0.404 nM/sec of DA for MPP^+ with E_2 stimulated responses ($p = < 0.0001$). This represents an 89.7% reduction in the rate of MPP^+-stimulated dopamine secretion with the striatal application of estrogen along with MPP^+ (See figure 14). The rate of secretion for MPP^+-stimulated responses in the AcbC was 22.77 +/- 1.873 nM/sec of DA, verses 4.33 +/- 0.739 nM/sec of DA for MPP^+ with E_2 stimulated responses ($p = < 0.0001$). This represents an 81% reduction in the rate of DA secretion in the AcbC when E_2 was applied along with MPP^+ (See figure 14). When comparisons were made between the CPu and the AcbC a reduction in secretion rate of 61.4% for MPP^+ ($p = 0.0003$) and 29.1% for MPP^+ with estrogen ($p = 0.0367$) is seen in the AcbC when compared to the same treatments in the CPu (See figure 14).

T 50. The mean T½ time in the CPu for the MPP^+-stimulated group was 66.2 +/- 3.01 seconds as compared to 42.8 +/- 1.59 seconds for MPP^+ with estrogen stimulated responses ($p = < 0.0001$). This clearance value was shortened by 35.3% in the MPP^+ with estrogen group when compared to the responses stimulated by MPP^+ alone (See figure 15). In the AcbC the mean value for the MPP^+ group was 62.8 +/- 8.17 seconds and 37.7 +/- 2.80 seconds for the MPP^+ with E_2 group ($p = 0.0025$). This represents a 40%

reduction in the T-50 time in the MPP^+ with E_2 treatment group. When comparisons were made between the CPu and the AcbC, no significant differences were observed in either the MPP^+ or MPP^+ with E_2 treatment groups.

T 20-60. The T 20-60 time for MPP^+-stimulated responses in the CPu averaged 35.5 +/- 3.48 seconds while the average for the MPP^+ with estrogen group was 17.3 +/- 1.09 seconds, ($p = < 0.0001$). This data shows a significant difference between the two groups. Calculated values represent a 51.3% reduction in the time required for the DA signal to decay over this range when estrogen was present (See figure 16). The T 20-60 time in the AcbC for MPP^+ was 30.5 +/- 5.82 seconds and 11.1 +/- 1.25 seconds for MPP^+ with estrogen ($p = 0.0007$). This data shows a significant difference between the two groups and represents a 63.6% decrease in the time required for the DA signal to decay over this range when estrogen was present (See figure 16). When comparisons were made between the CPu and the AcbC no significant differences in the T 20-60 time were noted between the MPP^+ treatment groups. In the MPP^+ with E_2 treatment groups there was a 35.8% decrease in the T 20-60 time for the AcbC when compared to DA responses in the CPu ($p = 0.0036$), (See figure 16).

T 40-80. The T 40-80 time in the CPu for MPP^+-stimulated DA releases was 45.2 +/- 2.40 seconds and 19.8 +/- 1.61 seconds for the MPP^+ with estrogen group ($p = < 0.0001$). Calculated values show a substantial decrease, 56.2%, in the time required for the DA signal to decay over this range when estrogen is applied with MPP^+ (See figure 17). In the AcbC the T 40-80 time was 40.0 +/- 5.51 seconds for the MPP^+ treatment group and

11.7 +/- 1.49 seconds for MPP^+ with E_2 ($p = < 0.0001$). This represents a significant decrease of 71.8% in the T 40-80 time when estrogen was applied along with MPP^+ (See figure 17). When comparisons were made between the CPu and the AcbC no significant differences in the T 40-80 time were noted between the MPP^+ treatment groups. In the MPP^+ with E_2 treatment group there was a 40.9% decrease in the T 40-80 time for the AcbC when compared to DA responses in the CPu ($p = 0.0110$), (See figure 17).

Clearance Rate. Clearance rates In the CPu were calculated to be 19.14 +/- 1.442 nM/sec of DA for MPP^+-stimulated responses and 3.63 +/- 0.228 nM/sec of DA for MPP^+ with estrogen stimulated responses ($p = < 0.0001$). The clearance rate for MPP^+ with estrogen was decreased by 82.4% when compared to responses stimulated by MPP^+ alone (See figure 18). The clearance rate for MPP^+-stimulated responses in the AcbC was 13.20 +/- 1.925 nM/sec of DA, verses 3.20 +/- 0.472 nM/sec of DA for MPP^+ with E_2 stimulated responses ($p = < 0.0001$). This represents a 75.8% reduction in the rate of DA clearance in the AcbC when E_2 was applied along with MPP^+ (See figure 18). When comparisons were made between the CPu and the AcbC no significant differences were observed in either the MPP^+ or MPP^+ with E_2 treatment groups.

Total Time Course. The mean for MPP^+-stimulated releases in the CPu was 189.3 +/- 10.45 seconds and 72.3 +/- 3.41 seconds for the MPP^+ with estrogen group, ($p = < 0.0001$). The average time course for responses was reduced by 61.8% in the MPP^+ with estrogen group (See figure 19). In the AcbC the total time course was 123.0 +/- 16.56 seconds for the MPP^+

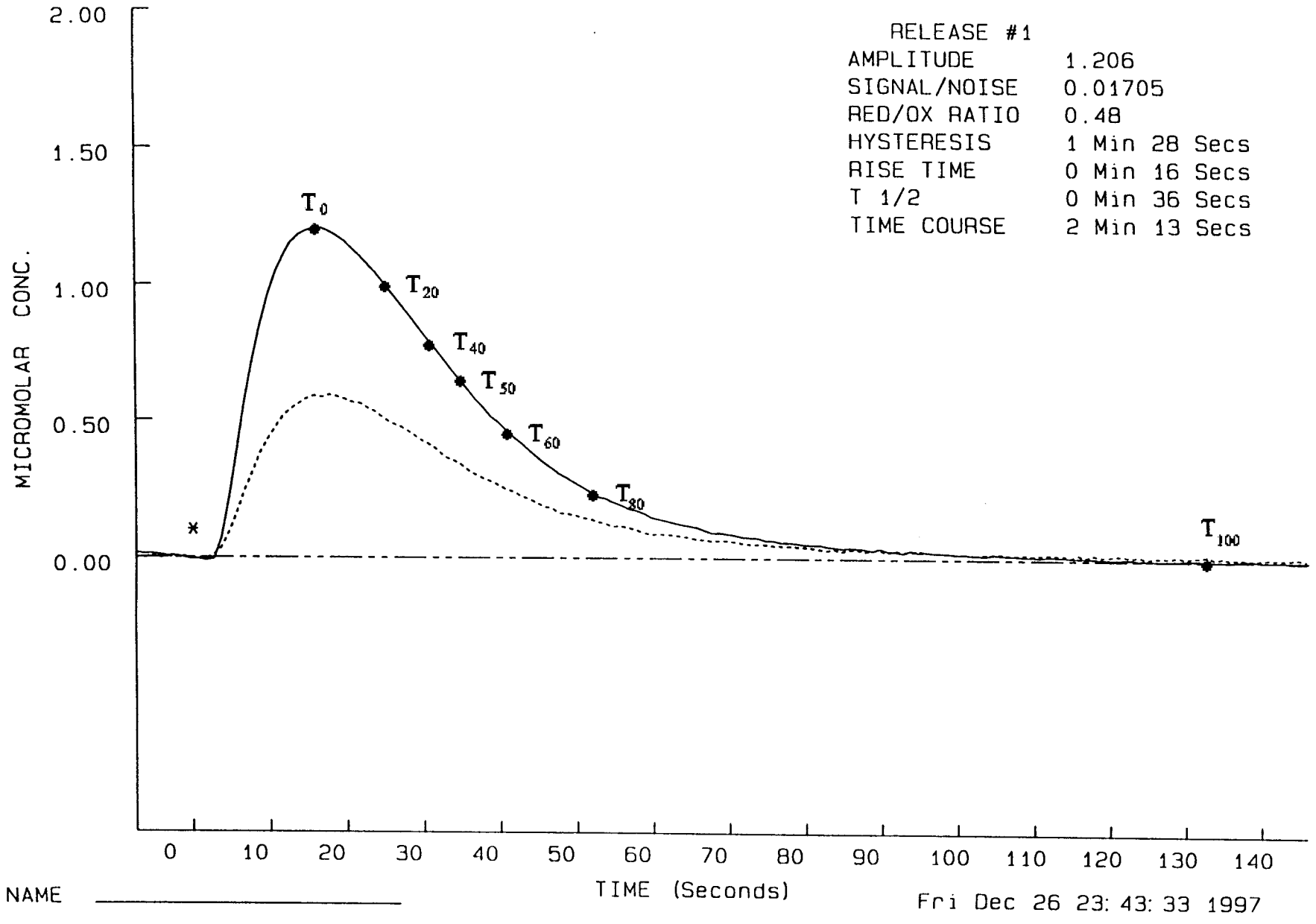
treatment group and 49.2 +/- 4.02 seconds for MPP^+ with E_2 ($p = < 0.0001$). This represents a significant decrease of 60.0% in the time course when estrogen was applied along with MPP^+ (See figure 19). When comparisons are made between the CPu and the AcbC a reduction in the time course of 35.0% for MPP^+ ($p = 0.0041$) and 31.9% for MPP^+ with estrogen ($p = 0.0005$) is seen in the AcbC when compared to the same treatments in the CPu (See figure 19).

While there was some variability between individual responses, calculated values for each parameter examined remained quite consistent within each group. This is very evident, as standard error bars for all parameters in both groups were relatively small.

The volume of test substances infused remained consistent throughout experimentation averaging between 150-175nL for both MPP^+ and MPP^+ with estrogen treatment group.

Figure 9. MPP$^+$ Induced Dopamine Electrochemical Signal.

This figure illustrates a typical response to the local application of 10^{-3} M MPP$^+$ into the corpus striatum of a female (OVX) Long-Evans rat. The oxidation current (solid line) and the reduction current (dashed line) were sampled at 5 Hz and the averaged over one second. The micromolar concentration of dopamine is given on the y-axis versus time on the x-axis. The signal was analyzed at several time points labeled T_0 to T_{100}.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AMPLITUDE 1.206
SIGNAL/NOISE 0.01705
RED/OX RATIO 0.48
HYSTERESIS 1 Min 28 Secs
RISE TIME 0 Min 16 Secs
T 1/2 0 Min 36 Secs
TIME COURSE 2 Min 13 Secs
MICROMOLAR CONC.
2.00
1.50
1.00
0.50
0.00
T0
T20
T40
T50
T60
T80
T100
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140
TIME (Seconds)
NAME
Fri Dec 26 23: 43: 33 1997

Figure 10. MPP^+ with Estrogen Induced Dopamine Electrochemical Signal.

This figure illustrates a typical response to the local application of 10^{-3} M MPP^+ with 10^{-5} g/ml estrogen into the corpus striatum of female (OVX) Long-Evans rats. The oxidation current (solid line) and the reduction current (dashed line) were sampled at 5 Hz and then averaged over one second. The micromolar concentration of dopamine is given on the y-axis versus time on the x-axis. The signal is analyzed at several time points labeled T_0 to T_{100}.

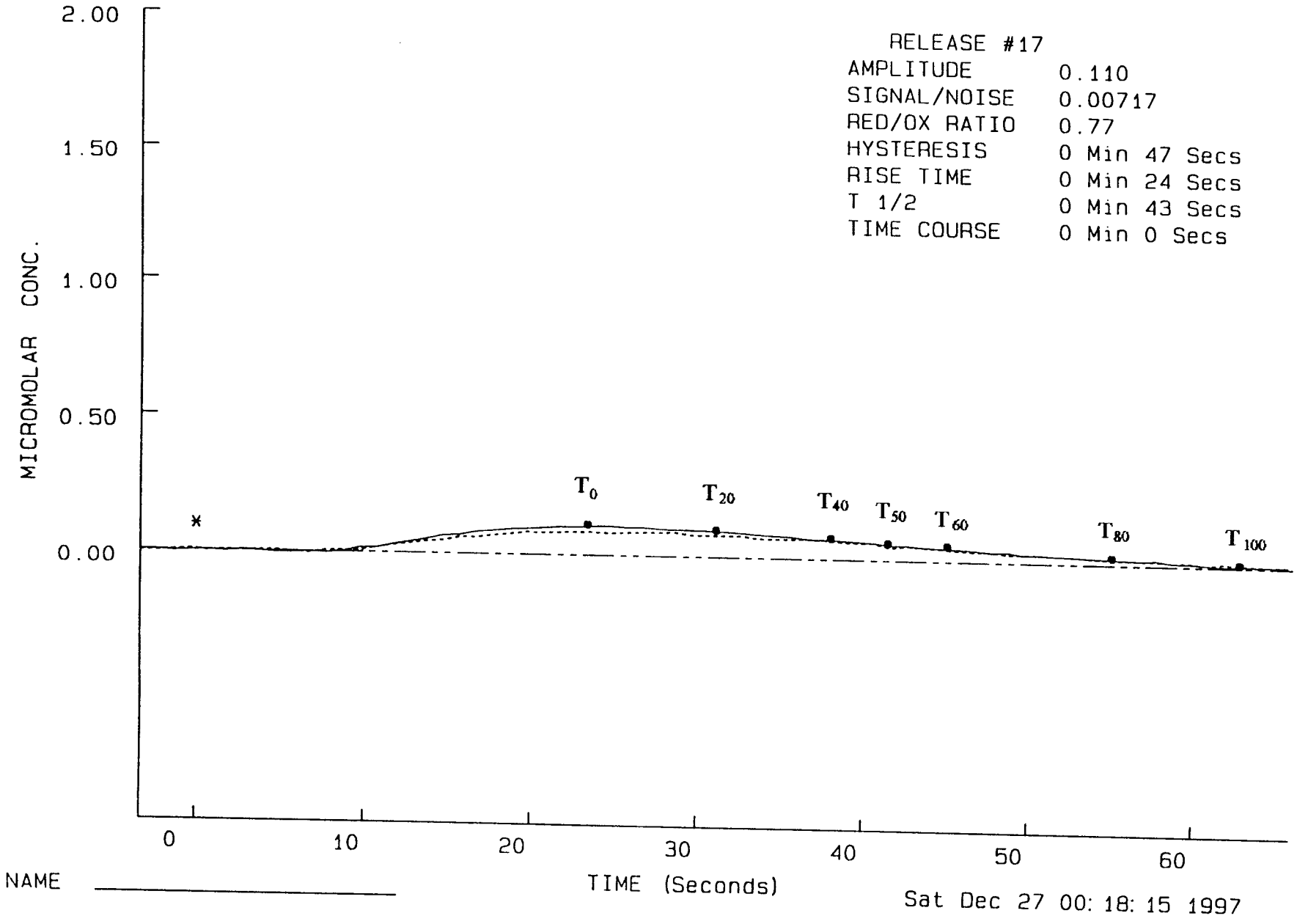
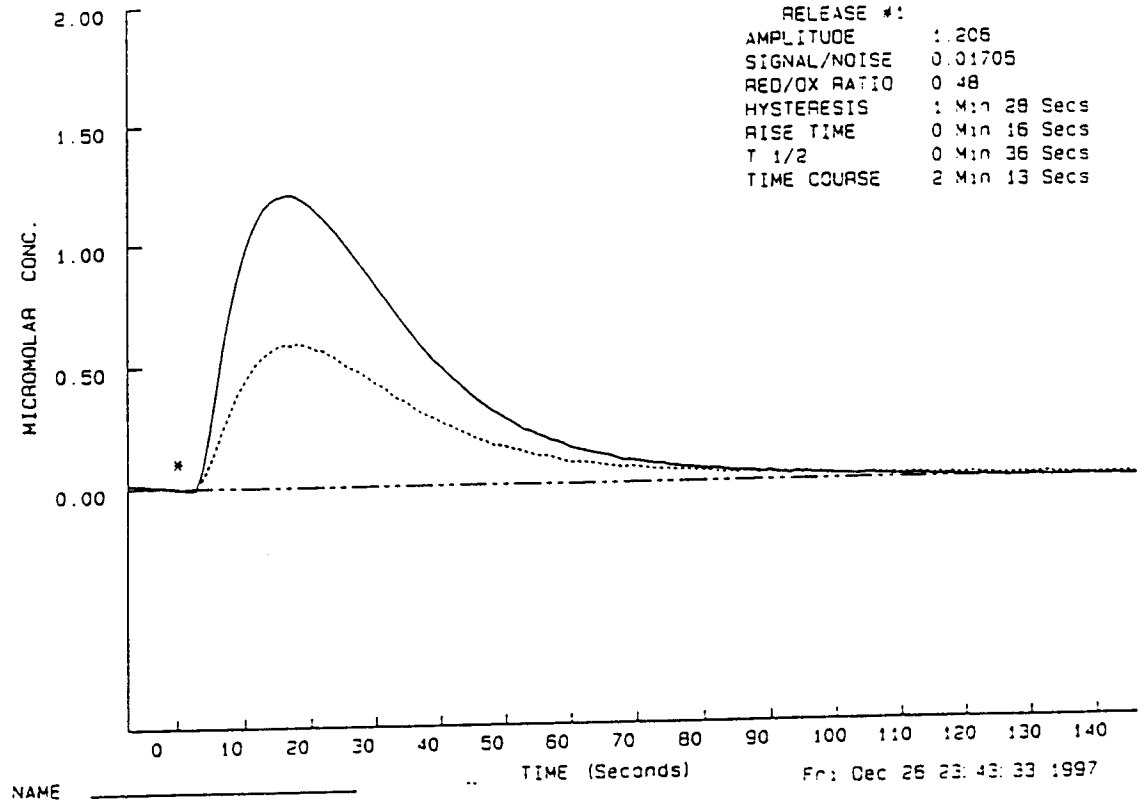

RELEASE #17
AMPLITUDE 0.110
SIGNAL/NOISE 0.00717
RED/OX RATIO 0.77
HYSTERESIS 0 Min 47 Secs
RISE TIME 0 Min 24 Secs
T 1/2 0 Min 43 Secs
TIME COURSE 0 Min 0 Secs
MICROMOLAR CONC.
2.00
1.50
1.00
0.50
0.00
T0
T20
T40
T50
T60
T80
T100
0
10
20
30
40
50
60
TIME (Seconds)
NAME
Sat Dec 27 00: 18: 15 1997

Figure 11. MPP$^+$ and MPP$^+$ with Estrogen Signal Comparison. This figure illustrates a comparison between a MPP$^+$ stimulated dopamine response (top) and a MPP$^+$ with estrogen stimulated dopamine response (bottom). Significant differences are apparent in micromolar concentration, rise-time, T½ time, and time course between the two signals.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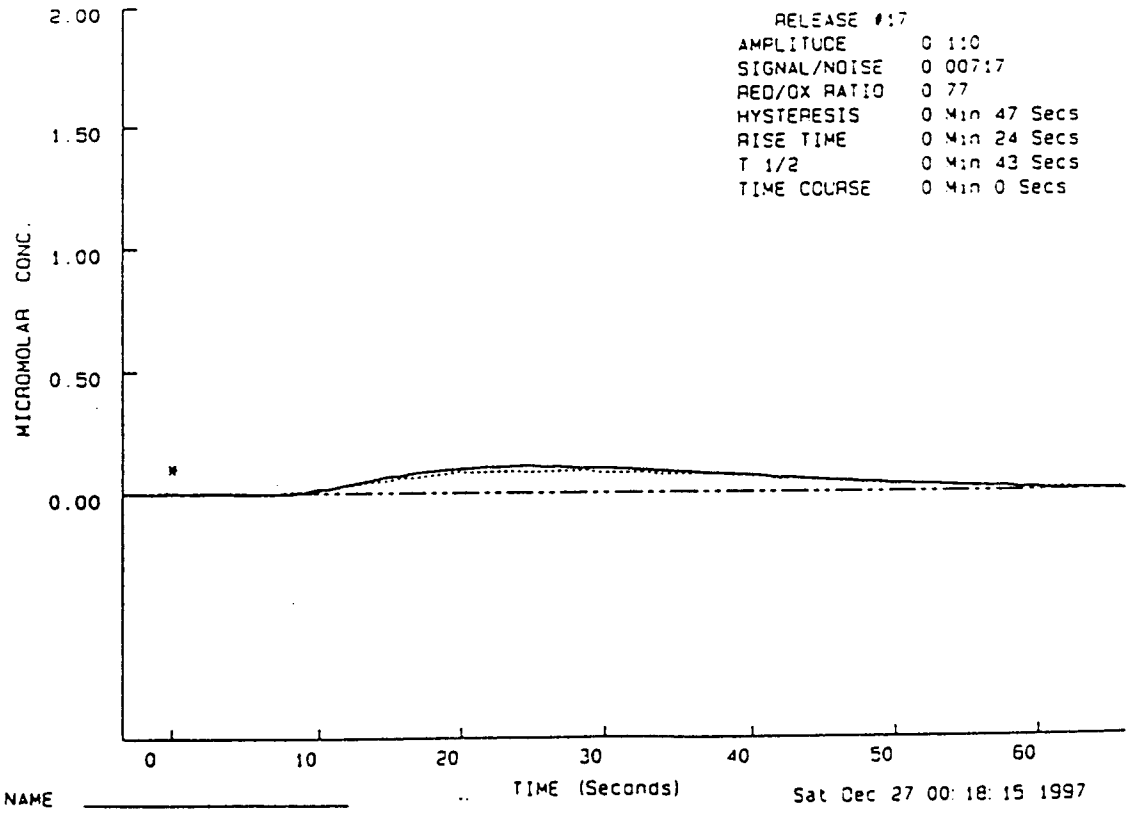
SIGNAL/NOISE 0.01705
RED/OX RATIO 0 48
HYSTERESIS 1 Min 28 Secs
RISE TIME 0 Min 16 Secs
T 1/2 0 Min 36 Secs
TIME COURSE 2 Min 13 Secs
MICROMOLAR CONC.
2.00
1.50
1.00
0.50
0.00
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140
TIME (Seconds)
Fri Dec 26 23:43:33 1997
NAME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SIGNAL/NOISE 0 00717
RED/OX RATIO 0 77
HYSTERESIS 0 Min 47 Secs
RISE TIME 0 Min 24 Secs
T 1/2 0 Min 43 Secs
TIME COURSE 0 Min 0 Secs
MICROMOLAR CONC.
2.00
1.50
1.00
0.50
0.00
0 10 20 30 40 50 60
TIME (Seconds)
Sat Dec 27 00:18:15 1997
NAME

Figure 12. Amplitude.

For figures 12-19 values are based on the total number (n) responses. For the CPu MPP^+ group (n = 142) and for the CPu MPP^+ with estrogen group (n = 139). For the AcbC MPP^+ group (n = 34) and for the AcbC MPP^+ with estrogen group (n = 41). Values given are the mean +/- the SEM. The individual p values are displayed in each figure legend while the error bars represent the standard error. Amplitude for MPP^+ and MPP^+ with estrogen is displayed in μM concentrations of dopamine.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = < 0.0001$).
3. Compares MPP^+ CPu vs. MPP^+ AcbC ($p = < 0.0001$).
4. Compares MPP^+ with E_2 CPu vs. MPP^+ with E_2 AcbC ($p = 0.0002$).

AMPLITUDE

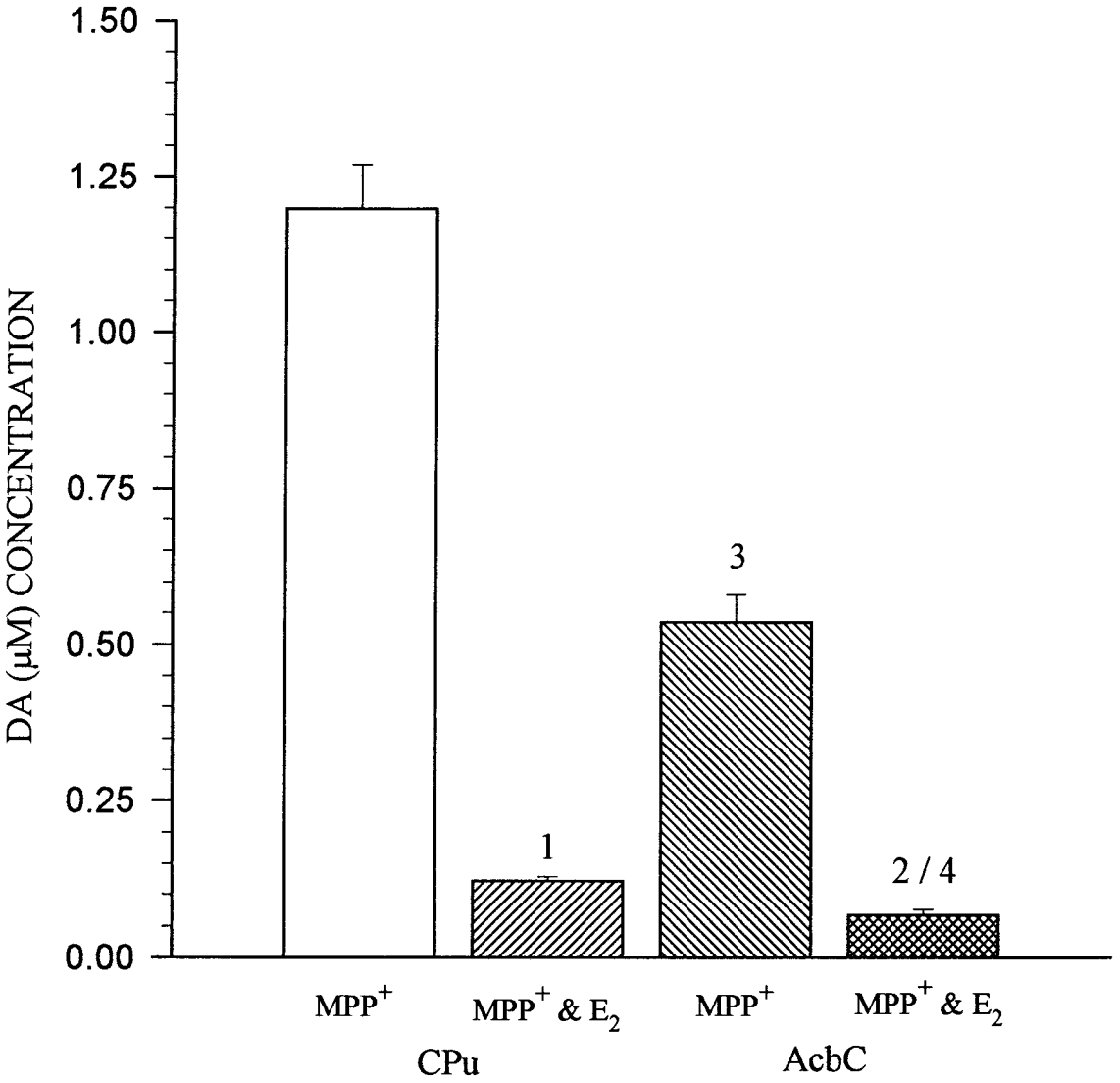

1.50
1.25
1.00
0.75
0.50
0.25
0.00
DA (μM) CONCENTRATION
1
3
2 / 4
MPP+
MPP+ & E2
MPP+
MPP+ & E2
CPu
AcbC

Figure 13. Rise-Time.

Rise-time, in seconds, for MPP^+ and MPP^+ with estrogen induced dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = 0.0251$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = 0.0074$).
4. Compares MPP^+ with E_2 CPu vs. MPP^+ with E_2 AcbC ($p = 0.0470$).

RISE TIME

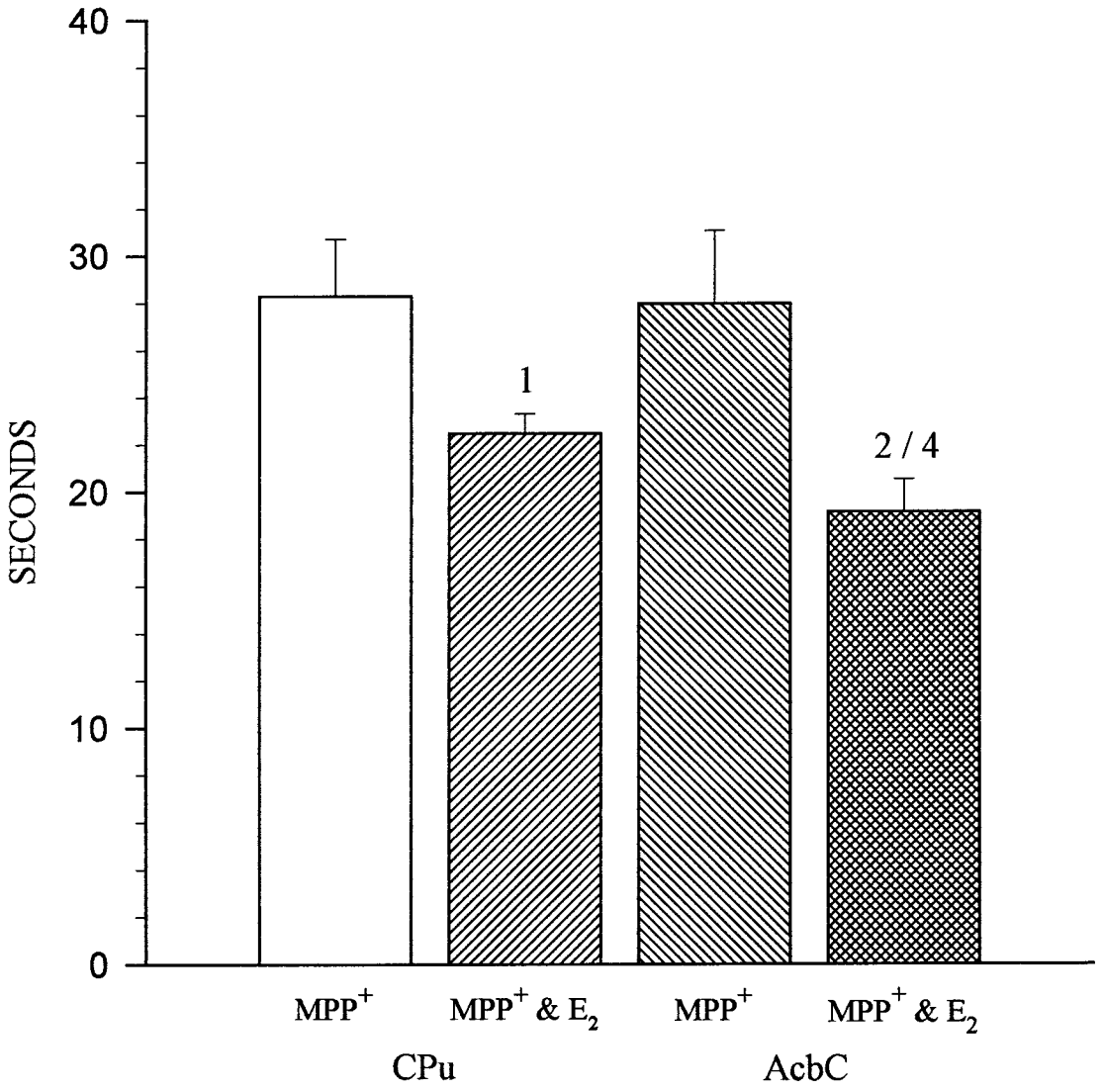


Figure 14. Secretion Rate.

Average secretion rates in nM concentrations for MPP^+ and MPP^+ with estrogen stimulated dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = < 0.0001$).
3. Compares MPP^+ CPu vs. MPP^+ AcbC ($p = 0.0003$).
4. Compares MPP^+ with E_2 CPu vs. MPP^+ with E_2 AcbC ($p = 0.0367$).

SECRETION RATE

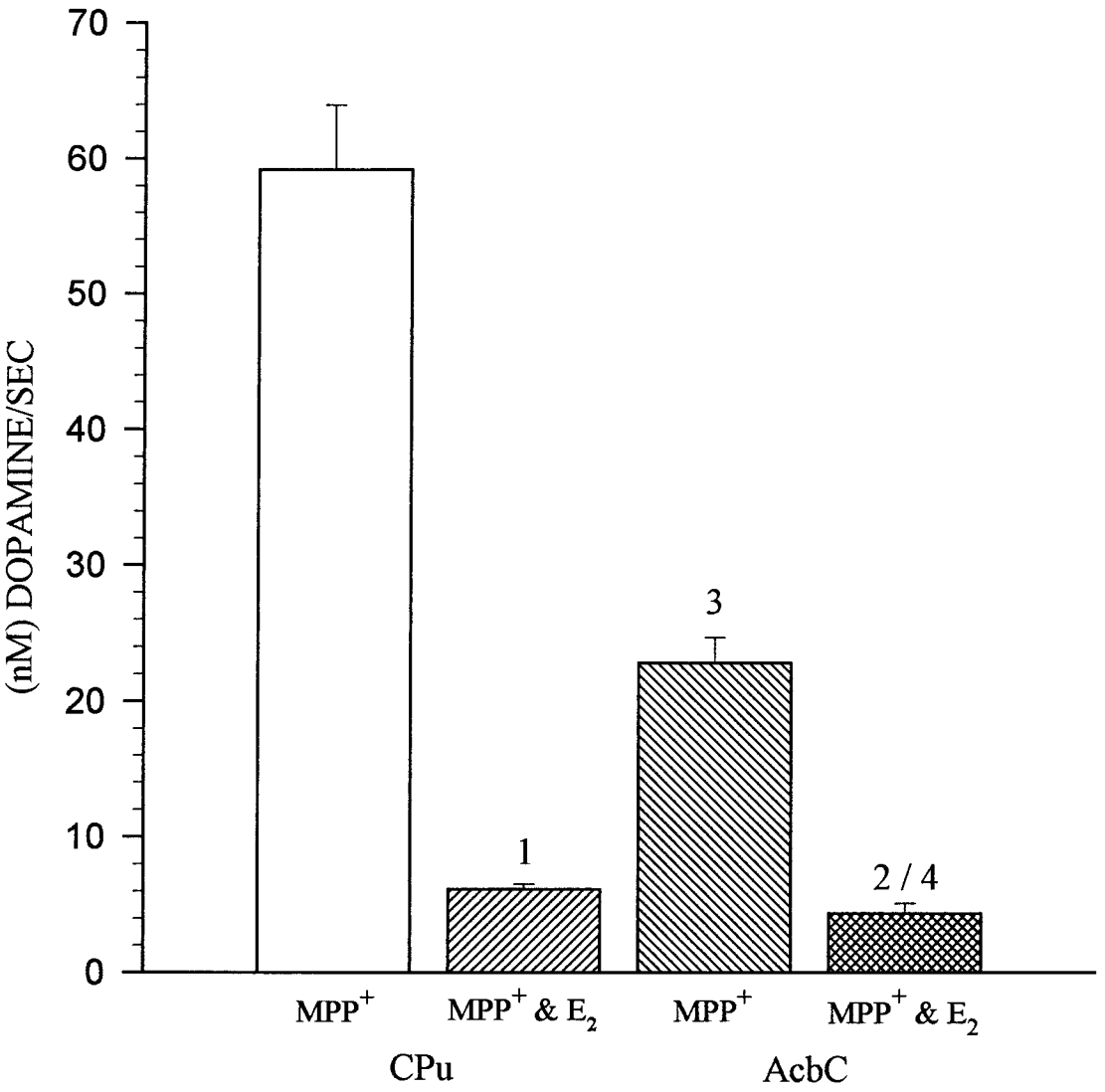


Figure 15. T 50.

Average T 50 times in seconds for MPP^+ and MPP^+ with estrogen stimulated dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = 0.0025$).

T - 50

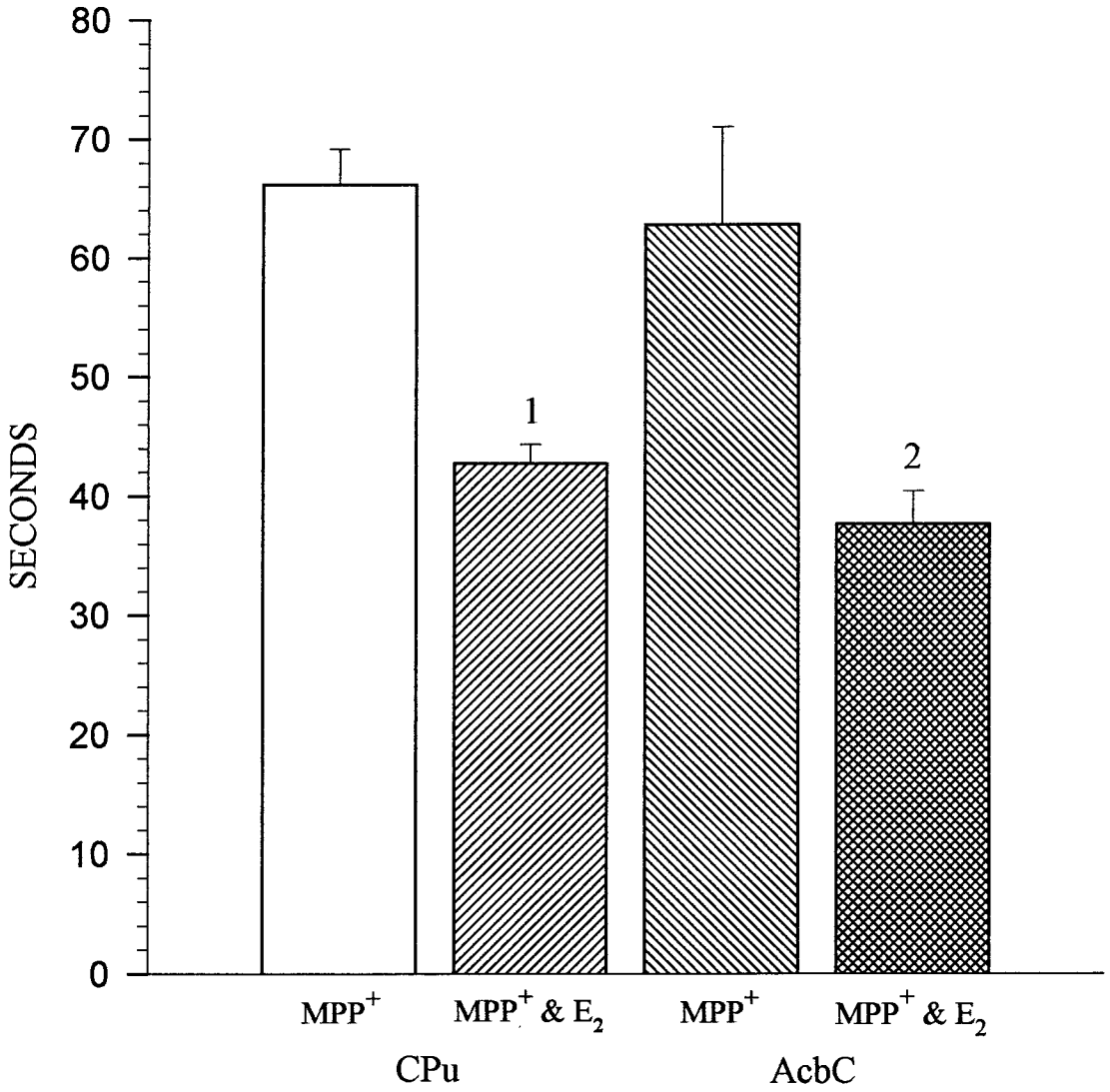


Figure 16. T 20-60.

Average T 20-60 times in seconds for MPP^+ and MPP^+ with estrogen stimulated dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = 0.0007$).
4. Compares MPP^+ with E_2 CPu vs. MPP^+ with E_2 AcbC ($p = 0.0036$).

T - 20-60

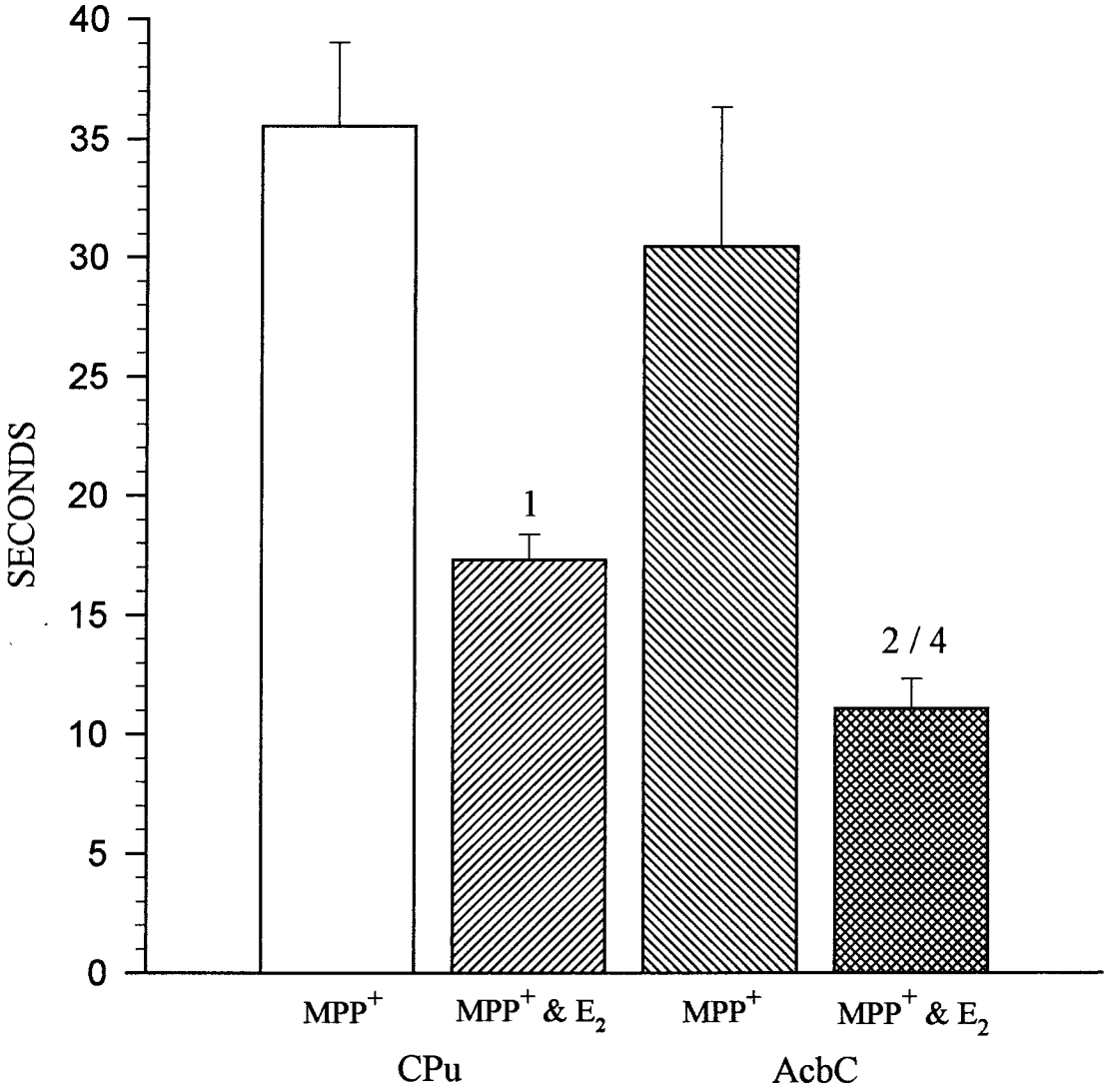


Figure 17. T 40-80.

Average T 40-80 times in seconds for MPP^+ and MPP^+ with estrogen stimulated dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = < 0.0001$).
4. Compares MPP^+ with E_2 CPu vs. MPP^+ with E_2 AcbC ($p = 0.0110$).

T - 40-80

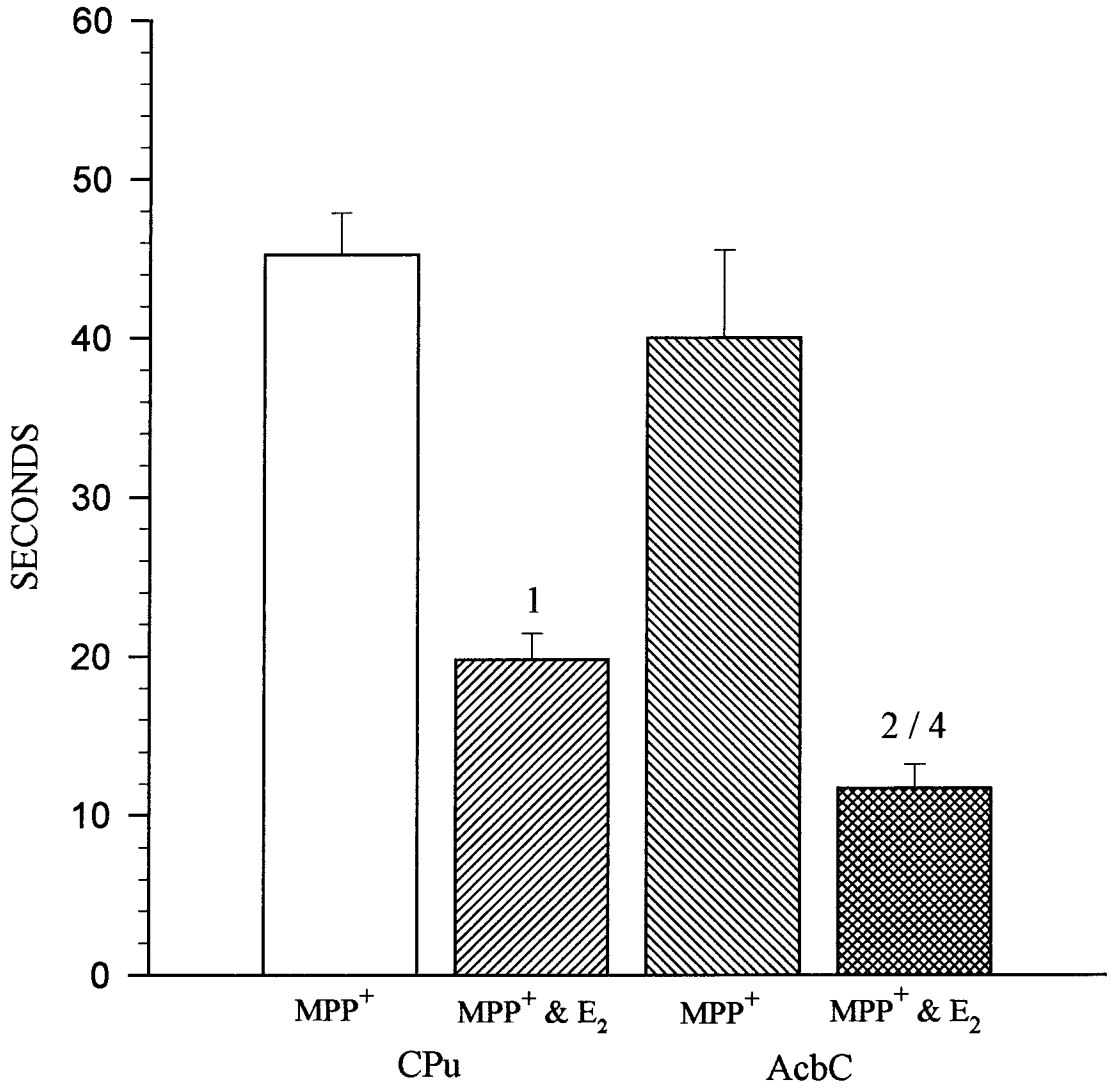


Figure 18. Clearance Rate.

Average clearance rates in nM concentrations for MPP^+ and MPP^+ with estrogen stimulated dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = < 0.0001$).

CLEARANCE RATE

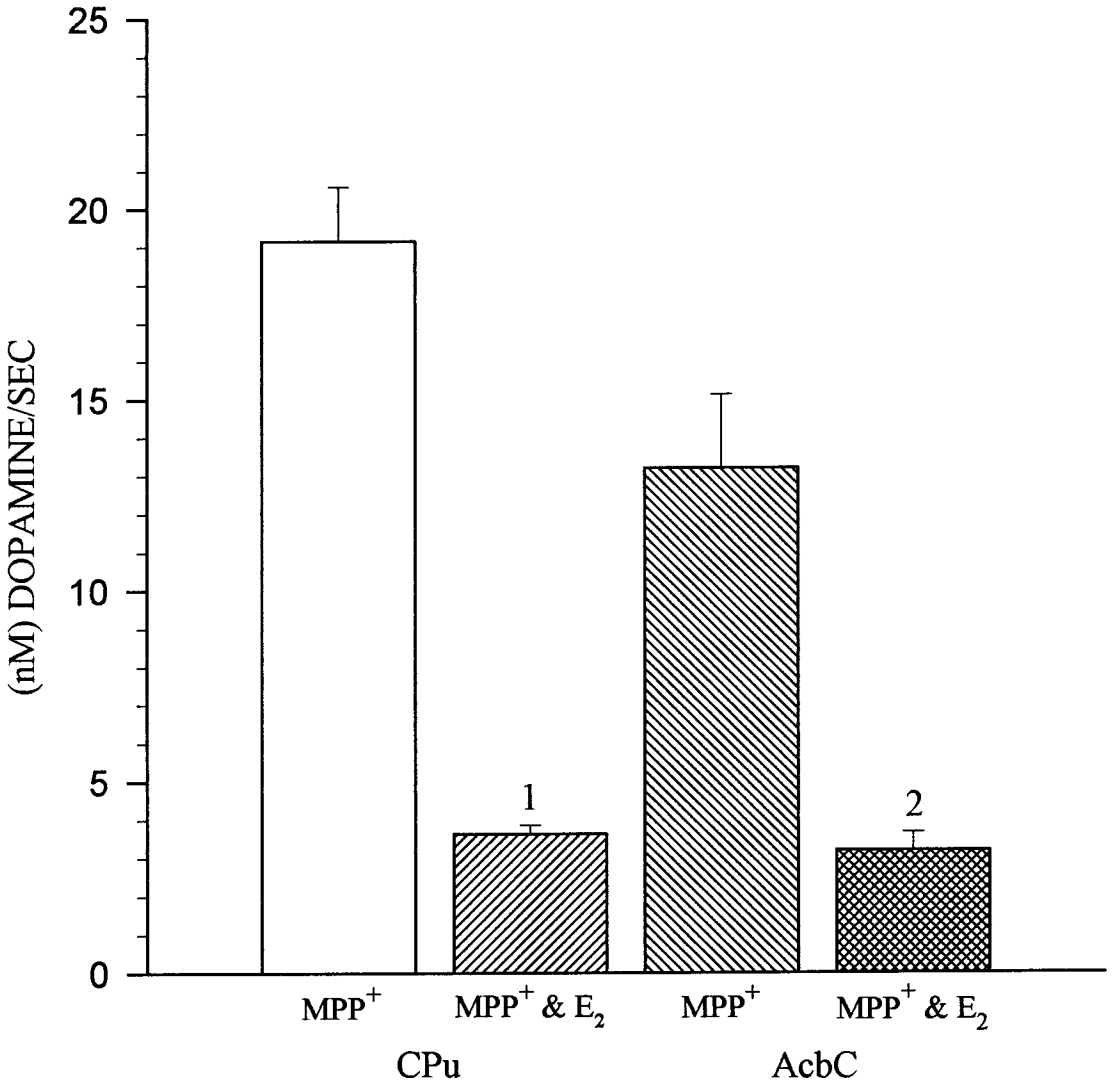


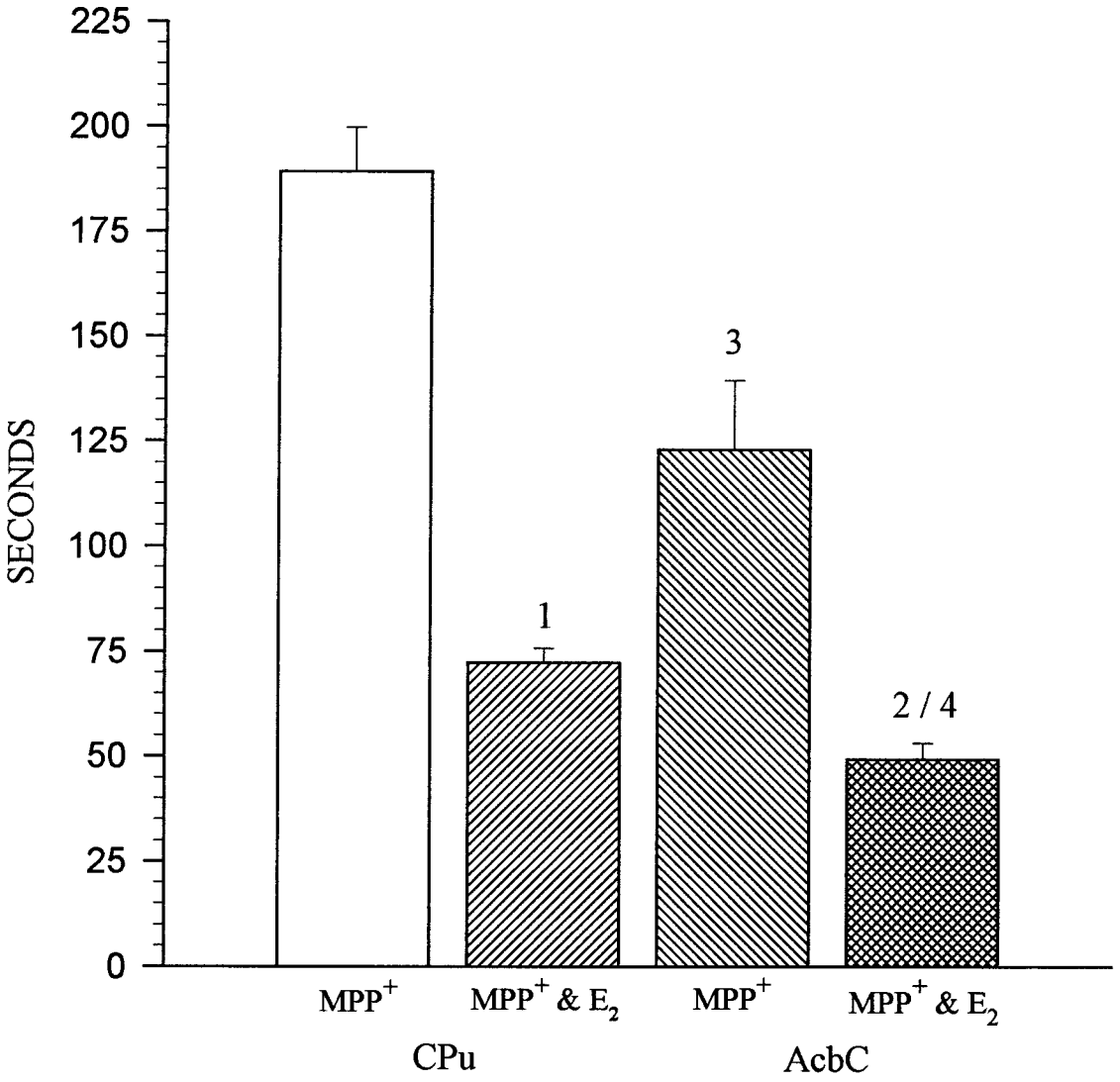
Figure 19. Total Time Course.

Average total time courses in seconds for MPP^+ and MPP^+ with estrogen stimulated dopamine responses in the CPu and the AcbC.

FIGURE LEGEND KEY

1. Compares MPP^+ CPu vs. MPP^+ with E_2 CPu ($p = < 0.0001$).
2. Compares MPP^+ AcbC vs. MPP^+ with E_2 AcbC ($p = < 0.0001$).
3. Compares MPP^+ CPu vs. MPP^+ AcbC ($p = 0.0041$).
4. Compares MPP^+ with E_2 CPu vs. MPP^+ with E_2 AcbC ($p = 0.0005$).

TIME COURSE


SECONDS
225
200
175
150
125
100
75
50
25
0
1
3
2 / 4
MPP+
MPP+ & E2
MPP+
MPP+ & E2
CPu
AcbC

CHAPTER IV

DISCUSSION

This study was designed to investigate the acute modulatory effects of estrogen on the dynamics of MPP^+-induced dopamine release in the corpus striatum and nucleus accumbens of the rat brain. The results of this experiment clearly demonstrate differences between MPP^+-stimulated and MPP^+ with estrogen stimulated dopamine secretion in both the corpus striatum and the nucleus accumbens. Comparisons between brain areas examined reveals considerable regional differences between the corpus striatum and nucleus accumbens, as to the effects of MPP^+ and estrogen upon dopamine release and clearance characteristics.

DOPAMINE RELEASE

Results from this study clearly demonstrate that the neurotoxin MPP^+ stimulates the acute release of dopamine from the CPu and the AcbC of

female OVX Long-Evans rats. Obata et al., (1992) reported the successful stimulation of dopamine secretion *in vivo* from striatal tissue using microdialysis. Similarly, Disshon and Dluzen (1997) and Dluzen et al., (1996) reported similar results *in vitro* using superfusion techniques. Through the use of *in vivo* voltammetry in this experiment, it was possible to detect dopamine electrochemical signals in both the CPu and AcbC following local administration of MPP^+. MPP^+-stimulated dopamine release is unlike normal physiologic or K^+-stimulated dopamine secretion, which occurs by way of classical Ca^{++} dependent exocytosis. DA release, secondary to MPP^+ stimulation is a result of dopamine efflux from the pre-synaptic terminal through the concentration dependent DA transporter (Chiba et al., 1985).

Parameters examined for dopamine electrochemical signals can be divided into two general categories, those that pertain to the release of dopamine and those that characterize clearance or removal of the dopamine signal. Release characteristics examined include amplitude, rise time and secretion rate. The amplitude, rise time and secretion rate of the MPP^+-induced dopamine signal were all suppressed with the striatal application of estrogen along with MPP^+. This effect was present both in the CPu and the AcbC but to different degrees. In the CPu amplitude, rise time and secretion rates were reduced by 89.9, 20.5 and 89.7% respectively when estrogen was applied along with MPP^+ (See fig. 12, 13 and 14). Similar decreases of DA secretion were observed in the AcbC with the application of estrogen with MPP^+. Reductions of 87.3, 31.8 and 81% were recorded for amplitude, rise time and secretion rate respectively (see fig. 12, 13, and 14). These results

demonstrate that estrogen is acting in an acute and presumably non-genomic manner to inhibit MPP^+-induced dopamine release in both the CPU and the AcbC. Estrogen's effect upon striatal dopamine release is not a completely new concept. Modulatory effects on dopamine release were postulated by Baker and Ramsey as early as 1980, and later by McDermott in 1993. At that time it was suspected that the long term effect of steroid hormones caused an increase in striatal dopamine release and an overall decrease in dopamine content. While still unclear, these effects may be linked to estrogen's genomic effects on protein synthesis. Since MPP^+'s site of entry into the dopaminergic neuron is the DA transporter, it is plausible that estrogen may be acting at this site to inhibit the entry of MPP^+ into the pre-synaptic neuron. While the exact mechanism of action for this inhibition is unclear, it is possible that estrogen may be competing with MPP^+ for receptor sites or affecting the affinity of the transporter for the neurotoxin MPP^+. Lastly, it is possible that estrogen could be acting directly on the MPP^+ molecule itself, altering it in some way thereby rendering it less effective in stimulating DA release. The exact mechanism by which estrogen is operating is yet to be determined but will be an important question to answer in future studies.

DOPAMINE CLEARANCE

In this study a number of parameters were investigated which correlate to the removal or clearance of the dopamine signal from the area of the recording electrode. T-50, T-20 to 60, T-40 to 80 and clearance rate were

all evaluated as to the effects of estrogen on the removal of the dopamine signal.

The clearance rate for dopamine was reduced by 82.4% and 75.8% in the CPu and the AcbC respectively, when estrogen was applied along with MPP^+. This reduction in the rate at which dopamine was being removed may again be linked to a direct effect of estrogen on the dopamine transporter. Michael et al., (1987) reported a decrease in dopamine uptake following treatment with estrogen or estrogen agonists. The effects of estrogen on the transporter itself, and/or the continued competition of MPP^+ with dopamine for transporter sites could result in a decreased rate of clearance of dopamine from the nerve terminal. MPP^+ competing for transporter space with DA was demonstrated in studies by Cooper et al., and Chiba et al., in 1985 and later in 1995 by Langston and others. It is difficult to determine the exact extent to which estrogen is effecting the rate of dopamine clearance in the presence of MPP^+. Numerous factors play a role in the removal of the neurotransmitter from the nerve synapse. If estrogen acts by preventing DA uptake via actions on the transporter, MPP^+'s entry into the neuron is also inhibited, then the neurotoxic effects of MPP^+ could be reduced.

T-50, T-20 to 60 and T-40 to 80 represent time periods during the clearance of the dopamine signal. Reductions in T-50, T-20 to 60 and T-40 to 80 were observed in both the CPu and the AcbC in the MPP^+ with estrogen treatment group. Therefore, less time was required for the dopamine signal to decay over these particular time intervals. Interestingly, the effects of estrogen on dopamine removal, seems more pronounced later

in the clearance event. Our data showed increasing differences between MPP^+ and MPP^+ with estrogen treatment groups as clearance of the dopamine signal progressed. Percentage differences between MPP^+ and MPP^+ with estrogen in the CPu was decreased by 35.3, 51.3, and 56.3% for T-50, T-20 to 60 and T-40 to 80 respectively. This trend was present to an even greater extent in the AcbC. Reductions of 40, 63.6, and 71.8% were observed for T-50, T-20 to 60 and T-40 to 80 in the MPP^+ with estrogen treatment group. This data reveals a more pronounced effect by estrogen in the later stages of dopamine clearance. This may be due to inherent differences between the CPU and the AcbC as to the effects of estrogen upon the dopamine transporter. It is also possible that the reduced values obtained for T-50, T-20 to 60 and T-40 to 80 were artificially reduced in the AcbC as a result of the overall reduction in dopamine release seen in this area.

The final parameter examined in this experiment was total time course. Again, significant reductions were observed for this time period within the MPP^+ with estrogen treatment groups. Reductions of 61.8 and 60% were observed for the CPu and AcbC. Decreases observed in the time course are a function of reductions in other previously mentioned parameters such as amplitude, secretion rate and the decay times. With such drastic reductions in the amount of dopamine being released within the MPP^+ with estrogen group, a decrease in the overall timing of the release event is expected.

REGIONAL COMPARISONS

Comparisons were also made between the CPu and the AcbC to determine if differences might exist in the effects of estrogen upon MPP^+-stimulated dopamine release. The CPu receives its dopaminergic input from the pars compacta of the substantia nigra. While input to the AcbC is from the mesolimbic dopaminergic system with cell bodies in the ventral tegmental area (Kandel et al., 1991). While the majority of cell loss in Parkinson's disease is seen in the substantia nigra resulting in dopamine depletion in the CPu, dopamine depletion in the AcbC has been documented as well.

Dopamine Release. When comparisons were made between the CPu and the AcbC, regional differences were observed in amplitude, secretion rate, and to a lesser extent rise time. Values obtained from the AcbC were reduced when compared to similar treatments in the CPu. For example, the average amplitude was decreased by 55.3% for MPP^+ and 56.2% for MPP^+ with estrogen in the AcbC, (See fig. 12). Regional differences were also observed for secretion rate. A reduction in secretion rate of 61.4% for MPP^+ and 29.1% for MPP^+ with estrogen was observed in the AcbC when compared to the CPu. There were only slight regional differences observed in rise times for the AcbC within the MPP^+ with estrogen treatment group. No regional differences were observed within the MPP^+ only treatment group (See fig 12,13).

While regional differences were not apparent in every parameter examined, significant differences in the dynamics of dopamine release and

clearance were observed between the CPu and the AcbC frequently enough to suspect a physiological distinction between the two areas as to the effects of MPP^+ and estrogen.

Dopamine Clearance. When comparisons were made between the CPu and the AcbC, no significant differences were noted in the MPP^+ only treatment group for T-50, T-20 to 60, T-40 to 80 or clearance rate. In contrast, T-20 to 60 and T-40 to 80 decay times were both reduced in the AcbC for MPP^+ with estrogen. This suggests a more pronounced effect of estrogen modulation upon these decay times within the AcbC. There was no significant difference noted between brain regions examined as to the rate of dopamine clearance. The total time course was substantially reduced for MPP^+ and MPP^+ with estrogen in the AcbC. Values obtained were decreased by one third for both treatment groups in the AcbC.

The results of this experiment would suggest at least some regional differences between the CPu and the AcbC as to MPP^+'s ability to stimulate dopamine secretion and estrogens effectiveness at blocking the action of MPP^+. Our data revealed that MPP^+ was less effective in stimulating dopamine release in the AcbC and estrogen was more effective in modulating the action of MPP^+ in the AcbC.

FUTURE STUDIES

While it seems clear that the steroid hormone estrogen can modulate nigrostriatal dopaminergic function in an acute manor, the exact mechanism at work remains unclear. Estrogen's effects on dopaminergic function are

complex, and likely two fold, with differences between acute and chronic modulatory influences. Additional investigations concerning the effects of estrogen on dopamine re-uptake are necessary since some studies report a dopamine depleting effect (McDermott 1992), while others (Disshon and Dluzen, 1997, and the present study) report a dopamine sparing or neuroprotective effect. The possible modulatory effects of other steroid hormones, in particular testosterone, progesterone and cortisol should be investigated in an attempt to reveal if the neuroprotective effects observed, are isolated to estrogen or are common to other similar steroid molecules. The effects of estrogen in aged versus young animals is an important issue as well since idiopathic Parkinson's disease effects people of late middle age and older. Male-female differences should be examined as well since it has been reported in several studies that males have anywhere from a 2-4 greater prevalence of Parkinson's disease (Diamond et al., 1990, Mayeux et al., 1992).

SUMMARY

In summary, the results of this *in vivo* electrovoltammetric study demonstrate that the neurotoxin MPP^+ stimulates the secretion of dopamine from both the corpus striatum and the nucleus accumbens of female OVX Long-Evans rats. These results also clearly demonstrate that estrogen acts in an inhibitory manor upon specific characteristics of MPP^+-induced dopamine release. This inhibitory effect was observed in both the corpus striatum and the nucleus accumbens. These results are also very suggestive

of regional differences between the corpus striatum and the nucleus accumbens, as to the actions of MPP^+ and the effectiveness of estrogen on modulating MPP^+-induced dopamine release. For example, MPP^+ is less effective in stimulating dopamine release in the AcbC while estrogen is more effective at modulating MPP^+'s action in the AcbC. Taken together, these results, suggest that estrogen may be acting in a neuroprotective role in the rat, against the acute neurotoxic effects of MPP^+.

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