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Studies on the mechanisms involved in the selective toxicity of MPTP, MPP+, and guanethidine to the central monoamine neurons in culture

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STUDIES ON THE MECHANISMS INVOLVED IN THE SELECTIVE TOXICITY OF MPTP, MPP+, AND GUANETHIDINE TO THE CENTRAL MONOAMINE NEURONS IN CULTURE.

by

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Abstract

STUDIES ON THE MECHANISMS INVOLVED IN THE SELECTIVE TOXICITY OF MPTP, MPP+, AND GUANETHIDINE TO THE CENTRAL MONOAMINE NEURONS IN CULTURE.

by

Linda Friedman

Adviser: Dr. Eugene Sachs

The recently discovered neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can induce parkinsonsim in humans, monkeys, and some rodents, by selectively destroying nigrostriatal dopamine (DA) neurons. As in Parkinson's disease, other catecholamine (CA) neurons, such as DA neurons of the ventral tegmental area (VTA) and norepinepherine (NE) neurons of the locus coeruleus (LC), are also affected by MPTP, although to a lesser extent. 1-Methyl-4-phenylpyridinium ion (MPP+), the product of MPTP oxidation by monoamine oxidase B (MAO-B), appears to be the toxin responsible for neuronal degeneration. The oxidation of MPTP probably occurs within glial cells and the toxin MPP+ accumulates in the DA neurons by the uptake pump.

We have used primary dissociated cultures of central CA or serotonergic (5HT) neurons and examined their response upon <u>in vitro</u> application of the specific neurotoxins MPTP, MPP+, and guanethidine, a compound known to cause degeneration of peripheral NE neurons to gain understanding of the cellular mechanisms underlying

Specific neurotoxins, such as MPTP, are valuable tools in studies of the cellular mechanisms underlying preferential cell death in neurodegenerative disorders. In the studies presented here we have used primary dissociated cultures of central CA neurons and examined their response upon *in vitro* application of the neurotoxins MPTP, MPP+, and guanethidine, a compound known to cause degeneration of peripheral NE neurons.

Cultures were established from the ventral mesencephalon or pons from rat embryos on the 14th gestational day. On the 8th day *in vitro* cultures were exposed to the neurotoxins MPTP or MPP+ for 1 week and guanethidine for 1 or 2 weeks at concentrations ranging from 0.01-200uM. Neurotoxicity was evaluated by CA histofluorescence after preincubation with alpha-methylnorepinepherine, tyrosine hydroxylase (TH) immunocytochemistry, or tritiated monoamine uptake. The effective concentration range differed for each toxin. In DA neurons, MPTP maximal toxicity occured between 5 and 10 uM, with a reduction in uptake values by 75-90%, and almost complete disappearance of neurons positive for CA histofluoresence and TH immunocytochemistry. Increasing the concentration of MPTP to 100-200 uM resulted in reduced toxicity, with a 20-40% reduction in DA uptake and about 50% decrease in surviving DA neurons. The decrease in MPTP toxicity at higher

concentrations was due to (1) reduction in MPTP oxidation due to the inhibitory effect of MPTP on MAO activity and (2) inhibition of the uptake of MPP+ by high concentrations of MPTP. Degeneration of DA neurons by MPTP was prevented by treatment with the MAO-B inhibitor deprenyl, but not by the MAO-A inhibitor clorgyline. MPTP was also toxic to NE neurons, but to a lesser extent. 5HT neurons were affected only by 100 uM MPTP, which caused a 50% decrease in uptake sites but no reduction in the number of 5HT neurons. On the contrary, 5HT levels increased in the cultures exposed to MPTP.

The metabolite of MPTP, MPP+ was a more potent toxin for the DA neurons and the toxicity increased with increasing concentrations (1-10 uM). 10 uM MPP+ reduced uptake sites by 97-99% and resulted in complete loss of DA neurons. Neurotoxicity was not influenced by deprenyl. However, uptake inhibitors partially protected the neurons from MPP+. NE and 5HT neurons were affected by MPP+, but their sensitivity was less than that of the DA neurons.

DA, NE and 5HT neurons were all affected by guanethidine after 2 weeks exposure and at high concentrations (100 uM). Lower concentrations produced a greater neurotoxic effect to NE neurons. One week exposure to guanethidine resulted in a selective toxicity to NE neurons, suggesting a higher affinity of this drug for the central NE neurons.

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List of Abbreviations

Ach ..... acetylcholine

CA ..... catecholamines

CNS ..... central nervous system

DA ..... dopamine

DDC ..... dopa decarboxylase

DRN ..... dorsal raphe' nuclei

5HT ..... serotonin

5HIAA ..... 5-hydroxy-indolacetic acid

HPLC ....high performance liquid chromatography

HVA ..... homovanilic acid

IP ..... idiopathic Parkinson's disease

LC ..... locus coeruleus

MAO ..... monoamine oxidase

MPTP 1-methyl-4-phenyl-tetrahydropyridine

MPP+ 1-methyl-4-phenylpyridine

nacc .....nucleus accumbens septi

NBM ..... nucleus basalis of Meynert

NGF ..... nerve growth factor

ODC ..... ornithine decarboxylase

SN ..... substantia nigra

TH ..... tyrosine hydroxylase

VTA ..... ventral tegmental area

I. INTRODUCTION

A. Neurodegenerative disease

Neurodegeneration occurs during normal aging. Many neurodegenerative diseases are associated with advanced age and are correlated with alterations in neurotransmitter systems. Most often, a specific brain region is predominantly affected and a variety of clinical symptoms result. Examples of some neurological disorders of advancing age include senile dementia of the Alzheimer's type, Parkinson's disease, and Huntington's disease. Lesions of the basal ganglia are found in these neurodegenerative diseases apart from senile dementia. Movement disorders and cognitive deficits are present in Parkinson's disease and a well defined neurotransmitter loss exists. Parkinsonism affects 1 person in 100 after the age of 50 (Irwin, 1986) and understanding its etiology has been the main impetus for the present research. The general approach taken in our laboratory is to understand the mechanism of preferential cell death that occurs in brain stem nuclei of parkinsonian patients.

Parkinson's disease, originally described by James Parkinson in 1817 was the first illness to be correlated to a specific neurotransmitter deficit (Ehringer and

Hornykiewicz, 1960) . This progressive neurodegenerative disease is characterized by symptoms of tremor, bradykinesia, and a unique rigidity. These symptoms are irreversible and may result from a variety of causes such as viral infection, tumor, stroke, carbon monoxide poisoning, or direct brain injury to various loci. However, most parkinsonian cases are of idiopathic origin where very little is known about the etiology. Idiopathic Parkinson's disease (IP) patients develop many symptoms including continuous loss of motor function that leads to stooped posture, shuffling gait, difficulty initiating movement, drooling, and mask-like faces. As the disease progresses, these patients may become virtually immobile. (see reviews: Alvord et. al., 1974; Forno, 1966; Forno et al., 1971; Jelling et al., 1986.)

Histological examination of postmortem parkinsonian brains has revealed reduced pigmentation and cell body loss in the area of the substantia nigra (SN) pars compacta, which is rich in dopamine (DA) neurons. Immunocytochemistry has revealed a lack of DA neurons belonging to other brain regions such as the ventral tegmental area (VTA) that projects to the nucleus accumbens septi (nacc) and cortical regions, mesolimbic fibers, and the hypothalamus (Javoy-Agid, et al., 1984). Cell loss also occurs in non-dopaminergic regions such as the locus coeruleus (LC) and sympathetic ganglia (Forno,

et. al., 1982), dorsal raphe nuclei (DRN) that give rise to ascending serotonergic pathways (Escourolle, et al., 1971), and the nucleus basalis of Meynert (NBM) in the substantia innominata, which gives rise to many cholinergic fibers (Arendt, et al., 1983; Candy, et al., 1983). However, these other regions and cell types are not affected as severely as the SN. Closer examination of the substantia nigra shows the presence of extraneuronal pigment, gliosis and eosinophilic inclusion bodies known as Lewy bodies. Lewy (1912) first described eosinophilic inclusions found in the dorsal motor nucleus of the vagus in parkinsonian patients. More recently, it has become clear that the SN and LC are two main characteristic sites for Lewy body development in Parkinson diseased patients (Forno, 1986).

Biochemical analyses demonstrate a dramatic reduction of dopamine (DA) levels in the striatum of parkinsonian brains (80-90%) (Bernheimer, et al., 1973; Bernheimer and Hornykiewicz, 1965), which has been traced directly to a corresponding loss of DA neurons of the SN. The DA depletion is usually greater in the putamen as opposed to the caudate nucleus (Bernheimer, et al., 1973). DA content is also reduced in other brain regions such as the nacc and cortical regions that receive projections from the ventral tegmental area (VTA) but to a lesser degree (Javoy-Agid, et al., 1984a; 1984b). Furthermore,

globus pallidus dopamine levels and norepinepherine (NE) levels in cortical areas receiving fibers from the LC are significantly diminished in parkinsonian brains (Hornykiewicz, 1966a). Reduced 5HT levels in the hippocampus and frontal cortex have also been reported (Scatton, et al., 1983). Moreover, the severity of the symptoms seen in parkinsonian patients is highly correlated with the degree of DA depletion measured in the neostriatum, nacc and frontal cortex (Javoy & Agid, 1980). In conjunction with the dramatic loss of DA, there is also a reduction in catecholamine (CA) synthesizing enzymes, tyrosine hydroxylase (TH) and dopa decarboxylase (DDC) (Lloyd et al., 1973; 1975). In addition, the specific uptake transport mechanisms for DA become impaired (Lloyd and Hornykiewitz, 1972). Finally, the major metabolite of DA in the central nervous system (CNS), homovanilic acid (HVA), is diminished (Bernheimer, et. al., 1973).

In response to the marked DA depletion, there is a compensation that occurs within presynaptic and postsynaptic cells (Schultz et al., 1982). Presynaptically, DA turnover increases measured by the ratio of HVA to striatal DA content. Therefore, the surviving DA neurons increase their activity to compensate the initial DA neuronal cell loss. Postsynaptically, the number of DA receptors located in the striatum rises resulting in a postsynaptic receptor sensitivity induced

by denervation. These compensatory mechanisms may reflect earlier stages of the disease, whereby enhanced post-synaptic sensitivity requires only small amounts of DA for maximal activation, so that clinical symptoms do not arise.

Aside from motor deficits, mental disturbances are often found in patients with Parkinson's disease (Agid, et. al., 1984). The most common alterations in mental functioning detected include bradyphrenia, depression, and dementia. Bradyphrenia is defined by a lack of concentration, an inability to associate ideas, a tendency to perseverate, and a general slowness of thought processes (Naville, 1922). Depression occurs in at least 50% of parkinsonian cases (Mayeux et al., 1981). In later stages of the disease, dementia, irreversible deterioration of intellectual functioning (i.e. memory loss, problem solving difficulty, ect.), develops in approximately one third of parkinsonian cases with a large percentage resembling senile dementia of the Alzheimer's type (Lieberman, et al., 1979).

Due to the enormous loss of DA in the nigrostriatal system, clinicians have attempted to reduce the parkinsonian symptoms by restoring DA levels with L-dihydroxyphenylalanine (L-dopa), (Birkmayer, et al., 1975) a precursor in DA synthesis. Amelioration of motor symptoms occurs for most patients undergoing L-dopa

therapy. However, severe side effects may develop several years following use of the drug, such as dyskinesia, psychosis, and "on/off" periods of intermittent muscle contraction. Unfortunately, drug therapy does not halt progression of the disease. Eventually, impoverished movement becomes frozen and often painful. In addition, L-Dopa therapy improves neuropsychological performance (Beardsley, 1971) indicating that some intellectual functions may be DA dependent. However, cognitive deterioration continues to progress.

Although other brain regions are affected, the major site of neuronal degeneration is within the nigro striatal pathway which appears to be responsible for the lack of ability to initiate voluntary movements. The differential anatomical connections between the SN and caudate or putamen and the caudate or putamen with cortical areas appear to have different functional capacities. For instance, the putamen is essentially linked with the pre-motor and motor-sensory cortex suggesting the putamen is associated primarily with motor functions (i.e. planning, programming, and execution of movements). Moreover, the striatal DA loss found in IP is greater in the putamen than it is in the caudate (Bernheimer, et al., 1973). Therefore, the tremor and rigidity seen may be due to a lack of inhibitory influence within the basal ganglia, which results in an increased

discharge of cells belonging to both the caudate and putamen. Research by Knoll (1983) supports this concept since 6-hydroxy-DA, injected into the SN of rats to induce a specific lesion to the DA neurons, increases significantly the rate of acetylcholine (Ach) released in the striatum due to the lack of presynaptic dopaminergic input. The hypersensitivity of dopamine receptors in the striatum that develops may be responsible for the dyskinetic movement, sine L-Dopa administration provokes dyskinesias (Javoy-Agid, et al., 1984a). Finally, alterations in the meso-cortico-limbic dopaminergic system may be responsible for neuropsychological deficits (Agid, et al., 1984), However, fibers of the cortico-striatal system, especially projections from the frontal cortex, may also be involved, since the caudate is connected with all parts of the frontal association cortex (DeLong, et al., 1983; Evarts, et al., 1984).

B. Selective Neurotoxins

1. MPTP and MPP+

Previous attempts to explain the selective DA cell death in the brain have implied several classes of environmental toxins. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a compound that has recently

been discovered to irreversibly induce parkinsonism in humans (Davis, et al., 1979) and has consequently become the focus of considerable interest in the effort to help understand the cause of this debilitating disease. MPTP was originally described as a potent neurotoxin by Ziering et. al., 1947, but MPTP-induced parkinsonism in humans has only recently been reported (Davis et. al., 1979; Langston et. al., 1983). A group of young drug addicts from northern California made an illicit and inexpert attempt to synthesize their own heroin and accidentally produced the meperidine analogue MPTP as a major contaminant of their synthetic heroin. Consequently, intravenous injection of the drug resulted in symptoms characteristic of IP (Langston et. al., 1983; 1984). L-dopa therapy successfully reversed the symptoms produced by MPTP. This unfortunate incident of drug abuse has allowed investigators to utilize this neurotoxin to create a model of IP in experimental animals.

Similar to IP, MPTP exposure results in dramatic decrements in striatal DA levels and in DA cell body loss of the SN. The selective toxicity to the nigrostriatal DA neurons appears to be species specific producing severe neuronal cell loss in humans (Davis et. al., 1979; Langston et. al., 1982) other primates (Burns et. al., 1984; Jenner et. al., 1984; Langston et. al., 1984) specific strains of mice (Hallman et. al., 1984; Heikkila

et. al., 1984a; 1984b) and more recently in the cat (Schneider and Markham, 1986). Attempts to selectively destroy dopaminergic neurons in the rat have been unsuccessful (Bradbury et. al., 1984; 1986a; 1986b; Markey et. al., 1984). However, neurochemical and behavioral alterations have been reported in response to systematic and intranigral administration of MPTP (Bradbury et. al., 1986; Chiueh et. al., 1984; Sayre et. al., 1986).

Due to the remarkable similarity between parkinsonism and MPTP toxicity in the destruction of the SN pars compacta and coexisting clinical symptoms, the MPTP animal model of Parkinson's disease has offered new hope to uncover the underlying mechanisms of neurochemical pathology that are responsible for the cell death and subsequent clinical symptoms common to IP. The utilization of an animal model of IP allows for both pharmacological manipulations and tissue transplantation that may lead to improved clinical treatment and possibly to reverse or prevent the disease.

Although only a few years of research has been invested in the MPTP-induced model of IP, a vast amount of literature has emerged (Bradbury et. al., 1985; 1986a; 1986b; Da Prada et. al., 1985; Denton & Howard 1986; Del Zompo et. al., 1985; 1986; Di Paolo et. al., 1986; Fries et. al., 1986; Fuller et. al., 1986; Gerhardt et. al., 1985; Heikkila et. al., 1984; 1985; Javitch et. al., 1985;

Lau & Fung 1986; Langston & Ballard 1984a; 1984b; Mitchell et. al., 1986; Mytilineou et. al., 1983; 1984; 1985; Perry et. al., 1985a; 1985b; Shen et. al., 1985; Wagner et. al., 1985; 1986; Zimmerman et. al., 1986; etc.). Numerous similarities exist among primates and rodents in the response to MPTP. Some of these similarities are persistant depletion of striatal DA levels (Burns et. al., 1983; Heikkila et. al., 1984; 1985; Langston et. al., 1985; Pileblad et. al., 1985; Ricurte et. al., 1986), and metabolites (Bradbury et. al., 1986), reduced mazindol binding (Javitch et. al., 1985), and an increase in the mumber of 3H spiperone striatal binding sites (Lau & Fung, 1986). Increases in D-2 receptor binding in the striatum supports the concept of development of DA receptor supersensitivity, (Creese et al., 1977; Ungerstedt, 1971) which has been demonstrated by a potentiation of apomorphine-induced stereotyped behavior (Unger stedt, 1975; Sayers, 1975; Tarsy, 1974). Recently, unilateral lesions produced by internal carotid artery infusions of MPTP are used for studying contralateral limb motor impairments (Bankiewiez et. al., 1986).

Current reports have suggested several mechanisms of MPTP toxicity. Interestingly, MPTP itself is not biologically toxic. Instead, MPTP is rapidly metabolized to its pyridinium congener, 1-methyl-4-phenylpyridine, (MPP+). The structure of MPTP and the oxidation pathway

to MPP+ is represented in Fig. 1. This biotransformation is necessary for the selective cell death of DA neurons both in vivo (Chiba, et. al., 1984; Heikkila, et. al., 1984; Javitch, et. al., 1985; Markey, et. al., 1984; Sundstrom, et. al., 1985) and in vitro (Cohen & Mytilineou, 1985; Mytilineou & Cohen, 1984). MPTP is a substrate for monoamine oxidase-B (MAO-B) since inhibition of its metabolic conversion occurs by deprenyl and not by clorgyline (specific MAO-B and MAO-A irreversible inhibitors, respectively). Therefore, inhibitors of MAO-B activity offer protection against MPTP nerotoxicity (Heikkila et al., 1984; Cohen, et. al., 1984; Langston, et. al., 1984). The accumulation of MPP+ within DA neurons is required to induce destruction (Javitch & Snyder, 1984; Pileblad & Carlsson, 1985; Ricaurte, et. al., 1985). Consequently, the neurotoxic effects of MPTP or MPP+ can be completely or partially blocked by catecholamine uptake inhibitors (Chiba, et al., 1984; Heikkila, et. al., 1984; 1985; Javitch & Snyder, 1984; Javitch et. al., 1985; Ricaurte, et. al., 1985; Schultz, et. al., 1986).

MPTP is a lipophilic substance which is not readily taken up into DA neurons (Javitch, et. al., 1985). Therefore, Javitch, et. al. (1985) have proposed that MPTP diffuses into all cells nonselectively and is converted to MPP+ by MAO-B containing cells. Subsequently, MPP+

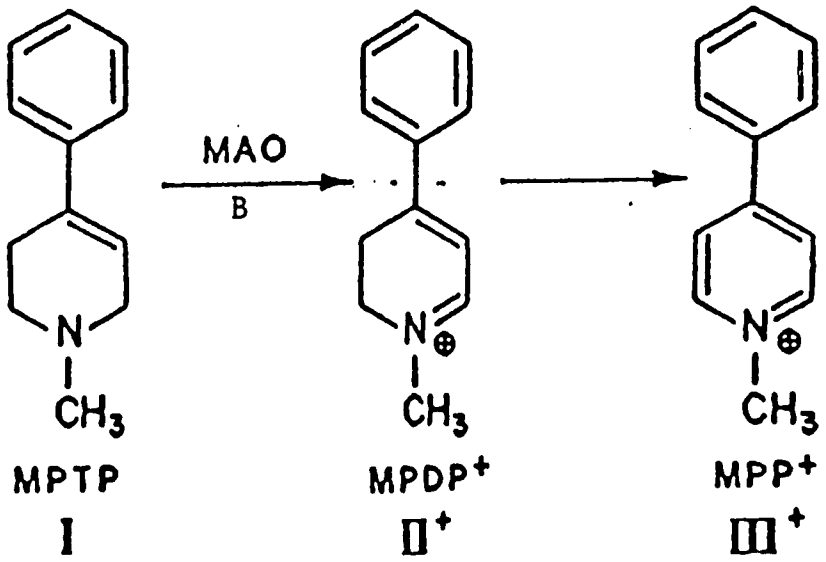


FIGURE 1. Oxidation of MPTP to MPP+ via MAO-B.

diffuses into limited extracellular space where it can accumulate within DA neurons by the high affinity uptake pump and induce destruction (Fig. 2). MPP+ accumulates also within other neurons, such as cortical noradrenergic terminals. (Javitch, et al.,1985). Hence, MPP+ is not selective for DA terminals and the mechanism of the sensitivity of DA neurons to MPTP toxicity requires further investigation.

Until recently, MPTP-induced toxicity has been considered to be an incomplete model of IP because the action of the drug was mistakenly thought to be restricted to the nigrostriatal pathway leaving VTA projections to the nacc and cortex and the LC cortical noradrenergic system unaffected. Mitchell et. al. (1985) showed that MPTP administration can affect the mesocortical system as well and produce a moderate-to-severe parkinsonian state in the macaque monkey. Reduced DA levels of the mesolimbic system have also been reported in mice (Melamed, et. al., 1985). In addition, several investigators have demonstrated a reduction in 5HT metabolism <u>in vivo</u> following MPTP administration (Enz, et al., 1984; Gupta, et. al., 1984) which has recently been confirmed <u>in vitro</u> (Friedman and Mytilineou, 1987).

In addition it appears that previous research studies may have produced a discrete lesion restricted to the nigrostriastriatal pathway because they used young

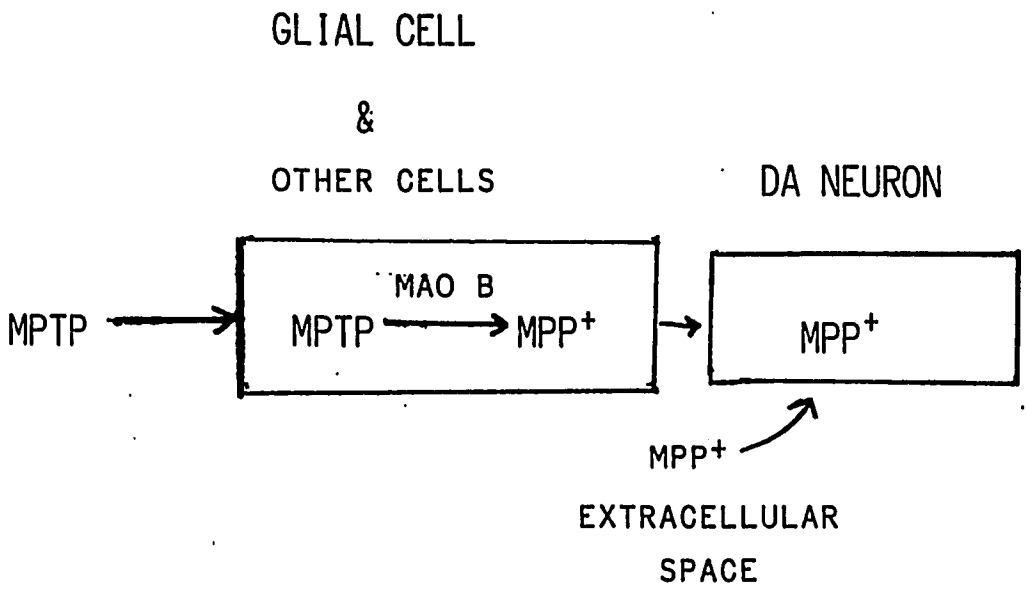


Figure 2. Proposed metabolic fate of MPTP.

experimental animals. In humans the age of onset of IP is usually over the age of 50. Presently the effect of MPTP is being studied in older animals and the results obtained from this work indicate that the lesions induced by MPTP in older animals resemble those of IP more closely (Heikkila, et al., 1987; Jarvis and Wagner, 1985; Langston et. al., 1986). The results of these experiments have shown an increased sensitivity to MPTP with increased age. Since the neurotoxic actions of MPTP are MAO-B dependent and MAO-B activity increases with age (Jarvis and Wagner, 1985; Langston et al., 1986), it has been postulated that the MPTP-induced toxicity in aged animals corresponds to the elevated MAO-B levels.

Several proposed mechanisms of destruction of the DA neurons by MPP+ have been discussed (see review: Langston and Irwim, 1986). Studies of the conversion of MPTP to MPP+ conducted <u>in vitro</u> have determined that biotransformation proceeds via an intermediate MPDP+, a dihydropyridinium intermediate (Fries, et. al., 1986; Sayre, et. al., 1985) and inhibition of MAO-B activity prevents this first metabolic step (Salach, et. al., 1984; Singer, et. al., 1985). Moreover, metabolism of MPTP via MPDP+ to MPP+ due to the enzymatic activity of MAO-B could result in the formation of peroxide, superoxide, or hydroxyradicals possibly leading to cytotoxicity.
Moreover, this intermediate was thought to act as a

covalent-binding agent (Chiba et al., 1985). However, this theory is not plausible since MPDP+ is mainly formed outside of the nigral neurons and therefore, non-nigral cells should be affected. Also, Sayre et. al. (1987) have shown MPDP+ to have a low potential to act as a covalent-binding agent.

It is known that the production of oxygen radicals may particitpate in cytotoxicity (Rolando, et. al., 1980; Tappel, 1986). Oxygen radical and hydroxyl radical damage was thought to be involved in the mechanism of toxicity due to the actions of the herbicide, paraquat (Lesco, et al., 1980; Ross, et al., 1979), which is very similar structurally to MPP+ (Perry, et al., 1986; Ody and Junod, 1985). However, paraquat undergoes redox cycling readily (Bus and Gibson, 1985) producing both superoxide radical ($O^{\cdot}_2^{-}$) and hydroxyl radical ($OH^{\cdot -}$), while MPP+ is a much more stable species where redox cycling is not likely to occur (Sayre, et al., 1987). On the other hand, there is some indirect evidence of oxidative stress produced by MPTP/MPP+ since pretreatment of animals with antioxidants such as tocopherol, beta-carotene, N-acetylcysteine (Perry, at al., 1985) ascorbate (Perry et. al., 1985; Wagner, et al., 1985), have partially or fully protected against MPTP-induced toxicity. However, several investigators have not been able to reproduce these data (Baldesseirini, et al., 1986; Martinovits, et al., 1986).

Metabolism of DA via MAO also results in the production of free radicals, toxic intermediates, or the formation of metabolites that could be correlated with some neurodegenerative diseases (Cohen, 1983). Therefore, it has been proposed that the formation of free radicals as a combined effect due to the metabolism of MPTP and DA may be in part, responsible for selective DA neuron destruction. Unfortunately, there are arguments against this hypothesis as well. For instance, efforts to reduce DA synthesis or block DA transmission have not interfered with MPTP toxicity (Fuller et al., 1985; Schmidt, et. al., 1985). In addition, increasing striatal DA with L-Dopa pretreatment did not increase but attenuated MPTP toxicity (Melamed, et. al., 1985).

Since MAO is a tightly bound mitochondrial enzyme (Greenawalt, 1970), several investigators have explored the effects of both MPTP and MPP+ directly on mitochondria (Heikkila, et. al., 1985; 1987; Nicklas et al., 1985; Ramsay and Singer, 1986; Ramsay et. al., 1986). MPP+ at high doses, but not MPTP was demonstrated to be a potent inhibitor of the mitochondrial enzyme NADH dehydrogenase (Nicklas et. al., 1985; Ramsay, et al., 1986). Inhibition of this key enzyme in cellular respiration may prove to be responsible for cytotoxicity especially since an active, energy-dependent uptake of MPP+ exists in mitochondrial brain preparations with an apparent Km=5mM (Ramsay and

Singer, 1986). However, inhibition of mitochondrial respiration does not explain the selective effects of the toxin on DA neurons. Presently, there is ongoing research on the respiratory effects of MPP+ in brain (Heikkila, et al., 1986; 1987).

2. Guanethidine

Guanethidine, (2-octahydro-1-azocinyl), ethyl guanine sulfate, is another well documented selective neurotoxin. Understanding how toxins selectively damage or destroy an individual cell type is important paticularly since it can lead to the understanding of the processes involved in cell death, aging, or neurological disorders. In parkinsonian patients NE neurons are also affected and a loss of neurons may occur in the LC and sympathetic ganglia. We therefore chose guanethidine, a neurotoxin known to destroy peripheral NE neurons, to study its effects on central catecholamine neurons in culture. Guanethidine, is an antihypertensive drug that depletes norepinepherine (NE) content in sympathetically innervated peripheral tissues (Burnstock et al., 1971; Johnson and O'Brien, 1976). Histological examination of the sympathetic ganglia has shown that chronic administration induces a partial (Nielsen, 1977) or complete sympathectomy in both neonate and adult rats

(Burnstock, et. al., 1971; Johnson and O'Brien, 1976; Johnson and Nielsen, 1976; Nielsen, 1977). The induced sympathetic lesion appears to be species specific (Johnson, et.al., 1977) and irreversible in neonate mice and rats (Angeletti, et. al., 1972; Johnson, et. al., 1976). A partial reinnervation of adrenergic fibers may occur in the adult rat, but the innervation pattern is abnormal and many of the regenerated fibers may be of the cholinergic phenotype (Evans, et. al., 1979).

The selective toxic effects guanethidine has on peripheral noradrenergic neurons are primarily due to the structure of the molecule which makes it a substrate for the catecholamine (CA) uptake pump (Fig. 3). Consequently CA uptake blockers prevent the neurotoxicity (Mitchell and Oats, 1970). The adrenergic neuronal cell death has been suggested to be due to an immunologically mediated mechanism, since the sypathetic ganglia become infiltrated with lymphocyte-like cells prior to axonal degeneration (Manning, et. al., 1983). Guanethidine-induced destruction may be prevented by immunosuppressive agents or by co-administration of nerve growth factor (NGF) (Manning, et. al., 1985).

Guanethidine is a complex compound and has been shown to have many effects in addition to the destruction of peripheral catecholamine neurons. Some of these effects are inhibition of action potential evoked

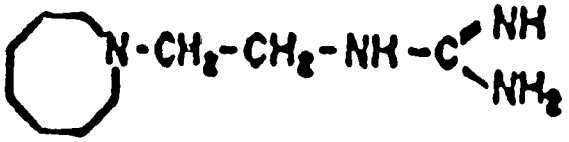


Figure 3 Structure of guanethidine.

neurotransmitter release, inhibition of neurotransmitter storage mechanisms, inhibition of nerve terminal uptake mechanisms and binding of neurotransmitters, inhibiton of drug-induced release of transmitter from nerve endings such as by tyramine, facilitation of spontaneous transmitter release, and ultrastructural damage to storage vesicles and mitochondria (see reviews: Johnson and Manning, 1984; Maxwell, 1982).

Since guanethidine is a highly charged agent and does not cross the blood brain barrier, the major focus of research has been in the peripheral nervous system. However, some investigators have studied the action of guanethidine in the CNS. Studies in the CNS have not reported any selective neuronal cell death. However, several alterations among CA neurons have been found. For example, high concentrations of guanethidine administered intacranially to a variety of brain regions significantly reduced CA histofluorescence levels of the LC and SN (Evans, et. al., 1975) and low concentrations depleted CA histofluorescence in many hypothalamic nuclei (Armstrong, et. al., 1973; Evans et al., 1975a; 1975b). Reduced CA brain levels (Cox and Maickel, 1969) and alterations in tyrosine hydroxylase (TH) and orthithine decarboxylase (ODC) activities (Bartolome', 1976) have been reported as well. To date guanethidine-induced-neuronal degeneration in the CNS has not been reported in

vivo.

The effects of guanethidine on sympathetic neurons have also been explored in vitro. Both dissociated and explant superior cervical ganglia (SCG) cultures, which are rich in NE neurons and contain a small population of DA interneurons have been treated with guanethidine. These NE neurons can accumulate 3[H]-guanethidine (Wakshull, et al., 1978), but gunanethidine-induced destruction appears to occur only under nonphysiological pH (8.0) (Johnson and Aloe, 1974; Wakshull, 1978; 1981) or under extremely high drug concentrations (250-400uM) (Hill et. al., 1973; Heath et al., 1974).

C. Tissue Culture

1. Background

The study of biological systems in tissue culture preparations has been developed in order to gain understanding of isolated cellular and molecular mechanisms involved in neural development and many cell functions. The culturing of neural tissue derived from frog neural tube was first described in 1907 by Ross Harrison. Harrison's work, which analyzed morphological aspects of the cultures, initiated much research adressing

a host of developmental questions. More recently, cell culture conditions have improved and a variety of neurobiological questions may be adressed more readily. General methods to analyze cultures today employ biochemical, electrophysiological, histochemical, immunocytochemical, and retrograde tracer techniques. Essentially, there are two types of neural tissue cultures: primary cultures and continuous cell line cultures. Primary cultures are derived from small isolated pieces of neural tissue from the prenatal brain and may be subdivided into three categories; explant, dissociated, and suspension cultures. Explant cultures are grown often on pretreated collagen coated coverslips as small excisions of discrete embryonic neural tissue and maintained for several months. The major advantage of using explants is that the cytoarchitecture of the immature brain is maintainted.

Dissociated cell cultures are derived from immature brain tissues which are dispersed into single cells by enzymatic or mechanical treatment. These isolated cells are plated in pretreated culture vessels to form a monolayer of heterogeneous cells. Individual neurons may be visualized in this type of preparation. In addition, cells may be grown in suspension where through rotation the cells may reaggregate into clusters without adhering to any surface. In these cultures non-neuronal cells do

not survive. Neural cell lines comprise a homogeneous population of cells that continuously proliferate, and therefore, may be maintained for long periods compared to primary cultures. Cultured cell lines may be derived from either normal or tumorous tissue (i.e. neuroblastoma, glioma, pheochromocytoma) which may be kept frozen for extended periods of time. Because cell line cultures can continuously divide, they have the advantage of having the ability of being cloned. In addition, they can be used for selecting mutants or variants.

2. Advantages of tissue culture.

Tissue culture serves as a powerful model in neurobiology because specific experimental conditions can be created that are simply not possible to achieve in vivo. This model may be used to answer many neurobiological questions because many molecular and developmental events mimic the in vivo situation. Each type of cell preparation has its own advantages and in each case the investigator has the ability to limit and control extrinsic factors. Experimental variables may be simplified so that micro-components of the nervous system may be more readily analyzed. In primary cultures, the

experimenter has the ability to partially separate the heterogeneity of cell types. Direct visualization of single neurons of a specific phenotype may also be achieved. It is possible to observe the cells under living conditions and analyze morphological changes that may occur.

In the event the investigator wishes to analyze the effects of a specific drug on a specific cell type, pharmacokinentics of systemic administration due to peripheral metabolism, penetration, absorption, and vascularization of the brain may be avoided. In addition, the investigator can control the drug concentration and the time of its exposure. Moreover, the tissue may be analyzed sequentially (i.e. 15min., 1 hr., 24hrs). Hence, the investigator can study possible relationships between drug effects and cellular growth, proliferation, differentiation, and other experimental variables.

The major components of a culture system that the experimenter controls include the culture medium, substratum, region of brain tissue, and the gaseous environment. Many of these details have already been worked out (Bottenstein and Sato, 1979; 1980; Botenstein, et. al., 1980; Botenstein, 1984). Briefly, culture medium in general contains a variety of amino acids, inorganic salts, vitamins, buffering agents, and an energy source (Bottenstein, 1983a). Neural cultures require a

compatible pH (7.2 to 7.6) and a constant osmolarity (approximately 325) for optimal survival. Neural cells survive better if non-neuronal cells' (i.e. glia, fibroblasts, ect.) proliferation rate is stunted, usually with an antimitotic agent such as fluro-deoxyuridine (FUDR). Moreover, culture medium is supplemented with several biological fluids such as serum (egs. horse, fetal, calf, rat), plasma, or embryo and placenta extract. Different proportions of these ingredients are supplied depending on which culture type is desired.

Two other major components that are necessary for neuronal survival are the substratum and gaseous environment which serve as an adhesive surface and energy supplier for the cells respectively. Examples of good surface matrices are collagen, polylysine, laminin, fibronectin, enactin, ect.. Previous studies in our laboratory have demonstrated that organotypic cultures derived from fetal rat brains serve as a suitable <u>in vitro</u> model for investigating the toxic effects of MPTP (Mytilineou and Cohen, 1984; 1986; Mytilineou, 1985). The use of the tissue culture model is powerful for us because it allows us to dissect out individual elements of the DA, NE, or 5HT phenotyope and subsequently study their susceptibility to each one of the previously described neurotoxins.

3. Monoamine neurons

Since the focus of our research has been on monoamine containing neurons, a short description of each cell type will be adressed here. Although neurons derived from fetal rat brain are immature, cytological and biochemical specificity appears early on during embryogenesis. Biochemical and histochemical identification of CA (Prochianz et al 1979; Berger et al., 1982) and 5HT (Azmitia and Whitaker-Azmitia, 1987) neurons have previously been characterized in dissociated primary cell cultures. In general, monoamine neurons may be identified in vitro when the regulatory enzyme (tyrosine or tryptophan hydroxylase) responsible for neurotransmitter synthesis appears embryonically. The enzyme, TH, may be labelled immunocytochemically with a specific antibody (Hokfelt, et. al., 1976; 1977). More recently antibodies have been raised against DA (Geffard, et al., 1984) and 5HT (Brusco et al., 1983; Consolazione et al., 1981; Nilsson et al., 1987) so that it is possible to directly localize DA and 5HT storage sites. Other indices for cell type characterization include fluorescence histochemistry for endogenous stores of catecholamines, fluorescence histochemistry for exogenously taken up catecholamines, autoradiography after labelling with 3[H] DA, 3[H] NE, or 3[H] 5HT, HPLC for measuring

endogenous levels of any of the monoamines and their corresponding metabolites and accumulation of trace amounts of radiolabeled ligands that utilize specific uptake mechanisms for each cell type. In this way it is possible to distinguish neuronal cell types by utilizing phenotypically distinct markers.

In the rat embryo, mitotic division of monoamine neurons begins on embryonic day 12 (E 12) and is complete by E 15 (Spect, et al., 1981; Lauder and Bloom, 1981). Lauder and Bloom (1981) utilized the technique of tritiated thymidine autoradiography which allows for precise dating of neuronal differentiation defined by the cessation of neural cell proliferation. Spect et al., (1981) have developed a fetal atlas based on specific immunocytochemical and cresyl-violet stained sections. They describe two groups of TH possitive neurons located in the rhombencephalon and large and intensley stained CA neurons belonging to the mesencephalon as early as E 12.5. By E 13.5 these cells have differentiated into at least 6 areas. By E 14, TH+ neurons have migrated from the intermediate to the ventricular zone. This stage of development has been shown to be the optimum for survival and growth of cultured monoamine neurons. Furthermore, increased survival, differentiation, growth and ability to accumulate neurotransmitter can be seen for all three monoamine cell types when co-aggregated with

their target cells (i.e. striatum, cortex, or hippocampus for DA, NE, 5HT neurons respectively) (Azmittia and Whitaker-Azmitia, 1987; Kotake et al., 1982; Hemmendinger et al., 1981; Porzio et al., 1980; Shalaby et al., 1983).

II. EXPERIMENTAL PROCEDURES

A. MATERIALS

1. Chemicals - Sources

MPTP-HCl and MPP+ were purchased from Research Biochemicals (Wayland, MA). L-Deprenyl was a gift from Dr. J. Knoll (Dept. of Pharmacology, Semmelweis Medical University, Budapest, Hungary). Clorgyline was obtained from May and Baker, Ltd. [3H] DA (29.0 Ci/mmol) and [3H] NE (11.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA) and [3H] 5HT and [14C] benzylamine were purchased from Amersham/Searle, Arlington Heights, Ill. Guanethidine was obtained from Ciba Pharmaceutical Co. (Summit, N.J.) and mazindol was obtained from Sandoz Pharmaceutical Co. (E. Hannover, N.J.). Desmethylimipramine (DMI) was purchased from USV Pharmaceutical Corp. (Tuckahoe, N.J.). Fetal calf serum, horse serum, Minimal Essential Medium (MEM) and balanced salt solution (BSS) were purchased from Gibco. Antibodies against TH were given as a gift from Dr. T. Joh (Cornell Med. Center, Westchester) or bought from Eugene Tech International, Inc. and antibodies against 5HT were obtained from Immunonuclear Corp. (Stillwater, Minn.). Citalopram was given to us as a sample gift from Dr. Richard Heikkila (Rutgers Med. School) and NGF was a gift

from Dr. K. Sonenfeld purified from mouse maxilary glands by Dr. P. Berd (Mt. Sinai, Med. Sch.).

2. Animals

Male Sprague-Dawley rats (150-200 grams) and female Sprague-Dawley pregnant rats were purchased from Zivic Miller Laboratories. The number of male rats used for both in vivo and in vitro studies was 100-150 animals. The number of pregnant rats used for setting up cultures was approximately 400 animals. Each pregnant animal carries 9-17 embryos so that approximately 4,000 embryos were used. 50,000-60,000 cultures can be set from this number of embryos. The animals were housed in the animal facilities at Mount Sinai School of Medicine and fed Purina Laboratory Chow ad libitum until the time of sacrifice. All pregnancies were keenly timed so that the embryos were the appropriate gestational age.

B. Solutions

1. Krebs Ringer Phosphate (pH 7.4)

The Krebs-Ringer phosphate buffer contained:

119 mM NaCl

4.7 mM KCL

1.3 mM EDTA

1.8 mM $CaCl_2$

1.2 mM $MgSO_4$

5.6 mM glucose

15.9 mM NaH_2PO_4

8.0 mM Na_2HPO_4

2. Tris Saline (pH 7.6)

The Tris-Saline buffer contained:

0.5 M Trizma Base

0.9% NaCl

The pH of Trizma Base solution was adjusted to 7.6 with concentrated HCL before addition of NaCl.

3. Phosphate Buffer 0.1M (pH 7.4)

The phoshate buffer contained:

19 ml 0.2M NaH_2PO_4

81 ml 0.2M Na_2HPO_4

100 ml distilled H_20

C. METHODS

1. Preparation of tissue culture

Pregnant Sprague-Dawley rats were sacrificed on the 14th day of gestation by ether anesthesia and the uteri were removed rapidly under semi-sterile conditions. The uteri were placed in petri dishes containing minimal essential medium (MEM) and the rest of the dissection was

conducted under complete sterile conditions under a laminar flow hood. The ventral mesencephalon, containing the DA neurons of SN and VTA, and the pontine area, containing the NE neurons of the LC and 5HT neurons of the raphe' were dissected out with the use of a micro-dissection microscope. The dissected tissues were collected in Dulbeco's Ca++ and Mg++ free buffered medium until the dissection was completed. The tissues were transferred to the feeding medium which consists of MEM containing glucose, sodium bicarbonate, glutamine, and phenol red as a pH indicator 10% fetal calf and 10% horse serum, and then dissociated by mild trituration with a small bore Pasteur pipette. Cells were plated in 35mm plastic Falcon (Primaria) dishes or poly-L-lysine coated dishes at a density of 1 or 3/4 brain area from each embryo per culture dish. In other words, out of a 10 embryo litter, we prepared 10 or 13 culture dishes for each brain area taken. Cells were counted with a hemocytometer just prior to cell plating yielding approximately 1/2 a million cells per dish. Non-living cells were labeled with trypan blue (4%) (1:1 dilution). Cell viability was between 95-97%. The number of cultures that could be set up depended on the number of embryos per animal, therefore, 50,000-60,000 cultures were set to conduct all the experiments. Incidence of infection was low, so that 15-20 animals were lost (about 2,000)

cultures. The feeding medium was supplemented with 10% bovine fetal calf and 10% horse serum during the first week and 10% horse serum thereafter. On the 7th day in vitro (DIV), the cultures were treated with an anti-mitotic agent, fluoro-deoxyuridine (FUDR) (13 ug/ml) and uridine (33 ug/ml) to prevent excess glial cell proliferation. The cultures were incubated at 37° C in an atmosphere of 90% air and 10% CO_2 and the feeding medium was replaced twice weekly.

2. 3[H] Monoamine uptake in the cultures

3[H] DA, 3[H] NE and 3[H] 5HT in the nanomolar range were used for measuring the uptake of DA, NE and 5HT neurons respectively. First, the culture medium was removed and the cultures were rinsed with Krebs Ringer's phosphate buffer (pH 7.4). Next, the cultures were incubated with one of the 3[H] monoamines at 37° C for 10 minutes in the same buffer supplemented with ascorbic acid (.2mg/ml). Following incubation with the respective tritiated amine, the cultures were washed twice with fresh buffer and incubated again for 10 more minutes. Cultures were also incubated at either 0° C in the presence of a potent monoamine uptake inhibitor such as mazindol, GBR-12909, desmethylimipramine (DMI), or citalopram in order to determine the non-specific uptake. After the

washes, the buffer was aspirated and the radioactivity was released from the tissue by adding 1 ml of 95% ethanol to the culture dishes and warming them to 37° C for 20-30 minutes. The ethanol with the extracted radioactivity was collected in scintillation vials containing 10ml of Liquiscent and counted in a Packard Tri-Carb Scintillation Spectrometer with 45% efficiency.

3. Catecholamine histofluorescence

The catecholamine histofluorescence technique, developed by Falk and Hillarp (1964) was used to determine the presence of catecholamine containing neurons and not their catecholamine endogenous content. In culture, the endogenous levels of catecholamines can be altered by the drugs used. Since our aim was to visualize catecholamine neurons in their entirety, a modified version was employed. All cultures were preincubated in 10 uM alpha-methyl-NE for 30 minutes in order to preload all the catecholaminergic neurons present in the cultures with a fluorescing amine. The cultures were washed and then fixed with 2% glyoxilic acid in 0.1 M phosphate buffer (pH 7.2) for 10 minutes at 4° C. The cells were then dried thoroughly by a stream of warm air provided by a hair dryer for 20 min. and treated with formaldehyde vapors generated from paraformaldehyde (70% relative humidity)

for 1 hour at 80° C. The cultures were readily examined with a fluorescence microscope and photographed with Kodak Ektachrome film (400 ASA). The combination of the glyoxylic acid and paraformaldehyde methods was utilized because it offers a consistent high fluorescence intensity with minimal diffusion.

4. Immunocytochemistry

The peroxidase antiperoxidase (PAP) method according to Sternberger (1979) was used for immunocytochemical localization of TH or 5HT. By this method, the catecholamine and 5HT neurons may be stained and visualized with bright-field or dark-field microscopy. The monolayered cell cultures were fixed in 4% formaldehyde in 0.1M phosphate buffer (pH 7.2) for 30 min. The cultures were rinsed with 0.5M Tris-saline buffer and subsequently treated with 0.2% Triton X-100 in order to facilitate penetration of the antibodies through the cell membranes. A 30 min incubation with goat serum (1:30 dilution in Tris-saline) was used to occupy non-specific antigen sites. Following a thorough wash, the cultures were incubated with the primary antibodies (1:1000 dilution) with the same concentration of Triton X-100 overnight at 4° C. The second day the dishes were washed with Tris-saline buffer and 1% normal goat serum followed by a 30 min. incubation with goat-anti-rabbit

serum (2.5%). The cultures were washed again and the PAP complex (2.0%) was added for 30 min. Finally, 3,3-diaminobenzidine (0.05%) was used as a chromogen in the presence of H_2O_2 (0.01%). The cells were examined by microscopy and kept with glycerine and glass cover slips.

In order to assure specific antibody labeling a control was established for both antibodies. In the TH immunocytochemistry group, several cultures were treated with the above procedure in the absence of the primary antibody. For the 5HT labeling specificity, 400 ul of anti-5HT (1:250) was incubated with 400 ul of 5HT-BSA at 4° C for 2 hours. 800 ul of anti-BSA (1:50) was added to the above solution and incubated for 1 hour at room temperature and then overnight at 4° C. The next day, the solution was centrifuged for 15 min at 16,000 rpm and the supernatant (approximately 1.5 ml) was used to incubate the control culture.

The TH+ or 5HT+ cells were counted as a quantitative index of monoamine cell survival following exposure to a toxin. Since dissociated cultures form a monolayer of cells across the culture dishes, it was possible to count all of the cells labeled with an antibody in drug treated and non-treated culture dishes with the aid of a grid to avoid counting a cell twice.

5. Uptake from synaptosomes and release from slices

The effects of guanethidine on the uptake and release of NE and DA were measured in synaptosomes or brain slices respectively prepared from Male Sprague-Dawley rats weighing 150-200 g. The rats were decapitated and the brains were immediately removed and placed in 0.9% saline. For the synaptosomal preparation the occipital cortex and striatum were dissected on ice and the tissues, pooled from four animals, were weighed and then homogenized in 0.32M sucrose (1:10 w/v). The homogenate was centrifuged for 10 min. at 3,000 r.p.m. (1100 x g) to remove blood and cellular debris. The supernatant was transfered to another centrifuge tube and spun at 15,000 rpm (27,000 x g) for 30 min. All centrifugations were performed in a Sorvall RC 2B refrigerated centrifuge with a SS 34 head. The supernatant was discarded and the pellet was resuspended in Krebs-Ringer phosphate buffer (pH 7.4) containing 0.3 mg/ ml ascorbic acid and 0.01 mg/ ml pargyline at a concentration of 100 mg/ ml. This preparation yields a synaptosomal suspension containing mitochondria but is relatively free of glia or other subcellular particles (Clark and Nicklas, 1970; Weinberger and Cohen, 1982). For uptake studies (Snyder and Coyle, 1969; Weinberger and Cohen, 1982) alliquots of homogenate (200ul) and varied doses of guanethidine were

added to flasks containing 5 ml of Krebs Ringer and preincubated for 10 min. at 37° C using a Precision Scientific GCA metabolic shaker. Guanethidine concentrations ranged from 0.1-100 uM for cortical synaptosomes and 1.0-500 uM for basal ganglia synaptosomes. Samples without guanethidine served as controls. All samples were done in triplicate. 3[H] NE was added to cortical synaptosomes and 3[H] DA to striatal synaptosomes at a final concentration of 1.4 x 10-8 M or 4.5 x 10-9 M respectivley and incubated for an additional 10 min. At the end of the incubation period 1 ml of the synaptosomal suspension was poured over 0.65 um sized Millipore filters in a millipore vacuum and rinsed twice with 1 ml of cold saline. The filters were collected and placed into vials containing 10 ml of Liquiscint scintilation fluid (National Diagnostics) and counted for radioactivity in a Packard Model 2450 Tricarb Scintillation spectrometer. Uptake values are expressed as percentage of control synaptosomes in the absence of guanethidine. Incubation medium without synaptosomes was used to obtain blank values.

Release studies were conducted in brain slices of the same brain regions studied for uptake. Brain slices were cut freely by hand at an approximate thickness of 1mm and area of $4mm^2$. The slices collected from 3 animals were preincubated for 10 min. at 37°C in Gouch crucibles

in 50 ml beakers containing 10 ml of Krebs buffer without EDTA then transfered to beakers containing the radioactive amine for 15 min for the uptake. Subsequently, the slices were transfered to beakers containing buffer for a 15 min wash. The slices were then transfered from the Gouch crucibles into 16mm well dishes individually and spontaneous release was measured every 5 min for 20 min. The tissues were then exposed to 50mM KCL for 5 min. then transfered again for 5 min for the final wash. The above paradigm was used in the presence of guanethidine at concentrations of 1, 10, and 100uM. Release was calculated from the radioactivity accumulated in the medium and expressed as the percentage of total radioactivity present in the tissue at the beginning of the incubation period. Statistical comparison was made by the Student t-test.

6. Monoamine Oxidase Activity

The MAO activity was measured in the cultures by the method described by Youdim (1975). 3[H] 5HT served as a substrate for MAO-A and 14[C] benzylamine for MAO-B activity. Clorgyline (1uM), a specific MAO-A inhibitor and deprenyl (10uM), a specific MAO-B inhibitor, were

present in samples during the assay to obtain the blank values. The tissue homogenate was prepared by washing the cultures with potassium phosphate buffer (.05M, pH 7.4) and scraping the cells of 4 cultures per sample group. The tissues were collected in diposable glass test tubes, then sonicated 3 times on ice for a total of 30 secs. Duplicate aliquots containing 200-400 ug protein were incubated with 0.2 mM 5HT (3 uCi) and 0.1 mM benzylamine (1.25 uCi) at a final volume of 0.5 ml for 10 min. at 37oC in a shaking water bath at 60 oscillations per min. The reaction was terminated by adding 0.3 ml 2N HCL. The oxidation products were extracted with 5 ml toluene-ethylacetate (1:1, vol/vol). Each sample was vortexed for 30 secs. for thoughrough mixing. To separate the organic and aqueous phases, the samples were centrifuged for 5 min. at 1000rpm. 3ml of the organic phase was removed and counted in 7 ml Liquiscint by liquid scintilation spectrometry. The protein content was determined by the method of Lowery, et al. (1951) using bovine albumen as the standard.

7. High performance liquid chromatography (HPLC)

Serotonin Assays

Cultures were placed on ice and ascorbic acid in perchloric acid (PCA) was added to the feeding medium at

a final concentration of 0.1 mM (0.4N PCA) to prevent oxidation of 5HT. The feeding medium was removed and the cells were scraped and collected in 1.5 ml centrifuge tubes and spun for 2 min at 10,000 g. The remaining feeding medium was removed and the cellular pellet was acidified (10:1) with 0.4N PCA (containing 40 mg/ l DTPA and 0.3 ng/ ml 3,4-dihydroxybenzylamine (Dhb) as an internal standard), homogenized, vortexed and then spun at 10,000 g for 10 min. The supernatants were analyzed by HPLC with electrochemical detection (Bioanalytic Systems, West Lafayette, Ind.). Standards contained 0.3 ng/ ml Dhb, and 5HT and 5HIAA (1 ng/ ml). A C-18 reverse phase column was used (5 um beads, 25 cm length) and the mobile phase contained 150 mM chloroacetic acid with 0.7 mM EDTA (pH 3.0) and 2.0 mM sodium octylsulfate and acetonitrile (at 40% final concentration). A glassy carbon electrode was used at +0.8 volts. Peak heights and retention times of standard solutions were compared with the samples to calculate the concentrations of 5HT and 5HIAA.

III. RESULTS

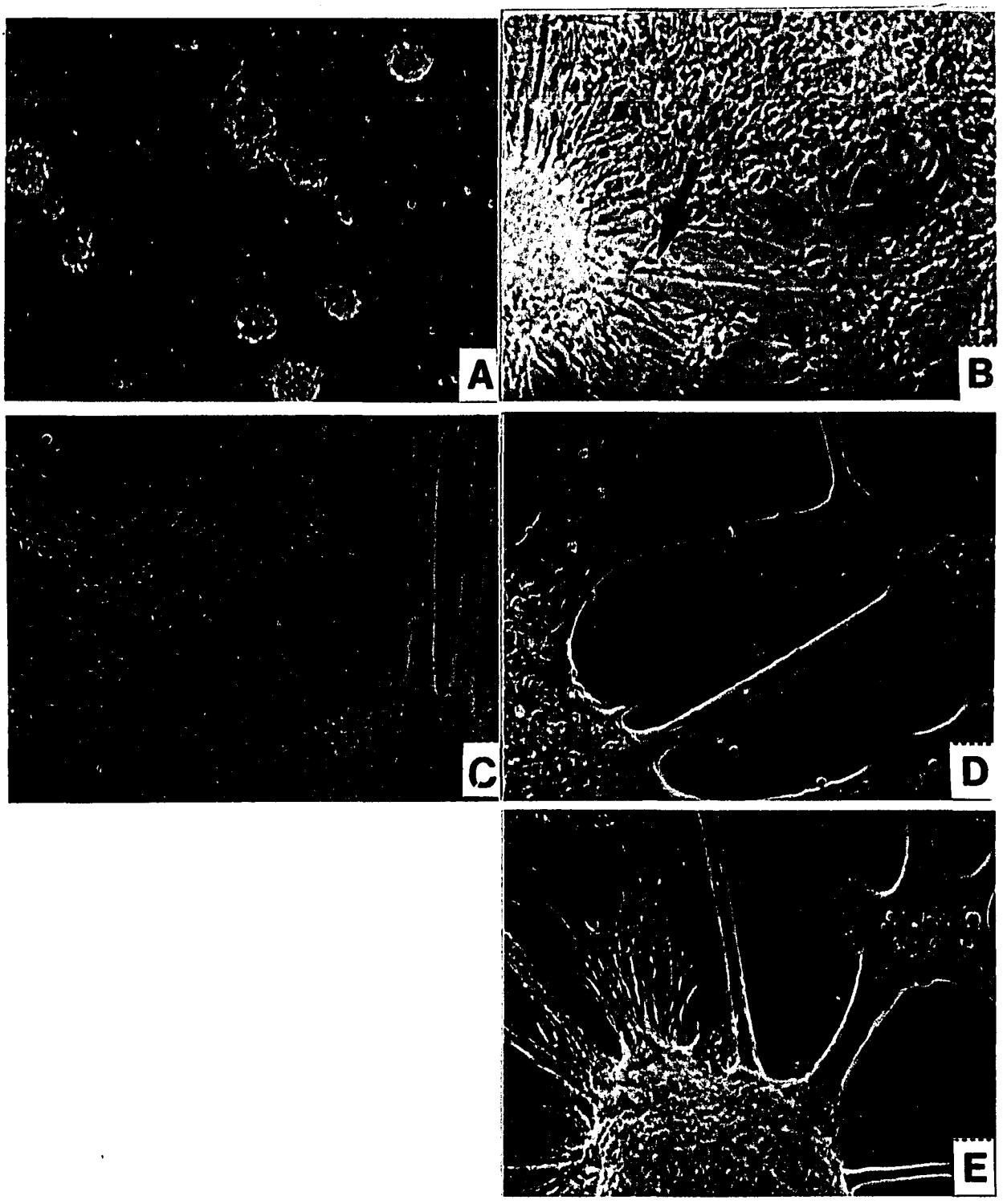
A. Developmental profile of monoamine neurons in culture.

We have characterized the development of catecholamine neurons belonging to the ventral midbrain and pontine region of fetal rat brains. We have also initiated studies on the development of serotonergic neurons located in the pons. The indices used to characterize these neurons were histofluorescence after incubation with a fluorescing amine, immunocytochemistry of TH and 5HT, and uptake of tritiated amines.

In our culture system, after the tissues are removed from the fetal rat brain, dissociated and plated, a reaggregation of dissociated cells occurs prior to adhering to the pretreated, Primaria (Falcon Trademark) culture dishes (Fig 4A). Primaria culture dishes have a positively-charged, growth enhancing surface that mimics the structure of protein by intrinsically incorporating amide- and amino functional groups. Immediately after plating the cell bodies of neuronal and non-neuronal cells may be visualized with phase contrast microscopy. Cell processes begin to emerge as early as 24-36hrs <u>in vitro.</u> Fig. 4A-4E shows how the cultures develop and grow under

Figure 4A-4E. DEVELOPMENT OF THE MIDBRAIN CULTURES OVER 4 WEEKS

Phase contrast microscopy of fetal neurons grown in culture for 24 hours, 1, 2, 3, and 4 weeks (A-E). (A) Neurons 24 hours after plating aggregate into clusters (arrows), adhere to the culture dishes, and begin to develop short processes. (B) After 1 week many processes appear and glial cells proliferate (arrow points to extended process with surrounding glial cells). (C) By the second week neuritic processes thicken and extend towards other cells (arrow). (D) By the third week, long, thickened processes (arrows) are formed throughout the cultures. (E) 4-week cultures appear similar to 3 week cultures but in many cases more fibers appear thicker and more extensive (arrow). x 98.


A
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D
E

phase contrast microscopy during one month *in vitro*. Notice the cultures become more populated with non-neuronal cells for the first two weeks and the neurons grow many processes that extend towards other cell aggregates. The morphological development of the DA neurons is represented in Fig. 5A-5H. Antibodies used against tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, reveal small cells with short processes by 5-7 DIV. By the second week the cell bodies are bigger and processes are longer but not yet fully grown. The DA neurons appear mature by the third week and continue to survive for several more weeks.

Antibodies against 5HT also reveal processes very early (Fig. 6A-6B). The cell bodies and processes labeled with 5HT antibodies are much more extensive and numerous in the pontine cultures when compared to the midbrain cultures (Fig. 6A, 6B) although numerous 5HT neurons of the dorsal raphe' nucleus are located in the ventral mesencephalon (Azmitia, 1978; Taber et al., 1960). Fig. 6C demonstrates the specificity of the antibody where no cells are selectively stained when the antibody is immunoprecipitated with anti-BSA. The colored cells represent backround staining.

For all three monoamine containing neurons studied,

cell body size and neurite outgrowth continues to increase reaching an apparent maturity by 3-4 weeks in vitro. The processes continue to grow at a higher rate than the somata. Visualization of the dendritic and axonal processes is possible by staining with antibodies against TH, DbH, and 5HT, but limiting, because many processes and branches which can be stained by CA histofluorescence are not labeled (Fig 5A-5H). DbH immunocytochemistry was of extremely limited value for us since only a few cells were labeled, so that a quantitative analysis between control and drug treated cultures was not possible.

As mentioned above, histofluorescence after preincubation with a fluorescing amine reveals a greater field of dendritic and or axonal arborization in cultures of the same age than those stained for TH for DA neurons (Fig. 5F-5H). Notice the extensive branching among the CA neurons of the midbrain and the great length of the processes when visualized after exposure to formaldehyde vapors. The long processes of the NE neurons seen in pontine cultures are reminiscent of the neurons that project from the LC in vivo (Dahlstrom and Fuxe, 1964). It is difficult to maintain the fluorescence reaction product formed with formaldehyde vapors and indoleamines, therefore, fluorescence of 5HT neurons was not used as a reliable index.

Figure 5A-5H. DEVELOPMENT OF THE DA NEURONS OVER 4 WEEKS

TH immunocytochemistry and CA histofluorescence were used to visualize the DA neurons and analyze their development for 4 weeks. (A) TH immunocytochemistry reveals DA neurons derived from the midbrain with small cell bodies and short processes begining to emerge after 1 week. x 168. B) At the end of 2 weeks the soma increase in size and the processes lengthen. x 168. (C) By the third week the DA neurons mature with large soma and extensive processes. x 168. (D) DA neurons during the 4th week appear similar to the 3rd week, however, some cells do appear to regress in size and process outgrowth. x 168. (E-H) CA histofluorescence reveals more extensive axonal and dendritic arborization when compared to TH+ neurons of the same age. The number of fluorescing fibers reaches a plateau between the 3rd and 4th week <u>in vitro</u> (G, H). x 200.

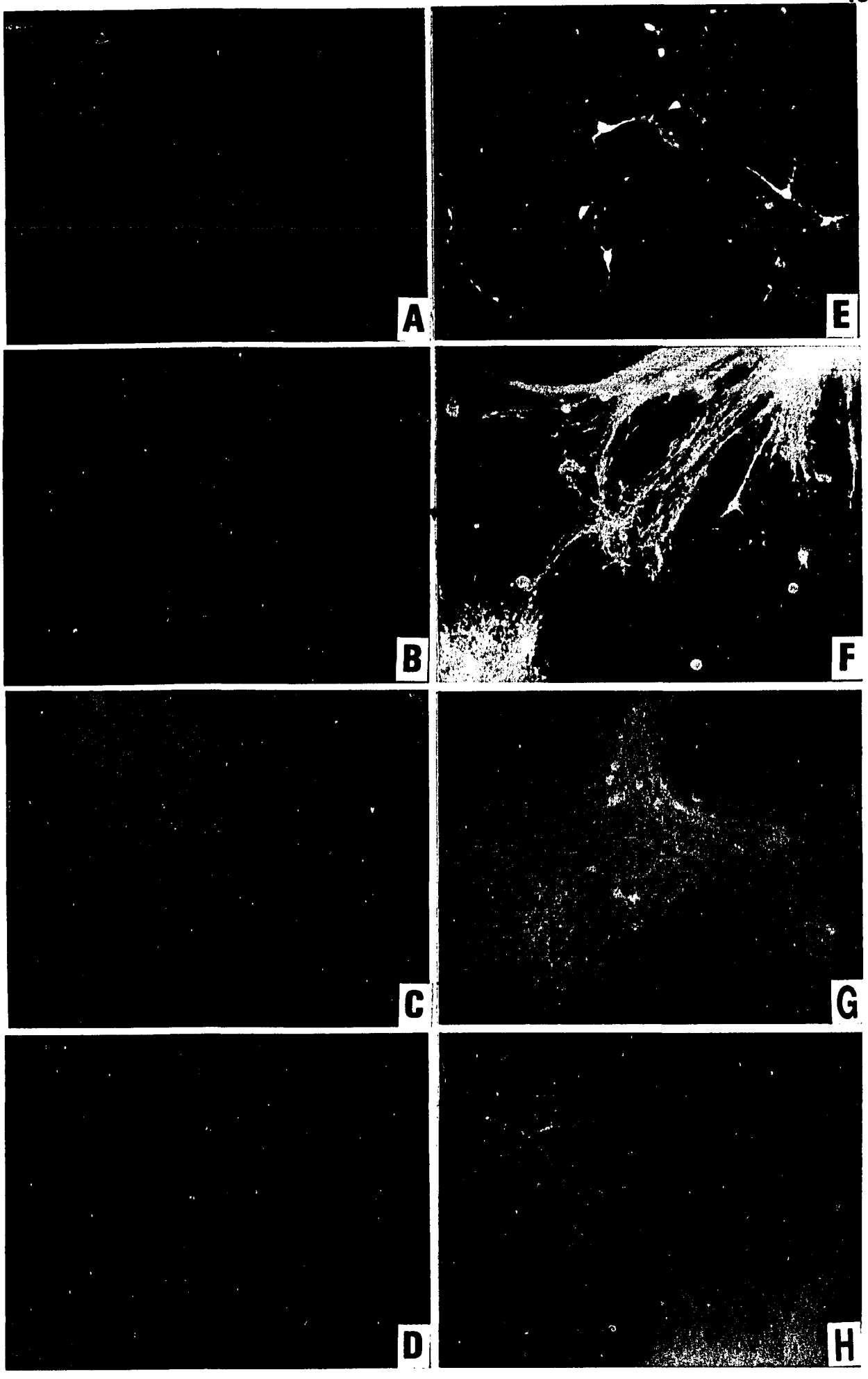
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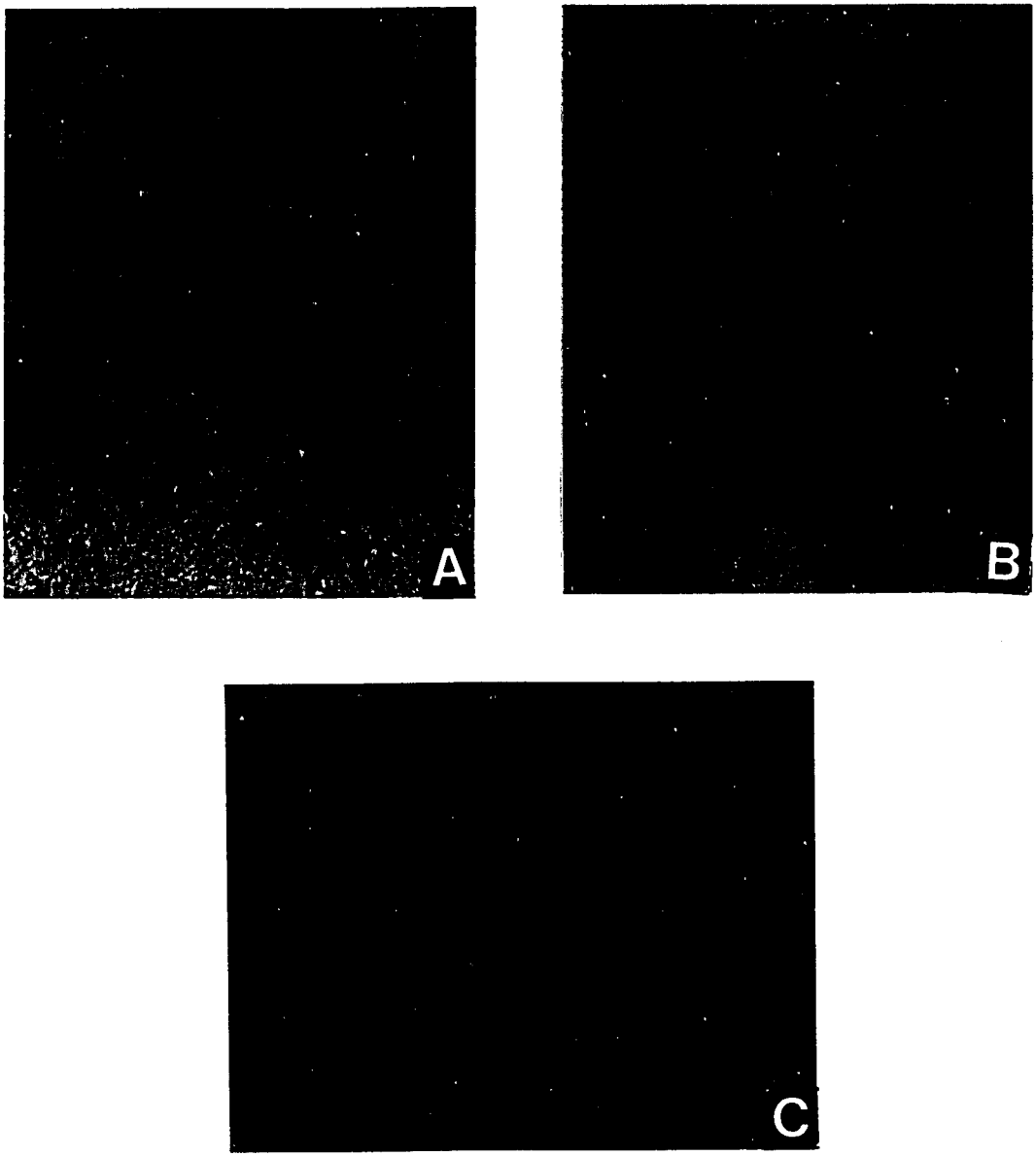
Figure 6A-6C. 5HT NEURONS IN MESENCEPHALIC AND PONTINE CULTURES

Immunocytochemistry with antibodies against 5HT conjugated to BSA reveal many 5HT+ neurons in cultures established from the rhombencephalon and few 5HT+ neurons from the mesencephalon. (A) One week old cultures established from rhombencephalic tissue contain many labeled 5HT neurons with well developed somata and processes. (B) Very few 5HT+ neurons are labelled (arrow) in the midbrain cultures and the short processes are hardly visible. (C) Immunoprecipitation of the conjugated antibody prevents any neurons from becoming labelled indicating that the antibody used is selective for 5HT. The colored cells represent backround staining throughout the culture. x 168.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The uptake mechanism of the monoamine neurons increases almost linearly for approximately 15 to 20 DIV and then growth becomes slower until it reaches a plateau after the 3rd week for both DA and 5HT neurons (Fig. 7). The uptake for NE neurons continued to increase linearly for the first 4 weeks. Either 3[H] DA or 3[H] NE was used to measure uptake in the ventral midbrain or pons respectively. Similarly, 3[H] 5HT has been used for analyzing the serotonin uptake mechanism in pontine cultures. The developmental profile of the 3[H] amine uptake on cultured midbrain and pontine neurons appears in Fig. 7. Notice that the amount of uptake increases markedly during the 2nd week and begins to level off during the 3rd week in vitro for both DA and 5HT neurons. The development of the uptake system resembles the morphological development. The absolute values of 3[H] amines accumulated per culture vary for the three amines used and depends on the number of the corresponding neurons present in the cultures. To examine whether the uptake of 3[H] DA represented accumulation specifically in the DA neurons, we pretreated cultures with 1 uM GBR-12909, a specific inhibitor of the high-affinity uptake pump of the dopamine neurons (Heikkila and Manzino, 1984). Pretreatment with GBR-12909 resulted in a greater than 95% inhibition of 3[H] DA accumulation by the cultures (control means $\pm$ S.E.M., 18892$\pm$4112, n=5;

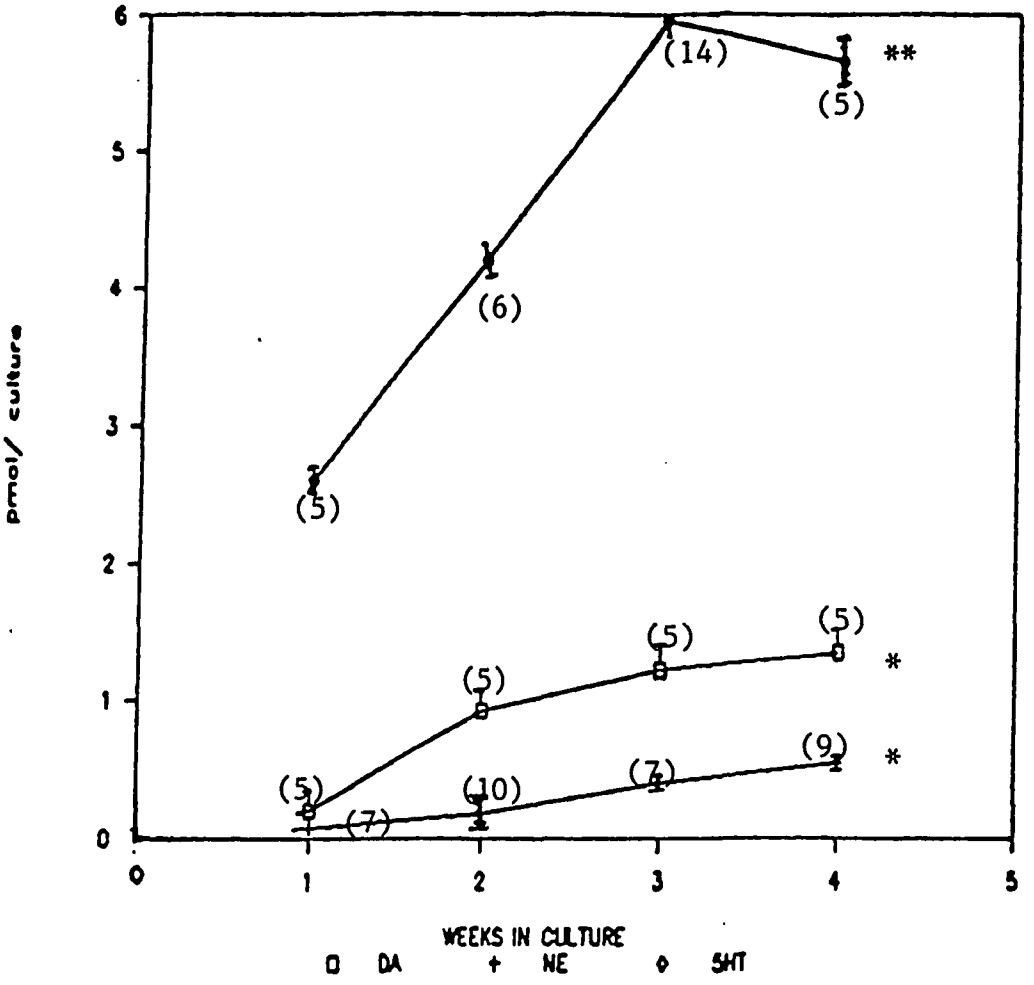


Figure 7. DEVELOPMENT OF MONOAMINE UPTAKE IN CULTURE

Uptake of 3[H] monoamines (DA , NE +, 5HT) in culture as a function of cultivation time. Uptake is shown for 4 weeks for all 3 monoamines. There is a marked increase in uptake between the 1st and 2nd weeks and the amount of uptake levels off for both DA and 5HT neurons between the 3rd and 4th weeks. NE uptake continues to increase linearly during the first 4 weeks. The amount of monoamines accumulated represent a quantitative index of neuronal terminal[s'] uptake capacity. Uptake is greatest among the 5HT neurons, then the DA neurons, and lastly among the NE neurons. The values are expressed in pmol 3[H] amine/ culture and represent the means $\pm$ S.E.M. of 5-14 cultures per cultivation week. Simple analysis of variance and pairwise comparisons were used to analyze the increase in uptake over time. (**F 0.01=4.64, $p< 0.01$; df=3, 26; *F 0.05=3.24; df=3, 16; *F 0.05=2.93, $p< 0.05$; df=3, 29. The amount of uptake was increased significantly over the 1st, 2nd and 3rd weeks for 5HT and between the 1st and 3rd weeks for DA and NE.

GBR-12909: 560±35, n=5: cpm/ culture/10 min). Similarly, 3[H] NE uptake specificity was defined with pretreatement with desmethylimipramine (DMI) (10 uM), a specific inhibitor of the high-affinity uptake pump of the NE neurons (Jonathan et al., 1982). 10 uM DMI produced a 94.3% inhibition of 3[H] NE accumulation by the rhombencephalic cultures (control means ± S.E.M., 23664±583, n=5; DMI (10 uM), 1581±22, n=5: cpm/ culture /10 min). Citalopram, a specific 5HT uptake inhibitor (Hyttel, 1982), was used to prevent 3[H] 5HT accumulation preferentially, where 10 uM reduced 5HT uptake by 97.8% (control means ± S.E.M., 155358±3979, n=5; citalopram (10 uM), 3475±180, n=5: cpm/ culture/ 10 min). Neither DMI nor citalopram had any inhibitory affect on the midbrain DA neurons at 10 uM. Since these indices are well characterized for DA, NE and 5HT neurons, we can compare any significant changes that may occur following exposure to a toxin.

B. Comparative effects of MPTP and MPP+ to dopamine neurons in culture.

1. Effects of MPTP on dopamine neurons in dissociated cultures.

The first index used to quantitate any changes that

may occur following MPTP exposure was the specific uptake of trace amounts of 3[H] DA in the cultures. In order to eliminate any acute effects of the toxin such as, inhibition on the CA uptake mechanism, we removed the drug and rinsed the cultures with fresh feeding medium for 24 hours. Removing MPTP for 24 hrs is sufficient time to eliminate the inhibitory effects MPTP has on the CA uptake pump (Mytilineou and Cohen, 1984) so that the uptake studies measured only the toxic effects of MPTP. In several experiments in which the cultures were exposed to various concentrations of MPTP, we observed that the toxic changes produced by 100 uM MPTP were less pronounced in comparison to 10 uM MPTP exposure. In order to investigate this phenomenon, we examined the toxicity of MPTP to dissociated cell cultures from rat embryo mesencephalon over a large range of concentrations (0.1 to 500 uM). Exact concentrations of MPTP were added to the feeding medium.

Fig. 8 shows the uptake of 3[H] DA by the mesencephalic neurons after 7 days exposure to various concentrations of MPTP followed by a 24 hour wash. An effect was seen at micromolar concentrations. At the lower concentrations of MPTP (from 0.1 to 5 uM) the uptake of 3[H]-DA was reduced and the effect was more pronounced with increasing concentrations of the toxin. By 1 uM the amount of 3[H] DA that could be accumulated was reduced

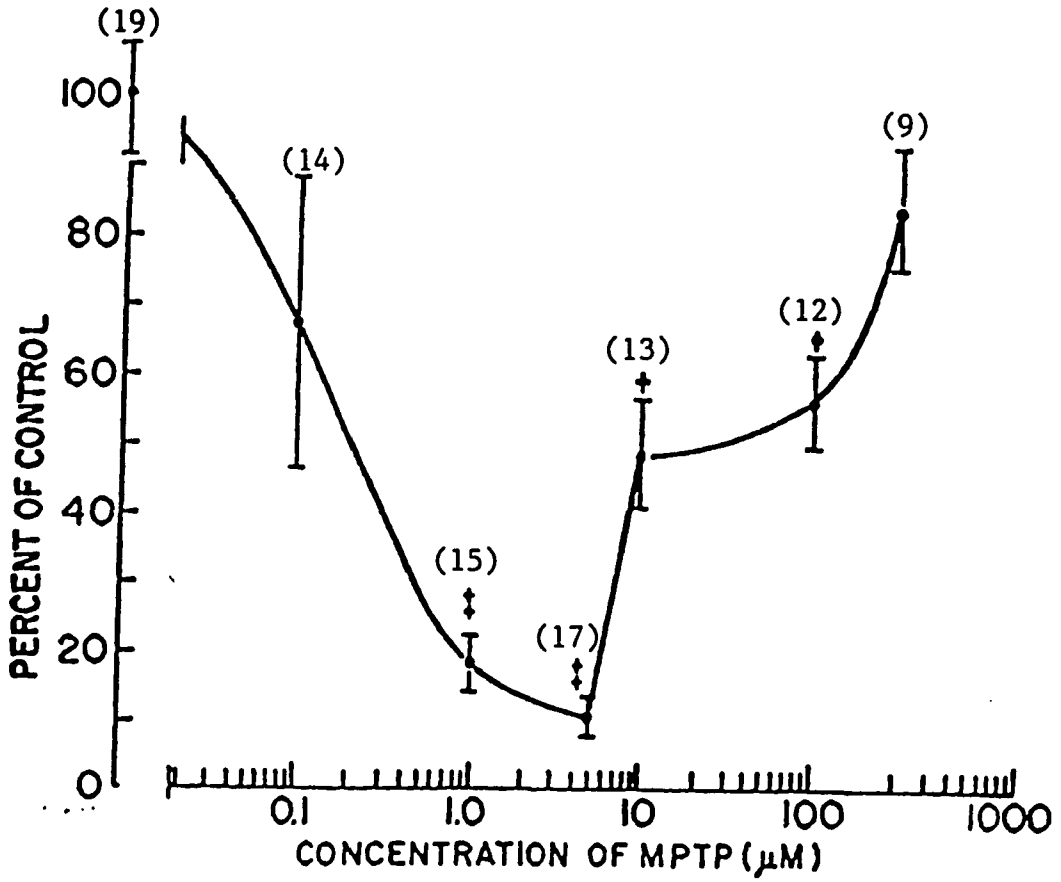


Figure 8. MPTP DOSE RESPONSE CURVE ON 3[H] DA UPTAKE IN MIDBRAIN CULTURES

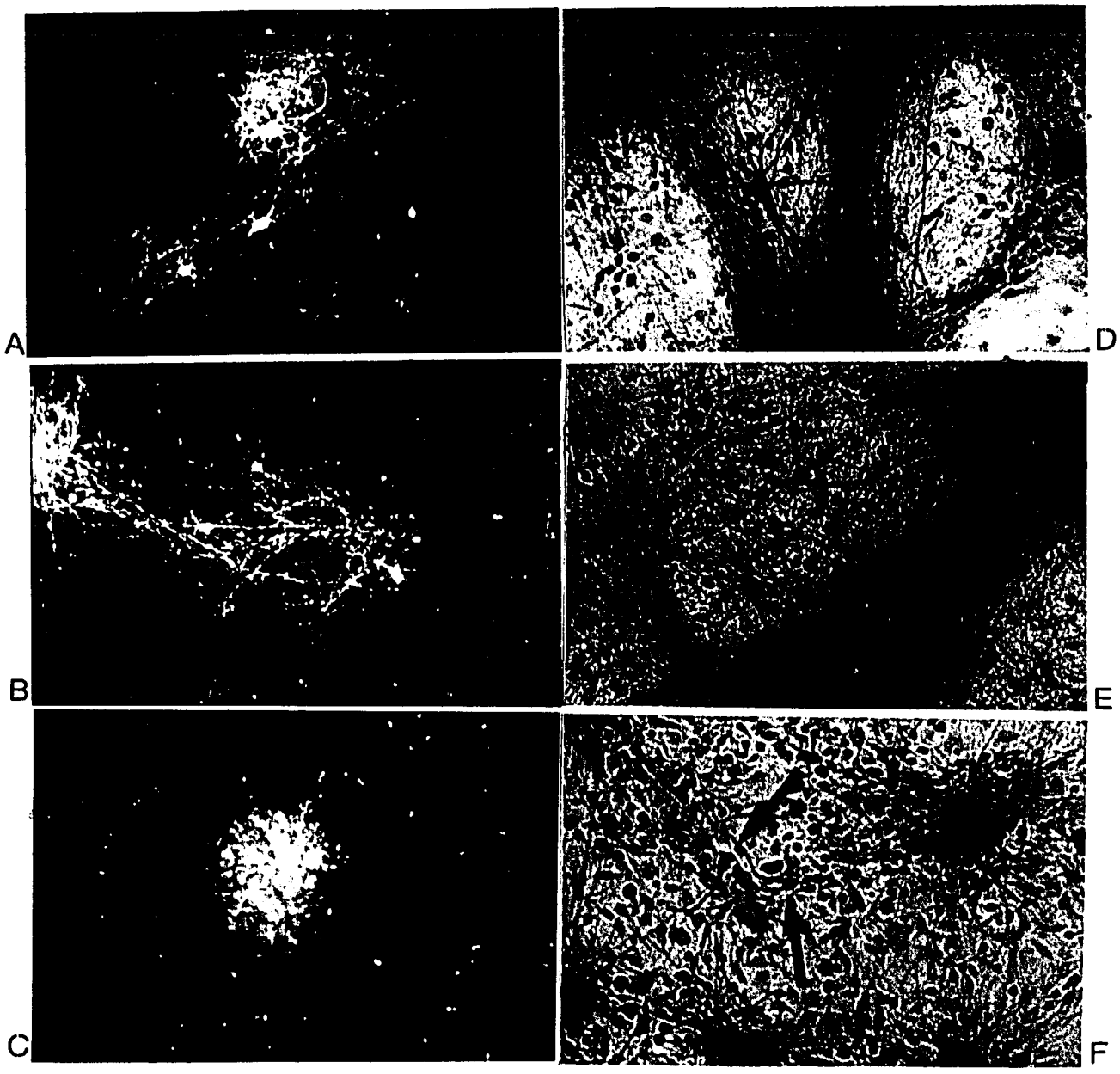
Figure 8. The effect of 7 day MPTP exposure on 3[H] DA uptake in midbrain cultures at varied concentrations (0.1-200 uM). MPTP reduced the uptake of 3[H] DA with increasing concentrations reaching a maximal toxic effect at 5 uM. Doses above 5 uM (10-200) produced a reduced toxic effect in uptake with the smallest effect seen at the highest dose (200 uM). Values equal the means ± S.E.M. of 9-17 cultures per group and each conentration is compared with the control group (no drug). The value for the control cultures was 0.924 ± 0.06 pmol 3[H] DA/ culture, +, $P < 0.01$; ‡, $P < 0.001$; Student's t-test.

by approximately 80% ($p < 0.001$). Maximum toxicity was observed at 5 uM MPTP, the uptake of 3[H] DA being reduced by 93%. When the concentration of MPTP was increased further (10 to 200 uM), the uptake of 3[H] DA began to return towards normal values. Cultures treated with 10 uM MPTP had reduced 3[H] DA accumulation that varied from one experiment to another ranging between 19.5 and 50.8% of the control uptake values. The uptake values obtained from 100 uM treated cultures were less variable and they were never decreased by more than 50 to 60%. Cultures incubated with 200 uM MPTP showed only a 12% reduction in the uptake values, which were not significantly different from the controls. Further increase in the concentration of MPTP to 500 uM resulted in generalized toxicity to all cellular elements present in the cultures. No significant effect was seen at doses equal to or below 0.1 uM MPTP.

In order to visualize the DA neurons remaining in the cultures after MPTP treatment, we used two techniques, which utilize different properties of the dopaminergic neurons. Catecholamine histofluorescence after preincubation with alpha-methyl-norepinepherine was used to demonstrate all DA neurons possessing a functional high-affinity uptake pump on their neuronal membrane. TH immunocytochemistry was used to demonstrate the DA neurons that contain enough TH molecules to produce a visible reaction product. Figure 9 shows the results obtained

Figure 9A-9F. MIDBRAIN CULTURES FOLLOWING 1 WEEK OF MPTP TREATMENT AT 100 AND 10 uM

Dissociated mesencephalic cultures were treated with MPTP at 100 and 10 uM MPTP for 1 week and washed for 24 hours. A-C are photomicrographs of midbrain cultures on the 16th day in vitro treated for CA histofluorescence after incubation with 10 uM alpha-methyl-norepinepherine at 37° C for 30 min. All DA neurons accumulate alpha-methyl-norepinepherine and become visible, irrespective of the endogenous DA levels. (A) Control culture shows fluorescing neuronal somata (arrow) and fibers. The presence of several DA neurons at the top of the picture makes the cell aggregate brightly fluorescent. x 168. (B) Culture treated with 100 uM MPTP and processed for CA histofluorescence 24 hr after removal of the drug has a similar appearance to the control. x168. (C) Culture treated with 10 uM MPTP shows a cell aggregate that contains a fluorescing neuron with no apparant processes. Two faintly fluroescing fibers can also be seen. x 168. D-F are photomicrographs of mesencephalic cultures processed for TH immunocytochemistry also on the 16th day in vitro. (D) Control culture shows several TH positive neurons and long processes. x 168. (E) Culture was treated with 100 uM MPTP and the TH positive neurons and processes appear normal . (F) 10 uM MPTP treated culture has some TH positive cells present but, they are greatly reduced in size and have very small or no processes (arrows). x 168.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by these techniques, in cultures treated with 10 and 100 uM MPTP for 7 days. Using both indices of dopaminergic integrity, we observed that 10 uM MPTP produced significant damage to the DA neurons (Fig. 9C, 9F). When the concentration of MPTP was increased to 100 uM, the damage was substantially reduced and the appearance of the DA neurons was similar to that of the control cultures (compare Fig. 9A and B to 9D and E). CA fluorescence of cell cultures treated with 1 and 5 uM MPTP were also examined (Fig. 10A-10C). Notice that the number of fluorescing fibers were decreased and remaining perykarya appeared damaged at 5 uM similarly to what was seen at 10 uM, however, there were more fluorescing fibers present at the lower dose (Fig 10B). 1 uM MPTP also reduced the amount of fluorescence, but many more surviving cells were present (Fig. 10C). Notice the MPTP treated cultures were indistinguishable from the controls under phase contrast microscopy (Fig. 11A-11C) indicating lack of generalized toxicity of the compound at these doses.

We also measured the total number of TH-positive neurons present in the cultures treated with MPTP (see table 1). The number of labeled cells were reduced by 78% at 10 uM MPTP when compared to the controls and only by 34% in the 100 uM treated cultures. Although the number of surviving DA neurons was higher than was predicted from the uptake studies at 5 and 10 uM MPTP,

Figure 10A-10C. CA HISTOFLUORESCENCE OF MIDBRAIN CULTURES TREATED WITH 5 AND 1 uM MPTP

Cultures were treated with two concentations of MPTP (5 or 1 uM) for 7 days and washed for 24 hours. (A) Control cultures appear bright with many fluorescing neurons and fibers. (B) Few somata remain in cultures treated with 5 uM MPTP compared with the controls and notice the damaged fibers (left arrow). The backround is lightened giving an increased impression of fluorescence. (C) 1 uM MPTP reduced the amount of fluorescence compared to the control cultures but, many surviving neurons are present. x 200.

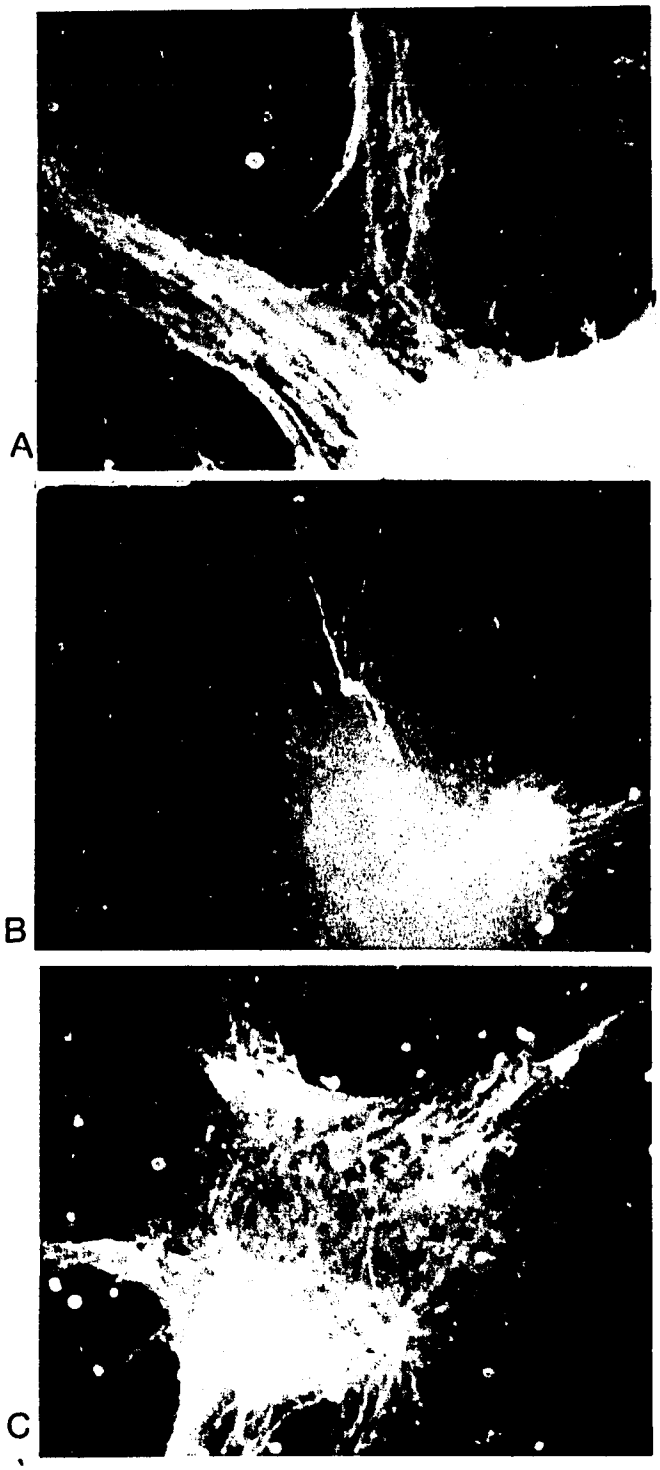
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Figure 11A-11C. PHASE CONTRAST MICROSCOPY OF MPTP TREATED CULTURES

Treatment of cultures with MPTP at all the concentrations used did not induce generalized destruction. (A) Control, (B) 100 uM MPTP, and (C) 10 uM MPTP cultures are indistinguishable under phase contrast microscopy, therefore, there is no apparent toxicity to the rest of the cellular elements in the cultures treated with 10 or 100 uM MPTP. Magnification = 168x.

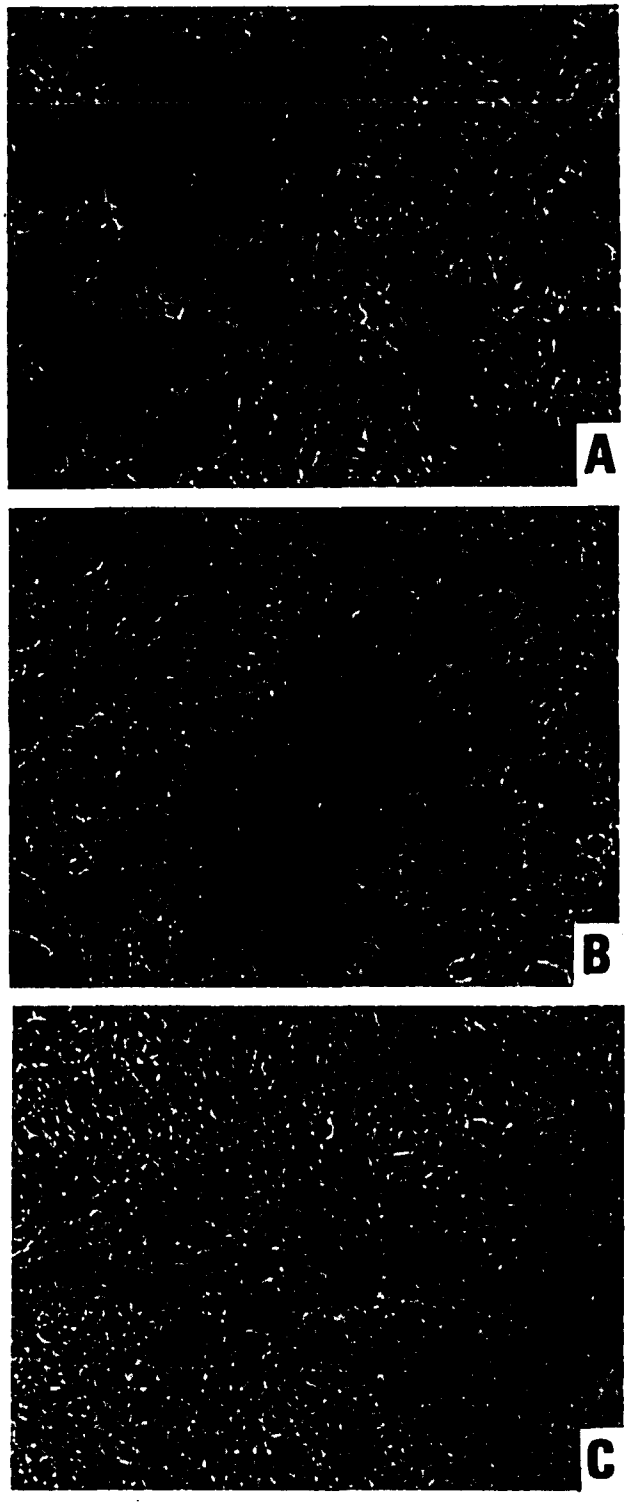
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TABLE 1

CELL COUNTS FOLLOWING 7 DAYS EXPOSURE TO VARIED CONCENTRATIONS OF MPTP (5-100 uM)

	Cell Description	cell count
Control (n=5)	TH+ neurons are darkly stained with normal somata and long processes.	426±49
MPTP (100 uM) (n=4)	Many surviving neurons that resemble controls, but less populated and some cells have swellings.	282±11
MPTP (10 uM) (n=3)	Not many surviving TH+ neurons remain. Many DA neurons have shrunken or swollen somata, reduced dendritic arborization, and truncated axons.	94±6+
MPTP (5 uM) (n=5)	Same description as 10 uM	139±13+
MPTP (5 uM) + mazindol (1 uM) (n=4)	About 50% appear as control TH+ neurons and 50% appear damaged.	246±5

Table 1. Midbrain cultures were exposed to 7 days of MPTP treatment and washed for 24 hours. The toxic effect of MPTP is reduced at high concentrations (100 uM) and partially protected by mazindol. All TH+ cells were counted with a grid in treated and non-treated groups. Values represent means ± S.E.M. of 3-5 cultures representitive of the effects produced by MPTP. Significance was measured with the Student's t-test, +, $P<0.001$.

most of these labeled cells appeared damaged. The DA neurons at 5 uM were either shrunken or swollen, or lacking processes (Fig. 10B).

In addition, we studied the possible mechanisms which could underlie the reduced toxicity of MPTP at higher concentrations. It has been shown that MPTP can act as an inhibitor or inactivator of MAO-B activity (Fuller et al., 1985; Singer et al., 1985). To test the possibility that at high concentrations MPTP might interfere with its own oxidation to MPP+, we measured the amount of MAO activity in mesenchephalic cultures during acute and chronic exposure to MPTP. In the acute treatment, MPTP was present only during the assay whereas during chronic treatment the drug was added to the cultures for 7 days. In addition, we also examined whether MPTP acts as an inhibitor of the uptake of MPP+. Inhibition of 3[H] MPP+ uptake into striatal synaptosomes by MPTP has been reported, with an apparent Ki of 3.3 uM (Javitch and Snyder, 1985).

The inhibitory effect of MPTP on MAO activity is seen in table 2. During acute exposure, high concentrations (100 uM) reduced both MAO-A and MAO-B activities with a greater inhibitory effect on MAO-A activity. On the other hand, lower concentrations (5 uM) of MPTP had very little effect on either MAO-A or MAO-B activities. With prolonged treatment (7 days), the

TABLE 2

MAO ACTIVITY IN MESENCEPHALIC CULTURES DURING ACUTE AND CHRONIC EXPOSURE TO MPTP

2 experiments were combined and 5HT and benzylamine were used as subsrtates. MAO activity was measured in nmoles/mg protein/hour.

	MAOA	MAOB	% control MAO-A	% control MAO-B
Controls (no drug)	27.0	6.95		
control + MPTP (100uM)	6.40	4.15	23.7	59.7
control + MPTP (5uM)	22.2	6.50	82.1	93.5
MPTP (100uM) 1 week	17.1	1.10	63.2	15.8
MPTP (100uM) + 100uM 1 week	4.99	0.63	18.1	9.0
MPTP (5uM) week	25.6	4.35	94.8	62.6
MPTP (5uM) + 5uM 1 week	20.6	4.20	76.5	60.4

Table 2. MAO activity in mesencephalic cultures after acute an chronic exposure to MPTP. Activity of both types A + B is reduced by 100 uM MPTP after acute (presence) treatment with a greater inhibition on MAO-A activity. Chronic exposure results in a greater inhibitory effect on MAO-B activity. Low doses have no effect. Samples were done in duplicate (n=2).

inhibitory effect of MPTP on MAO activity was much more dramatic. In cultures exposed to 100 uM MPTP and washed prior to the assay, both MAO-A and MAO-B activities were reduced with a much greater effect this time on MAO-B activity, which was reduced by 85%. MAO-A activity was decreased by only 37%. Addition of 100 uM MPTP to 100 uM treated cultures decreased the activity further (by 91% for MAO-B and by 82% for MAO-A). Prolonged treatment of 5 uM MPTP followed by wash of the drug prior to the assay did not alter MAO-A activity. However, MAO-B activity was considerably lower than the controls. Addition of 5 uM MPTP to these cultures decreased the MAO-A activity, while MAO-B activity was not reduced any further. The results show that very little MAO-B activity remains in the cultures during prolonged exposure to high concentrations of MPTP. Furthermore, it appears that the inhibitory effects MPTP has on MAO-A activity are reversible, consistent with a competitive type of inhibition, while the effects on MAO-B increase with the length of exposure and appear irreversible, suggesting a slowly occuring irreversible inactivation. Therefore the reduced toxicity at high concentrations of MPTP could be the result of inactivation of MAO-B which would result in reduced MPP+ formation.

An alternative explanation for the reduced toxicity at high concentrations could also be that MPTP has an

inhibitory effect on the accumulation of MPP+ into the DA neurons, since its accumulation is required for cell death to occur. To test this possibility we measured the uptake of 3[H] MPP+ by the cultures in the presence of increasing concentrations of MPTP. Fig. 12 shows the results of this experiment. The presence of MPTP inhibited the uptake of MPP+ by the DA neurons. At the concentrations of MPTP that result in reduced toxicty (100-200 uM), the inhibition was very pronounced (more than 80%). The amount of 3[H] MPP+ accumulated in the presence of 10 uM MPTP was inhibited by 50% and at 1 uM by only 17%. Therefore, the amount of inhibition on the CA uptake pump increases with increasing concentrations of MPTP. Thus, we have defined a very specific dose range (5-10 uM) that is required to induce maximal MPTP toxicity.

3. Protection against MPTP toxicity.

a. MAO inhibition

Since MPTP is a substrate for MAO-B and inhibition of MAO-B activity by specific inhibitors such as deprenyl prevents MPTP-induced toxicity <u>in vivo,</u> we have preincubated the cultures for 15 min with the specific MAO-B inhibitor deprenyl (10 uM), the specific MAO-A inhibitor clorgyline (1 or 10 uM) or a combination of the two and then added MPTP to the feeding medium at the most toxic concentrations (5-10 uM) for the DA neurons. A

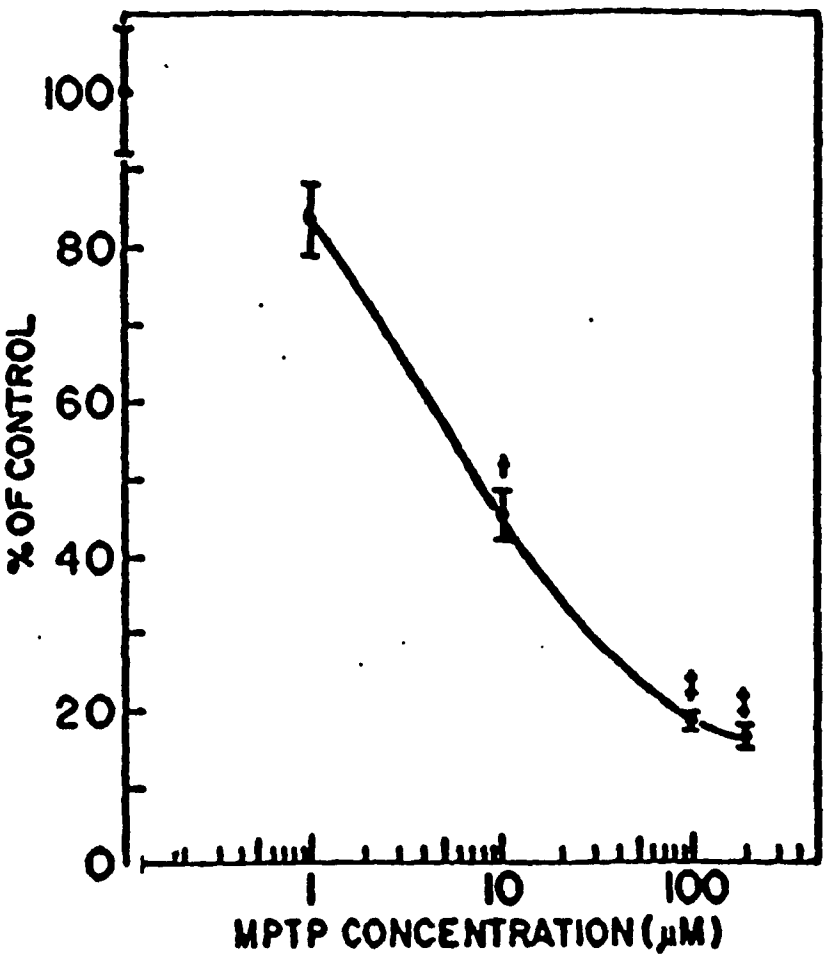


Figure 12. EFFECT OF MPTP ON 3[H] MPP+ UPTAKE BY MESENCEPHALIC NEURONS IN CULTURE

Midbrain cultures were incubated with 3[H] MPP+ for 10 min. at 37° C in the presence of varied concentrations of MPTP (1-100 uM). The values are the mean ± S.E.M. from 14-16 cultures per group and expressed as % of 3[H] MPP+ uptake by the control(no MPTP present in the incubation medium). 3[H] MPP+ uptake by the control cultures was 65 ± 4 fmol/culture. Significantly different from control, + $P < 0.01$; ‡ $P < 0.001$; Student,s t-test.

partial protection of neuronal cell damage or destruction occured when the cultures were exposed to deprenyl (1 uM) and MPTP (5 uM). Tritiated DA uptake values increased from 18 to 65-85% of the controls. Deprenyl (10 uM) coincubated with 10 uM MPTP resulted in complete protection whereby uptake of 3 [H] DA was indistinguishable from the controls. The complete protection by deprenyl at the higher concentration of MPTP (10 uM) probably reflects the inhibitory action of MPTP itself on MAO-B. In contrast, clorgyline introduced at concentrations that are specific for MAO-A inhibition (1 uM) produced no protection against MPTP toxicity when combined with 5 uM MPTP. However, higher concentrations of clorgyline (10 uM) that inhibit both forms of MAO, fully protected against MPTP-induced toxicity when the concentration of MPTP in the cultures was 10 uM (Figure 13). On the other hand, when the concentration of MPTP was 5 uM, 10 uM clorgyline displayed only a partial protection in uptake (52.5% of control). Furthermore, deprenyl (1 uM) and clorgyline (1 uM) introduced into the feeding medium together prevented the reduction of tritiated amine uptake completely. These results suggest that a partial conversion of MPTP to MPP+ via MAO-A is possible and they may explain the partial protection seen with deprenyl at the lower concentration of MPTP. Neither deprenyl nor clorgyline protected against MPP+ toxicity.

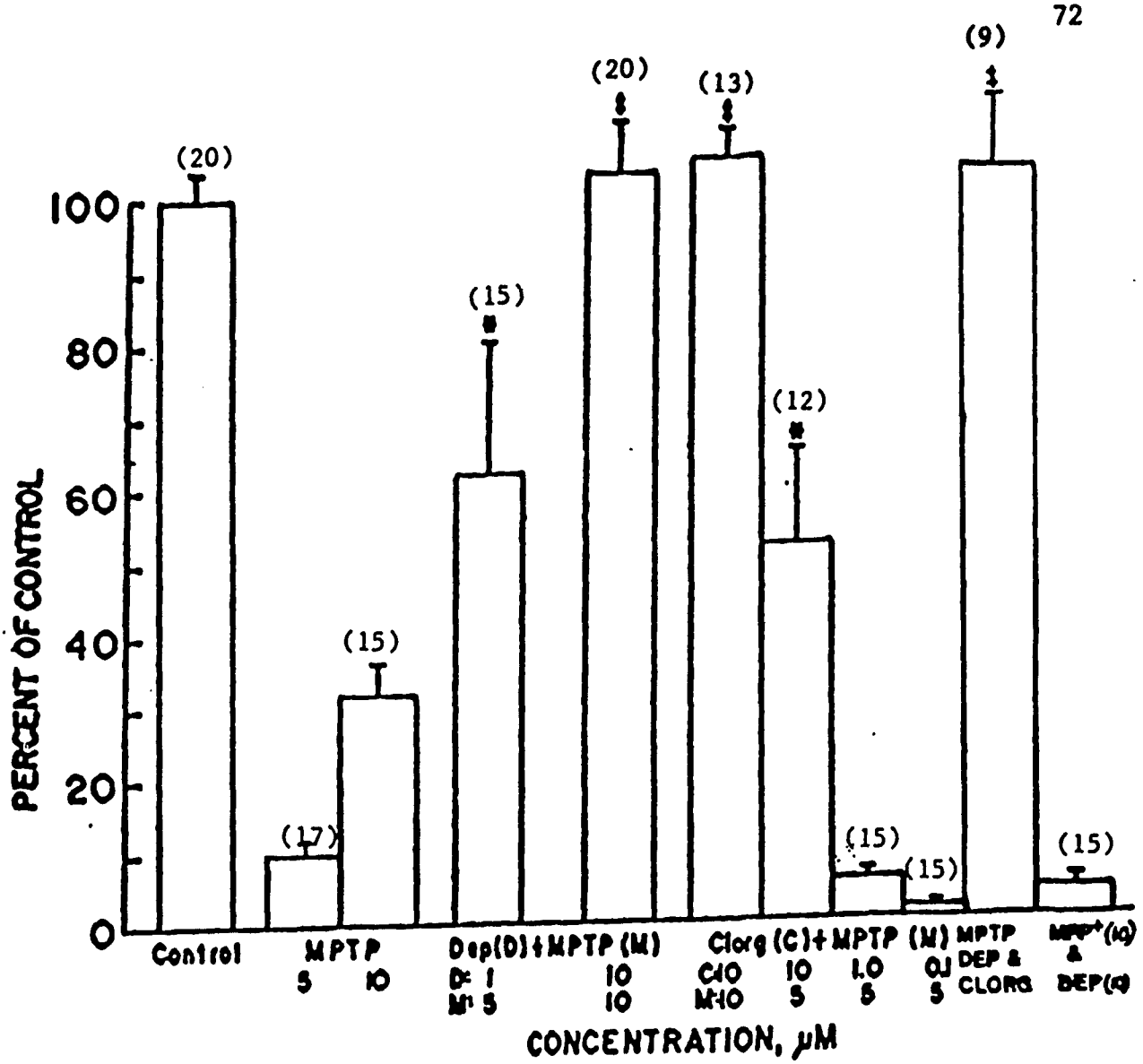


Figure 13. 3[H] CA UPTAKE FOLLOWING MPTP OR MPP+ TREATMENT IN THE PRESENCE OF DEPRENYL OR CLORGYLINE

Figure 13. Effect of MPTP (5 or 10 uM) alone or after pretreatment with deprenyl (1 or 10 uM) or clorgyline (0.1, 1.0 or 10 uM) on 3[H] DA uptake. Statistical comparison was made on absolute values between combined treatment and MPTP or MPP+ alone among 15-20 cultures per group. *, $P < 0.05$, ++, $P < 0.001$, Student's t-test.

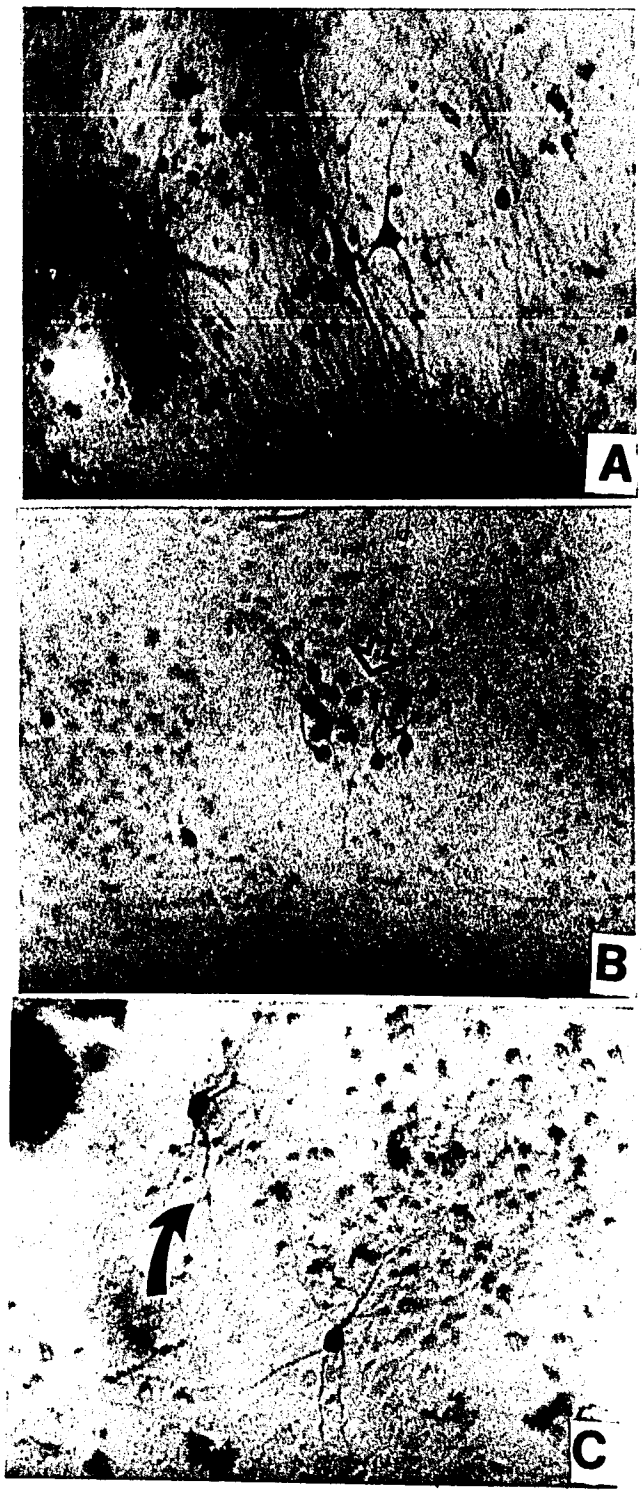
b. Inhibition of CA uptake mechanisms.

Since the accumulation of MPP+ into the DA neurons is necessary for cell destruction to occur and in vivo studies have demonstrated protection with uptake ihibitors, we incubated the cultures with 5 uM MPTP in the presence of mazindol (1 uM), a specific CA uptake inhibitor, for 1 week. When 3[H] DA was used as an index of neuronal integrity, mazindol did not appear to protect the DA neurons, since 3 [H] DA uptake was reduced even further from 7 to 5.1% of the controls.

In order to see if the cells were still protected against destruction, several of the cultures treated with MPTP in the presence or absence of mazindol were incubated with antibodies against TH to visualize the DA neurons. The number of TH positive neurons remaining in those cultures treated with 5 uM MPTP and 1 uM mazindol exceeded the number of cells surviving in cultures treated with 5 uM MPTP alone (see table 1). In addition, many more of the surviving neurons appeared normal in the mazindol co-treated group (Fig 14A-14C). Therefore, a partial protection against cell death was afforded by mazindol, even though the uptake mechanisms became impaired.

Figure 14A-14C. PROTECTION OF MPTP-INDUCED DESTRUCTION BY MAZINDOL

DA neurons were treated with 5 uM MPTP in the presence or absence of mazindol (1 uM). (A) Control TH+ neurons appear darkly stained with large somata and long processes. (B) MPTP (5 uM) reduced the number of DA neurons and those cells that did survive appear to have shrunken somata and few processes remaining (arrow). (C) When cultures were preincubated with mazindol (1 uM), 5 uM MPTP did not cause destruction to the majority of DA neurons. Many cells appeared normal although the number of TH+ neurons was reduced (arrow). x 168.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3. The effects of MPP+ on DA neurons.

The effects of MPP+ to the DA neurons were more pronounced when compared to MPTP. The dose response curve for the effect of MPP+ on the uptake of 3[H] DA appears in figure 15. Varied concentrations of MPP+ (0.1-50 uM) were introduced into the feeding medium for 7 days beginning on the 8th DIV. Uptake of 3[H] DA following 1 week exposure and removal of the drug for 24 hours decreased with increasing concentrations of MPP+ with a maximal reduction seen at 50 uM. Uptake values were close to backround at concentrations between 10 and 50 uM MPP+ and a generalized destruction to all cells occurs at concentrations above 50 uM. To visualize the DA neurons, CA histofluorescence and immunocytochemistry were used again (Fig. 16A-16F). At concentrations equal to 10 uM, cultures were devoid of TH+ cells (see table 3). Very few cells were labeled with CA histofluorescence (Fig. 16D) and lacked processes. Many cells that survived the 1 uM MPP+ dose looked severely damaged, with either swollen or shrunken cell bodies and processes or without processes at all (Fig. 16B). In addition, 1 uM MPP+ reduced the number of surviving TH positive neurons compared with the controls (67% of control). When the cultures were pretreated with a CA uptake inhibitor such as mazindol (1 uM) and 1 uM MPP+, the number of TH positive neurons increased toward control levels (66.8%) from almost being

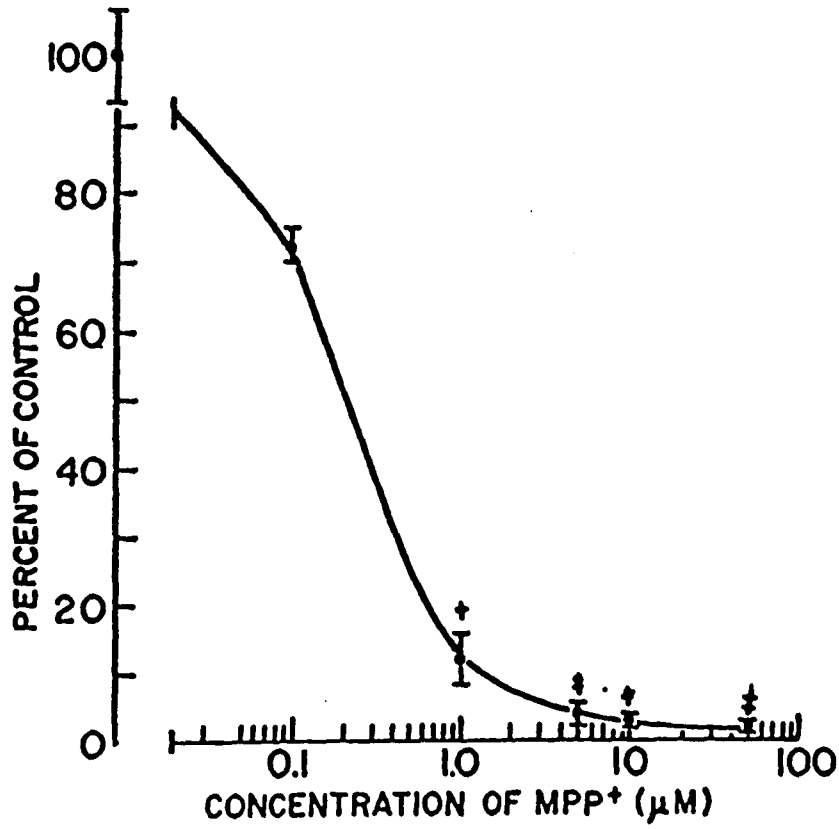

FIGURE 15. 3[H] DA UPTAKE AFTER 7 DAYS EXPOSURE TO MPP+

Figure 15. The effect of 7 day exposure of MPP+ at varied concentrations (0.1-50 uM). In the SN of the midbrain culutres there is a dose dependent decrease in the amount of 3[H] DA uptake. Values are the means ± S.E.M. of 26 cultures per group. +, P< 0.01, ++, P< 0.001, Student's t-test.

Figure 16A-16D. MIDBRAIN CULTURES TREATED WITH MPP+

TH immunocytochemistry and CA histofluorescence were used to visualize the DA neurons after exposure to 10 or 1 uM MPP+. Control cultures (A, C) are rich in DA neurons with well defined perikarya and neuritic processes, x 168; x 80 respectively. Cultures treated with 10 uM MPP+ (D) are devoid of fluorescing fibers and contain very few fluorescing cell bodies. Notice one shrunken cell body is present in (D) (arrow). x 80. 1 uM treated cultures (B) contain TH+ cells, however, many of these neurons are damged with shrunken somata, axonal truncations, or reduced dendritic arborization. x 168.

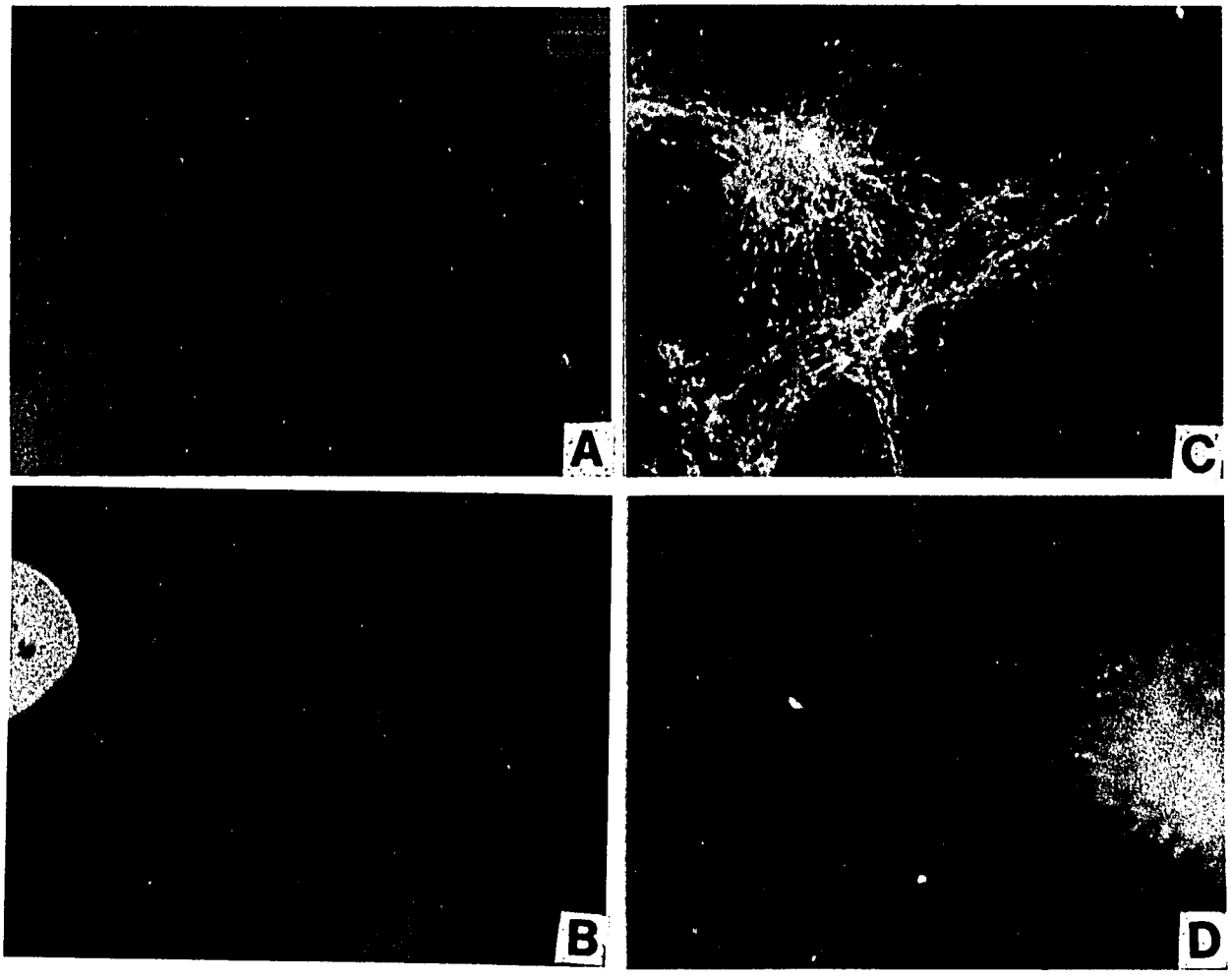
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TABLE 3

MPP+ EXPOSURE TO MIDBRAIN CULTURES FOR 7 DAYS

	Cell Description	cell count
Control (n=5)	Many TH+ neurons with normal appearance: well defined somata, long axons and processes.	404±28
MPP+ (10 uM) (n=4)	Very few DA neurons survive. Only shrunken or swollen somata remain.	13±4++
MPP+ (1 uM) (n=3)	Low TH+ cell count with many damaged neurons: process swellings, reduced arbor, or trucated axons.	130±11+
MPP+ (1 uM) Mazindol (1 uM) (n=5)	Reduced cell count, but majority of TH+ neurons appear as controls.	212±58

Table 3. Midbrain cultures were treated with MPP+ for 7 days and rinsed for 24 hours. TH+ neurons are reduced by both concentrations of MPP+ to a greater extent than MPTP treated cultures and protected by mazindol. Values represent means ± S.E.M. of 3-5 cultures. +, $P<0.001$; ++, $P<0.0001$, Student's t-test.

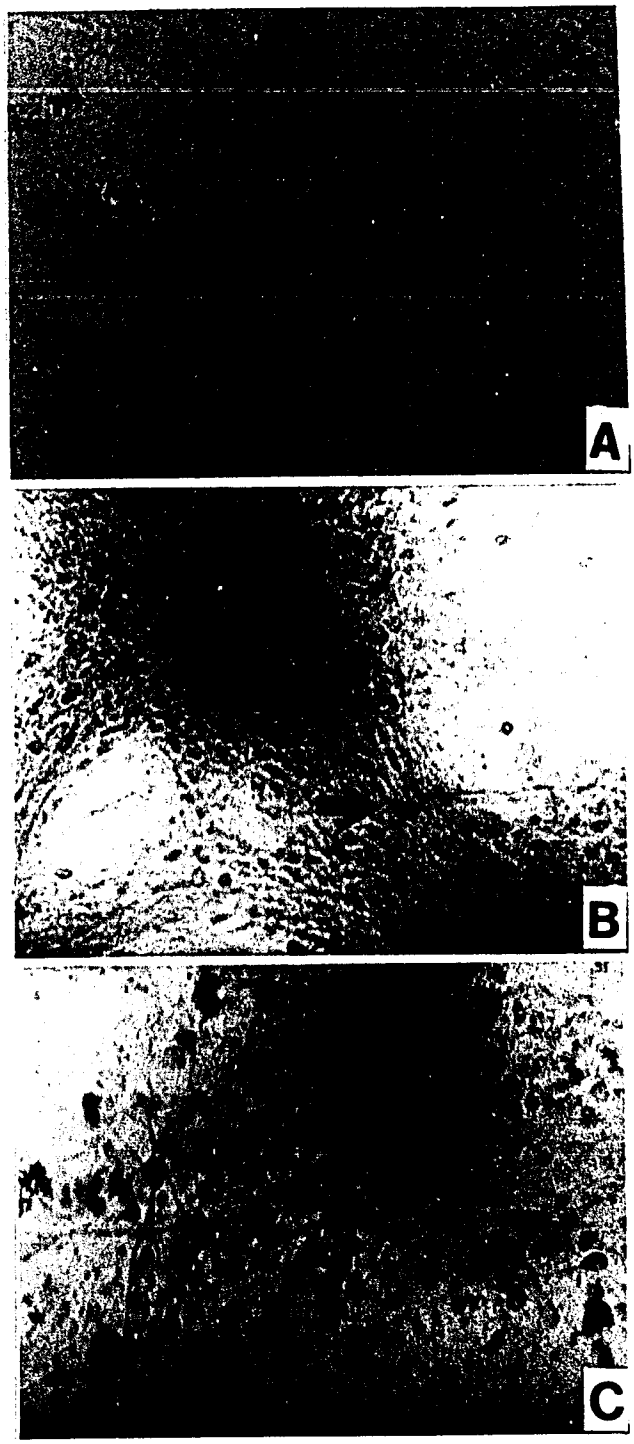
wiped out (3.2%), but were still significantly lower than in the control cultures. Many cells in the protected group (10 uM MPP+, 1 uM mazindol) appeared more similar to the controls (Fig. 17A-17C). Therefore, mazindol partially protected against DA cell destruction by MPP+. However, treatment of MPP+ in the presence of mazindol for 7 days did not prevent the impairment of the uptake mechanisms just as with MPTP.

C. Effects of MPTP and MPP+ on NE neurons.

MPTP or MPP+ was added to the feeding medium of pontine cultures at varied concentrations for 7 days. The drug was removed 24 hours prior to noradrenergic neuronal analysis. The cultures were analyzed by measuring the uptake with trace amounts of tritiated NE in the presence of a specific 5HT uptake inhibitor, citalopram in order to prevent nonspecific accumulation of 3[H] NE into the 5HT neurons. The dose response curve of MPTP on NE neurons is shown in figure 18. The amount of NE uptake seen was significantly reduced with exposures between 1.0 to 100 uM MPTP, with the greatest reduction seen in the 100 uM MPTP treated cultures (41.2% of control). Compared to the DA neurons the reduction in uptake at lower concentrations of MPTP was not as pronounced, indicating that these neurons are not as

Figure 17A-17C. PROTECTION FROM MPP+ TOXICITY BY MAZINDOL

Midbrain cultures were treated with MPP+ in the presence or absence of mazindol for 7 days and washed for 24 hours. (A) Control cultures are rich in DA neurons while MPP+ treated cultures (1 uM) (B) contain many damaged neurons (arrows). Many of the TH+ neurons appear like the controls when they are preincubated with mazindol (1 uM) (C). Magnification = 168 x.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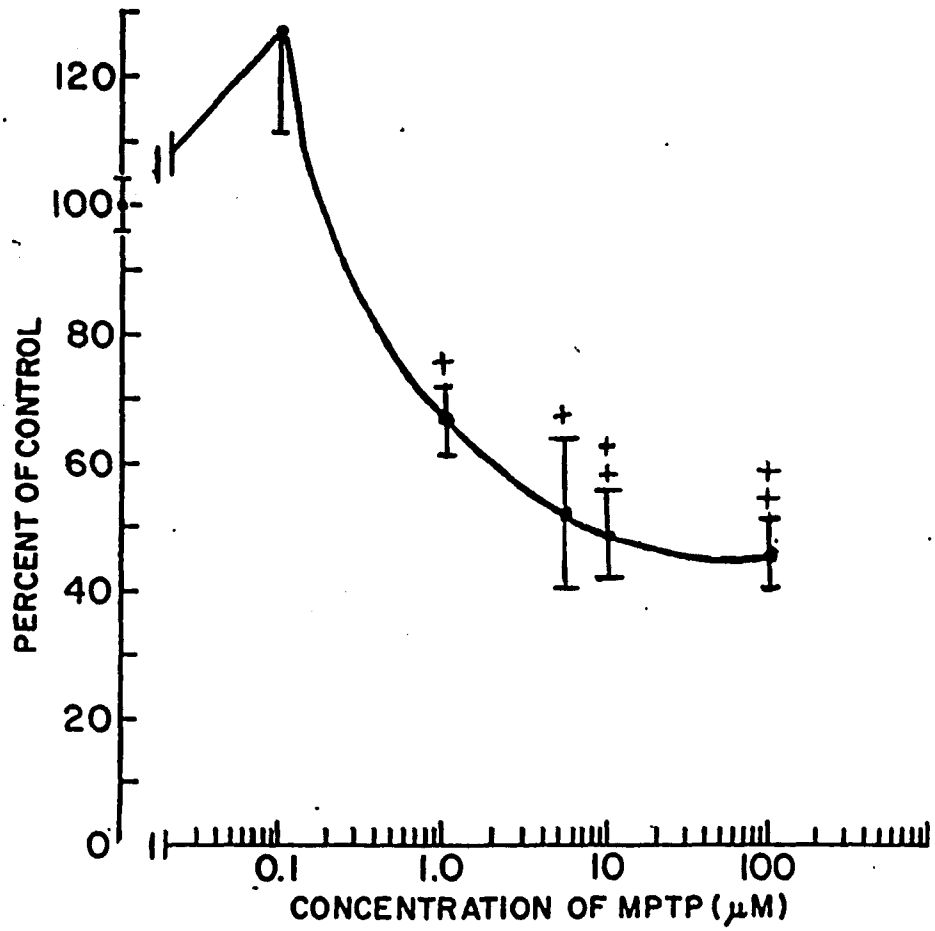


Figure 18. MPTP DOSE RESPONSE CURVE ON 3[H] NE UPTAKE IN PONTINE CULTURES

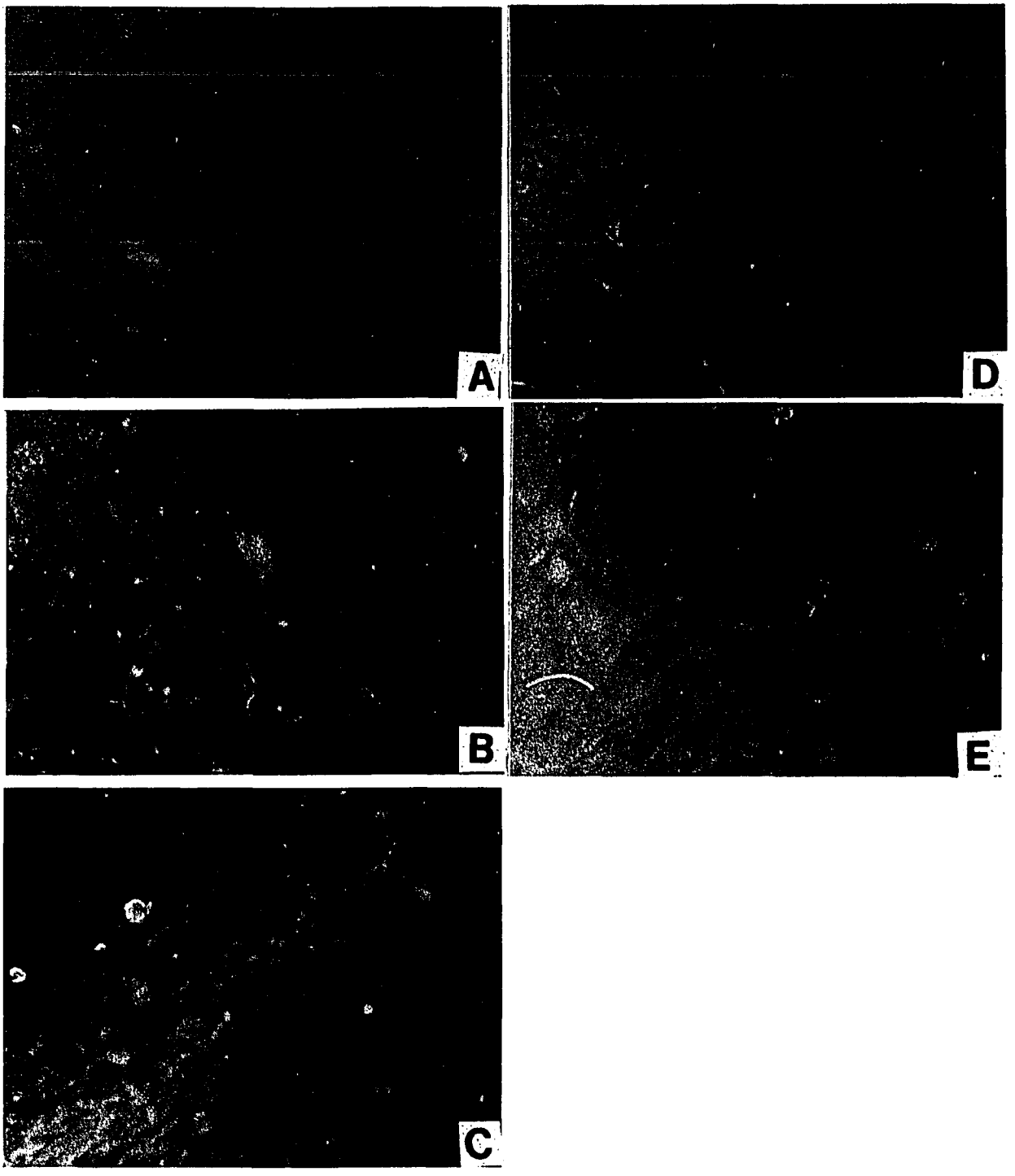
Uptake of 3[H] NE by rhombencephalic neurons after exposure to varied concentrations of MPTP for 7 days and 24 hours wash was measured. As with the midbrain treatment began on the 8th day *in vitro*. The values represent the average ± S.E.M. of 3[H] NE uptake of 15 cultures. They are expressed as % of uptake by the control (untreated) cultures. The value for the control cultures was 0.39±0.04 pmol 3[H] NE/ culture, + $P < 0.01$; ‡ $P < 0.001$; Student's t-test.

sensitive to the toxin. Interestingly, the amount of fluorescing fibers and cell bodies remaining after 100 uM MPTP were few compared to the midbrain cultures containing DA neurons (Fig. 19B) at the same MPTP dose. This may be due to a difference in the inhibitory effect of MPTP on the MPP+ uptake between DA and NE neurons. The amount of fluorescing NE fibers present was reduced to a similar degree in cultures treated with concentrations ranging between 5 and 10 uM MPTP (Fig. 19C, D). Many fluorescing fibers and cell bodies were present in 1 uM MPTP treated cultures and it was difficult to distinguish them from control cultures (Fig. 19E).

Pontine cultures treated with MPP+ at doses ranging from 5 to 10 uM had markedly decreased values of 3[H] NE uptake (i.e. approximately by 75%) (Fig. 20). In addition, there was a 58% reduction in 3[H] NE uptake at 1 uM MPP+. Concentrations above 10 uM induced generalized destruction to the cultures. CA histofluorescence after exposure to various concentrations of MPP+ is shown in figure 21A-21D. Notice very low levels of fluorescence with few remaining cells at both concentrations of MPP+ (5 and 10 uM) (Fig. 21B, C). 1 uM MPP+ did not produce any apparent decrement in fluorescing cell bodies or fluorescing processes. These data indicate that both MPTP and MPP+ are toxic to NE neurons grown in culture but some quantitative differences appear to exist between the

Figure 19A-19E. CA HISTOFLUORESCENCE IN PONTINE CULTURES TREATED WITH MPTP (1-100 uM)

Rhombencephalic cultures were treated with varied concentrations of MPTP for 1 week and rinsed for 24 hours. (A) Control cultures are abundant in fluorescing processes and large cell bodies. (B) The amount of fluorescing cell bodies and fibers after 100 uM MPTP treatment were greatly reduced when compared to the control cultures. The arrow points to one of only several surviving cells. Much of the remaining fluorescence is backround staining. (C, D) More fluorescing fibers appear in cultures treated between 10 and 5 uM MPTP respectively, however, fluorescence levels are lower than the controls. (E) Lower concentrations (1 uM) did not produce any apparent decrement in the amount of fluorescence. Magnification = 200 x for A-E.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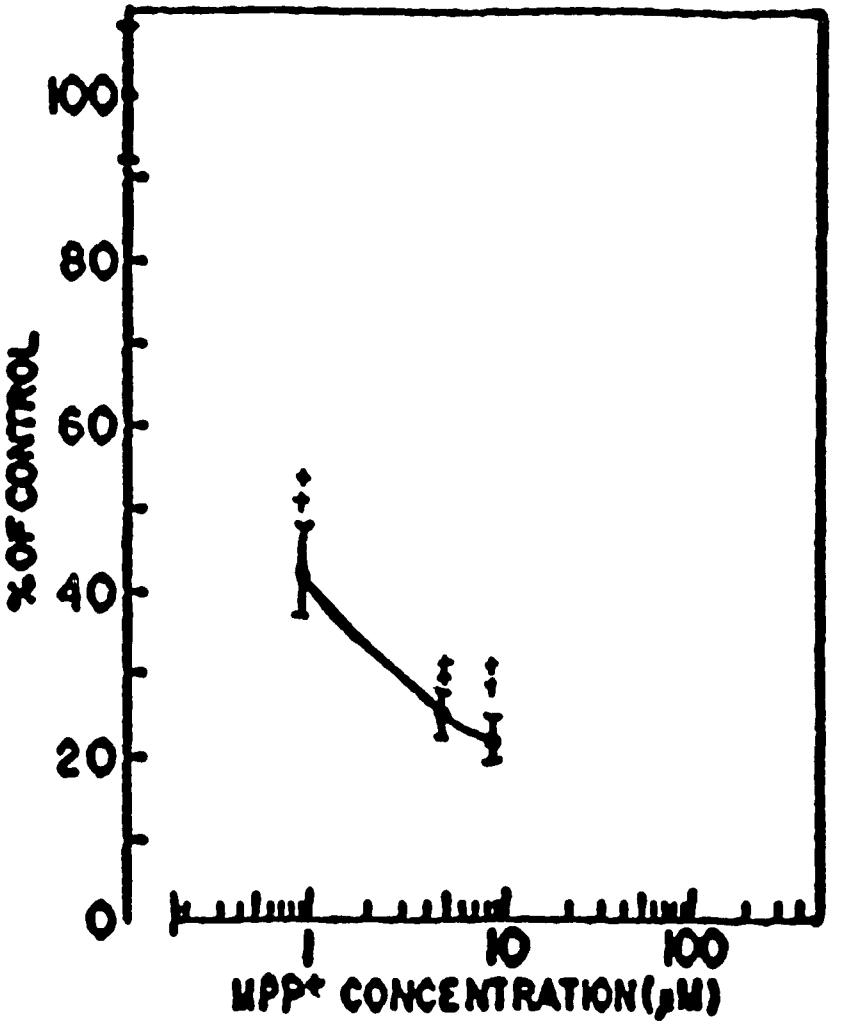
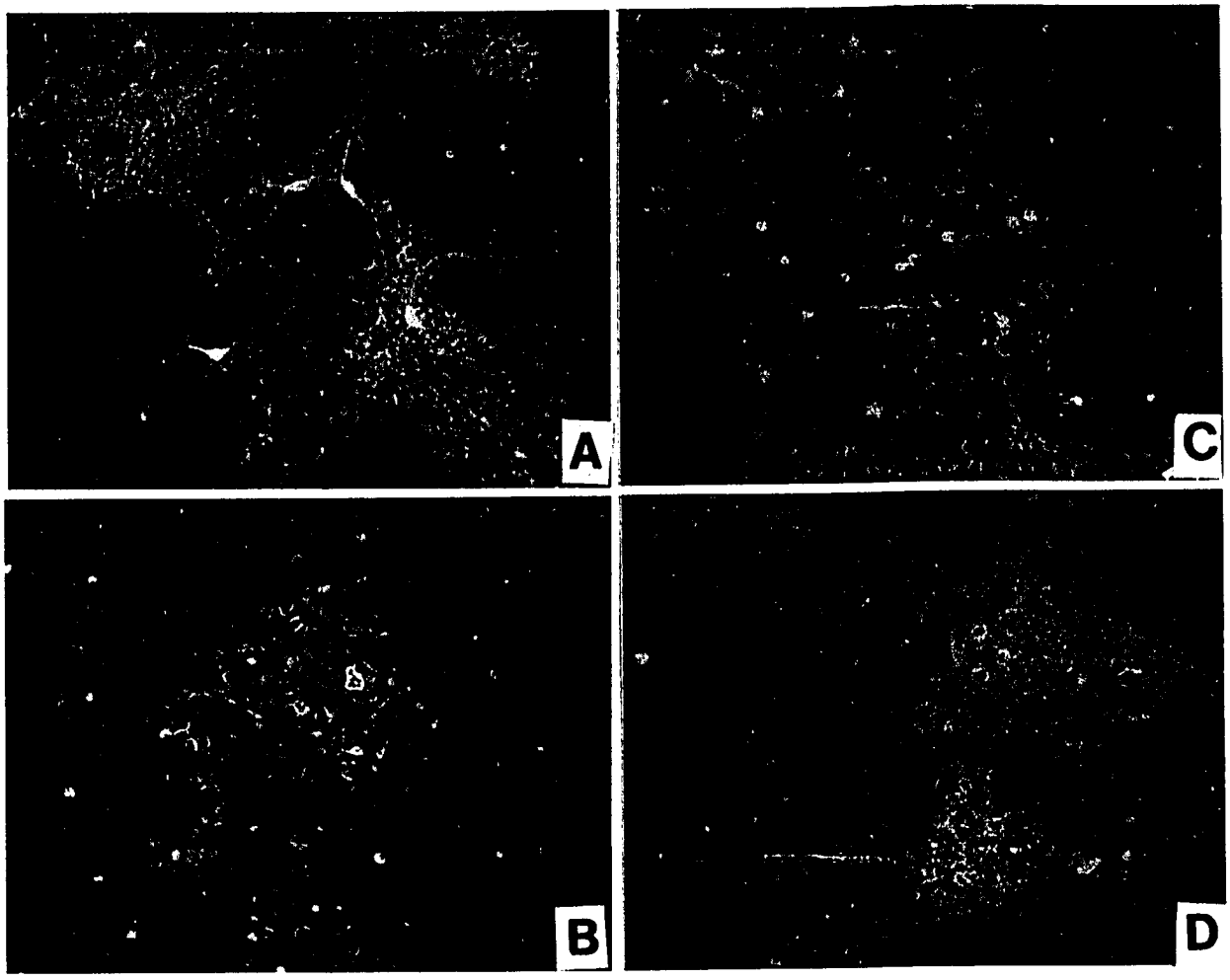


Figure 20. MPP+ DOSE RESPONSE CURVE ON 3[H] NE UPTAKE IN PONS

Rhombencephalic cultures were treated with varied concentrations of MPP+ for 1 week and washed for 24 hours. Uptake of 3[H] NE by noradrenergic neurons in the pontine cultures was dose dependently decreased with increasing concentraions of MPP+. The values are the average ± S.E.M of 3[H] NE uptake of 15 cultures. They are expressed as % of uptake by the controls. The value for the control cultures was 0.4±0.04 pmol 3[H] NE/ culture. Significantly different from control, + P< .01; ‡, P< 0.001; Student's t-test.

Figure 21A-21D. CA HISTOFLUORESCENCE IN PONTINE CULTURES TREATED WITH MPP+ (1-10 uM)

Rhombencephalic cultures were exposed to various concentrations of MPP+ for 1 week and washed for 24 hours. (A) Control cultures have a rich field of dendritic arbor and many cell bodies. (B) Cultures treated with 10 uM MPP+ appear dim and devoid of neurons. In (C) fluorescence levels increase but only a few cell bodies are visible after 5 uM MPP+ exposure (arrows). (D) 1 uM MPP+ treated cultures appear similar to the controls. Magnification = 80x.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response of NE and DA neurons to the toxins.

D. Effects of MPTP and MPP+ on 5HT neurons.

Since serotonin (5-HT) neurons appear to be resistant to MPTP and MPP+ toxicity in vivo, we decided to see if 5-HT neurons may be affected by either drug in the tissue culture model where they could be exposed directly to the toxins. Uptake of 3[H] 5HT following exposure of varied concentratons of MPTP/MPP+ for 7 days was measured in the presence of a specific NE uptake inhibitor, DMI. Following 7 days of MPTP exposure and a 24 hr. washout, the uptake of 3[H] 5HT was reduced significantly by 51% only at high MPTP (100 uM) concentrations (Fig. 22). Lower concentrations did not affect the 3[H] 5HT uptake. In order to determine whether the reduced 5HT accumulation reflected nerve terminal destruction or persistant inhibition of uptake by MPTP, we measured the uptake of 3[H] 5HT in cultures treated with 100 uM MPTP that were washed for 6 days with 2 changes of feeding medium. Uptake values were increased from 49 to 72% of the controls. However, the uptake was still significantly lower than control values.

In contrast, MPP+, the oxidation product of MPTP, decreased the uptake of 3[H] 5HT significantly at all concentrations used. 1 uM MPP+ reduced the uptake to 59%,

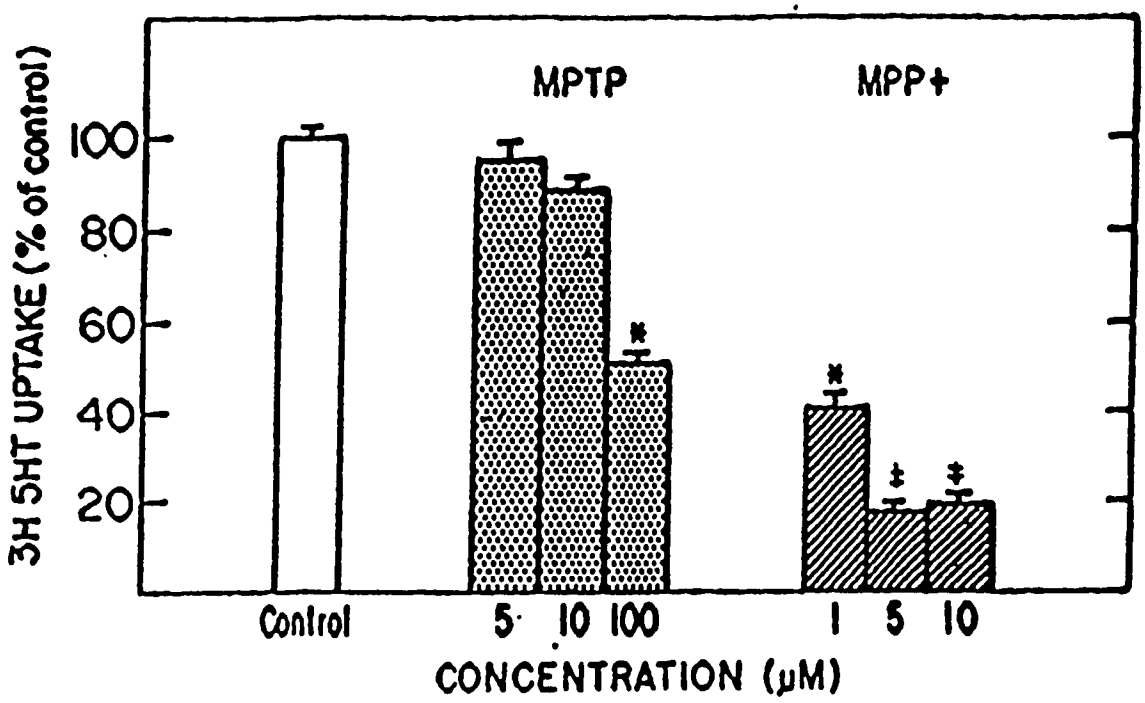

Figure 22. 3[H] 5HT UPTAKE FOLLOWING EXPOSURE OF MPTP OR MPP+ FOR 1 WEEK

Pontine cultures were treated with either MPTP or MPP+ for 7 days and rinsed for 24 hours. 3[H] 5HT uptake was reduced only at high concentrations (100 uM), while MPP+ reduced the uptake with increasing concentrations. Values are the average ± S.E.M. of 20 cultures and expressed in % of control. Control uptake was 6.0±0.2 pmol 3[H] 5HT/ culture, *, Significantly different from control, ++ P< 0.001; Student's t-test.

5 uM to 17%, and 10 uM to 19% of control, indicating that the toxic effect is maximal by 5 uM. Concentrations above 10 uM MPP+ induced generalized destruction to the cultures.

Anti-5HT antibodies were used to visualize the 5HT neurons in order to determine possible morphological damage or changes. (Fig. 23A-23D). Enough 5HT molecules were present endogenously in control cultures to produce a visible reaction product. In figure 23 (A, B) a control culture is compared with a culture treated with 100 uM MPTP for 7 days. Untreated cultures were lightly stained while 100 uM MPTP treated cultures revealed darkly stained neurons with apparently larger neuronal soma with extensive and visible processes. The number of 5HT positive neurons after 100 uM MPTP treatment was not different from control values. (Table 4). Cultures treated with 5 uM MPTP also displayed enhanced staining with more visible processes compared with the control cultures. However, the size of the somata and the extent of their processes did not appear to be as long and thickened as those cultures treated with 100 uM MPTP (Fig. 23C).

In figure 24B, cultures treated with MPP+ (10 uM) revealed few surviving 5HT positive neurons (13.6% of control) and most of these neurons were damaged. Cell bodies were enlarged, often enucleated, and the processes

Figure 23A-23D. ANTI-5HT IMMUNOCYTOCHEMISTRY IN PONTINE CULTURES TREATED WITH MPTP (5-100 uM).

Pontine cultures were treated with MPTP for 7 days and washed for 24 hours. 5HT neurons were analyzed with antibodies raised against 5HT conjugated to BSA. (A) Control cultures appear lightly stained and processes are difficult to visualize. (B) 100 uM MPTP treated cultures revealed darkly stained neurons with an apparently larger neuronal soma with extensive and visible processes (arrow). (C) Cultures treated with 5 uM MPTP also displayed enhanced staining with more visible processes compared to the control cultures, however the size of their somata and the extent of their processes did not appear as long as those cultures treated with 100 uM MPTP. In (D) cultures were treated with 5 uM MPTP in the presence of citalopram (3 uM). Notice the cell bodies appear darker and larger, however, the processes are not greatly visible as seen in the controls. Magnification = 168x.

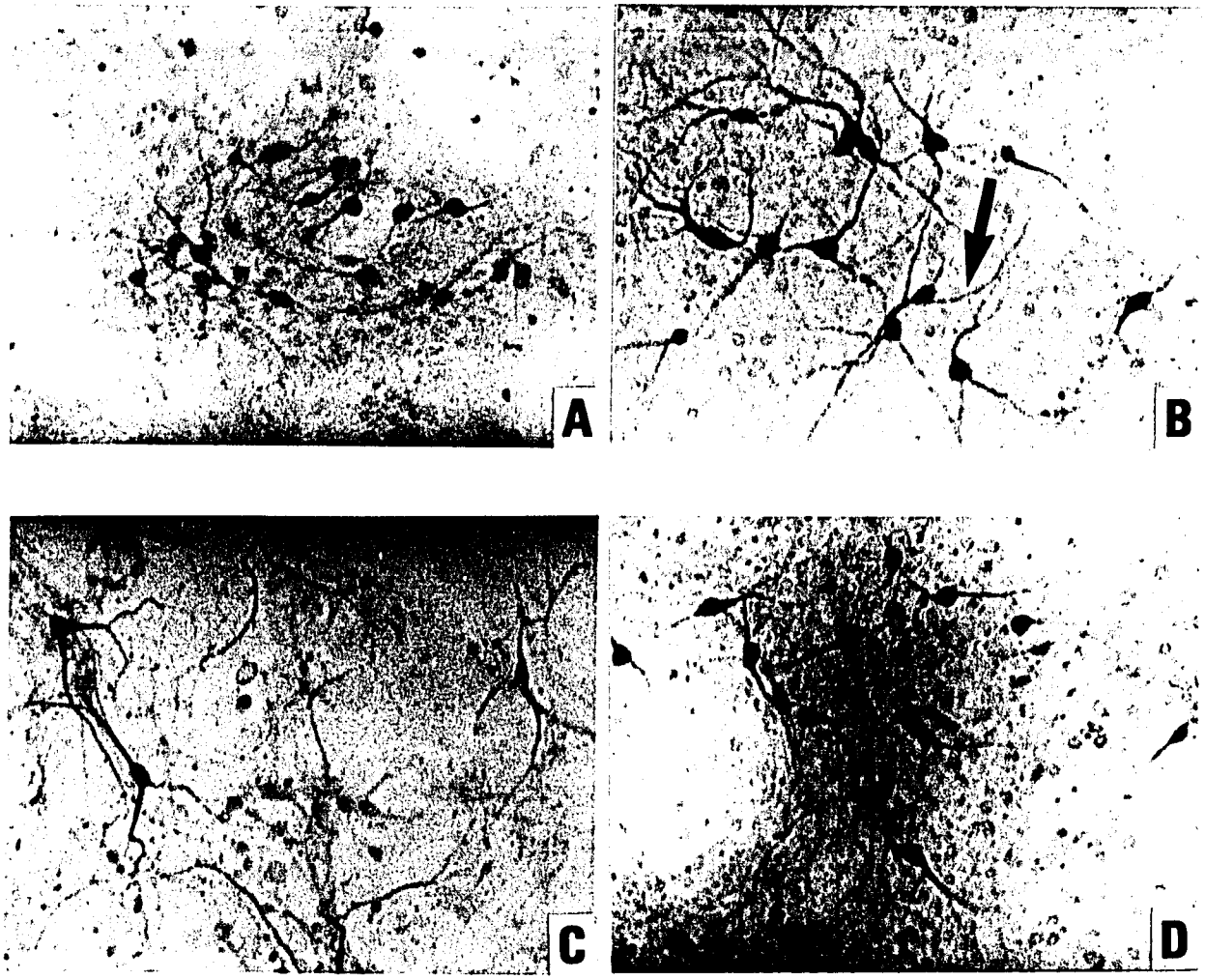
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Figure 24A-24C. ANTI-5HT IMMUNOCYTOCHEMISTRY IN PONTINE CULTURES TREATED WITH MPP+ (1-5 uM)

Pontine cultures were treated with MPP+ for 7 days and rinsed for 24 hours. (B) This photomicrograph shows one of the few surviving 5HT neurons in a culture treated with 10 uM MPP+. Most of these neurons were severely damaged when compared to the controls (A). (C) 1 uM MPP+ treated cultures contained fewer 5HT+ cells and these cells were darker than the controls. Furthermore, many of these cells were partially damaged (arrows). Magnification = 300x.

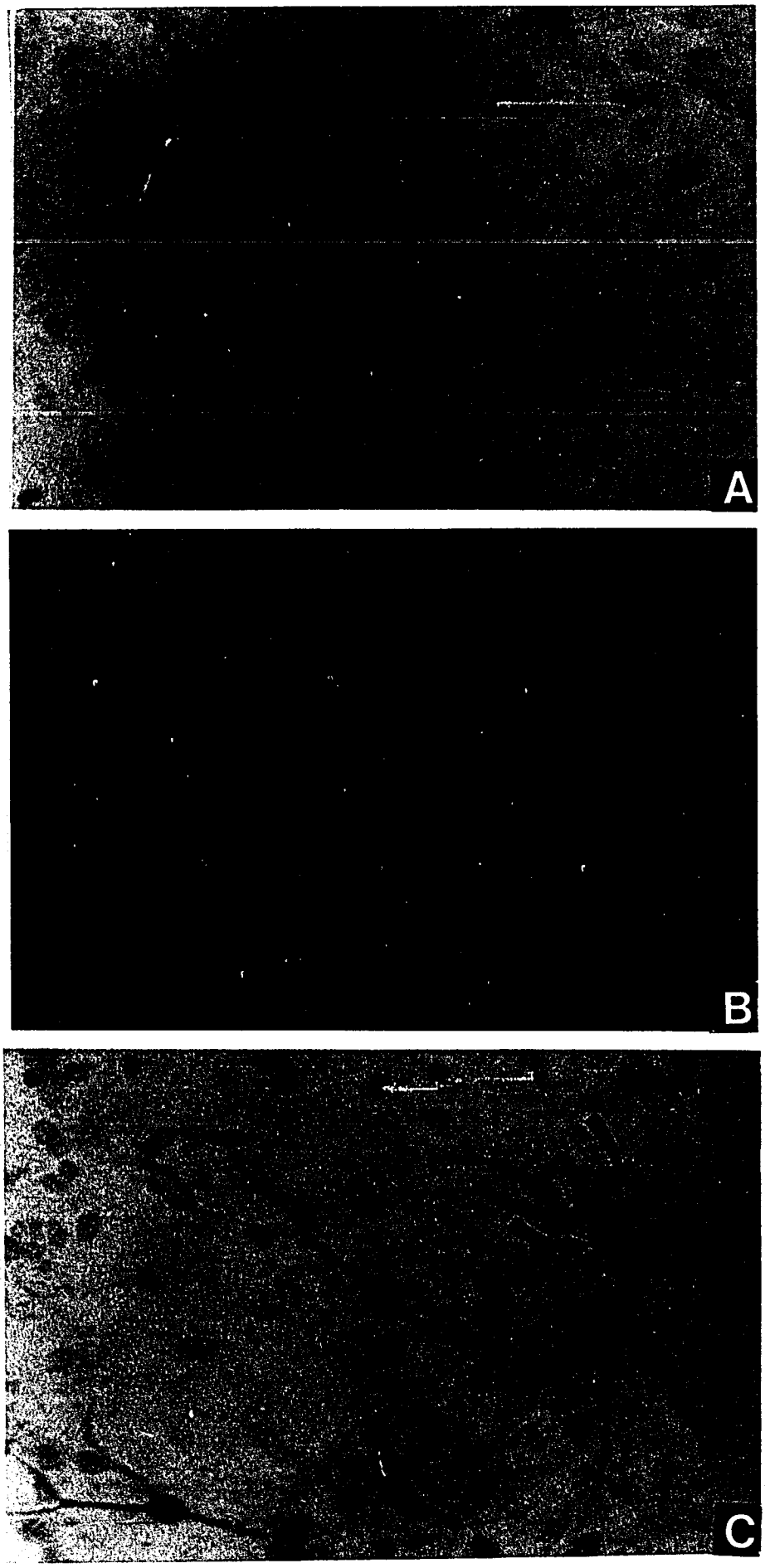
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TABLE 4

CELL COUNTS OF 5HT+ CULTURED NEURONS

	cell count	% control
Control	2320±336	
MPTP (100uM)	2333±379	100.6
MPP+ (10uM)	315±379	13.6+
MPP+ (1uM)	675±148	29.0+
MPP+ (1uM) + cital. (2uM)	1876±138	80.8

Table 4. Cell counts of 5HT+ neurons after MPTP/MPP+ treatment. 5HT+ neurons are wiped out by MPP+, but not by MPTP. Values represent means ± S.E.M. of 6 cultures. +, P< .001, Student's t-test.

appeared swollen. 1 uM MPP+ treated cultures also had a dramatic reduction in 5HT+ neurons (29% of control) with signs of neurodegeneration (Fig 24C). The 5HT+ neurons in these cultures were also darker than the controls, but, less than in cultures treated with MPTP. Also, the extent of nerve processes appeared more similar to the controls.

Cell counts for all treated and untreated cultures appear in table 4. Citalopram (1 uM) a highly selective 5HT uptake inhibitor was preincubated with cultures treated with 1 uM MPP+ and present during the entire drug treatment period. Citalopram protected these cultures from 5HT cell death and cell counts increased to close to control levels (80% of control).

To determine the reason for the increased immunostaining of 5HT neurons among all treated cultures, especially those treated with MPTP, we measured 5HT levels and its metabolite 5HIAA by HPLC (Table 5). Cultures treated with 5 and 100 uM MPTP demonstrated an increase in 5HT levels and a decrease in 5HIAA levels suggesting a reduction in 5HT metabolism. In cultures treated with 10 uM MPP+, both 5HT and 5HIAA levels were non-detectable, a result consistent with the low survival of 5HT neurons after this treatment. In table 6, pontine cultures were treated with MPP+ for 3 days. At both 5 and 1 uM MPP+, 5HT levels increased and 5HIAA levels decreased. The uptake inhibitor, citalopram, prevented this effect. In

TABLE 5

NEUROCHEMICAL CHANGES OF SEROTONIN METABOLISM
IN RHOMBENCEPHALIC CULTURES FOLLOWING MPTP EXPOSURE

5HT and its metabolite 5HIAA were measured by HPLC. Values shown represent 2 experiments and are expressed in ng/mg of protein. Cultures were treated for 1 week and washed for 24 hours.

	5HT	5HIAA	% control	
			5HT	5HIAA
Controls (n=2)	3.0	2.38		
MPTP (100uM) (n=2)	5.2	0.73	177.4	30.9
MPTP (5uM) (n=1)	5.4	1.06	181.2	44.9
MPP+ (10uM)	n.d.	n.d.		

n.d. = not detectable

TABLE 6

NEUROCHEMICAL CHANGES IN 5HT METABOLISM IN RHOMBENCEPHALIC CULTURES FOLLOWING MPP+ EXPOSURE FOR 3 DAYS

Cultures were treated for 3 days and the cultures were rinsed just prior to assay.

	5HT	5HIAA	% Control	
Controls (n=2)	7.31	6.37		
MPP+ (5uM) (n=1)	12.67	2.55	173.3	40.0
MPP+ (1uM) (n=2)	11.32	3.85	154.9	60.4
MPP+ (1uM) + citalopram (1uM) (n=2)	6.92	4.38	94.6	68.7

5HT levels rise and 5HIAA levels decline. The effect is removed by citalopram. Due to a small n, statistical analysis was not performed.

order to determine if the reduction in 5HT metabolism was related to MPTP-induced inhibition of MAO activity, we examined the enzymatic activity in the rhombencephalic cultures following acute and chronic treatment of MPTP. These data are represented in table 7. The effects on MAO activity resemble the results seen in mesencephalic cultures. MPTP at high doses (100 uM) inhibited both types of MAO activity with a greater inhibitory action on MAO-A when present only during the assay. Chronic exposure of 100 uM MPTP resulted in a more pronounced inhibitory effect on MAO-B activity. Lower doses (5 uM) produced no significant reduction in either MAO-A or MAO-B activities with prolonged exposure (1 week) unless an additional 5 uM MPTP was added during the assay. In this case only MAO-B activity was significantly reduced (to 65% of control). In addition, we analyzed MAO activity in response to MPP+ (Table 8). MPP+ inhibited only MAO-A activity during acute exposure at the higher dose (5 uM). Prolonged exposure did not reduce MAO activity, unless an additional 5 uM MPP+ was added during the assay. Although 5HT is a preferred substrate for MAO-A, MAO-B may also metabolize 5HT, therefore, a reduced 5HT turnover rate would be expected by inhibition of either form of the enzyme. This would explain the increased anti-5HT immunostaining seen in the cultures treated with MPTP or MPP+ since 5HT levels would rise producing more molecules

TABLE 7

MAO ACTIVITY IN RHOMBENCEPHALIC CULTURES DURING ACUTE AND CHRONIC EXPOSURE TO MPTP

5 experiments were combined and 5HT and benzylamine were used as substrates. Values represent means ± S.E.M. MAO activity was measured in nmoles/mg protein/hour. *, P< 0.05, +, P< 0,001, Student's t-test.

	MAO-A	MAO-B	% control MAO-A	% control MAO-B
Control (no drug)	41.9+7.1	4.3+0.7	100	100
control + MPTP (100 uM)	8.4+1.1	2.3+0.5	20+	53+
Control + MPTP (5 uM)	34.2+5.6	3.7+1.0	82	86
MPTP (100 uM) 1 week	29.8+4.1	1.5+0.2	71*	35+
MPTP (100 uM) + 100 uM 1 week	8.2+1.3	0.5+0.1	20+	12+
MPTP (5 uM) 1 week	47.2+6.5	3.0+0.5	113	70
MPTP (5 uM) + 5 uM 1 week	38.4+5.3	2.8+0.4	92	65*

TABLE 8

MAO ACTIVITY IN RHOMBENCEPHALIC CULTURES DURING ACUTE AND CHRONIC EXPOSURE TO MPP+

4 experiments were combined and 5HT and benzylamine were used as substrates. MAO activity was measured in nmoles/mg protein/hour.

	MAO-A	MAO-B	% control MAO-A	% control MAO-B
Control (no drug)	43.1+8.8	4.5+0.9	100	100
control + MPP+ (5 uM)	21.3+4.0	3.7+1.1	49*	82
control + MPP+ (1 uM)	35.4+6.8	4.1+1.2	82	91
MPP+ (5 uM) 1 week	51.6+6.7	4.1+1.2	120	91
MPP+ (5 uM) + 5 uM 1 week	26.7+2.7	3.8+1.1	64	84
MPP+ (1 uM) 1 week	46.0+6.4	4.0+0.7	107	89
MPP+ (1 uM) + 1 uM	35.9+4.8	2.9+0.9	83	64

Table 8. MAO activity in rhombencephalic cultures after MPP+ exposure for 7 days. Inhibition of MAO-A activity occurs only in the presence of 5 uM MPP+. The values are the means $\pm$ S.E.M. of 4 experiments done in duplicates. *, $P < 0.05$, significantly diferent from controls, Student's t-test.

to react with the 5HT antibodies.

E. Effects of guanethidine in the CNS.

1. The effects of guanethidine on CA uptake and release mechanisms in a synaptosomal preparation.

a. Uptake in synaptosomes

Before treating the cultures with guanethidine, it was necessary to know if DA neurons could accumulate guanethidine. Since radiolabelled guanethidine was not available we measured the competition of guanethidine for the uptake sites of DA and NE neurons. Therefore, we measured the uptake of 3[H] DA or 3[H] NE in synaptosomal preparations derived from adult rat striatum or occipital cortex respectively, in the presence of varied concentrations of guanethidine. The effects of guanethidine on the uptake of 3[H] catecholamines in the occipital cortex and striatum, two brain regions rich in NE and DA terminals respectively (Fuxe, 1965; Moore et al., 1971) is demonstrated in Fig. 25. The uptake of 3[H] NE in the occipital cortex was inhibited by guanethidine at concentrations ranging from 1 to 100 uM. The amount of inhibition observed increased with increasing concentrations of guanethidine. The IC50 of guanethidine for NE

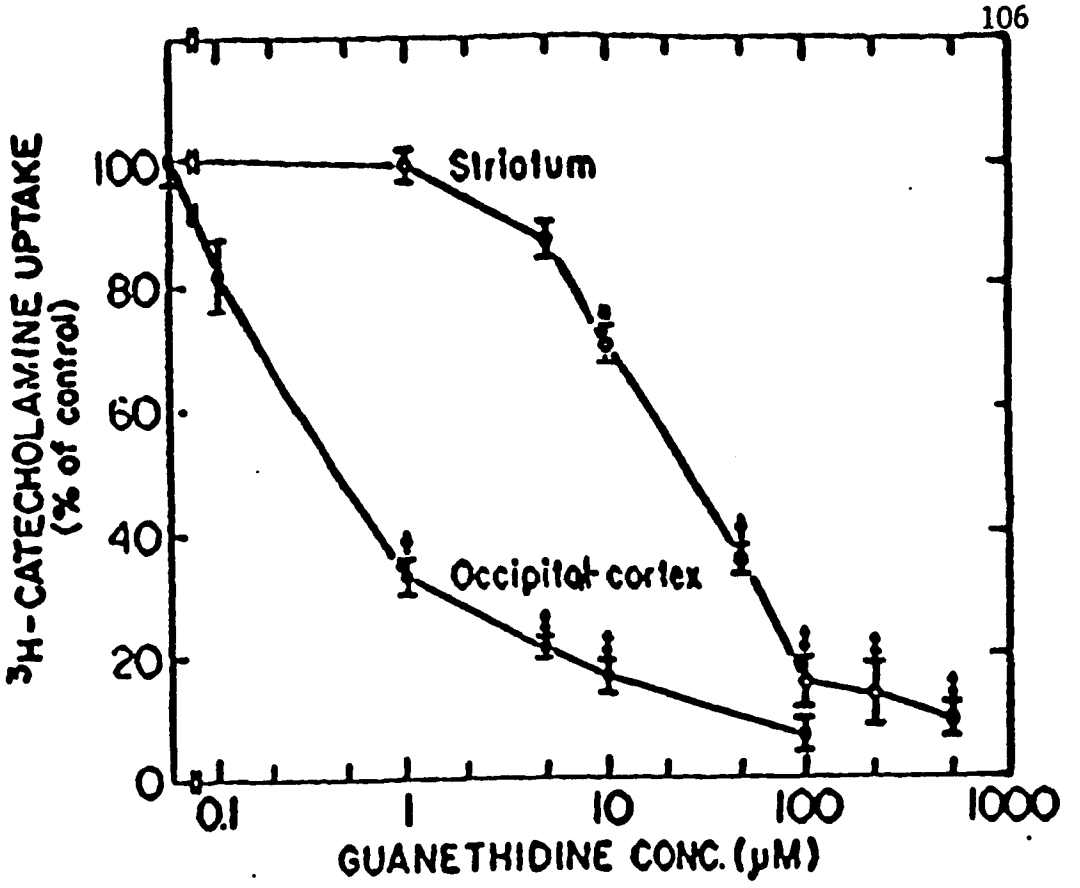

Figure 25. EFFECT OF GUANETHIDINE ON THE UPTAKE OF 3[H] CATECHOLAMINES INTO SYNAPTOSOMES OF OCCIPITAL CORTEX AND STRIATUM

Guanethidine was present in the incubation medium at concentrations ranging from 0.1 to 500 uM. Uptake values are expressed as percentage of control (no drug) uptake. Occipital cortex (closed circles) IC50 0.5 uM and striatum (open circles) IC50 33.6 uM. IC50 values are the concentrations of guanethidine where 1/2 of the uptake is inhibited. Values are the mean ± S.E.M. of 3 experiments and all sample were done in duplicate. Student's t-test was used for statistical comparisons. + $P < 0.01$; + $P < 0.001$.

uptake was 0.5 uM. IC50 values represent the concentrations required to inhibit 1/2 of the uptake for either CA. The uptake value of 3[H] NE by the occipital cortex in the absensce of guanethidine was 0.34 fmol/mg prot/min). In the striatum, the uptake of ^{3}[H] DA showed little change at guanethidine concentrations up to 10 uM. However, higher concentrations (50-500 uM) resulted in significant inhibition on 3[H] DA utpake (IC50=33.6uM). The inhibition of uptake by guanethidine for NE neurons was 67 times more potent than that of DA neurons. These data indicate that guanethidine at low concentrations is a selective inhibitor of the CA uptake mechanisms for NE neurons. The high concentrations (above 50 uM) required to inhibit DA uptake could be the result of a non-specific effect of guanethidine on these neurons.

b. Release

The effect of guanethidine on the spontaneous and K+ stimulated release of catecholamines were measured in slices from occipital cortex and striatum. A differential effect was again seen between the two brain regions (Figs. 26 and 27). Figure 26 shows the spontaneous and K^{+} evoked release of the ^{3}[H] amines in occipital cortex and striatum in the presence or absence

of 100 uM guanethidine. Tissue slices preincubated with 3[H] NE or 3[H] DA for 15 min, underwent a 15 min wash plus an additional 5 min wash period in fresh buffer to allow for an equilibration period. Tissues were transfered from 1 well (plate position) to the next, containing fresh aliquots (1 ml) of buffer, at 5 min intervals. The amount of released radioactivity was measured every 5 min from each plate position containing 1 ml of buffer. The spontaneous release was calculated from the values of ^{3}[H] amines released in the buffer during each 5 min interval. The K+ induced release was calculated from the 5th well containing 50 mM K^{+} (plate position number 5 in fig. 26). in a similar manner. Figure 27 compares the effect of all guanethidine concentrations used (1, 10 and 100 uM) on the spotaneous and K^{+} induced release in occipital cortex
and striatal slices. In occipital-cortical slices preincubated with 3[H] NE (0.1 uM) for 15 min., guanethidine increased significantly the spontaneous efflux of 3[H] NE by 27% at 1 uM, 88% at 10uM guanethidine and 105% at 100 uM (Fig. 27). On the other hand, the spontaneous release of 3[H] DA from striatal slices was not significantly affected by any concentration of guanethidine (1, 10, or 100 uM). The effect of guanethidine on K+ stimulated release was similar for
both neuronal populations (Figs. 26 and 27). High

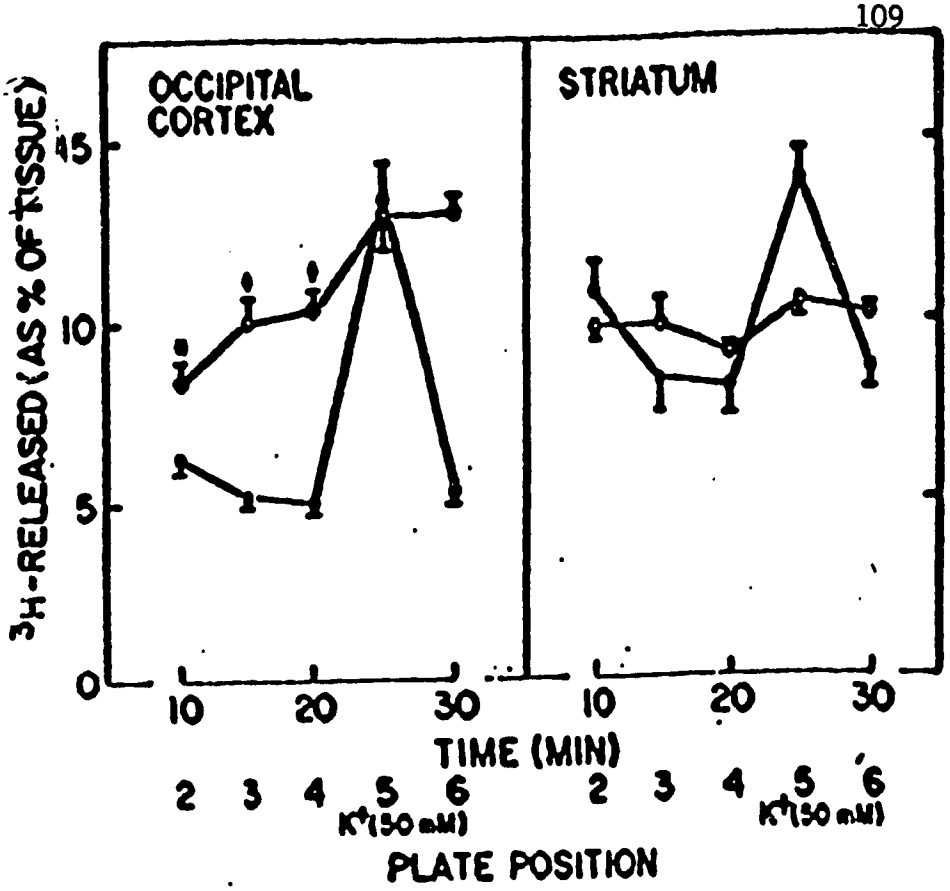

Figure 26. EFFECT OF GUANETHIDINE ON SPONTANEOUS AND K+ STIMULATED RELEASE OF CAs FROM OCCIPITAL CORTEX AND STRIATUM

The effect of guanethidine (100 uM) on the spontaneous and K+ stimulated release of 3[H] catecholamines from brain slices. The occipital cortex was labelled with 3[H] NE and the striatum with 3[H] DA. Following the 3[H] catecholamine incubation and wash period, the tissue slices were transferred to fresh aliquots of buffer at 5 min intervals and the buffer with the released tritium was collected and counted. In the drug treated samples, guanethidine was present during all of the time intervals. Data from the first time interval are not presented because it was considered as an equilibration period. The tritium collected from the medium for each sample was expressed as a percentage of the total tritium of the tissue at the beginning of each time interval (total radioactivity = radioactivity removed from the tissue + the total radioactivity released into the medium). Data points are the means ± S.E.M. of quadruplicate samples from 2 experiments. Student's t-test * $P < 0.05$, + $P < 0.01$, ‡ $P < 0.001$, Student's t-test.

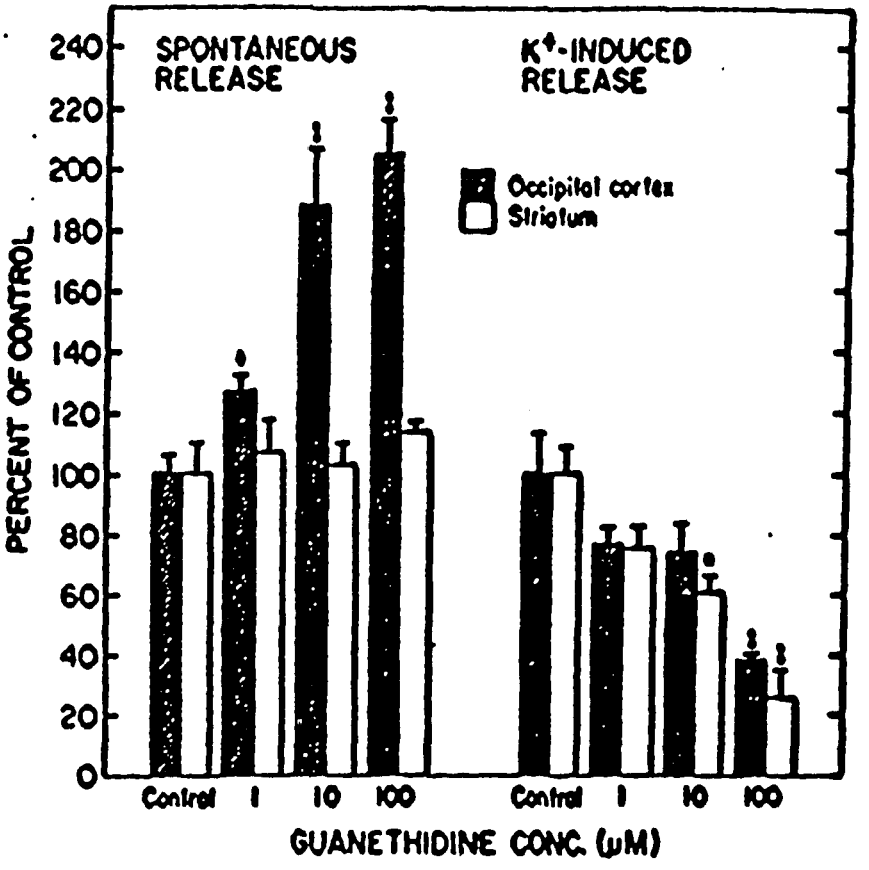

Figure 27. EFFECT OF GUANETHIDINE ON SPONTANEOUS AND K+ STIMULATED RELEASE OF CAs FROM OCCIPITAL CORTEX AND STRIATUM

See figure 26 for explanation. Release values are expressed as percentage of the release of control (untreated) slices. * P< 0.05, + P< 0.01, + P< 0.001, Student's t-test.

concentrations (100uM) produced a marked inhibition of the K+ induced release 61.2% in occipital cortex and 73.7% in striatum) while lower concentrations (1 and 10uM) had a much smaller effect (Fig. 27).

2. Toxic effects of guanethidine to central monoamine neurons grown in culture.

a. Guanethidine and CA neurons.

1. Acute inhibition of uptake by guanethidine.

Since our <u>in vitro</u> studies indicated that guanethidine was a selective inhibitor of the CA mechanisms for NE neurons and can accumulate within DA neurons only at high concentrations, (100 uM), we were able now to compare the toxic effects this drug may have on central monoamine neurons grown in culture. Previously guanethidine has been shown to have a toxic effect only to peripheral catecholamine neurons. Manning et al (1985) have suggested that the guanethidine-induced cell death may be due to an autoimmune response where lymphocytic-like small cells and macrophages infiltrate the sympathetic ganglia and destroy the neurons. We were interested to see if the CA neurons of the CNS could be affected in the absense of an immune system.

When using the uptake system as a measure of neurotoxicty, it is important to be sure the drug itself

under investigation does not have an inhibitory effect on the uptake sites. We found when guanethidine was introduced into the feeding medium of the cultures for a short exposure period that it was an extremely potent inhibitor of tritiated amine uptake prior to any damage of the CA neurons. For example, if guanethidine was present in the midbrain cultures for only 24 hours at high concentrations (100 uM), the uptake of 3 [H] DA was reduced by 64%. Following a 24 hour washout, the uptake mechanism continued to be inhibited by the same degree (66.6%). Since a 24 hr drug exposure plus a 24 drug free period revealed a strong, lingering inhibition of the CA uptake mechanism we incubated the cultures for an additional 24 hrs in the absense of guanethidine. An additional 24 hour rinse, removed the inhibitory effect completely (Fig. 28). Therefore, uptake analysis was conducted after guanethidine was removed for 48 hours.

2. Toxicity after 2 week exposure to guanethidine.

Analysis of toxicity to the DA and NE neurons was done by uptake measurement, histochemical, and immunocytochemical techniques. Mesencephalic and rhombencephalic cultures were treated with guanethidine beginning on the 8th DIV for 2 weeks with 4 changes of feeding medium. Guanethidine was removed 48 hours prior

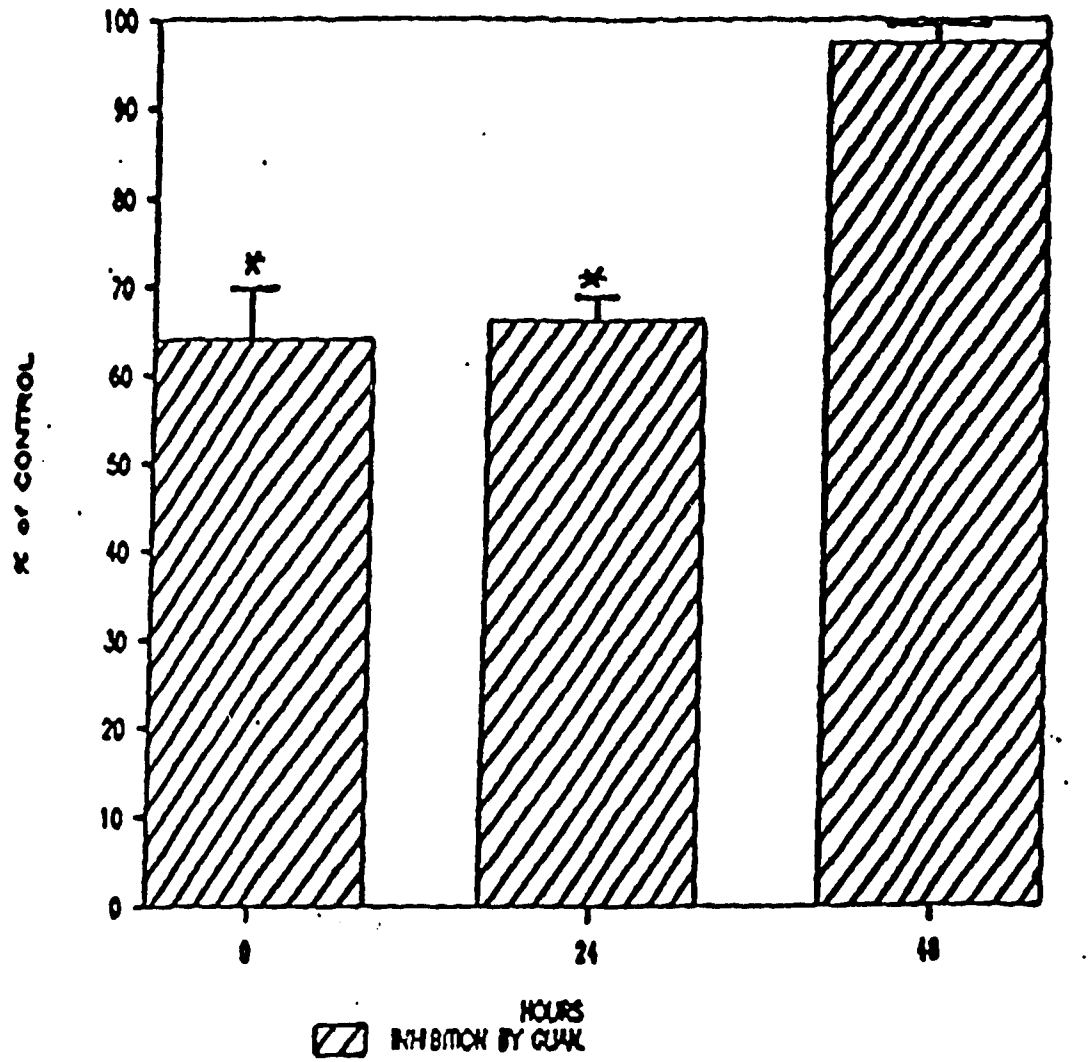

Figure 28. PROLONGED INHIBITION BY GUANETHIDINE

Guanethidine (100 uM) was introduced into the feeding medium of midbrain cultures for 24 hours. Uptake of 3[H] DA was measured immediately after removal of the drug, 24 hrs later, and after 48 hrs. Notice 48 hrs removal of guanethidine is required to eliminate its prolonged inhibitory effect. Uptake values are taken as % of controls (n=8). Control uptake was 0.19±0.04 pmol 3[H] DA/ culture. *, Significantly different from controls, $P < 0.05$, Student's t-test.

to uptake analysis. 3 [H] DA and 3 [H] NE were used in the corresponding cultures.

The catecholamine uptake capacity was measured as an index of cell damage or death once the inhibitory effects of guanethidine were eliminated. The amount of tritiated amine that could enter these neurons was markedly reduced at increasing concentrations ranging from 1-100 uM of guanethidine (Fig. 29). At 10 uM guanethidine uptake values were significantly reduced by 67.2% for midbrain and by 84% for pontine preparations of DA and NE neurons. The effect was not increased further at higher concentrations (100 uM) for both DA and NE neurons. Lower concentrations (1 uM) slightly reduced 3[H] DA uptake from control values, however, the reduction was not significant. In contrast, 3[H] NE uptake was significantly inhibited with 1 uM guanethidine by 52.6%. In addition, guanethidine treated cultures did not undergo generalized toxicity at any of the concentrations used and growth of supporting glial cells was normal.

Fluorescence and immunocytochemical techniques employed in midbrain cultures revealed shrunken or swollen somata, axonal swellings, reduced dendritic arborization, and truncated neurons where only the perykarya remained visible (Figs. 30A-30D and 31A-31C). Decreased cell counts (Table 9) indicated DA cell death by guanethidine. Since DbH only labeled a small number of NE neurons,

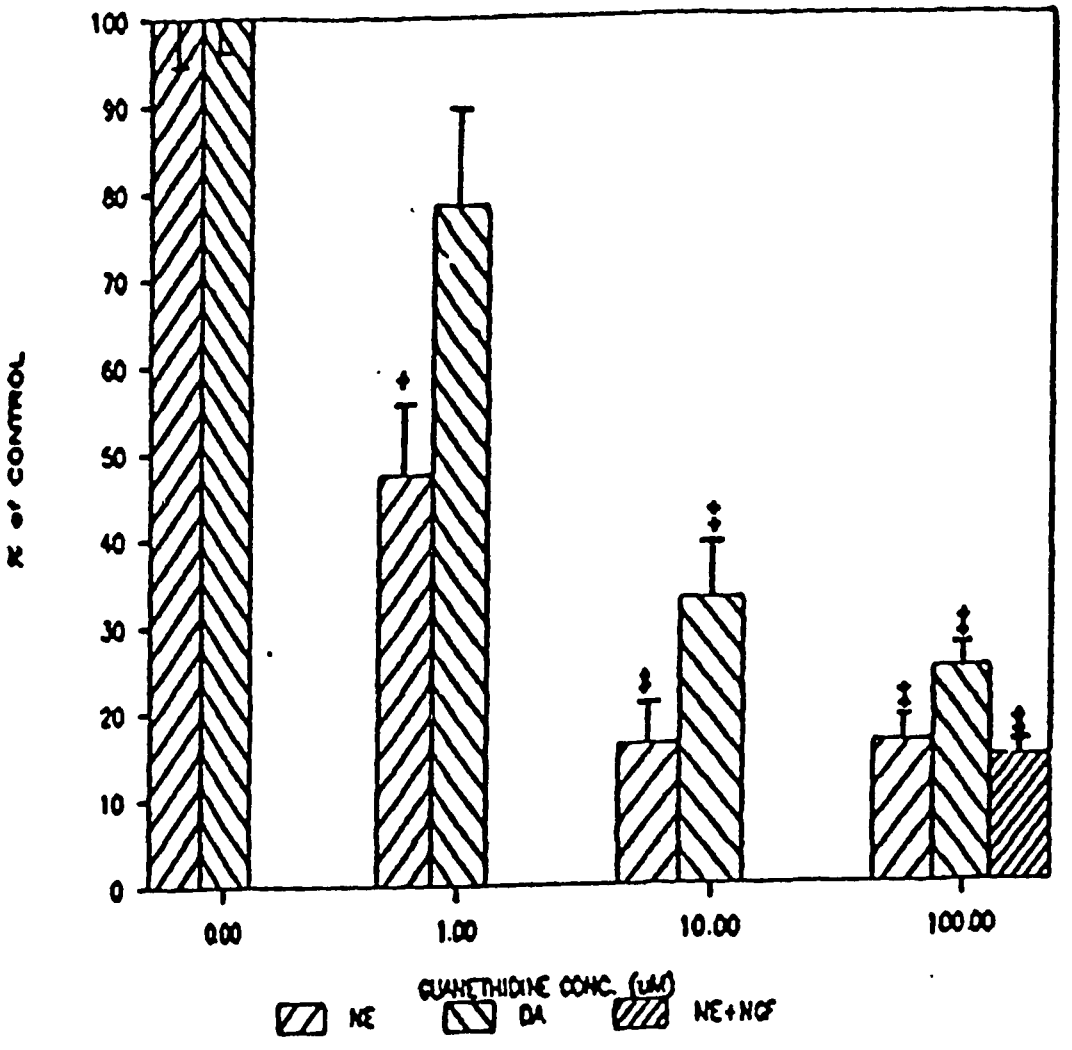

Figure 29. 3[H] CATECHOLAMINE UPTAKE AFTER 2 WK GUANETHIDINE EXPOSURE AND 48 HR WASH

Midbrain and pontine cultures were treated with guanethidine at varied concnetrations (1-100 uM) for 2 weeks and washed for 48 hours. Uptake values are expressed as % of control. Both the uptake of 3[H] DA and 3[H] NE were greatly reduced following prolonged exposure to guanethidine. Control uptake was 1.34± 0.01 pmol 3[H] DA/ culture or 0.51±0.02 pmol 3[H] NE/ culture (n=12) Significantly different from controls, +, $P< 0.01$, ++, $P< 0.001$.

Figure 30A-30D. TOXIC EFFECTS OF GUANETHIDINE TO MIDBRAIN CULTURES AFTER 2 WEEKS EXPOSURE

TH immunocytochemistry was employed to analyze toxicity induced by guanethidine to DA neurons after 2 weeks exposure and 48 hour washout. (A) Control cultures represent typical TH+ neurons after 3 weeks in vitro. (B) Cultures that were treated with 100 uM guanethidine contained TH+ DA neurons with shrunken somata, truncated axons, and very few or no processes. (C) 10 uM guanethidine produced shrunken or swollen somata, axonal swellings, reduced staining, and reduced dendritic arborization to the DA neurons. (D) 1 uM treatment reduced the number of TH+ neurons present in the cultures and some cells were more lightly stained and asimilar to the controls indicating an effect at this low dose and chronic exposure. Magnification = 168x.

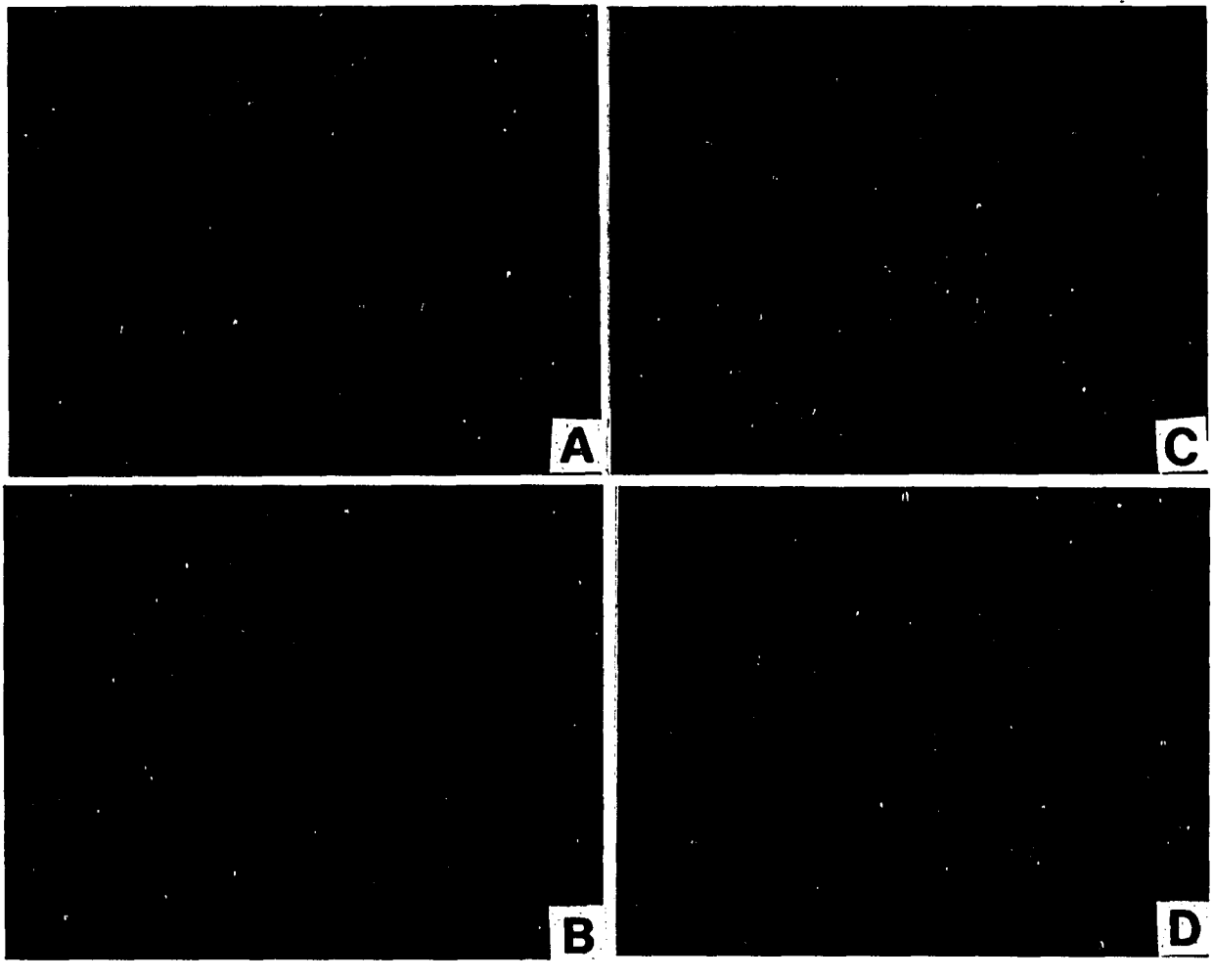
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Figure 31A-31C. CA HISTOFLUORESCENCE IN MIDBRAIN CULTURES AFTER 2 WEEK GUANETHIDINE EXPOSURE

CA histofluoresence was used to analyze the toxic effects induced by guanethidine after 2 weeks and a 48 hour washout. (B) Cell bodies are deficient and smaller at 100 uM guanethidine with no visible processes as seen with the TH+ neurons. (C) 10 uM guanethidine reduced the number of fluorescing cell bodies and the amount of fluorescing fiber markedly when compared to the controls (A). Magnification = 80x.

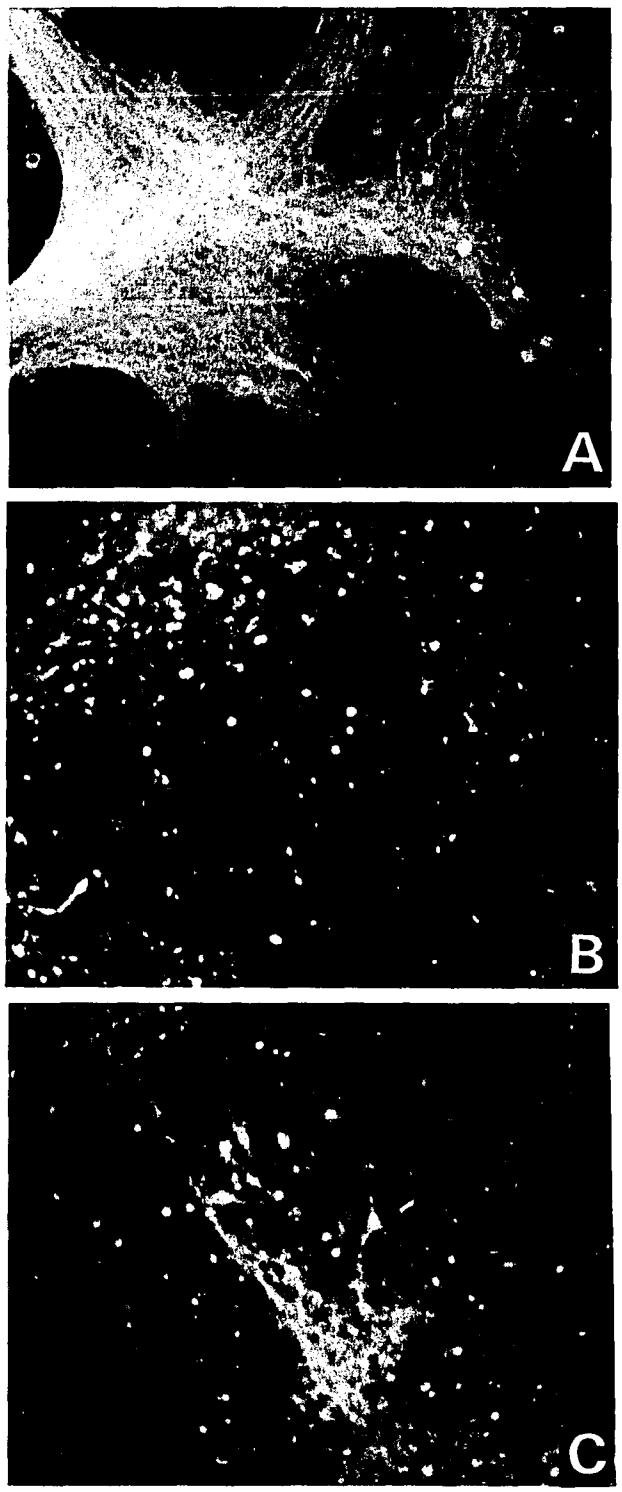
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TABLE 9

CELL COUNTS OF TH+ NEURONS IN MIDBRAIN CULTURES FOLLOWING 2 WEEKS EXPOSURE TO GUANETHIDINE

	cell count	% control
Control	547.8±4	100
Guanethidine (100 uM)	110.5±3.5	20+
Guanethidine (10 uM)	305±19	55*
Guanethidine (1 uM)	389.6±46	71.2

Table 9. TH immunocytochemistry was employed to visualize CA containing neurons belonging to the ventral midbrain following prolonged exposure (2 weeks) to guanethidine. The number of TH+ cells decreased with increasing concentrations of guanethidine. Values represent the means ± S.E.M. of 4 cultures. *, $P< 0.05$, +, $P< 0.01$, Student's t-test.

morphological analysis of NE neurons was examined by CA histofluorescence (Fig. 34A-34D). Little to no fluorescence was visible in 100 uM treated cultures and at 10 uM guanethidine, an extremely sparse amount of NE cells could be seen. The percentage of surviving neurons in the mesencephalic cultures that were possitive for TH exceeded that of the rhombencephalon at all concentrations studied suggesting that the toxic effect to NE neurons was more selective. Since nerve growth factor (NGF) protects against guanethidine-induced toxicity among sympathetic neurons, we have treated midbrain and pontine cultures with guanethidine for 2 weeks in the presence of NGF (1ng/ ml). No protective effect was seen in the CNS (Fig. 29) whereby 3 [H] CA uptake was reduced by over 80%. These data indicated that guanethidine can be toxic to central CA neurons with a more selective effect on NE neurons in the absence of an immune system.

Since short exposure of guanethidine (24hrs) resulted in such a prolonged inhibition on the CA uptake system, lasting for more than 24 hrs after withdrawal of the toxin, we thought that an increased exposure time (2 wks) may lead to an even longer inhibitory effect on the uptake. Therefore, we treated both sets of cultures with guanethidine for 2 weeks followed by one week of drug free medium (Figs. 32 and 33) (3 [H] DA and 3[H] NE uptake

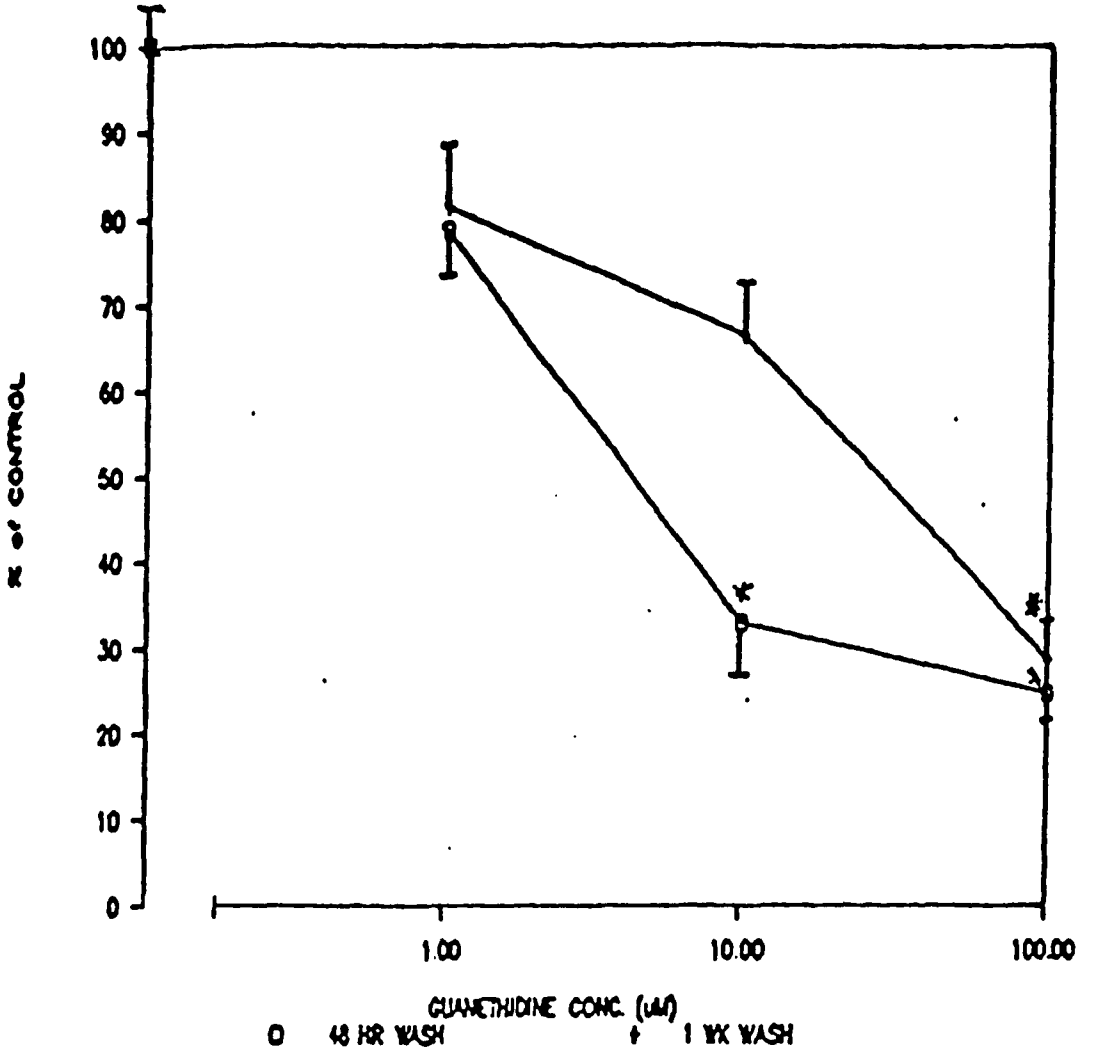


Figure 32. 3[H] DA UPTAKE: GUANETHIDINE WASHOUT FOR 2 OR 7 DAYS

Mesencephalic cultures were treated with guanethidine for 2 weeks and the drug was removed for 2 or 7 days. The inhibitory effect produced by guanethidine is similar at high (100 uM) and low (1 uM) concentrations regardless of the extent of washing. The inhibitory effect of guanethidine at 10 uM was attenuated after removing the drug for 1 week. Uptake values are taken as % of controls and are the means ± S.E.M. of 12-18 cultures. *, significantly different from the controls, $P < 0.01$, Student's t-test.

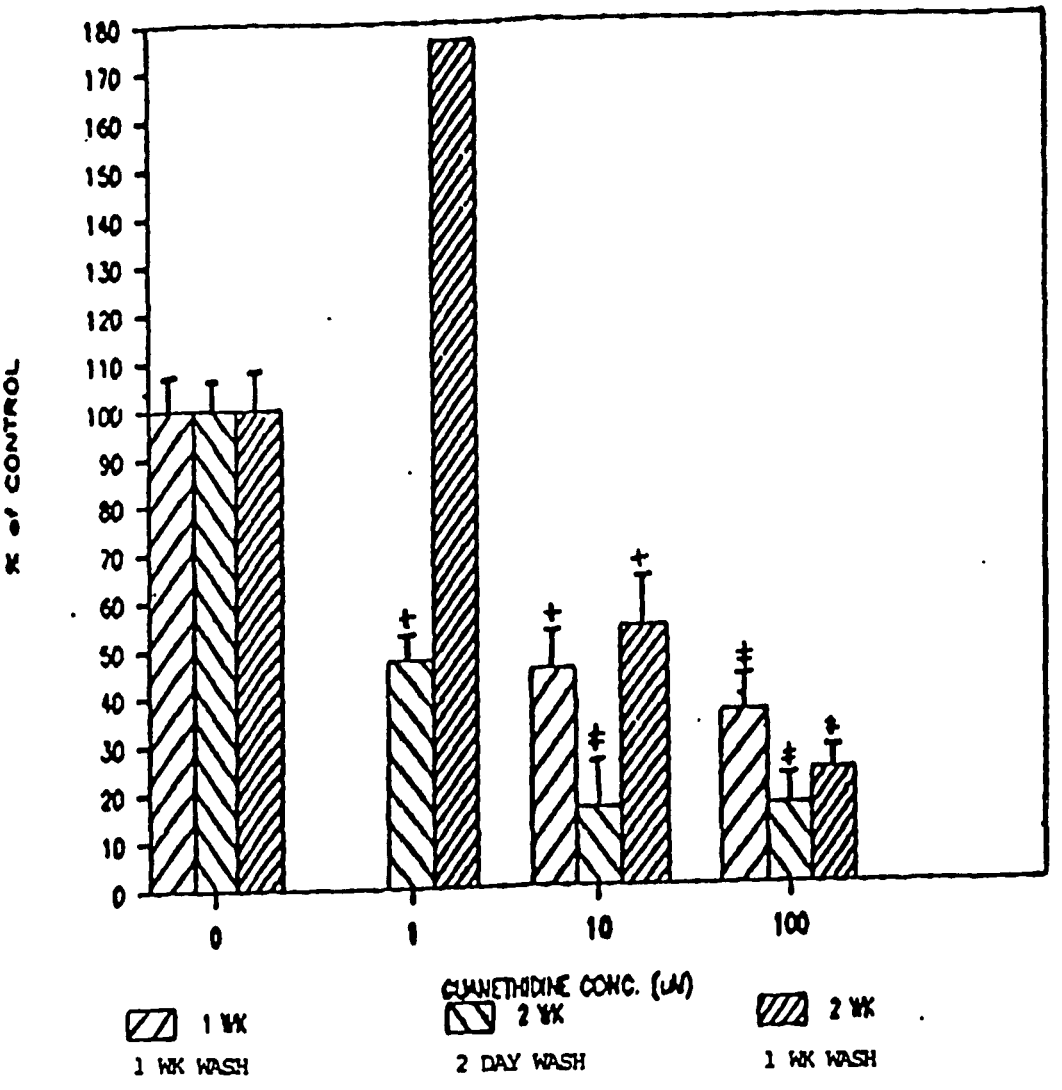


Figure 33. 3[H] NE UPTAKE: GUANETHIDINE EXPOSURE OVER TIME

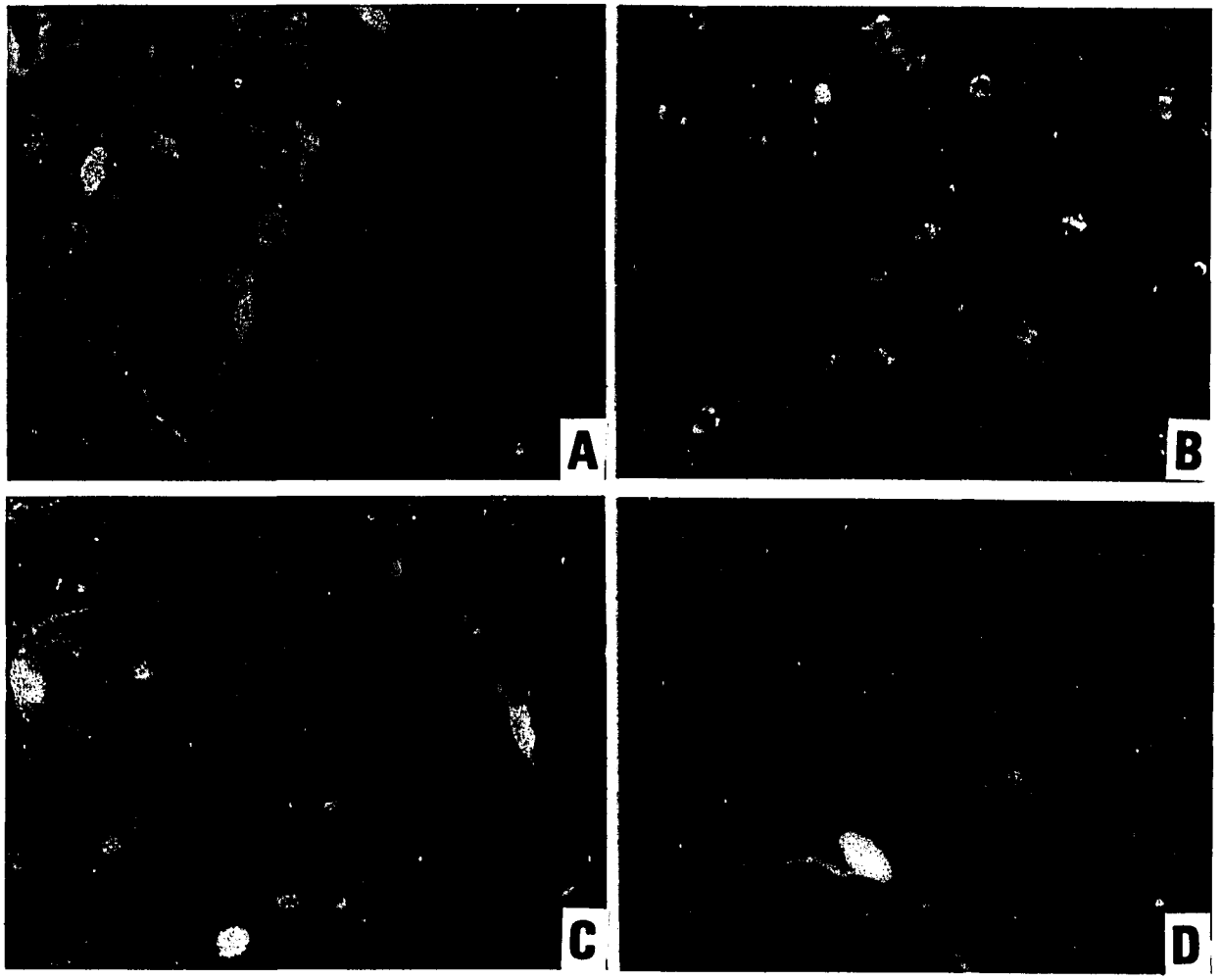
The uptake of 3[H] NE after guanethidine treatment over time is summarized. Removal of guanethidine for 1 week following a 2 week exposure time increased 3 [H] NE uptake at 10 and 100 uM slightly, and markedly at 1 uM. Uptake values are expressed as % of control for 10 cultures per group. +, $P < 0.01$, ++, $P < 0.001$, Student's t-test.

after 1 week washing respectively). Interestingly, the inhibitory effect on the CA uptake mechanism was attenuated after a 1 week recovery period, however, at concentrations beteen 10 and 100 uM the reduction in CA uptake was still significantly lower than the control values. Recovery of uptake at the higher concentrations of guanethidine (100 uM) was seen, but the effect was not pronounced (3 [H] DA uptake rose from 24.7% after 48 hrs to 32.2% and 3 [H] NE uptake rose from 16.2% after 48 hrs 23.4% of controls). At 10 uM guanethidine 3 [H] DA uptake increased from 32.8 to 66.4% and 3 [H] NE increased from 16.1 to 52.7%, suggesting that either a residual inhibitory effect of guanethidine on the uptake was eliminated after a one week washout or that a certain amount of regrowth of surviving neurons occurred. Low concentrations of guanethidine (1 uM) appear to support the latter suggestion at least for NE neurons since 3 [H] NE uptake increased by 76% above the control values (Fig. 33).

Figure 34A-34D represents the effects produced by guanethidine to the NE neurons following 2 weeks exposure and 1 week wash. Taken together, the above findings demonstrated a persistent reduction in both 3 [H] DA and 3 [H] NE uptake following prolonged removal of guanethidine at doses ranging between 10 to 100 uM. Low concentrations of guanethidine, even after a prolonged

Figure 34A-34D. GUANETHIDINE-INDUCED TOXICITY TO PONTINE CULTURES AFTER 2 WEEKS EXPOSURE

Pontine cultures were treated with guanethidine at varied concentrations for 2 weeks and washed for 1 week and processed for CA histofluorescence. (A) Control cultures contain many cells with large perykarya and many processes. (B) Cultures treated with 100 uM gaunethidine were devoid of NE neurons. (C) 10 uM guanethidine reduced the number of fluorescing neurons and processes without changing the size of the remaining somata. (D) 1 uM guanethidine also decreased the fluorescence levels but the neurite outgrowth appeared to increase in length which may suggest axonal sprouting (arrows). Magnification = 168x.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exposure period were not toxic to either DA or NE neurons.

3. Toxicity after 1 week exposure to guanethidine.

We also investigated the effects of guanethidine after 1 week exposure and 1 week removal of the drug. Since 2 weeks of 1 uM exposure of guanethidine did not inhibit the CA uptake pump, cultures were treated with either 10 or 100 uM only. The effect of this schedule of treatment was less pronounced for DA neurons. However, both uptake studies and immunocytochemistry revealed toxicity to DA and NE neurons. In the midbrain, uptake was not significantly reduced at the lower dose (10 uM) (68.3% of control), however, 100 uM guanethidine reduced the uptake to 35.4%. On the other hand, 3 [H] NE uptake was decreased significantly by both 10 and 100 uM guanethidine (to 45.1% after 10 and to 35.7% after 100 uM respectively (Fig. 35). Finally, TH immunocytochemistry in the midbrain revealed damaged DA neurons only at the high dose of guanethidine (100 uM) (Fig. 36A-36C) but with an increased number of DA neurons survived when compared to the 2 week treatment. CA-histofluorescence also demonstrated a reduction in fluorescence only at the higher dose (100 uM) as well (Fig. 37A-37C). Midbrain cultures treated with 10 uM guanethidine appeared bright and full of fibers similar to the control cultures and

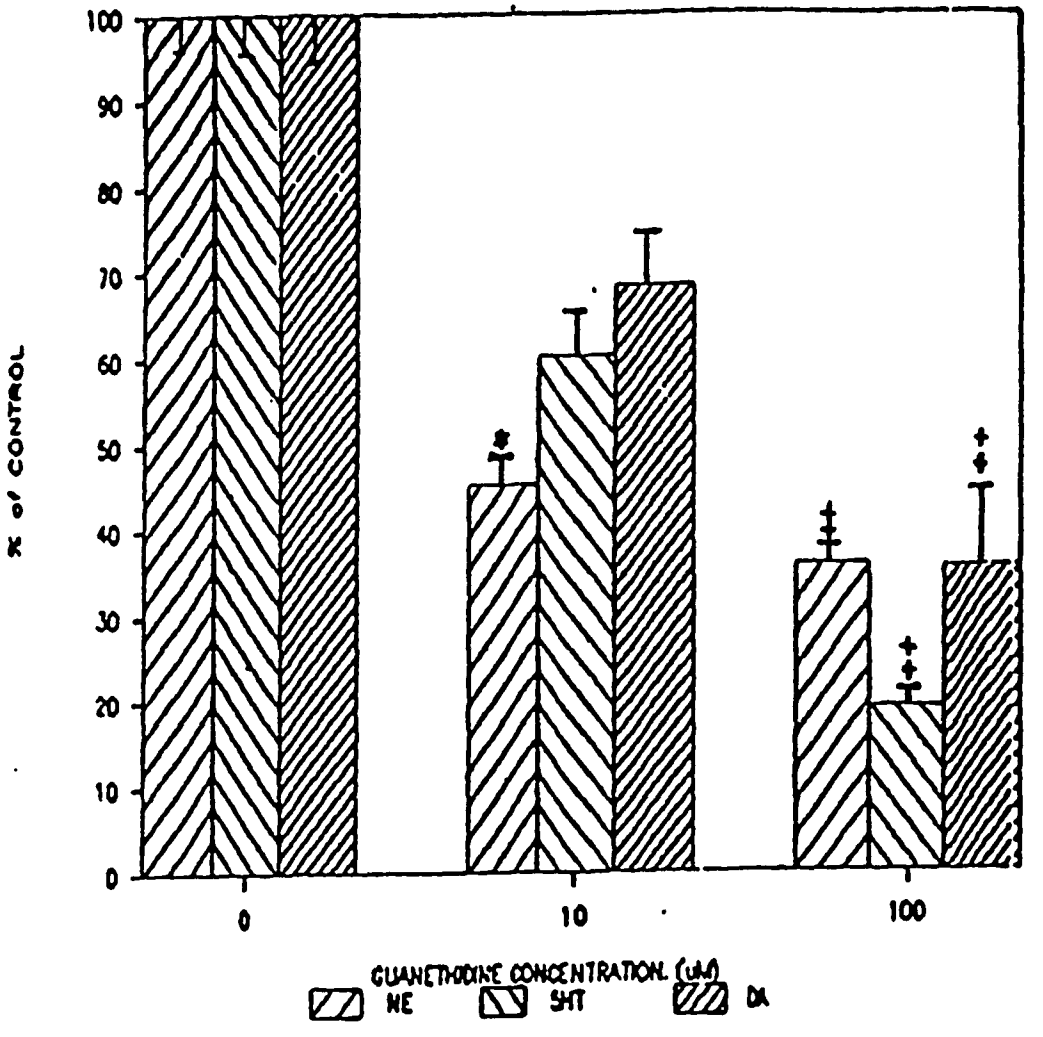

Figure 35. 3[H] MONOAMINE UPTAKE: 1 WEEK GUANETHIDINE EXPOSURE 1 WEEK WASH

Guanethidine was introduced into the feeding medium for 1 week and removed for 1 week. Uptake values are the means ± S.E.M. (n=10/ group) for all 3 tritiated amines (controls are 1.22, 0.4, 5.95 pmol/ culture for DA, NE, 5HT respectively). *, P< 0.5; +, P< 0.001, Student's t-test.

Figure 36A-36C. MIDBRAIN CULTURES EXPOSED TO GUANETHIDINE FOR 1 WEEK

Midbrain cultures were treated with guanethidine for 1 week, washed for 48 hours and analyzed for toxicity with TH immunocytochemistry. (B) Treatment of 100 uM guanethidine destroyed many TH+ DA neurons. Surviving neurons were either lightly stained or lacking processes, but the cell body size was not altered. (C) 10 uM treated cultures did not appear different from the controls (A). Magnification = 168x.

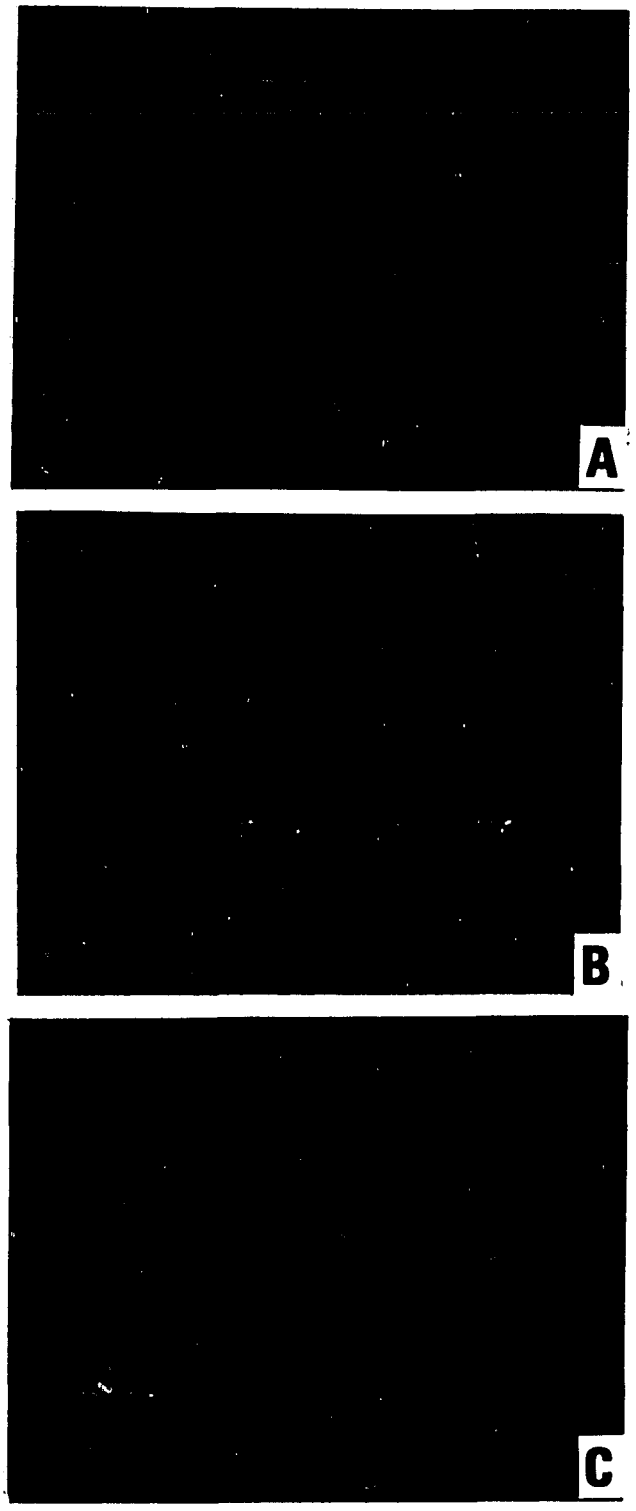
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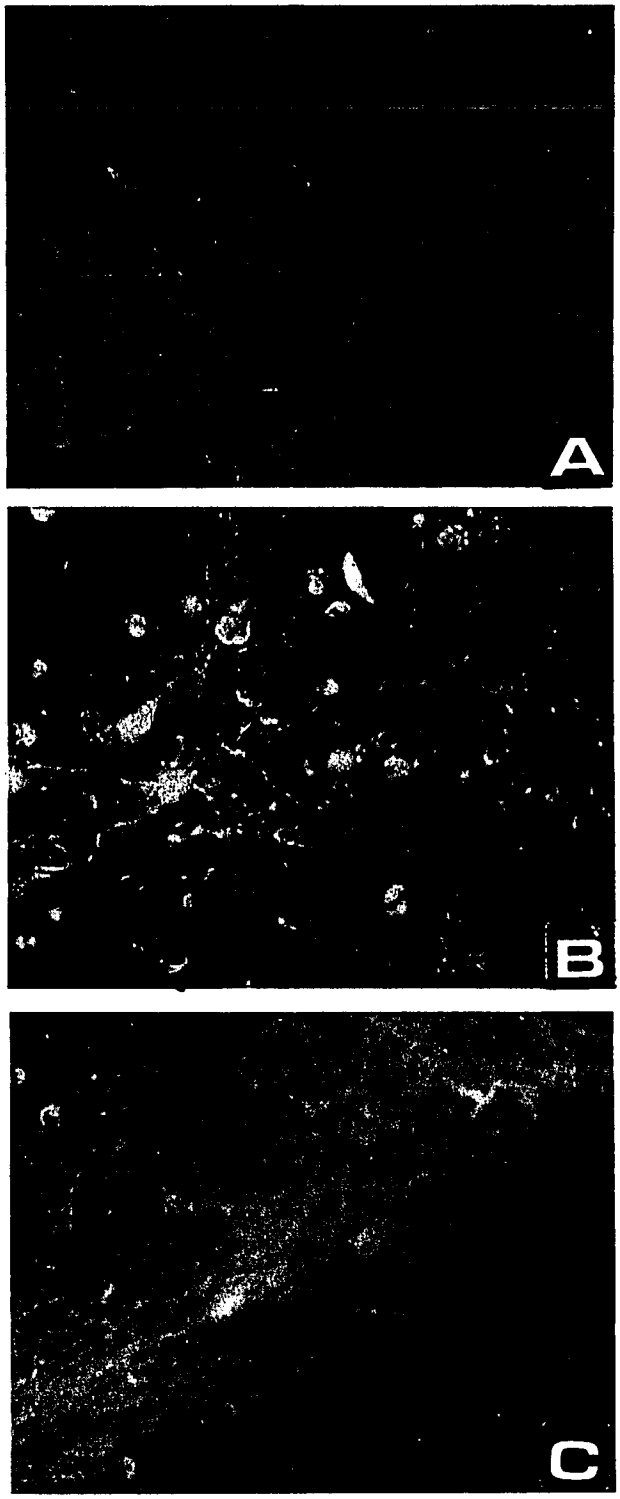
Figure 37A-37C. CA HISTOFLUORESCENCE OF MIDBRAIN CULTURES TREATED WITH GUANETHIDINE FOR 1 WEEK

Midbrain cultures were treated with guanethidine for 1 week, washed for 1 week, and analyzed for toxicity with CA histofluoresence. (B) The number of fluorescing cells were greatly reduced at the high dose as seen with the TH+ neurons, however, more processes are visible when compared to cultures washed for only 48 hrs suggesting some neurite regrowth after 1 week removal of the drug. (C) 10 uM treated cultures were similar to the controls (A) in appearance. Magnification = 168x.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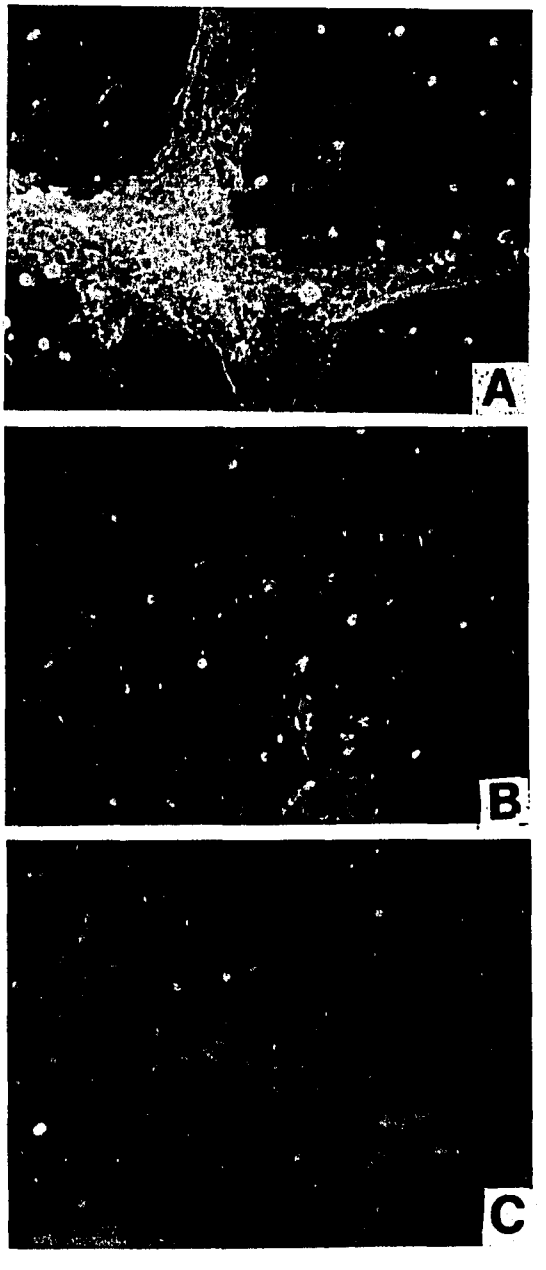
consistant with the uptake values. In contrast, pontine cultures were nearly devoid of any fluorescing neurons at 100 uM and very few fibers remained in the 10 uM guanethidine treated cultures (Fig. 38A-38C) consistant with the uptake studies.

b. The effects of guanethidine on 5HT uptake mechanisms compared to NE neurons.

Since an affinity of guanethidine for central 5HT neurons has been shown, the effects of guanethidine on the 5HT uptake system was also investigated. Rhombencephalic cultures were treated for either 1 or 2 weeks with guanethidine and rinsed for 1 week. After a 2 week exposure period, the effects on 3[H] 5HT uptake in the presence of a NE uptake inhibitor (DMI) resembled the inhibition on the CA uptake (Fig 39). 3[H] 5HT uptake was reduced to 10% at 100 uM and to 52.6% of controls at 10 uM. No effect was seen at 1 uM. One week exposure had a pronounced reduction of uptake at 100 uM (by 80%) while 10 uM guanethidine reduced the uptake by only 30%. (by 64.3% for NE). Thus, 1 week exposure of guanethidine at lower doses (10 uM) produced a greater reduction in uptake for NE neurons when compared to either 5HT or DA neurons.

Figure 38A-38C. CA HISTOFLUORESCENCE OF PONTINE CULTURES TREATED WITH GUANETHIDINE FOR 1 WEEK

Pontine cultures were treated with guanethidine for 1 week, washed for 1 week, and analyzed for toxicity with CA histofluoresence. (B) Very few or no fluorescing NE neurons remained in 100 uM guanethidine treated cultures and (C) there was a paucity of fibers in the 10 uM treated group when comared to the controls (A). 168x.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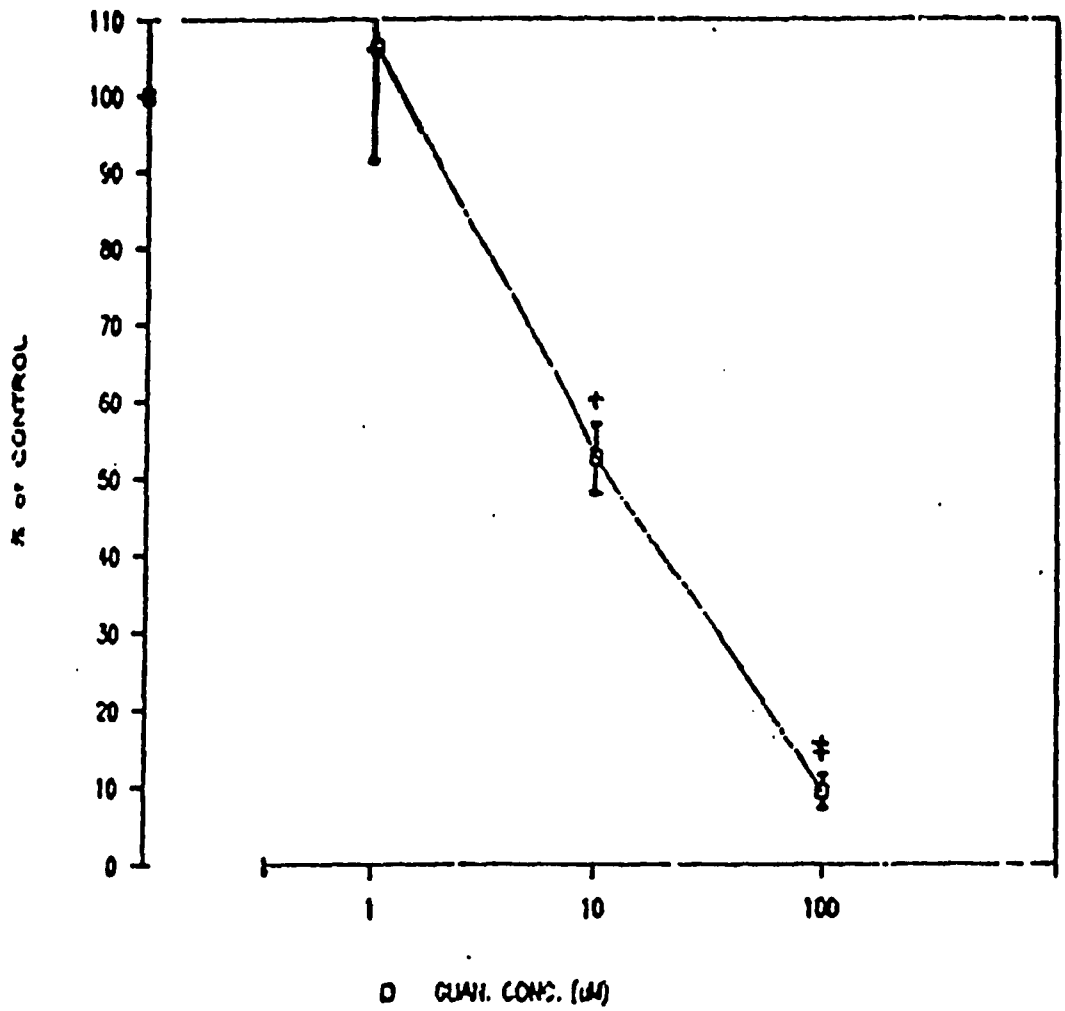

Figure 39. 3[H] 5HT UPTAKE: 2 WEEK GUANEHTIDINE EXPOSURE 1 WEEK WASH

Pontine cultures containing 5HT neurons were treated with guanethidine and analyzed by 3[H] 5HT uptake following 2 weeks exposure and 1 week wash. Uptake was reduced at 10 and 100 uM significantly. Control uptake was 5.7± 0.8 pmol/ culture and expressed as % of control (n=6). +, P< 0.01, ‡, P< 0.001 Student's t-test.

IV. DISCUSSION

A. Summary of results

The research reported here has resulted in several new findings by three selective neurotoxins, MPTP, MPP+ and guanethidine:

(1) DA, NE, and 5HT neurons removed from embryonic rat brain on the 14th gestational day survive for several months in dissociate cell cultures plated on Primaria dishes without the addition of extracellular matrix. The neurons develop several of their phenotypic characteristics, including the monoamine uptake pump and neurotransmitter synthesizing and metabolizing enzymes.

(2) Exposure of the dissociated cultures to the neurotoxin MPTP results in toxicity, similar to that observed <u>in vivo</u>, i.e. degeneration of DA and NE neurons with higher sensitivity for the dopaminergic cells, and lack of neurodegeneration for 5HT neurons. (3) MPTP toxicity to DA neurons is influenced by the interactions of the neurotoxin with MAO and uptake mechanisms of the neurons. As a result of these interactions MPTP has maximal toxicity only at a specific range (between 5 and 10 uM) with a decreased toxicity at higher concentrations. (4) MPTP applied to the cultures is a potent reversible inhibitor of MAO-A and a slowly acting irreversible inactivator of MAO-B, particularly at higher concentrations (100 uM). (5) MPP+ applied directly to the cultures causes degeneration to all monoamine neurons studied, with a potency for DA > NE > 5HT. (6) Guanethidine has

a higher affinity for the uptake pump of central NE neurons compared to DA neurons in adult rats. (7) Guanethidine applied directly to the cultures is toxic to all monoamine neurons studied with a higher selectivity for NE neurons.

B. Development of monoamine neurons in culture.

Prior to investigating the effects of a specific toxin in a dissociated tissue culture preparation of the CNS, it was necessary to examine individual properties that characterize DA, NE, and 5HT neurons (i.e. development of the uptake mechanisms, appearance of the synthesizing enzyme) and be sure that the neurons could survive and grow in Primaria dishes. Our studies have shown that all three monoamine neurons do survive and continue to grow for several weeks in these specially treated culture dishes. Thereafter the cultures may be maintained for 2 or 3 months. The monoamine neurons develop a specific uptake mechanism that may be blocked by classical selective inhibitors such as GBR-12909 for DA neurons, DMI for NE neurons, and citalopram for 5HT neurons. In addition, we have seen that antibodies against TH and 5HT allow us to selectively visualize DA and 5HT neurons respectively in the cultures. CA histofluorescence offers visualization of DA and NE

neurons which have been separated anatomically by microdissection of the ventral mesencephalic and pontine areas and grown in isolated, culture dishes.

C. Toxic effects of MPTP/MPP+ to cultured central DA neurons.

The discovery that MPTP is a parkinson-inducing agent in several species including humans has led to a vast amount of research regarding the mechanism of action of the drug. After in vivo MPTP administration, an extensive loss of DA neurons occurs in primates (Burns, et al., 1983; Langston, et al., 1983) and several other species (Heikkila et al., 1984). Our laboratory has previously described the reduction of DA neurons, and levels of DA and metabolites (Mytilineou et al., 1985; Mytilineou and Cohen 1986) in explant cultures associated with MPTP treatment. In the present studies we used the dissociated culture model to analyze separately neurons of a distinct phenotype namely DA, NE and 5HT.

We have observed with both quantitative and qualitative approaches, that in primary dissociated neuronal cultures, exposure to MPTP results in a decrease in DA cell survival and impaired dopaminergic mechanisms (ie. uptake, axonal elongation, and dendritic branching). Our results demonstrate that in cultures MPTP requires a

specific concentration for the expression of maximal toxicity to the DA neurons above and below which toxicity is attenuated. Moreover, it is nescessary to expose the cultures to MPTP for a prolonged period (4-7 days) to induce neurotoxic effects (Mytilineou and Cohen, 1984).

We have observed that a 24 hour MPTP exposure, with a subsequent 24 hour wash, does not produce any changes in the dopaminergic indices examined. Similarly, Sanchez-Ramoz et al (1986) have demonstrated that MPTP does not cause destruction of rat DA neurons in dissociated cultures following 24 hour treatment measured by TH immunocytochemistry. The prolonged exposure required for the manifestation of MPTP toxicity probably reflects the fact that MPTP itself is not toxic and requires metabolic oxidation by the cellular elements of the cultures in order to build toxic levels of the metabolite in the relatively large volume of the feeding medium. In this respect, MPTP differs from other known neurotoxins specific for catecholamine neurons, such as 6-hydroxydopamine as well as MPP+ (Friedman and Mytilineou, 1987).

High concentrations of MPTP (100-200 uM) resulted in a reduction of toxicity to the DA neurons. We have shown that the reduced toxicity is probably the result of two distinct properties of MPTP. First, we have shown that MPTP (100 uM) results in a profound inhibition of MAO-B

activity after acute and especially after chronic exposure to the toxin. Since MAO-B is the enzyme that is predominantly responsible for the conversion of MPTP to its toxic metabolite, MPP+, a certain amount of MAO-B activity is necessary for sufficient levels of MPP+ to accumulate in the feeding medium and induce destructiom of DA neurons. High levels of MPTP that is continuously present at relatively stable concentrations in the tissue culture feeding medium -less than 50% of MPTP is metabolized in 4 days (Mytilineou and Friedman, submitted) - appear to produce a slowly occuring irreversible inactivation of MAO-B. Consequently when cultures are incubated with high concentrations of MPTP, insufficient biotransformation of MPTP takes place and the DA neurons are protected from destruction. An irreversible, product initiated, inhibition of MAO-B by MPTP has also been demonstrated in vitro (Singer et al., 1985).

On the other hand, low doses of MPTP (5 uM) did not affect the MAO-B activity after acute exposure (during the assay) and the reduction of MAO-B activity after prolonged exposure was not nearly as severe as the high dose (37.4% reduction in activity as compared to 84.2% respectively). Thus, it appears that sufficient conversion to MPP+ occurs in the presence of 5 uM MPTP and results in DA neuron destruction. The variability of the amount of toxicity produced by 10 uM MPTP from one

experiment to another may be due to differences in relative inhibition on MAO-B activity between experiments.

In addition to the interaction of MPTP with MAO, we have also shown that high concentrations of MPTP inhibit the accumulation of MPP+ in the DA neurons, while lower concentrations have very little effect. Since accumulation of MPP+ into the DA neurons by the CA uptake pump is required for neurotoxicity, inhibition of the CA uptake mechanism would protect the cells from degenerating. Therefore, the reduced toxicity of high concentrations of MPTP could be due to the independent effects of the MPTP molecule on MAO-B activity and on the monoamine re-uptake pump. These properties of MPTP could explain reports of failure to produce damage to DA neurons after local infusion of high doses of MPTP to rat substantia nigra in vivo (Bradbury, et al., 1986).

Evidence that high doses of MPTP prevent DA cell destruction by inactivation of MAO comes also from our protection studies with the well known MAO inhibitors, deprenyl and clorgyline. Our results show that deprenyl (MAO-B inhibitor) alone at 1 uM could only partially protect the DA neurons from MPTP-induced toxicity when the concentration of MPTP was low (5 uM). On the other hand, when MPTP concentration was increased to 10 uM and co-incubated with deprenyl (1 uM), we observed a complete protection measured by 3[H] DA uptake. Similarly,

nonspecific doses of clorgyline (10 uM) that inhibit both types of MAO (A + B) fully protected against the toxic effects of MPTP when the concentration of MPTP in the cultures was equal to 10 uM but not at 5 uM. Finally, deprenyl (1 uM) and clorgyline (1 uM) combined, at a regiment that inhibits both MAO-A and MAO-B protected the DA neurons at either concentration of MPTP (5 or 10 uM).

Our data with specific MAO inhibitors also suggest several ways in which MPTP interacts with MAO to induce its toxic effects: (1) Low concentrations of clorgyline (1 uM) that inhibit specifically only MAO-A activity, do not protect against against MPTP toxicty, indicating that MAO-B is responsible for the majority of the metabolic oxidation of MPTP to MPP+. (2) A combination of deprenyl (1 uM) and clorgyline (1 uM) added to the feeding medium which inhibits maximally both MAO-A and MAO-B and protects the DA neurons from MPTP toxicity fully. This suggests that some MAO-A bioactivation of MPTP occurs in addition to the metabolic oxidation by MAO-B in cultures and has an additive effect on MPTP toxicity. MPTP has been shown to be a substrate and also inhibit both forms of MAO activity (Fuller et al., 1985; Salach et al., 1984; Singer et al., 1985) (3) Higher concentrations of clorgyline (10 uM) that inhibit both MAO-A and MAO-B activities combined with lower doses of MPTP (5 uM) only partially protect against toxicity because probably enough MPP+ can still be

converted by MAO-B and accumulate in the neurons to have a partial effect. (4) Higher concentrations of MPTP (10 uM) may itself exert a partial inhibition on both MAO-A and MAO-B activities so that when combined with the higher dose (10 uM) of either MAO inhibitor, it results in full protection.

The regional localization of the mitochondrial bound enzyme MAO in vivo appears to be very important determining the toxicity of MPTP in different animal species (Schneider and Markham, 1987). It has been shown that the SN is rich in MAO-B containing glia cells in certain species such as in primates (Murphy et al, 1979; Westlund et al., 1985) and cats (Schneider and Markham, 1987). Thus, large amounts of MPP+ may be produced in the immediate vicinity of the SN and gain access to the high affinity uptake system of the DA neurons. In vitro, however, the glial cells are dispersed and proliferate so that all of the DA containing neurons are surrounded by glia. This could possibly explain why DA neurons from rat embryos in culture appear to be vulnerable to the toxin, while the adult rat is resistant to MPTP exposure.

We have examined the metabolic conversion of MPTP to MPP+ in the cultures under different conditions (Mytilineou and Friedman, 1987 submitted for publication). Our results demonstrate after incubation with MPTP, that the metabolite of MPTP, MPP+, diffuses readily into the

feeding medium and its concentration increases over time. The amount of MPP+ that is formed parallels the degree of toxicity to the DA neurons. In addition, increasing the number of cells plated in the culture dishes increases the concentration of MPP+ formed, therefore, increasing the toxicity. Furthermore, decreasing the number of glial cells present in the cultures with FUDR, reduces the metabolism of MPTP. Finally, incubation of cultures with toxic concentrations of MPTP, which are toxic to the DA neurons, does not affect the survival of glial cells.

The results of these experiments can explain the lack of toxicity after incubation for 24 hours. A prolonged exposure period is required because the rate of metabolism of MPTP by the cultures is slow. Several days are necessary to accumulate MPP+ within the culture medium to induce neurotoxicity. Also, the reduced metabolism of MPTP when the number of glial cells are diminished supports the concept that the distribution of glia is important in the selective effects of the toxin, since MAO-B is predominantly localized within glial cells, presumably astrocytes (Levitt et al., 1982).

Furthermore, since MPP+ diffuses readily into the feeding medium, it can exist outside of the glial cells in which it was formed, and accumulate in increasing concentration in the extracellular space. Hence, the toxic metabolite can gain access to the CA high affinity uptake pump to

induce destruction. Therefore, our studies present direct evidence to support the theory postulated by Javitch et al (1985) that MPTP is metabolized mainly by MAO-B to MPP+ outside of the DA neurons, is released in the extracellular space and subsequently taken up avidly by the DA neurons.

Protection of MPTP-induced toxicity with CA uptake inhibitors also supports the concept that the high affinity uptake mechanism is directly involved in the selective action of MPTP/MPP+ (Pilebad and Carlsson, 1985; Ricaurte et al., 1985; Schultz et al., 1986). Our results demonstrated a partial protection against DA cell death by mazindol, indicated by an increased survival of TH+ neurons and a healthier appearance of those neurons (Fig. 14A-14C). However, mazindol did not protect the neurons fully, since 3[H] DA uptake values were reduced to even lower levels than in those cultures treated with MPTP alone. Perhaps treatment with MPTP in conjuction with a potent CA uptake blocker caused damage to the uptake pump in a model where constant, direct exposure to the drugs was possible.

The toxicity of MPP+ to the DA neurons was more potent when compared to MPTP. This was to be expected since MPP+ is presumably the major toxic metabolite of MPTP that is responsible for DA neuronal destruction. MPP+ is a fairly stable species (Sayre, et al.,1987)

so that it has the ability to remain active in a biological system for quite some time before further breakdown or metabolism occurs. Hence the accumulation of MPP+ into the DA neurons occurs readily and toxicity prevails. Protection against MPP+ toxicity was also attempted with mazindol, but again only a partial protection was seen. Higher number of TH+ cells were present in the cultures co-treated with MPP+ and mazindol when compared with MPP+ alone. However, as with MPTP, the uptake mechanisms were greatly impaired in both groups of cultures. Perhaps an uptake inhibitor cannot prevent a direct toxicity of MPTP or MPP+ to the DA uptake pump.

D. Effects of MPTP and MPP+ on non-dopaminergic neurons.

1. Cultured NE neurons.

Since its discovery, the central focus of MPTP neurotoxicity has been on its ability to selectively destroy DA containing neurons of the nigrostriatal system similar to neurodegeneration that is observed in parkinsonian brains. Although selective dopaminergic lesions reflect the major neuropathology of Parkinson's disease, other brain regions and neurotransmitter systems are affected. For instance, non-nigrostriatal DA neurons belonging to both mesocortical and mesolimbic

pathways may be affected, (Mitchell, et al., 1985; Melamed, et al., 1985) and reduced catecholamine neurons of the LC and sympathetic ganglia have been reported (Javoy-Agid, et al,. 1984.) Mitchel et al (1985) have also reported lesions by MPTP to NE neurons of the LC. Moreover, in parkonsonian patients, reduced 5HT levels in the hippocampus and frontal cortex (Scatton, et al., 1983) have also been demonstrated.

Our studies show that MPTP/MPP+ can induce toxic effects to the NE neurons upon direct exposure. However, the toxic effects seen were less pronounced when compared to the DA neurons indicating that these neurons are more resistant to the toxin. Interestingly, in contrast to the effect on DA neurons, high doses of MPTP (100 uM) did not show reduced toxicity on the NE uptake pump, when compared to lower concentrations of MPTP. Uptake values were decreased by 50 to 60% of the controls. In addition, fluorescing fibers and cell bodies remaining after 100 uM MPTP were few when compared to the midbrain cultures containing DA neurons at the same MPTP dose. Although our experiments do not provide an explanation for the different response of NE and DA neurons to 100 uM MPTP, it may possibly be due to a difference in the inhibitory effect of MPTP on the MPP+ uptake between NE and DA neurons. There is no difference in the effect of MPTP on MAO activity between mesencephalic and pontine cuyltures.

Lower concentrations of MPTP destroyed many more DA neurons belonging to the midbrain than NE neurons suggesting that the DA neurons are more sensitive than the NE neurons at lower MPTP concentrations when MAO-B activity is only slightly inhibited.

MPP+, after it has been washed out, does not have any significant inhibitory effect on the CA uptake system, therefore, the toxic effects expressed were more easy to interpret. Uptake values were decreased with increasing concentrations of MPP+ and very few fluorescing perikarya and processes remained, indicating destruction. Low doses (1 uM) of either MPTP or MPP+ did not have any apparent effect on the histofluorescence for NE neurons, whereas this dose did reduce both fluorescence levels and TH+ neurons in the midbrain cultures, suggesting the DA neurons are more sensitive to both toxins at low concentrations.

2. Cultured 5HT neurons

In the MPTP model of Parkinson's disease, other systems aside from the DA nigrostriatal system are also affected. Damage to CA containing neurons of the NACC, VTA, LC (Mitchell et al., 1985) and hypothalamus (Gibb et al., 1986) have been documented. In addition, a reduction in central 5HT metabolism has been reported (Di Paolo,

1986; Enz et al., 1984; Fine et al., 1985) without any noticeable morphological damage or alteration in 5HT+ neurons of the dorsal and median raphe nuclei (Gupta et al., 1984). The results of our experiments, where direct exposure of a specific neuronal population to a neurotoxin is possible, suggest that MPTP does not cause destruction of the 5HT neurons in vitro. However, high doses (100uM) of MPTP do appear to damage the serotonergic high affinity uptake mechanisms since extensive washout did not restore uptake values to control levels. Furthermore, MPP+ introduced into the feeding medium (1-10 uM) destroyed or severely damaged many of the 5HT neurons in the cultures. However, the extent of 5HT neuronal degeneration was less than that produced by the same MPP+ concentration in DA neurons.

Possible explanations to why MPTP is essentially not toxic to the 5HT neurons while MPP+ is toxic are: (1) low concentrations between 1 and 10 uM MPTP may not produce high enough levels of MPP+ in the rhombencephalic cultures to induce a toxic effect. (2) prolonged treatment with high concentrations of MPTP inhibits MAO-B activity dramatically whereby the conversion of MPTP to MPP+ is severely impaired, thereby protecting the 5HT neurons from cell death and increasing their staining ability due to reduced 5HT metabolism, (3) High concentrations of MPTP inhibit the uptake of MPP+ by 5HT neurons by approximately

50% (unpublished data) thereby protecting the 5HT neurons.

We have found that the activity of MAO in both mesencephalic and rhombencephalic cultures is inhibited in a similar way by MPTP (tables 2 + 7), therefore, possibility (1) is probably not correct. We have seen that at low concentrations (5 uM MPTP), MAO-B activity is slightly inhibited, but DA neurons are affected by the amount of MPP+ that is formed at that dose. Possibilty (2) may be correct and (3) probably is in part responsible for the survival of 5HT neurons after exposure to 100 uM MPTP. However, MAO-B has been consistantly localized within the 5HT neurons of rats (Levitt et al., 1982), therefore, intraneuronal conversion of MPTP to MPP+ should occur locally. Inhibition of MAO-B at high concentrations of MPTP depends on product formation (Singer, et al., 1986) and is time dependent. Therefore, MPP+ should be formed at least initially within the 5HT neurons. Hence, it is more likely that the mechanisms responsible for the lack of toxicity are the inhibition of MPP+ uptake by MPTP at the high dose and possibly an intrinsic, higher resistance of 5HT neurons to MPTP toxicity.

E. Effects of guanethidine in the CNS.

1. Effects on uptake and release.

The studies of guanethidine on uptake

and release were conducted to define the affinity of the compound for central CA terminals. Our data show a selective inhibitory effect of guanethidine (at concentrations of 1 - 5 uM) on the uptake of ^{3}[H]-NE by the cortical noradrenergic processes. There is no effect on the uptake of DA in the striatum at these concentrations. The inhibition of uptake could result from the competition of guanethidine for the amine uptake pump of the noradrenergic neurons of the CNS. Guanethidine has an affinity for the peripheral adrenergic neuron pump, demonstrated by the inhibition of guanethidine effects by catecholamine uptake inhibitors such as desmethyl- imipramine (Stone et al., 1964; Kaumann et al. 1965) and cocaine (Nasmyth and Andrews, 1959). An interaction of guanethidine with the central NE neurons is also indicated by the observation that it causes a significant reduction in NE levels after intraventricular administration (Cox and Maickel, 1969).

At higher concentrations (10 - 100uM) guanethidine inhibited the membrane uptake pump in both NE and DA neurons. However, the NE neurons were more sensitive to the inhibitory effect of guanethidine at all concentrations studied (IC50 0.5 uM and 33.6 uM for norepinephrine and dopamine neurons respectively). Thus, guanethidine appears to have a differential effect on the uptake pump of the NE and DA neurons in the CNS, a

property that makes it a useful tool for pharmacological studies of these two neuronal populations.

The increase in spontaneous NE release in the presence of guanethidine in the cortical slices is similar to that found in the periphery (Maxwell, 1982) and appears to be specific for central noradrenergic neurons since 3[H] DA spontaneous release in striatal slices was unaffected by guanethidine. It is interesting that, even at concentrations that inhibit the uptake of DA into striatal synaptosomes, guanethidine did no increase the spontaneous efflux of ^{3}[H] DA in striatal slices. One possible explanation could be that guanethidine, which probably induces release of NE through its affinity for both neuronal and storage vesicle membrane, as is the case in the peripheral adrenergic neurons (Chang et al. 1964; Brodie et al. 1965), has no affinity for the storage vesicles of the DA neurons. This would be consistent with the observation that the membrane pump of the storage vesicles has more specific substrate requirements than the neuronal membrane amine pump (Shore and Giachetti, 1966).

In our experiments, guanethidine did not have a selective effect on the blockade of K^{+} depolarization evoked release. Only high concentratons (100uM) could block the tritium overflow from slices preloaded with their respective amine. Schlicker and Gothert (1983) have reported guanethidine blockade of electrically evoked

release of NE labeled occipitoparietal slices at lower concentrations (10uM). The lack of effect, seen in our experiments at lower concentrations of guanethidine, could be due to the use of K^+ depolarization to induce release. A difference in the effectiveness of guanethidine to inhibit the release of ^{3}[H] NE after electrical stimulation and K^+ induced depolarization has been shown in the guinea pig vas deferens (Stutzin et al., 1982). Guanethidine may interfere with K+ conductance channels since it has been recently proposed, that guanethidine inhibits NE release by activating a hyperpolarizing calcium-activated potassium conductance in the membrane of noradrenergic neurons (Stutzin et al., 1983; Ferreira et al., 1985) .

2. Effects of guanethidine on catecholamine neurons.

Since we found that guanethidine has affinity for the uptake system of central CA neurons (NE neurons have a higher affinity than DA neurons), we were able to select the appropriate dose range of guanethidine to introduce into the culture feeding medium. In contrast to previous suggestions (Bartolome', et al., 1976. Evans, et al. 1985,), we have demonstrated that guanethidine can be toxic to central monoamine neurons in a primary culture

system of discrete brain regions.

Aside from the ability to induce peripheral sympathectomy in rats, guanethidine has many pharmacological actions such as competing for the NE uptake sites in sympathetic neurons (Lee et al., 1980). We have found in the cultures that guanethidine had a potent inhibitory effect on the CA uptake pump when exposed for only 24 hours. In other words, removal of guanethidine for 24 hrs after a 24 hr exposure period did not eliminate the inhibitory action of the drug on the CA uptake pump (Fig. 28). Whether this effect is the result of persistent binding of guanethidine to the uptake sites or of reversible damage to the pump is not evident from our experiments. Consequently, in order to determine survival of CA fibers during the analysis of experiments on guanethidine toxicity, it was necessary to remove guanethidine from the culture medium for at least 48 hours.

a. Exposure to guanethidine for 2 weeks

When the cultures were exposed to high concentrations of guanethidine (100 uM) for 2 weeks and drug free for 48 hours there was a similar effect on the reduction of uptake on all 3 monoamine containing neurons studied (DA, NE, 5HT) where the remaining uptake ranged between 10 to 22.3% of the controls. Since lower

concentrations of guanethidine (10 uM) reduced the amount of 3[H] CA uptake by the same amount as the higher dose for both DA and NE neurons, it can be concluded that the toxic effect by guanethidine was maximal at 10 uM under the longer exposure period (2 wks). Even lower doses of guanethidine (1 uM), reduced the uptake by the NE neurons to a greater extent when compared to the DA neurons. Hence, NE neurons appear to be more sensitive to guanethidine toxicity at lower concentrations when compared to DA neurons. The increased sensitivity at the lower dose indicates the affinity of guanethidine for the NE neurons is greater than for DA neurons, which we have also seen in adult brain synaptosomes. The morphological changes produced by guanethidine to both DA and NE neurons after prolonged and direct exposure, demonstrate the first example of guanethidine-induced toxicity to central CA neurons. Morphological changes, probably precursors to neuronal degeneration, were evident by the description of the surviving CA neurons (i.e. cell bodies were either reduced in size or swollen, axons and dendrites often were swollen in several places or lacking altogether at the higher doses (10-100 uM) for both DA and NE neurons). Additional evidence of a higher selective neurotoxic effect to the NE neurons was demonstrated by the increased survival of DA neurons when compared to the NE neurons seen by CA histofluorescence at all concentrations of

guanethidine studied. Hence, we show a clear effect of toxic changes to both DA and NE central neurons by guanethidine with a higher specificity toward the NE neurons.

The neuronal destruction produced by guanethidine to NE containing sympathetic neurons has been completely prevented by concomitant treatment with NGF (1-10 mg/kg) in neonatal rats (Johnson and Aloe, 1974; Manning et al., 1983; 1985). NGF also protects neuronal degeneration in the superior cervical ganglion in response to 6-hydroxy-dopamine (Levi-Montalcini, et al., 1975) or axotomy (Hendry and Campbell, 1976). It has been suggested (Johnson and Aloe, 1974; Manning et al., 1985) that following guanethidine accumulation into the sympathetic neurons to high concentrations (0.5-1 mM), an immune response is triggered by the synthesis or presentation of antigens on sympathetic membrane surfaces. Thus, the immune system recognizes these antigens as foreign, and subsequently produces T-lymphocytes to destroy these neurons.

Although CNS catecholamine neurons lack NGF receptors (Thoenen and Barde, 1980), and may not share the same response as peripheral adrenergic neurons, we examined the effect of NGF on central guanethidine neurotoxicity. NGF did not protect NE or DA neurons from guanethidine-induced toxicity. NGF protection is

proposed to be due to the suppression of antigen formation (Manning et al., 1983; 1985). However, in a culture system of the CNS where an immune system does not exist, we could still observe CA neuronal cell death after exposure to guanethidine.

The persistent inhibitory effect on the CA uptake pump produced by short guanethidine exposure time (24 hrs) implicated an irreversible effect on the CA uptake transport system. Removal of guanethidine from the culture medium for an increased period (1 wk) should allow enough time to evaluate an irreversible effect.

Our results showed that the inhibitory effect on the CA uptake mechanism was attenuated after a 1 week recovery period, however, at concentrations between 10 and 100 uM the reduction in CA uptake was still significantly lower than the control values. On the other hand, low concentrations of guanethidine (1 uM) after a 1 week wash, resulted in a marked increase in 3[H] NE uptake in pontine cultures, higher than the uptake in the control cultures. These data suggest that either a residual inhibitory effect of guanethidine on the uptake after a 1 week washout was eliminated or that a certain amount of regrowth of surviving neurons occured. Cultures subjected to low concentrations (1 uM) of guanethidine support the latter suggestion for NE neurons, since the 3[H] NE uptake was significantly higher than controls after 1 week

washout. Other neurotoxins such as kainic acid (Peichl and Bolz, 1983) that produce neuritic lesions or lesions produced by electro-coagulation (Scheff, et al., 1977) can result in axonal sprouting in addition to neuronal degeneration. Therefore, it is possible that certain neurotoxins at specific doses may have the ability to induce neural growth and create new functional circuitry.

b. Exposure to guanethidine for 1 week

Cultures exposed to guanethidine for only 1 week demonstrated a clearer differential effect between DA and NE neurons which appears to mimic the in vivo situation (Evans, et al., 1985). Uptake inhibition by guanethidine was similar for DA and NE neurons at the high doses and few cells were labelled by CA histofluorescence for both groups of cultures. In contrast, 10 uM guanethidine reduced the uptake of 3[H] NE to a greater extent than 3[H] DA uptake. Furthermore, the fluorescence levels in the midbrain cultures were similar to the controls at this dose, while pontine cultures had few fluorescing cells remaining. These results show that the toxic effects induced by lower concentrations (10 uM) were selective towards the NE neurons after a shorter exposure time (1 week) and DA neurons are more resistant to guanethidine toxicity. However, longer exposure time (2

weeks) affects both DA and NE neurons greatly, with more pronounced toxic changes occuring to the NE neurons.

3. Effects of guanethidine on 5HT neurons

Aside from catecholamines, serotonergic neurons also have an apparent affinity to guanethidine (Cox and Maickel, 1969). Our data showed that 3[H] 5HT uptake could be significantly reduced after exposure of pontine cultures to guanehtidine (10-100 uM) for 2 weeks. One week of guanethidine treatment (100 uM) also had a dramatic effect on the uptake capacity of the 5HT neurons. The reduction in uptake was significantly different from controls after 10 uM guanethidine as well. Therefore, we have shown that 5HT neurons can be susceptible to guanethidine toxicity as demonstrated by the reduction in uptake capacity. In conlusion, guanethidine can be toxic not only to NE neurons that have a higher affinity for the drug, but also to other central monoamine neurons in a model where these neurons have direct and constant exposure to guanethidine.

SUGESTIONS FOR FUTURE RESEARCH

(1) Since we found that MAO activity peaks during the 3rd week *in vitro*, it would be interesting to compare the toxic effects of MPTP over time in the cultures when the oxidative enzyme is undergoing different stages of maturation.

(2) Shorter exposure periods of MPP+ could be investigated to see the minimum time required to see an effect.

(3) MPP+ exposure in the presence of MPTP over time may reveal more information on the mechanisms of MPTP toxicity.

(4) Study of the role of the mitochondria and respiration in the cultures after exposure to either MPTP or MPP+ should be useful since MPP+ has been shown to inhibit NADH dehydrogenase, and hence interfere with respiration.

(5) The toxic effects of guanethidine in the CNS have not been well established, therefore, many other studies could be conducted such as protection of neurotoxicity with uptake inhibitors.

(6) Guanethidine has also recently been shown to have direct effects on respiration, therefore, mitochondrial studies in the cultures could also be employed.

REFERENCES

Agid, Y., Ruberg, M., Dubois, B. and Javoy-Agid,F. Biochemical substrates of mental disturbances in Parkinson's Disease. Adv.in Neurol., v.40, ed. by R.G. Hassler and J.F.Christ, Raven Press, N.Y., pp. 221-217, 1984.

Alvord, E.D., Forno, L., Kusske, J.A., Kaufman, R.J., Rhodes, J., and Goetowski, C.R. The pathology of parkinsonism. A comparison of degeneration in cerebral cortex and brain stem. Adv. Neurology 5:175-193, 1974.

Angeletti, P.U., Levi-Montalcini, R, and Caramia, F. Structural and ultrastructural changes in developing sympathetic ganglia induced by guanethidine. Brain Research, 43:515-525, 1972.

Arendt, T., Bigl, V., Arendt, A., and Tennstedt, A. Loss of neurons in the nucleus basalis of Meynert in Alzheimer's disease, paralysis agitans and Korsakoff's disease. Acta Neuropathol. (Berl.) 61:101-108, 1983.

Azmitia, E.C. The serotonin producing neurons of the midbrain median and dorsal raphe nuclei. In:The Handbook of Psychopharmacology, ed. by L. Iversen, S.D. Iversen, S.H. Snyder, 9:233-314, Plenum Press, N.Y., 1978.

Azmitia, E.C. and Whitaker-Azmitia, P.M. Target cell stimulation of dissociated serotonergic neurons in culture. Neuroscience, 20:1, pp. 47-63, 1987.

Bakay, R.A., Fiandaca, M.S., Barrow, D.L., Schiff, A. and Collons, D.C. Preliminary report on the use of fetal tissue transplantation to correct MPTP-induced Parkinson-like syndrome in primates. Appl. Neurophysiol. 48:1-6, 1985.

Baldessarini, R.J., Kula, N.S., Francoeur, D. and Finklestein, S.P. Antioxidants fail to inhibit depletion of striatal dopamine by MPTP, Neurology, 36:735, 1986.

Bankiewicz, K.S. Oldfield, E.H., Chiuh, C.C., Doppman, J.L. Hemiparkinsonism in monkeys after unilateral internal carotid artery infusion of 1-methyl-4-phenyl-tertahydropyridine (MPTP). Life Sciences 39:7-16, 1986.

Bartolome', J., Bartolome', M, Seidler, F.J., Anderson, T.R. and Slotkin, T.A. Effects of early postnatal guanethidine administration on adrenal medulla and brain of developing rats. Biochem. Pharm., 25:2387-2390, 1976.

Beardsley, J.V.and Puletti, F. Personality (MMPI) and cognitive (WAIS) changes after levodopa treatment. Arch. Neurol., 25:145150, 1971.

Berger, B., DiPorzio, U., Daguet, N.C., Gay M., Vigny, A., Glowinski, J. and Prochiantz, A. Long-term development of mensencephaloc dopaminergic neurons of mouse embryos in dissociated primary cultures: morphological and histochemical characteristics. Neuroscience, 7:193-205, 1982.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O. Jellinger, R., and Steitelberger, F. Brain dopamine and the syndromes of Parkinson and Huntington. J. Neurol. Sci. 20:415-455, 1973.

Bernheimer, H. and Hornykiewicz, O. Herabgesezte konzentration der homovanillinsaure im gehirn von Parkinsonkranken menschen als ausrruck der storung des zentralen dopaminstoffwechsels. Klin. Wochenschr., 43:711-715, 1965.

Birkmayer, W., Riederer, P., Yardim, M.B.H., and Linauer, W. The potentation of the antiakinetic effect after L-Dopa treatment by an exhibitor of MAO-B, Deprenil. J. Neural Transm., 36:303-326, 1975.

Bottenstein, J. Growth requirements of neural cells in vitro, in Advances in Cellular Neurobiology. Volume 4, ed. by S Fedoroff and L. Hertz, Acedemic Press, New York, pp. 333-379, 1983.

Bottenstein, J. Culture methods for growth of neuronal cell lines in defined media in Cell Culture Methods for Molecular and Cell Biology. vol. 4, ed. by D. Barnes, D. Sirbasku and G. Sato, Liss, New York, pp. 3-13, 1984.

Bottenstein, J. Growth of primary neural cells in chemically defined media, in: Growth and Differentiation of Cells in Defined Environment. ed. by H. Murakami, I. Yamane, D. Barnes, J. Mather, I. Hayashi, and G. Sato, Springer-Verlag, Berlin, 1984.

Bottenstein, J., and Sato, G. Growth of a rat neuroblastoma cell line in serum-free supplemented media. Proc. Natl. Acad. Sci. USA, 76:514-517, 1979.

Bottenstein, J. and Sato, G. Fibronectin and polylysine requirement for proliferation of neuroblastoma cells in defined medium. Exp. Cell Res., 129:361-366, 1980.

Bottenstein, J., Mather, J., and Sato, G. Growth of neuro-epithelial-derived cell lines in serum-free hormone-supplimented media. CSH Conf. Cell Prolif., 6:531-544, 1979.

Bottenstein, J., Skaper, S., Varon, S., and Sato, G. Selective survival of neurons in chick sensory ganglionic cultures utilizing serum-free supplimented medium. Exp. Cell Res., 125:183-190, 1980.

Bradbury, A.J., Brossi, A., Costall, B., Domeney. A.M., Gessler, W.and Naylor, R.J. Biochemical changes caused by the infusion into the substantia nigra of the rat of MPTP and related compounds which antagonize dihydropyridine reductase. Neuropharmacol. 25:583-586, 1986a.

Bradbury, A., Costall, B., Jenner, P., Kelly, M., Marsdan, C. The effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on striatal and limbic catecholamine neurons in white and black mice. Neuropharmacol. 25:897-904, 1986b.

Bradbury, A.J., Costall, B., Jenner, F.G., Kelly, M.E., Marsden, C.D., and Naylor, R.J. MPP+ can disrupt the nigrostriatal dopamine system by acting in the terminal area. Neuropharmacol. 25:939-941, 1986c.

Bradbury, A.J., Costall, B., Janner, P.G., Kelly, M.D., Marsden, C., Naylor, R. The neurotxic actions of MPP+ are not prevented by deprenyl treatment. Neuroscience Lett. 58: 177-181, 1985.

Brodie, B.B., Chang, C.C., and Costa, E. On the mechanism of action of guanethidine and bretylium, Br. J. Pharmacol. 25, 1965.

Brusco, A., Peressini, S., Pecci Saavedra, J. Serotonin-like immunoreactivity and anti-5-hydroxytryptamine (5-HT) antibodies: ultrastructural application in the central nervous system. J. Histochem. Cytochem. 31:524, 1983.

Burns, R.S. Chiueh, C.C., Markey, S.P., Ebert, M.H., Jacobowitz, D.M., and Kopin, I. A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurobiol., 80:4546-4550, 1983.

Burnstock, G., Doyle, A.E., Gannon, B.J., Gerkens, J.F., Iwayama, T. and Mashford, M.L. Prolonged hypotension and ultrastructural changes in sympatheic neurons following guanicline treatment. Eur. J. Pharmacol. 13:175-187, 1971.

Bus, J.S., Aust, S.D., Gibson, J.E. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem. Biophys. Res. Commun., 58:749-755, 1985.

Candy, J.M., Perry, R.H., Perry, E.K., Irving, D., Blessed, G., Fairbairn, R.F., and Momlinson, B.E. Pathologica changes in the nucleus of Meynert in Alzheimer's and Parkinson's disease. J. Neurol. Sci. 54:277-289, 1983.

Chang, C.C., Costa, E. and Brodie,B.B. Reserpine induced release of drugs from sympathetic nerve endings, Life Sci. 3, 839, 1964.

Chiba, K., Trevor, A. and Castagnoli, N., Jr. Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. Biochem. Biophy. Res. Commun., 120:574-578, 1984.

Chiba, K. Trevor, A. and Castagnoli, N., Jr. Active uptake of MPP+, a metabolite of MPTP by brain synaptosomes. Biochem. Biophy. Res. Commun., 128:1228-1232, 1986.

Chiuh, C.C., Markey, S.P., Burns, R.S., Johannessen, J.N., Pert, A. and Kopin, I.J. Neurochemical and behavioral effects of systemic and intrnigral administration of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the rat. Eur. J. Pharmacol. 100:189-194, 1984.

Clark, J. and Nicklas, W.J. The metabolism of rat brain mitochondria, J. Biol. Chem. 245, 4724, 1970.

Cohen, G. The pathobiology of Parkinson's disease: biochemical aspects of dopamine neuron senescence. J. Neural Trans., Supp. 19:89-103, 1983.

Cohen, G. and Mytilineou, C. Studies on the mechanism of action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Life Sci. 36:237-242, 1985.

Cohen, G., Pasik, P., Cohen, B., Liest, A., Mytilineou, C. and Yahr, M.D. Pargyline and deprenyl prevent the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in monkeys. Eur. J. Pharmacol., 106:209-210, 1984.

Consolazione, A., Milstein, C., Wright, B., Cuello, A.C. Immunocytochemical detection of serotonin with nomoclonal antibodies. J. Histochem. Cytochem., 29:1425, 1981.

Cox, R.H. Jr. and Maickel, R., Effects of guanethidine on rat brain serotonin and norepinepherine. Life Sci. 8:1319, 1969.

Creese, I., Burt, D.R. and Snyder, S.H. Dopamine receptor binding enhancement accompanies lesion-induced behavioral supersensitivity. Science 197:596-598, 1977.

Cropper, E.C., Eisenman, J.S. and Asmitia, E.C. 5-HT-Immunoreactive fibers in the trigminal muclear complex of the rat. Expl. Brain Res., 55:512-522, 1984.

Dahlstrom, A. and Fuxe, K. Evidence for the existance of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta. Physiol. Scand. (Suppl. 232), 62: 1-55, 1964.

Davis, G.C., Williams, A. C., Markey, S. P., Ebert, M. H., Caine, E.D., Reichert, C. M. and Kopin, I.J. Chronic parkinsonism secondary to intravenous injection of meperidine analogues. Psychiat. Res., 1:249-254, 1979.

De Longo, M.R., Georgopopalos, A.P., and Crutcher, M.D. In: Neural Coding of Motor Performance, Exp. Brain Res., Suppl. 7, eds. J. Massion, J. Paillard, W. Schultz, and M. Wiesendanger, pp. 29-40. Springer-Verlag, Berlin, 1983.

Denton, T. and Howard, B.D. Inhibition of dopamine uptake of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a cause of parkinsonism. Biochem. Psychopharm. 37:255-263, 1984.

Del Zompo, M., Piccardi, M.P. Bernardi, F., Bonucelli, U. and Corsi. Involvement of monoamine oxidase enzymes in the action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a selective neurotoxin,in the squirrel monkey: binding and biochemical studies. Brain Res. 378:320-324, 1986.

Del Zompo, M. Pintus, S. Zuddas, A. and Corsini, G.U. Deprenyl selectively inhibits 3H MPTP binding sites in monkey brain. Eur. J. Pharmacol. 107:285-286, 1985.

Di Paolo, T., Bedard, P., Daigle, M. and Boucher, R. Long-term effects of MPTP on central and peripheral catecholamine and indolamine concentrations in monkeys. Brain Res. 379:286-293,1986.

Di Porzio, U., Daguet, M., Glowinski, J.and Prochiantz, A. Effect of striatal cells on in vitro maturation of mesencephalic dopaminergic neurones grown in serum-free conditions. Nature, 200, pp. 370-373, 1980.

Ehringer, H., and Hornykiewicz, O. Verteilang von Noradrenaline and Dopamin (3-hydroxytyramin) im Gehirn des Menschen und ihr erhalten Dei Erkrankungen des Extrapyamidalen Systems. Klin. Wochenschr., 38:1336-1339, 1960.

Enz, A., Hefti, F. amd Frick, W. Acute administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) reduces dopamine and serotonin but accelerates norepinephrine metabolism in the rat brain. Effect of chronic pretreatment with MPTP. Eur. J. Pharmacol., 101:37-44, 1984.

Escourolle, R., Recowdo, J. de, and Gray F. Etude anotomo-pathalogique des syndromes parkinsoniens. In:Monoamines et Noyaux Gris Centraux, ed. by J.A. Juriaguerry and G. Gauthier, pp. 173-229. Georg-Masson, Geneve, Paris, 1971.

Evans, B.K., Singer, S.A.G., and Burnstock, G. Intra-cranial injection od drugs: Comparison of diffusion of 6-OHA and guanethidine, Pharmacol. Biochem. Behav. 3, 205, 1975a.

Evans, B.K. Singer, G. Armstrong, S., Saunders, P.E., and G. Burstock. Effects of chronic intracranial injection of low and high concentration of guanethidine in the rat. Pharmacol Biochem Behav. 3, 219, 1975b.

Everts, E.V., Kimura, M., Wurtz, R.H., and Hikosaka, O. Behavioral correlates of activity in basal ganglia neurons. Trends in Neuroscience, 7:447-453, 1984.

Fagervall, I., and Ross, S. B. A and B forms of monoamine oxidase within the monoaminergic neurons of the rat brain. J. Neurochem., 47:569-576, 1986.

Falk, B. Hillarp, N.A., Thieme, G. and Thorp, A. Flourescence of catecholamines and related compounds condensed with formaldehyde. J.Histochem. Cytochem., 10:348-354, 1962.

Ferreira, J., Gil, L, Stutzin, A. and Orrego, F. Effects of guanethidine on electron transport and proton movements in rat heart, brain and liver mitochondria, Biochem. Pharmacol. 34:2507, 1985.

Forno, L.S. Pathology of Parkinsonism. J. Neurosurg., 24:Suppl. II:266-271, 1966.

Forno, L.S. Pathology of Parkinson's disease. In:Movement Disorders, ed. by C.D. Marsden, S. Fahn, Butterworth Scientific, London, 25-40, 1982.

Forno, L.S. and Alvord, E.C., Jr. The pathology of Parkinsonism. In:Recent Advances in Parkinson's Disease, ed. by F.H. McDowell and C.H. Markham, pp. 120-130. F.A. Davis, Phila., 1971.

Forno, L.S., De Lanney, L.E., Irwin, I., Langston, J.W. Neuropathology of MPTP-treated monkeys: comparison with the neuropathology if human idiopathic Parkinson's disease. In: A Neurotoxin Producing a Parkinsonian Syndrome, ed. by S.P. Markey, N. Castignoli Jr., A.J. Trevor, I.J Kopin,119-140, New York: Academic Press, 1986.

Friedman, L.K., Mytilineou, C. The toxicity of MPTP to dopamine neurons in culture is reduced at high concentrations. Neurosci. Lett., 79:65-72, 1987.

Friedman, L.K., Mytilineou, C. Cultured serotonin neurons are sensitive to MPP+ toxicity but resistant to MPTP. Soc. for Neurosci. Abstr., 220.4, 1987.

Fries, D.S., Fries, J., Hazelnoff, B. and Horn, A. Synthesis and toxicity toward nigrostriatal neurons of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). J. Med. Chem. 29:424-427, 1986.

Fuller, R.W. Characterization of the neurotoxic potential of m-methoxy-MPTP and the use of its N-ethyl analogue as a means of avoiding exposure to a possible parkinsonism causing agent. J. Med. Chem. 29:1517-1520, 1986.

Fuller, R.W. and Hemrick-Luecke, S.K. Inhibition of types A and B monoamine oxidase by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. J. Pharmacol. Exp. Ther.232:696-701, 1985.

Fuller, R.W. Robertson, D.W. and Hemrick-Leucke, S. Persistent depletion of striatal dopamine in mice by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Biochem. Pharmacol. 35:143-144, 1986.

Gerhardt, G. Rose, Stromberg, G. Obson, L. Jonsson, G. and Hoffer, B. Dopaminergic neurotoxicity of 1-mthyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouswe: An in vitro electerochemical study. J. Pharmacol. Exp. Therp. 253:259-265, 1985.

Gibb, W.R.G., Lees, A.J., Jenner, P., Marsden, C.D. The dopamine neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces histological lesions in the hypothalamus of the common marmoset. Neurosci. Lett., 65:79-83, 1986.

Greenawalt, J.W., Schnaitner, C. An appraisal of the use of monoamine oxidase as an enzyme marker for the outer membrane of rat liver mitochondria. J. Cell Biol., 216:173-179, 1970.

Gupta, M., Felten, D.L., Felten, S.Y. MPTP alters monoamine levels in systems other than the nigrostriatal dopaminergic system in mice. MPTP: a neurotoxin producing a parkinsonian syndrome., ed. by Markey, S., Castagnoli, N. Jr., Trevor, A.J., Koplin, I.J., New York: Academic Press, 58:177-181, 1986.

Hallman, H.L., Olson, L. and Jonsson, G. Neurotoxicity of meperidine analogue N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. Eur. J. Pharmacol. 97:133-136, 1984.

Harrison, R. Oberservations on the living developing nerve fiber. Anat. Rec. 1:116-118, 1907.

Hausler, G. and Haefely, W. Modification of release by adrenergic neuron blocking agents and agents that alter the action potential, In: Paton, P.M. (Ed.), The release of catecholamines from adrenergic neurons, Pergamon Press, Oxford. p. 185, 1979.

Heath, J. W., Hill, C.E. and Burnstock, G. Axon retraction following guanethidine treatment: studies of sympathetic neurons in tissue culture. J. Neurocytol. 3:263-276, 1974.

Heikkila, R. E., Hess, A. and Duvoisin, R. Dopaminergic neuro-toxicy of 1-methyl-4-phenyl-4-1,2,3,6-tetrahydro-pyridine in mice. Science, 224:1451-1453, 1984a.

Heikkila, R.E. and Manzino, L. Behavioral properties of GBR 12909, GBR 13069 and GBR 13098: specific inhibitors of dopamine uptake. Eur. J. Pharmacol., 103:241-248, 1984.

Heikkila, R. E., Manzino. L., Cabbat, F.S. and Duvoisin, R. Protection against the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by MAO inhibitors in mice. Nature, 311:467-469, 1984b.

Hemmendinger, L.M., Garber, B.B., Hoffmann, P.C., Heller, A. Target neuron-specific process formation by embryonic mesencephalic dopamine neurons in vitro. Proc. Natl. Acad. Sci. USA, 78:2, pp. 1264-1268, 1981.

Hendry, T.A. and Campbell, J. Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. J. Neurocytol., 5:351-360, 1976.

Hill, C.E., Mark, G.E., Eranko, O., Eranko, L. and Burnstock, G. Use of tissue culture to examine the actions of guanethidine and 6-hydroxydopamine. Eur. J. Pharmacol. 23:162-174, 1973.

Hokfelt, T., Johansson, O., Fuxe, K., Goldstein, M. and Park, D. Immunohistochemical studies on the localization and distribution of monoamine neuron systems in the rat brain. I. Tyrosine hydroxylase in the mes- and diencephalon. Med. Biol., 54:427-453, 1976.

Hokfelt, T., Johansson, O., Fuxe, K., Goldstein, M. and Park,D. Immunohistochemical studies on the locatization and distribution of monoamine neuron systems in the rat brain. II Tyrosine hydroxylase in the telencephalon. Med. Biol., 55:21-40, 1977.

Hornykiewicz, O. Dopamine ((3-hydroxytyramine) and brain function. Pharmacol. Rev., 18:925-964, 1966.

Irwin, I. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP: a key to Parkinson's disease? Pharm. Res., 3:7 1986.

Javoy-Agid, F. and Agid, Y. Is the mesocortical dopaminergic system involved in Parkinson's disease? Neurology 30:1326-1330, 1980.

Javoy-Agid, F., Reiberg, M., Taguet, H., Boboza, B., and Agid, Y. Biochemical neuropathology of Parkinson's Disease. Adv. Neurology, 40:189-197, 1984.

Javoy-Agid F., Taquet, H., Cesselin, F., Epelbaum J., Grouselle, S., Manborgne, A., Studler, J. M., and Agid, Y. Neuropeptides in Parkinson's Disease. In: Catecholamines: Neuropharmacology and the Central Nervous System-Therapeutic Aspects, ed. E Usdin, pp. 35-42, A.R. Liss, New York, 1984.

Javitch, J.A. and Snyder, S.H. Uptake of MPP+ by dopamine neurons explains selectivity of Pakinsonism-inducing neurotoxin, MPTP. Eur. J. Pharmacol. 10:455-456, 1985.

Javitch, J.A., D'Amato, R.J., Strittmatter, S. M. and Snyder, S.H. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: Uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc. Natl. Acad. Sci. U.S.A., 82:2173-2177, 1985.

Jellinger, K. Pathology of Parkinsonism. In: Recent Developments in Parkinson's Disease; eds. S Fahn, C.D. Marsden, P. Jenner & P. Teychenne, pp. 33-66, Raven Press, N.Y., 1986.

Jellinger, K. and Seitelberg, F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical morphological and neurochemical correlations. J. Neurolog. Sci.,20:415-455,1973.

Johnson, E.M. and Aloe, L. Supression of the in-vitro and in vivo cytotoxic effects of guanethidine in sympathetic neurons by nerve growth factor. Brain Research, 81:519-532, 1974.

Johnson, E.M. Jr. and O'Brien, F. Evaluation of the permanent sympathectomy produced by the administration of guanethidine to adult rats. J. Pharmacol. Exp. Therap. 196:53-61, 1975.

Johnson, E.M. Jr., O'Brien, F. and Werbitter, R. Modification of the permanent sympathectomy produced by the administraton of guanethidine to new born rats, 1972.

Johnson, E. M., Marcia, R.A. and Yellin, T.O. Marked difference in susceptibility of several species to guanethidine-induced chemical sympathectomy. Life Sciences 20:107-112, 1977.

Johnson , E.M. and Manning, P.T. Guanethidine-induced destruction of sympathetic neurons. Inter. Rev. Neurobiol. 25:1-37, 1984.

Kaumann, A., Basso, N. and Aramendia, P. The cardiovascular effects of N-(gamma-methylamino-propyl-imino-dibenzyl)-HCl (desmethylimipramine) and guanethidine, J. Pharmacol Exp. Ther. 147:54, 1965.

Kish, S., Rajput, A., Gilbert, J., Rozdilsky, B., Chany, L.J., Shannak, K. and Hornykiewicz, O. Elevated gamma-aminobutyric acid level in striatal but not extrastriatal brain regions, Ann. Neurol., 20:26-31, 1986.

Knoll, J. Deprenyl (Selegiline): the history of its development and pharmacological action. Acta Neural. Scand. [Suppl.] 95:57-80, 1983.

Kotake, Connie, Hoffmann, P.C. and Heller, A. The biochemical and morphological development of differentiating dopamine neurons co-aggregated with their target cells of the corpus striatum in vitro, J. of Neurosci., 2:9, pp. 1307-1315, 1982.

Langston, J.W. Ballard, P., Tetrud, J.W. and Irwin, I. Chronic parkinsonism in humans due to a product of meperidine-analogue synthesis. Science, 219:979-980, 1983.

Langston, J.W. and Ballard, P. Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): Implications for treatment and the pathogenisis of parkinson's disease. Can. J. Neurol. Sciences, 11:160-165, 1985.

Langston, J.W., Forno, L.S., Rebert, C.S. and Irwin, I. Selective nigral toxicity after systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the squirrel monkey. Brain Res. 292:390-394, 1984a.

Langston, J.W. Irwin, I. and Langston, E.B. and Forno, L.S. Pargyline prevents MPTP-induced parkinsonism in primates. Science, 225:1480-1482, 1984b.

Lau, Y.S. and Fung, Y.K. Pharmacological effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on striatal dopamine receptor system. Brain Res. 369-311-315, 1986.

Lauder, J.M. and Bloom, F.E. Ontogeny of monoamine neurons in the locus coeruleus, raphe nuclei and substantia nigra of the rat. J. Comp. Nuer., 155:469-482, 1981.

Lee, C.H., Strosberg, A.M.and Warren, L.A. The importance of catecholamine uptake inhibition in the reversal of guanethidine blockage of adrenergic neurons. Res. Commun. in Chem. Pathol. and Pharmacol., 30:1, pp. 3-14, 1980.

Lee, M. Javitch, A., and Snyder, S.H. Characterization of 3[H] Desipramine binding associated with neuronal norepinephrine uptake sites in rat braim membranes., J. of Neurosci., 2:10, pp. 1515-1525, 1982.

Lesko, S.A., Lorontzen, R.J. and Iso, P.O.P. Role of suproside in dioxyribonuclaic and strand scission. Biochemistry, 19:3025-3028, 1980.

Levi-Montalcini, R., Aloe, L., Mugnaini, E., Oesch, F. and Thoenen, H. Nerve growth factor induces volume increase and enhances tyrosine sympathetic ganglia of newborn rats. Proc. Natl. Acad. Sci., 72:595-599,1975.

Levitt, P., Pintar, J. and Breakefield, X. Immunocytochemical demonstration of monoamine oxidase B in brain astrocytes and serotonergic neurons. Proc. Natl. Acad. Sci., 79:6385-6389, 1982.

Lewy, F.H. Paralysis agitans. Pathologicale Anatomie. In: Handbuch der Neurologie, ed. M. Lewandovsky, Springer, Berlin, pp. 920-933, 1912.

Lewy, F. H. Zur Pathologischen Anatomie der Paralysis Agitans. Dtsch. Z. Nervendeilk, 50:50-55, 1913.

Lieberman, A., Dziatolowski, M., Kupersmith, M., Goodgold, A., Korein, J. and Goldstein, M. Dementia in Parkinson Disease. Ann. Neurol., 6:355-359, 1979.

Lloyd, K. G., Mohler, H., Hertz, P., and Bartholini, G. Distribution of choline acetyltransferase and glutamic acid decarboxylase within the substantia nigra and other brain regions from control and parkinsonian patients. 1975.

Lloyd, K.G. and Hornykiewicz, O. L-Glutanic acid decarboxylase in Parkinson's disease: effect of L-Dopa therapy. Nature, 243:521-523, 1973.

Lloyd, K.G. and Hornykiewicz, O. Clinical evidence for the involvement of catecholamines in the control of movement, Catecholamines and Behavior, 1:42-54, 1975.

Lowry, O.H., Rosebrough, N.J., Farr, A. L. and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265-275, 1951.

Mann, D. M. A. Yates, P. O., and Hawkes, J. The pathology of the human locus coeruleus. Clin. Neuropathol., 2:1-7, 1983.

Manning, P.T., Powers, C.W., Schmidt, R.E. and Johnson, E.M. Jr. Guanethidine-induced destruction of peripheral sympathetic neurons occurs by an immune-mediated mechanism. J. Neurosci., 3:4, pp. 714-724, 1983.

Manning, P.T., Russell, J.H., Simmons, B.and Johnson, E.M. Jr. Protection from guanethidine-induced neuronal destruction by nerve growth factor: effect of NGF of immune function. Brain Research, 340:61-69,1985.

Markey, S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S. and Herkenman, M.A. Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. Nature 311:464-467, 1984.

Martinovits, G., Melamed, E., Cohen, O., Rosenthal, J. and Uzzan, A. Systemic administration of antioxidants does not protect mice against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). Neurosci. Lett., 69:192-197, 1986.

Maxwell, R.A. Guanethidine after twenty years: A pharmacologist's perspective, Br. J. Clin Pharmac. 13:35-44, 1982.

Mayeux, R., Stern, Y., Rosen, J., and Leventhal, J. Depression, intellectual impairment, and Parkinson disease. Neurology, 31:645-649, 1981.

Mitchell, I.J., Cross, A.E., Sambrook, M.A. and Crossman, A.R. Sites of the neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the macaque monkey include the ventral tegmental area and the locus coeruleus. Neuroscience Lett. 61:195-200, 1985.

Mitchell, I.J., Cross, A.E., Sambrook, M.A. and Crossman, A.R. Neural mechanisms mediating 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine-induced parkinsonism in the monkey. Neuroscience Lett. 63:61-65, 1986.

Mitchell, J.R., Cavanaugh, J.H., Arias, L. and Oates, J.A. Guanethidine and related agents III. Antagonism by drugs which inhibit the norepinephrine pump in man. J. Clin. Invest., 49:1596-1604, 1970.

Murphy, D.L., Redmond, D.E., Garrick, N. and Baulu, J. Brain region differences and some characteristics of monoamine oxidase type A and B activities in the vervet monkey. Neurochem. Res., 4:53-62, 1979.

Mytilineou, C. and Cohen, G. 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine destroys dopamine neurons in explants of rat embryo mesencephalon. Science 225:529-531, 1985.

Mytilineou, C. and Cohen, G. Deprenyl protects dopamine neurons from the neurotoxic effect of 1-methyl-4-phenylpuridinium ion (MPP+). J. Neurochem. 1985.

Mytilneou, C. Cohen, G. and Heikkila, R.E. 1-methyl-4-phenyl-pyridine (MPP+) is toxic to mesencephalic dopamine neurons in culture. Neurosci. Lett. 57:19-24, 1985.

Mytilineou, C., Dembiec-Cohen, D., Van Woert, M. and Hwang, E. Explant cultures of dog substantia nigra and striatum: a model for the study of nigro-striatal dopamine neurons. J. Neural Transm., Suppl. 19 (1983) 37-51.

Nash, C.W., Costa, E. and Brodie, B.B. The actions of reserpine, guanethidine and metaraminol on cardiac catecholamine stores, Life Sci. 3:441, 1964.

Nasmyth, P.A. and Andrews, W.H.H. The antagonism of cocaine to the action of choline alpha,6-xylyl ether bromide at sympathetic nerve endings. Br J. Pharmacol. 14:477, 1959.

Naville, F. Etudes sur les complications et les sequellesmentales de l' encephalite epidemique la bradyphrenie. Encephale. 17:369-375;423-436,1922.

Nicklas, W.J., Vyas, I., and Heikkila, R. E. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenylpyridine, a metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Life Sci., 36:2503-2508, 1985.

Nilsson, O. Dahlstrom, A., Geffard, M., Ahlman, H. and Ericson, L.E. An improved immunocytochemical method for subcellular localization of serotonin in rat enterochromaffin cells. J. Histochem. Cytochem., 35:3, pp. 319-326, 1987.

Peichl, L. and Bolz, J. Kainic acid induces sprouting of retinal neurons. Science, 223, pp. 503-504, 1984.

Perry, T.L. Young, V.W. Jones, K. Wall, R.A. Clavier, R.M. Foulks,J.G. and Wright, J.M. Effects of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and its metabolite, N-methyl-4-phenyl-pyridinium ion, on dopaminergic nigrostriatal neurons in the mouse. Neurosci. Lett. 58:321-326, 1985a.

Perry, T.L., Yong, V.W., Clavier, R.M., Jones, K., Wright, J.M., Foulks, J.G. and Wall, R.A. Partial protection from the dopaminergic neurotoxin N-Methyl-4-phenyl, 1,2,3,6-tetrahydropyridine by 4 different antioxidants in the mouse. Neurosci. Lett. 60:109-114, 1985b.

Prochiantz, A., Di Porzio, U., Kato, A., Berger, B. and Glowinski, J. In vitro maturation of mesencephalic dopaminergic neurons from mouse embryos is enhanced in the presence of their striatal cells. Proc. Natl. Acad. Sci. 76:5387-5391, 1979.

Pileblad, E., Fornstedt, B., Clark, D. and Carlsson, A. Acute effects of 1-methyl-4-phenyl-tetrahydropyridine on dopamine metabolism in mouse and rat striatum. J. Pharm. Pharmacol. 37:707-712, 1985.

Ramsay, R.R., Salach, J.I., Dadgar, J., Singer, T.P. Inhibition of mitochondrial NADH dehydrogenase by pyridine derivatives and its possible relation to experimental and idiopathic parkinsonism. Biochem. Biophys. Res. Commun. 135:269-275, 1986.

Ramsay, R.R. and Singer, T.P. Energy-dependaent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. J. Biol. Chem., 261:7585-7587, 1986.

Ricaurte, G.A., Langston, J.W. Delanney L.E., Irwin, I. and Brooks, J.D. Dopamine uptake blockers protect against the dopamine depleting effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the mouse striatum. Neurosci. Lett. 59:259-264, 1985.

Ricaurte, G.A., Langston, J.W., Irwin, I. and Forno, L.S. The neurotoxic effect of MPTP on the dopaminergic cells of the substantia nigra inmice is age-related. Soc. Neurosci. Abstr., 11:631, 1985.

Ross, W. Block, E.R. and Chang, R. Paraquat induced DNA damage in mammalian cells. Biochem. Biophys. Res. Commun., 91:1302-1308, 1979.

Salach, J.I., Singer, T.P., Castagnoli, N. Jr., and Trevor, A. Oxidation of the neurotoxic amine 1-methyl-4-phenyl-1,2,3,6tetra-hydropyridine (MPTP) by monoamine oxidases A and B and suicide inactivation of the enzymes by MPTP. Biochem. Biophys. Res. Commun., 125:831-835, 1984.

Sanchez-Ramos, J.R., Barnett, J.N., Goldstein, M., Weiner, W.J. and Hefti, F. 1-Methyl-4-phenylpyridinium (MPP+) by not 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively destroys dopaminergic neurons in cultures of dissociated rat mensecephalic neurons. Neurosci. Lett. 72:215-220, 1986.

Sannerstedt, R. and Conway, J. Hemodynamic and vascular responses to antihypertensive treatment with adrenergic blocking agents: A review, Am Heart J. 79, 122, 1970.

Sayre, L.M. Arora, P.K., Feke, S.C. and Rubach, F.L. Mechanism of induction of parkinson's disease by 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP). Characterization of a geminal-dimethyl-blocked analogue of a postulated toxic metabolite. J. Am. Chem. Soc. 108:2464-2466, 1986.

Scatton, B. Javoy-Agid, F. Rouquier, L., Dubois, B. and Agid, Y. Reduction of cortical dopamine, noradrenaline, serotonin and their metabolites in Parkinson's disease. Brain Research, 275:321-328, 1983.

Schanker, L.S. and Morrison, A. S. Physiolkogical disposition of guanethidine in the rat and its uptake by heart slices, Int J. Neuropharacol. 4:27, 1965.

Scheff, S., Benardo, L., and Cotman, C. Progressive Brain Damage accelerates axon sprouting in the adult rat. Science, 197:795797, 1977.

Schneider, J.S. and Markham, C.H. Neurotoxic effects of N-methyl-4-phenyl (MPTP) in the cat. Tyrosine hydroxylase immunocytochemistry. Brain Res. 373:258-267, 1986.

Schultz, W. Depletion of dopamine in the striatum as an experimental model of parkinsonism: direct effects and adaptive mechanisms. Prog. Neurobiol., 18:121-166, 1982.

Schultz, W., Scarnati, E., Sundstrom, E. Tsutsumi, T. and Johnson, G. The catecholamine uptake blocker nomifensine protects against MPTP-induced parkinsonism in monkeys. Exper. Brain Res. 63:216-220, 1986.

Schneider, J.S., Markham, C.H. Immunohistochemical localization of monoamine oxidase-B in the cat brait: clues to understanding N-Methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) toxicity. Exp. Neurol., 97:465-481, 1987.

Shen, R., Abell, C.N. Gessner, N. Brossi, A. Serotonergic conversion of MPTP and dopaminergic accumulation of MPP+. Fed. Eur. Biochem. Soc. 189:225-230, 1985.

Shore, P.A. and Giachetti, A. Dual actions of guanethidine on amine uptake mechanisms in adrenergic neurons, Biochen. Pharmacol. 15: 899, 1966.

Singer, T.P., Castagnoli, N. Jr., Ramsay, R.R., and Trevor, A.J. Bopchemical events in the development of Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. J. Neurochem., 49:1-8, 1987.

Singer, T.P., Salach, J.I., Castagnoli, N. Jr., and Trevor, A.J. Interactions of the neurotoxic amine MPTP with monoamine oxidases. Biochem. J., 235:785-789, 1986.

Singer, T.P., Salach, J.I. and Crabtree, D. Reversible inhibition and mechanism-based irreversible inactivation of monoamine oxidases by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Biochem. Biophys. Res. Commun., 127:707-712, 1985.

Snyder, S.H. and Coyle, J.T. Regional differences in 3H-norepinepherine and 3H-dopamine uptake into rat brain homo-genates, J. Pharmacol. Exp. Ther. 165: 78, 1969.

Specht, L.A., Pickel, V,M., Joh, T.H. and Reis, D.J. Light microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. I Early Ontogeny. J. Comp. Neurol., 199:233-253, 1981.

Specht, L.A., Pickel, V.M., Joh, T.H. and Reis, D.J. Light microscopic immunocytochemical localization of tyrosine hydroxylase in prenatal rat brain. II Late Ontogeny. J. Comp. Neurol., 199:255-276, 1981.

Stone, C.A., Porter, C.C.,Stavorsky, J.M., Luden C.T. and Totaro, J.A. Antagonism of certain effects of catecholamine-depleting agents by antidepressant and related drugs, J. Pharmacol Exp. Ther. 144:196, 1964.

Stutzin, A., Paravic, F., Ormeno, G. and Orrego, F. Guanethidine effects on the guinea pig vas deferens are antagonized by the blockers of calcium-activated conductance, apamin, methylene blue, and quinine, Molec. Pharmacol. 23:409.

Sundstrom, E., and Jonnson, G. Pharmacological interference with the neurotoxic actions fo 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholaminergic neurons in the mouse. Eur. J. Pharmacol. 110:293-299, 1985.

Taber, E., Brodal, A. and Wallberg, F. The raphe nuclei of the brain stem of the cat. I. Normal topography and cytoarchitecture and general discussion. J. comp. Neurol., 114:161-188, 1960.

Tarsy, D. and Baldessarini, R.J. Behavioral supersensitivity to apomorphine following chronic treatment with drugs which interfere with the synaptic fuction of catecholamines. Neuropharmacol. 13:927-940, 1974.

Teitelman, G., Baker, H., Joh, T.H. and Reis. Appearance of catecholamine-synthesizing enzymes during development of rat sympathetic nervous system:passible role of tissue environment. Proc. Natl. Acad. Sci., 76:509-513, 1979.

Thoenen, H. and Barde, Y.A. Physiology of nerve growth factor. Physiol. Rev., 60:1284-1335, 1980.

Thoenen, H., Hurlimann A. and Haefely, W. Interaction of phenoxybenzamine with guanethidine and bretylium at the sympathetic nerve endings of the isolated perfused spleen of the cat, J. Pharmacol. Exp. Ther. 151:189, 1966.

Ungerstedt, U. Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigrostriatal dopamine system. Acta physiol. Scand. Suppl. 367:69-78, 1971.

Ungerstedt, U. Mechanisms of dopamine neurons. Pharmacol. Ther. 2:27-40, 1975.

Wagner, G.C., Jarvis, M.F. and Carelli, R. Ascorbic acid reduces the dopamine depletion induced by MPTP. Neuropharm. 24:1261-1262, 1985.

Wagner, G.C. Jarvis, M., Rubin, G. L-Dopa reverses the effects of MPP+ toxicity. Psychopharm. 88:401-402, 1986.

Wakshull, E., Johnson, M.I., and Burton, H. Persistance of an amine uptake system cultured rat sympathetic neurons which use acetylcholine as their transmitter. J. Cell Biol. 79:121-131, 1978.

Watanabe, I., Vachal, E. and Tomita, T. Dense core vessicles in incidental Parkinson's disease: an electron microscopic study. Acta Neuropathol. 39:173-175, 1977.

Weinberger, J. and Cohen, G. The differential effect of ischemia on the active uptake of dopamine, gamma-amino butyric acid, and glutamate by brain synaptosomes, J. Neurochem. 38:963, 1982.

Westlund, K., Denney, R., Kochsperger, L., Rose, R. and Abell, C. Distinct monoamine oxidase A and B populations in primate brain. submitted, 1985.

Whetsell, W.O., Jr., Mytilineou, C. Shen, J. and Yahr, M.D. The development of the dog nigrostriatal system in organotypic cultures. J. Neural. Transm. 52:149-161.

Youdin, M.B.H. Modification of methods, Research Methods in Neurobiology, 3:167-207, ed. by N. Marx, R. Bolnight, N.Y. 1975.

Zimmerman, D.M. Cantrel, B.E. Reel, J.K. Hemrick-Luecke, S.K., Fuller, R.W., Characterization of the nuurotoxic potential of m-methoxy-MPTP and the use of its N-ethyl analogue as a means of avoiding exposure to a possible Parkinsonism-causing agent. J. Med. Chem., 29:1517-1520, 1986.