Androgen Modulation of Dopamine Transporter Function in the Corpus Striatum of Male Rats

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ABSTRACT

This thesis examined the relationship between the presence or the absence of testosterone on the function of the dopamine transporter system (DAT) in adult male rats. Eighteen male rats were assigned to control, castrated (GNX), and castrated with testosterone replacement (GNX+T) groups. Using an in vitro superfusion technique, the corpus striatum from each rat was divided and treated with dopamine, MPP^+, and NMF infusions. Striatal dopamine and DOPAC levels were measured during baseline conditions and after drug infusion. Results of our experiments demonstrated that dopamine and DOPAC recovery were greatest in the GNX rats and were decreased in the intact or the GNX+T animals. Testosterone appears to be an important steroid that modulates the function of the DAT. Testosterone acts to enhance DAT activity while the loss of testosterone reduces DAT function. With respect to activity of neurotoxins (such as MPP^+) to cause symptoms of PD, our results suggest that men may be more prone to this disease due to the facilitatory actions of testosterone on the DAT activity. Further experiments are needed to examine in detail the mechanisms of action of testosterone on DAT function and to explore the relationship DAT function and neurodegenerative diseases.

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INTRODUCTION

Men and women differ in the risk factors that expose them to Parkinson's disease (PD), according to a recent analysis. It is not clear why men are more prone to getting the disease, but it is clear that it is related to a lack of female hormone, estrogen (Dluzen; McDermott 2000). The general population that is at greater risk for developing PD are people older than fifty. The disease is related to a motor system disorder that causes the patient's hands, legs, jaw, face, and arms to tremor. Parkinson's disease may also cause bradykinesia (slowness of movement) as well as a loss of balance, and difficulty in doing ordinary daily activities such as walking, talking, eating, and writing (Calne and Calne, 2001). Parkinson's disease occurs when certain nerve cells, or neurons, in an area of the brain known as the *substantia nigra* die or become impaired. Normally, these neurons produce an important brain chemical known as dopamine. Dopamine is a chemical messenger responsible for transmitting signals between the substantia nigra and the next "relay station" of the brain, the corpus striatum, to produce smooth, purposeful muscle activity. Loss of dopamine causes the nerve cells of the striatum to fire out of control, leaving patients unable to direct or control their movements in a normal manner. Studies have shown that Parkinson's patients have a loss of 80 percent or more of dopamine-producing cells in the substantia nigra. The cause of this cell death or impairment is not known but significant findings by research scientists continue to yield fascinating new clues to the disease. One theory holds that free radicals — unstable and potentially damaging molecules generated by normal chemical reactions in the body — may contribute to nerve cell death thereby leading to Parkinson's disease. Free radicals are unstable because they lack one electron; in an attempt to replace this missing electron, free radicals react with neighboring molecules (especially metals such as iron), in a process called oxidation. Oxidation is thought to cause damage to tissues, including neurons. Normally, free radical damage is kept under control by antioxidants, chemicals that protect cells from this damage. Evidence that oxidative mechanisms may cause or contribute to Parkinson's disease includes the finding that patients with the disease have increased brain levels of iron, especially in the substantia nigra, and decreased levels of

ferritin, which serves as a protective mechanism by chelating or forming a ring around the iron, and isolating it.

Parkinson's disease (PD) is a progressive neurodegenerative disorder, of unknown cause, found mostly in elderly patients. In this disorder, dopamine neurons located in the substantia nigra are damaged and are gradually destroyed, and therefore, the nigrostriatal pathway degenerates. The substantia nigra and the striatum are part of the basal ganglia, which is the motor-center of the brain. As the cells in the substantia nigra die, the levels of dopamine in the striatum also diminish. When these damaged cells are examined under a microscope one can distinguish pink stained spheres within them. These spheres are called Lewy bodies and are considered a reliable indicator of PD. What these bodies are, or how or why they come about, is still unknown. Individuals with PD have difficulty initiating and performing complex, sequential movements. PD produces a constellation of symptoms including rigidity, hypokinesia, and tremor often accompanied by dementia and depression (Fisher, Hanin et al. 1986)

In the early stages of PD, the brain can counteract the effects of missing dopamine by increasing the number of dopamine receptors in the striatum, however, once 80% of the brain's normal level of dopamine is lost, Parkinsonian symptoms begin to appear. Without the crucial brain messenger dopamine, produced by the neurons in the substantia nigra, critical brain signals go awry, resulting in symptoms that vary widely from individual to individual (Koller 1995). Although the most prominent neuropathlogical abnormality in PD is destruction of the nigrostriatal dopaminergic neurons, other cell groups, including noradrenergic cells of the locus coeruleus, dopaminergic cells of the ventral tegmental area, and cholinergic neurons of the nucleus basalis of Meynert, are also affected in many cases (Koller 1995).

About one in three patients also experience depression and nearly three of every four ultimately see some decline in their cognitive skills. Stooped posture, shuffling gait, loss of balance, fatigue, and difficulty speaking and swallowing are common (Koller 1995). Tasks like brushing one's teeth, handwriting, shaving, fastening small buttons, and stirring coffee gradually become more and more difficult and patients may end up in a wheelchair or bedridden (Calne and Calne 2001).

Dopamine Circuits in the Brain

In addition to the dopamine neurons found in the retina of eye, there are three main divisions of dopamine neurons in the brain. One of these circuits is found in the hypothalamic-pituitary axis. The neuron cell bodies are found in the hypothalamic region and they project their axons towards the pituitary gland. This division is considered to be part of the endocrine system.

The second division, are dopaminergic neurons located next to the substantia nigra in the ventral tegmental area. They send their axons to the cerebral cortex and the limbic system. This pathway is involved in one of the most devastating mental illnesses known, schizophrenia.

The third division is the best understood one of all. It is the projection of dopaminergic neurons from the substantia nigra (*dark substance*), located in the lower part of the midbrain, deep below the cerebral cortex in the cerebrum. This circuit represents about three-quarters of all dopaminergic neurons found in the brain and more importantly these dopaminergic neurons play a very important role in movement regulation (Thompson, 1985). The substantia nigra is a brown and black-pigmented region in the brain. Histological studies done on the substantia nigra and locus coeruleus in the human brain verified that the pigmentation of that region is similar to melanin, so it was called neuromelanin. As mentioned earlier, an indicative cause of PD is the selective death of the neurons of the substantia nigra. The death of these neurons can be linked to neurotoxic compounds, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and paraquat an herbicide, which has a similar structure to MPTP. MPTP causes cell death by its conversion to MPP^+ through monoamine oxidase type B, which stops the respiratory chain at the NADH-CoQ1 reductase stage (Zecca, 2001).

Following the dopaminergic neurons from the substantia nigra down the axon into the nerve terminal we arrive at a region called the basal ganglia. The direct correlation between PD and this region of the brain is that the basal ganglia is involved in modifying minute-to-minute voluntary movements and maintaining posture. The basal ganglia consists of the corpus striatum, the subthalamic nucleus, and the substantia nigra. The corpus striatum is the largest structure of all and it is divided into three components, the caudate nucleus, the putamen, and the globus pallidus. The striatum is considered to be a

relay station for the neurons projecting from the substantia nigra. Since dopamine is the neurotransmitter synthesized in the substantia nigra and released in the corpus striatum, it is responsible for producing smooth and voluntary muscle activity. The reward pathway is a neural network in the middle of the brain that prompts good feelings in response to certain behaviors, such as relieving hunger, quenching thirst or having sex, and it thereby reinforces these evolutionarily important drives. However, the circuit also responds to drugs of abuse, such as heroin, cocaine, amphetamine and nicotine, which seem to hijack the circuitry, altering the behavior of its neurons (Creutz and Kritzer, 2002).

Dopamine as a Chemical Messenger

Dopamine is a chemical messenger that tells the body how to move and what action to take, and it is responsible for how we think and act. Some dopamine present in the brain activates the frontal lobes, which integrates thoughts, feelings, and sensory information. The frontal lobes then choose which action the body has to take next. Therefore, if dopamine is missing, a person becomes unfocused and easily distracted (Filley 1995). These symptoms can be so faint that they are hardly noticed, but they can also be very serious. A lack of dopamine can lead to brain dysfunctions such as PD. It has also been suggested that an abnormal increase in the amounts of dopamine can lead to schizophrenia. It is also known that a dysfunction of the dopamine system is responsible for drug addiction (William 1995).

A quarter century ago, in 1975, it was suggested that dopamine was responsible for depression. This deficiency of dopamine in our body can be treated with nutrients and amino acids, which are the raw materials that our body uses to make this neurotransmitter naturally. If that doesn't work, prescribing a drug called amphetamine, which is also known as speed, can also treat it. However this treatment may be worse than the symptoms. Some say that amphetamines are one of the most dangerous medications ever discovered (Fisher 1986). Amphetamines are similar to dopamine, but they can injure tissue, interfere with growth and development, cause sleep problems or aggressive and depressed moods. Dopamine is associated with feelings of pleasure and elation. A hug, a kiss, a word of praise or a winning poker hand can elevate your mood. Scientists say that

dopamine is a chemical that transmits pleasure signals. It is also the master molecule of addiction. Drug addiction may result from malfunctions of the dopamine neuron neurotransmission system and lead to mental illness (Hadley 1996).

Synthesis of Dopamine

Dopamine synthesis, like that of all catecholamines in the nervous system, originates from the amino acid tyrosine, which must be transported across the blood–brain barrier and into the dopamine neuron. Once L-tyrosine gains entry into the neuron it is converted to L-3,4dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase. DOPA is subsequently converted to dopamine by L-aromatic amino acid decarboxylase. This latter enzyme turns over so rapidly that DOPA levels in the brain are negligible under normal conditions (Amara, 1998). Because of the high activity of this enzyme and the low endogenous levels of DOPA normally present in the brain, it is possible to significantly enhance the formation of dopamine by providing this enzyme with increased amounts of this substrate (Figlewicz, 1999) (see illustration 1).

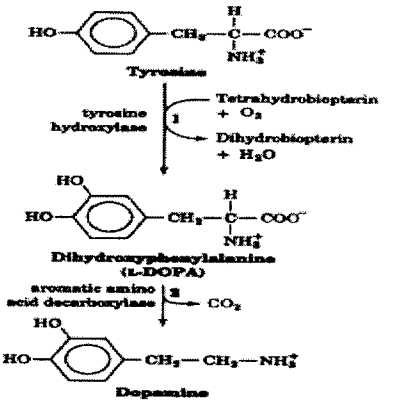


Illustration (1) Internet Source

After dopamine is synthesized, it is stored in vesicles at the active zone of a presynaptic neuron. Upon the arrival of an action potential it opens voltage gated Ca^{+2}

channels located on the neuronal membrane, allowing the influx of positive calcium ions. Extracellular Ca^{+2} diffuses into the terminals by means of these voltage gated Ca^{+2} channels (Brunger 2000). Before exocytosis, the vesicle first moves from the cytoplasm to the plasmalemma. Second, the vesicle becomes attached to the plasma membrane, a process often referred to as docking. Third, activation involving metabolic energy also referred to as priming occurs (Skehel, Wiley 1998). Through exocytosis, the vesicles discharge their contents (dopamine), into the synaptic cleft. The extent to which dopamine is released depends on the rate and pattern of the neuron's activity (Garris, Walker and Wightman 1997). Once returned to the sending neuron by the reuptake system, dopamine is subject to an enzyme named monoamine oxidase (MAO). MAO usually breaks down dopamine. If no other factors were at work, MAO would keep the amount of "used" dopamine fairly low. However, dopamine taken back into the nerve ending can return to the vesicle for storage. Once inside the vesicle, dopamine is protected from MAO.

A drug named reserpine prevents the reuptake of dopamine and some other neurotransmitters. Administering reserpine causes dopamine to remain exposed within the cell and broken down by MAO. This profoundly reduces the available dopamine.

Changing the action of MAO can help us treat diseases that involve dopamine transmission. For instance, the drug deprenyl inhibits MAO. This increases the stores of dopamine and slows the progression of Parkinson's disease. In higher doses, deprenyl enhances the effects of dopamine on behavior.

Interestingly, one form of MAO actually protects dopamine. This form of MAO, found in dopamine neurons, acts on substances in the neuron other than dopamine. Here MAO protects the "purity" of neurotransmission by breaking down other neurotransmitters

Treatments for Parkinson's Disease

Currently available drugs offer temporary relief from the symptoms of the disorder, but do not stop or reverse the neuronal degeneration caused by this disease. Available drugs are Levodopa and Carbidopa. Both of these drugs decrease the rigidity,

tremors, and other symptoms associated with Parkinson's disease. Levodopa or L-Dopa (L-3,4-dihydroxyphenylalanine) is the metabolic precursor of dopamine, which crosses the blood-brain barrier, and be converted to dopamine in the brain. This replaces the dopamine that has been lost due to Parkinson s disease. Meanwhile, Carbidopa is an enzyme inhibitor that works to protect L-Dopa in the peripheral circulation so more can reach the target tissue and be converted into the desired molecule (Sourkes, 1971). Other drugs are also available such as Bromocriptine, Amantadine, and Deprenyl. These drugs are dopamine agonistic drugs that target postsynaptic dopamine receptors. They mimic the effect of dopamine on the postsynaptic neurons and thus are considered in the treatment of Parkinson's disease (Pearce, 1978).

Bromocriptine, pergolide, pramipexole and ropinirole. These four drugs mimic the role of dopamine in the brain, causing the neurons to react as they would to dopamine. They can be given alone or with levodopa and may be used in the early stages of the disease or started later to lengthen the duration of response to levodopa in patients experiencing wearing off or on-off effects. They are generally less effective than levodopa in controlling rigidity and bradykinesia. Side effects may include paranoia, hallucinations, confusion, dyskinesias, nightmares, nausea, and vomiting (Pearce, 1978). Treating Parkinson's disease with surgery was once a common practice. But after the discovery of levodopa, surgery was restricted to only a few cases. Currently, surgery is reserved for patients who have failed to respond satisfactorily to drugs. One of the procedures used, called *cryothalamotomy*, requires the surgical insertion of a supercooled metal tip of a probe into the thalamus (a "relay station" deep in the brain) to destroy the brain area that produces tremors. This and related procedures, such as thalamic stimulation, are coming back into favor for patients who have severe tremor or have the disease only on one side of the body. Investigators have also revived interest in a surgical procedure called *pallidotomy* in which a portion of the brain called the globus pallidus is lesioned. Some studies indicate that pallidotomy may improve symptoms of tremor, rigidity, and bradykinesia, possibly by interrupting the neural pathway between the globus pallidus and the striatum or thalamus

A great deal of work has been done in the past several years to attempt to find treatments as an alternative to drug therapy. It has been shown that transplantation of

various kinds of catecholamine producing tissues into the striatum of PD patients can be beneficial as a treatment. The most favorable results have been obtained with the transplantation of fetal mesencephalic (nigral) tissue into the caudate nuclei of monkeys rendered Parkinsonian with MPTP. Significant motor behavioral improvement was observed in these animals (Garris, 1997).

The improvement seems to be due in part to dopamine derived from the fetal dopaminergic graft. It has been shown that MPTP selectively destroys the dopaminergic cells in the substantia nigra but spares the mesolimbic system originating from the ventral tegmental area. Because the effects of MPTP so closely mimic true idiopathic PD, it is reasonably safe to assume that the same occurs in PD brains (Fisher, 1986). The implantation of the tissue apparently stimulates these nearby remaining dopaminergic neurons to develop new dopaminergic fibers into the caudate and a pathway is thus established to carry host dopamine to the striatum. The transplantation of human fetal nigral tissue into the striatum of PD patients has been attempted a few times and has been relatively successful in combination with immunosuppressive therapy. However, widespread clinical application of human fetal tissue implantation obviously presents serious immunological and ethical problems (Kish, 1998).

Distribution of Dopamine Receptors and Autoreceptors in the Brain

The Dopamine Receptors

In the striatum, dopamine interacts with two major subfamilies of receptors, the D_1 subfamily and D_2 subfamily. Both subfamilies of receptors are located on postsynaptic neurons. The D_1 subfamily consists of two different types, the D_1 receptor and the D_5 receptor. This subfamily of receptors act via adenylate cyclase. The D_2 receptor subfamily contains the D_2, D_3, and D_4 subtype receptors and are found in high levels in the brain. D_2 and D_3 receptors are found in the striatum in greater abundance than the D_4 receptor. The D_4 receptor is mainly distributed in limbic areas of the brain (Young at el. 1999). The D_2 subfamily receptors have each been shown to inhibit adenylyl cyclase when expressed in recombinant cells. Although the signal via the D_3 receptor has been more difficult to demonstrate and it is generally found at lower levels than the other receptor subtypes.

Overall the D_2, D_3, and D_4 receptors exhibit pharmacological properties similar to those of the originally defined D_2 receptor. They all show high affinities for drugs such as the butyrophenones, e.g. haloperidol, and the substituted benzamides, e.g. sulpiride, and these classes of drugs provide selective antagonists for the D_2 subfamily receptors (Inase, Li and Tanji 1997). The D_2 like receptors also show high affinities for phenothiazines and thioxanthines. Each D_2 subfamily receptor does have its own pharmacological profile so that there are some differences in affinities of drugs for the individual D_2 subfamily receptors. For example, raclopride shows a high affinity for the D_2 and D_3 receptors, but a lower affinity for the D_4 receptor. Clozapine shows a slight selectivity for the D_4 receptor. The D_2 subfamily receptors show moderate affinities for typical dopamine agonists with the D_3 receptor generally showing higher affinities for agonists than the other subtypes. There are compounds available that are selective agonists for the D_2 subfamily receptors, e.g. NO 437 and quinpirole (Araki, Tanji, Kato and Itoyama 1998).

Dopamine Autoreceptor

Dopamine autoreceptors are receptors located on the presynaptic nerve terminal that produce feedback inhibition of dopamine synthesis and release. There are three kinds of autoreceptors classified according to their effects, I) impulse modulating autoreceptor, II) release modulating autoreceptor; and III) synthesis modulating autoreceptor. All of these autoreceptors show similar pharmacological profiles, but data suggests a difference in the second messenger system, which is responsible for the signal transduction cascade that mediates the autoreceptor response (Perachon, Schwartz and Sokoloff, 1999).

The dopamine autoreceptors are members of the D_2 receptor subfamily. They have a higher binding affinity compared to the effects of dopamine on the postsynaptic receptors. Several drugs have been synthesized that agonize or antagonize the autoreceptor. One of these is Pramipexole, a new dopamine receptor agonist with preference for D_3 compared to D_2 receptors. This drug is considered to be potent against the dopamine autoreceptor and at the same time it shows no affinity to other receptors of the D_2 subfamily. Given this fact, researchers suggest that such a drug would have limited side effects when treating PD, due to it's high binding affinity to the D_3 receptor and not to any other receptor. This drug also has been reported to have a neuroprotecting effect

for PD and may slow the progressive destruction of dopamine neurons (Grandas, 1999). Pramipexole produces its neuroprotection by interacting with hydroxyl free radicals in MPP^+ infused rats. It may also protect against oxygen free radicals produced in solutions and protect red blood cell membranes from lipid peroxidation, which have been reported in with patients with PD. These patients may refrain from taking L-dopa for several years as long as they are receiving pramipexole (Grandas, 1999).

On the other hand, antagonist drugs of the dopamine autoreceptor such as raclopride are known to block the receptor leading to an increase in dopamine neuronal activity (firing rate, synthesis, and release). These studies showed that raclopride binds to the dopamine autoreceptor with high affinity in the rat striatum, which in turns provides an indirect measure of changes in synaptic dopamine (Usidn et al. 1991; Shimada et al. 1992).

Monoamine Transporters

Monoamine transporters are plasma membrane transporters, also known as uptake pumps. They are one of the most efficient means of controlling extracellular catecholamine concentrations. There are monoamine transporters for dopamine (DAT), serotonin (SERT), and norepinephrine (NET). These are selectively expressed on the corresponding neurons and serve as targets of many psychostimulants, antidepressants, and neurotoxins.

The Dopamine Transporter (DAT)

The dopamine transporter is a plasma membrane protein located on the presynaptic nerve terminal of dopamine neurons. It is responsible for the termination of dopaminergic neurotransmission through transmitter reuptake from the synaptic cleft (Hitri et al., 1994; Kuhar, 1998). The DAT works through a concentration gradient, where released dopamine in the synaptic cleft has a higher concentration. This high concentration of dopamine is sensed by the DAT and dopamine is transported from the synaptic cleft back into the neuron terminal to be recycled and/or degraded. The importance of the DAT lies with its regulatory activity as a synaptic modulator of

dopamine levels. The re-uptake of dopamine by a presynaptic transporter protein is the primary mechanism for inactivating dopamine's synaptic effects (Figlewicz, 1999). The DATs are members of a family of Na^+ and Cl^- dependent neurotransmitter transporters responsible for the rapid clearance of dopamine from synaptic clefts. The primary sequence of the dopamine carrier contains multiple phosphorylation sites in the putative intracellular domains for cAMP dependent protein kinase, protein kinase C (PKC), and Ca^{+2}- calmodulin-dependent protein kinase. Numerous studies have examined the effects of phosphorylating conditions on DAT activity. The evidence for DAT modulation by phosphorylation is most compelling in the case of PKC. Activation of PKC inhibits DAT-mediated uptake through the rapid sequestration/internalization of DAT protein. Recent studies have demonstrated basal (P^{32}) orthophosphate incorporation into the DAT, which is increased by PKC activation (Zhu et al., 1997; Pristupa et al., 1998; Melikian and Buckley, 1999). The DAT undergoes endogenous phosphorylation in striatal synaptosomes that are regulated by activators of protein kinase C. The activation of protein kinase C involves sequestering the reaction components into membrane domains. (Vrindavanam et al., 1996; Huff et al., 1997; Vaughan et. al., 1997), and down-regulation of transport activity (Kitayama et al., 1994; Huff et al., 1997; Vaughan et al., 1997; Zhang et al., 1997).

Studies indicate that the dopamine transporter requires molecules that possess a phenyl ring with a primary ethylamine side chain for optimal activity, and the ß-rotamer of the extended conformation of catecholamines is transported preferentially (Meiergerd and Schenk, 1994). Recent transport studies on the cloned human dopamine transporter suggest that, although ß- or phenolic ring hydroxylation of a substrate results in a change in the K_m over a wide range, the presence of a phenolic hydroxyl group is not a perquisite for optimal function of the transporter. Compounds without a phenolic hydroxyl group such as ß-phenethylamine, amphetamines or MPP^+, can bind to the carrier and be transported with same the V_{max} (speed) as dopamine (Chen and Justice, 2000). Therefore, phenethylamine seems to be the most important structural element accommodated by the dopamine transporter (Giros and Caron, 1993).

Drugs Acting on the Dopamine Transporter

The Neurotoxin MPTP (MPP^+)

MPTP is a contamination of synthetic heroin and 1-methyl-4-phenylpyridinium (MPP^+) is metabolite of MPTP oxidized by the enzyme monoamine oxidase type B (MOA-B). MPP^+-induced dopaminergic neuronal degeneration is similar to that observed in PD. The amount of MPP^+ produced from MPTP depends on the amount of the MOA-B present. The site of activation of this neurotoxin is thought to be the glial cells found outside the nerve terminal. This is supported by studies that demonstrated MPTP was taken up by cultured astrocytes and metabolized into MPP^+ (Marin, 1999).

MPP^+ is considered to be a major neurotoxin that can mimic the neurodegeneration of neurons seen in PD. The effects of this neurotoxin that mimics PD are related to the chronic presence of MPP^+ in the neuron's powerhouse, the mitochondria. MPP^+ selectively accumulates inside dopaminergic neurons and causes the death of these neurons (Irwin and Langston, 1985). The mechanism of action of MPP^+ is thought to be through the generation of superoxide. When MPP^+ is found in sufficient amounts, it can overcome the powerhouse by inhibiting the mitochondrial complex I of the electron transport system leading to the generation of reactive oxygen species and eventually cell death (Marin, 1999).

MPP^+ also has an acute effect; once it reaches the inside of the neuron it causes the displacement of dopamine from their synaptic vesicles into the intracellular cytoplasm. Since the DAT moves dopamine down its concentration gradient, the displaced dopamine in the active zone of the neuron activates the DAT and causes the dopamine to be transported out of the nerve terminal and into the synaptic cleft leading to dopamine depletion (Qu, 1988).

In a study by Mandavilli and Van Houten (2000), the levels of dopamine and its metabolites, DOPAC and HVA, were measured by HPLC equipped with electrochemical detection. DNA damage was also measured by quantitative PCR in both mitochondrial and nuclear (beta-polymerase) targets from the caudate-putamen, substantia nigra, and cerebellum regions of control and MPTP-treated mice. They found MPP^+ treatment led to damage in both mitochondrial and nuclear DNA of the substantia nigra, while there was no damage in either the cerebellum or caudate putamen (Mandavilli, 2000). These

findings indicate that MPP^+ enters dopaminergic neurons of the nigrostriatal system via the dopamine reuptake system, where it is concentrated within mitochondria by active transport. MPP^+ inhibits NADH-ubiquinone reductase, which is the first enzyme-protein complex of the mitochondrial respiratory chain. By inhibiting this complex, MPP^+ probably induces nigrostriatal cell death by depleting cellular ATP levels (Dluzen 1996).

As mentioned before, MPP^+ is selective for the nigrostriatal system but it is not well understood why. The degeneration of dopamine neurons are detected in the nigro-striatum in brains with PD (Iwata, Keikilni and Gengy 1997). Also during aging, the number of dopamine neurons in the substantia nigra is known to decrease at a rate of about 5-10% neurons per decade, suggesting that dopamine neurons in this region are more vulnerable than in other regions and other types of neurons (Goulet, et. al., 1999). It has been thought that aging might account for the death of selective neuronal populations in certain progressive neurodegenerative disorders, including Alzheimer's disease and PD (Goulet, et. al., 1999).

Cocaine

Cocaine is a popular drug of abuse that possesses the properties of a local anesthetic and psychomotor stimulant. Cocaine blocks the DAT resulting in an increase in the extracellular concentration of dopamine and the accompanying physiological effects associated with cocaine (Ritz et al., 1987). This includes to the enhancement of sympathetic activity and potentiation of the action of dopamine and other neurotransmitters (Amara and Sonders, 1998). Chronic cocaine users eventually face depletion of their striatal dopamine, which also leads to some of the neurological and psychiatric complications which increase the risk for narcoleptic movement disorders (Wilson, 1996). The depletion of dopamine triggers the vicious cycle of craving for more cocaine. The behavioral effects of cocaine result from a powerful stimulation of the cortex and the brain stem. Cocaine acutely increases mental awareness and produces a feeling of well-being and euphoria, but it can also cause hallucinations, delusions and paranoia. It increases motor activity and at high doses causes tremors and convulsions followed by respiratory and vasomotor depression. Addiction to cocaine, once fully developed, may represent a true biological dependency on the drug. Cocaine can also

temporarily relieve the neurological defects seen with PD. This occurs because cocaine blocks the DAT and thus more dopamine is present in the synaptic cleft, which relieves some of the symptoms associated with PD (Amara and Sonders, 1998).

The DAT and the serotonin reuptake transporter (SERT) are the only monoamine transporters that can facilitate cocaine reward in chronic users. Neurotransmitter rearrangements in single and double knockout mice demonstrate compensations for transporter deletion. Similar changes might also follow long-term DAT or SERT blockade by drugs. Researchers have investigated the influence of cocaine in single DAT or SERT knockout mice that can retain some of the cocaine reward and in double knockout mice that do not experience a cocaine reward. The data obtained by the investigators indicates that the presence of dopaminergic neurons and the DAT are more important for cocaine reward than are serotonergic neurons (Sora, 2001).

Nomifensine

Nomifensine (NMF) is an antidepressant drug that possesses the ability to block the DAT. In contrast to cocaine, nomifensine does not have an addictive side effect to it. Thus, this drug potently inhibits the reuptake of dopamine, by inhibiting presynaptic DAT as well as the autoreceptor activities. Blockade of the DAT causes increased levels of synaptic dopamine, which results in prolonged postsynaptic receptor activation and ultimately motor activation (Garris, 2003).

Research done by Disshon and Dluzen (1999), demonstrated that after infusion of NMF into superfused striatal tissue the outcome showed an increase in dopamine recovery. Chronic infusion of d-amphetamine (AMPH) causes dopamine depletion, degeneration of nigrostriatal dopaminergic neurons, decreased activity of tyrosine hydroxylase and reduces in the number of DAT of rats. Treatment with NMF protects against AMPH-induced long-term dopamine depletion (Dluzen, 2000).

Gender Related Factors Contributing to Neural Protection

Monoamine transporters are known to be sensitive to changes in sex steroids and any variation in the levels of these hormones may alter the expression of these

transporters, which is known to be related to the pathophysiology of movement disorder, depression, schizophrenia, and obsessive compulsive disorder (Rehavi, 2000).

The impact of the female gonadal hormone estrogen upon the DAT has been well documented. Estrogen acts in the striatum to rapidly inhibit dopamine clearance from the synaptic cleft by inhibiting the DAT (Dluzen, 2000). Estrogen also decreased amphetamine (AMPH)-induced dopamine release from striatal tissue (McDermott and Liu 1994), enhanced dopamine turnover (Kelly and Lagrange 1999), and decreased dopamine receptor density in the striatum (Garris and Walker 1997). The Estrogen metabolites, estrone, estriol, and the non-steroid analog diethylstilbestrol do not produce these effects (Ekue and Boulanger 2002). Thus the steroidal pattern and hydroxylation on the A-ring of estrogenic compounds may be important determinates of ligand binding to the putative estrogen-binding site in the striatum (Lindford and Wade 2000). Estradial conjugated to bovine serum albumin mimics the effect of estradial to enhance stimulated straital release. These findings suggest that the modulatory effects of estrogen are mediated by a specific membrane-bounded receptor mechanism (Kelly et. al., 1999).

Numerous studies have demonstrated that estrogen provides neuroprotectivity within the dopaminergic system. One of the ways that estrogen exerts its neuroprotection is by acting as an inhibitor of the DAT. Since the DAT does not discriminate between dopamine and neurotoxins such as MPP^+, transport of MPP^+ with dopamine into the presynaptic neuron leads to destruction (neurodegeneration) of the neuron. Estrogen acts as a neuroprotectant by inhibiting the dopamine transporter function. This influence leads to the blockade of the transporter and therefore, the DAT is unable to recover the neurotoxin and the dopamine, which are found in the cleft (Dluzen, 2000).

Evidence that estrogen blocks the DAT comes from experiments examining the effects of methamphetamine on the nigrostriatal dopaminergic system and therefore the DAT. Methamphetamine, like MPP^+, induces dopamine displacement from synaptic vesicles and is transported out of the presynaptic vesicle via the DAT. Infusion of methamphetamine into striatal tissue significantly increases dopamine release, an effect that is prevented in the presence of estrogen, indicating that estrogen is acting to inhibit DAT functioning (Myers, 2003). The DAT is therefore unable to recover the neurotoxin and dopamine, which remain in the synaptic cleft (Dluzen, 2000).

Additional support for the hypothesis that estrogen acts as a dopamine uptake inhibitor comes from experiments examining the effect of estrogen in male animals. After castrating male rats and treating them with estrogen, it has been observed that estrogen exerts neuroprotective effects upon the nigrostriatal dopaminergic system (Gao and Dluzen 2001). Estradiol effect on the DAT has been suggested to be mediated by a membrane bound receptor, rather than having a genomic action. This suggestion is supported by in *vivo* electrochemistry experiments, where researchers explored the effect of administering MPP^+ in the presence or absence of estrogen into the corpus striatum of rats. The amplitude of the dopamine release was decreased by ten fold when MPP^+ was administered with estrogen, which implied an inhibitory effect of estrogen on the DAT activity. The researchers also measured the clearance rate of dopamine after MPP^+ alone and MPP^+ with estrogen treatment. They found that there was a significant reduction in the clearance rate of dopamine after treatment with estrogen. These results suggest that estrogen exerts its actions very rapidly, which is most likely due to a membrane action rather than by classical genomic effects in protein synthesis (Arvin, 1998).

As mentioned before, PD is a neurodegenerative disease that is more prevalent in older populations versus younger ones. Another study done by Leipheimer and Arvin (2000) examined the effects of infusing MPP+ alone or in combination with estrogen on the release and clearance of dopamine in the corpus striatum of aged female versus young female rats. Their in *vivo* electrochemistry experiments demonstrated that estrogen was effective in aged animals and significantly inhibited the MPP+ induced release of dopamine and significantly inhibited the clearance rate of dopamine from the extracellular fluid. This study provides evidence that estrogen retains its inhibitory action on DAT activity in aged animals.

The action of estrogen of appears to be inhibitory to DAT activity, however the site of estrogen acting is still a matter of debate. To address this issue a study was done by Leipheimer and Arvin (2001) to further understand the specificity of estrogen action. This electrochemistry study was done with female rats infused with dopamine alone, dopamine plus estrogen, or the combination of dopamine plus estrogen plus tamoxifen (a dopamine antagonist) in the corpus striatum. In this study, tamoxifen completely reversed the inhibitory action of estrogen on the clearance rate of dopamine. These results suggest

that estrogen is acting via a specific receptor mechanism to inhibit the activity of the DAT.

Studies done at Harvard University explored the effects of low levels of testosterone on depression in man. They found that the low levels of testosterone are directly related to depression and to the expression of the serotonergic and dopaminergic transporters. Furthermore, it was found that depressed men receiving regular injections of testosterone had significant mood improvement which suggests the importance of testosterone in regulating the expression of dopaminergic and serotonergic transporters on psychological conditions (Treatment up date, 2000).

Although much evidence is accumulating concerning the role if estrogen in the modulating neurotransmitter function, there is little information concerning the role of testosterone.

A study done by Leipheimer, Fedrokova, and Arvin (1999) explored the effects of castration on the K^+ or MPP^+-stimulated dopamine release from the nucleus accumbens and the corpus striatum of male rats. Using real time recording of both the release and reuptake of dopamine, it was found that castration changed the dynamics of the dopamine transporter especially in the nucleus accumbens after MPP^+ infusion. These results suggests that the effects of androgens on the DAT activity vary with specific brain areas, and that testosterone may have greater effects on DAT activity in the nucleus accumbens than in the corpus striatum.

In a study done by Dluzen and Ramirez (1989) Testosterone and DHT was investigate and compared to estrogen action on MPP+-induced dopamine depletion. Studies have shown that the male gonadal steroid hormone testosterone did not weaken the effects of MPP+-induced dopamine release nor did DHT. Meanwhile, estrogen showed a reduction in striatal dopamine concentrations (Dluzen and Ramirez 1989). Furthermore, striatal specific binding to the DAT was measured using [125I] RTI-121 and [3H] dihydrotetrabenazine autoradiography. With MPP^+ treatment the DAT concentration was significantly reduced in control rats, meanwhile estrogen prevented this reduction in the concentration of the DAT but testosterone failed to do the same. The role of testosterone was also explored in the substantia nigra with MPP+ treatments and the observed results suggested that androgen did not prevent MPP-induced decrease of

the DAT mRNA, while estrogen significantly prevented MPP+-induced depletion of DAT mRNA.

Furthermore, a recent study revealed that testosterone did not have the same modulatory effects as estrogen upon amphetamine evoked dopamine release from the superfused corpus striatum. This study emphasized the effects of estrogen in producing neuroprotectency upon the striatal tissue, meanwhile it suggested that infusing testosterone had no protective effects against the depletion of dopamine from the striatal tissue (Myers, 2003). In another study, investigators found that estradiol treatment reduced MPTP induced neurotoxicity and lowered dopamine depletion in mice. However, treatment with testosterone or DHT did not reduce MPTP-induced neurotoxicity. These androgen treated mice had dopamine depletion similar to control mice treated with MPTP alone (Ekue, 2002).

In conclusion, steroid hormones, estrogen and testosterone, seems to exert differential modulatory effects upon dopamine and DOPAC output from striatal tissue, and this could be the basis for the difference in occurrence of this disease between males and females.

Specific Aims of the Study

The main focus of this thesis was to investigate the role of testosterone (T) on dopamine transporter function. Using *in vitro* superfusion techniques, the dopamine transporter was studied under three conditions. These included intact male animals (control), castrated or gonadectomized male animals (GNX), and castrated males with testosterone replacement (GNX+T).

Striatal tissue isolated from rats in each group was treated with the neurotoxin MPP^+, with nomifensine a DAT inhibitor, or with dopamine. The amount of dopamine released from the tissue and recovered in the superfusion solution served as an indicator of DAT activity. Increased dopamine release and recovery indicated a decrease in DAT function. We hypothesized that testosterone has a facilitatory effect on the DAT. Therefore it was expected that castrated male rats (GNX) would show increased dopamine recovery when compared to control rats or castrated male rats with testosterone replacement.

METHOD

Animals

Eighteen male Sprague Dawley rats about 3-5 months old, with an approximate weight of 125g, were housed in groups of two or three per cage. The animals were kept on a twelve-hour light/dark cycle, with lights on at 0600. Food and water was available *ad lib*. Six animals remained intact and were used as the control group. Twelve animals were castrated and six of these received testosterone replacement therapy. These experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Northeastern Ohio Universities College of Medicine (NEOUCOM.).

Surgical Procedures

Castration

Animals were weighed and given an intramuscular injection of a mixture of Ketamine (60mg/kg) and Xylazine (13 mg/kg). Ketamine or ketamine hydrochloride, is a non-barbiturate, rapid-acting disassociative anesthetic used on both animals and humans. Xylazine is a clonidine analoque, an agonist that acts on the α_2 adrenergic receptors on the presynaptic and postsynaptic neurons in the central and peripheral nervous system, which causes the muscles to relax. One disadvantage of Xylazine that it causes cardiopulmonary depression, so it was found that a combination or a mixture of both Ketamine, which is, considered a good cardiovascular stimulant and Xylazine, which provides long periods of anesthesia, can be good and suitable for surgery (Zandieh, 2000)

.

Following anesthesia, the animals were checked for reflex withdrawal reactions to ensure complete anesthesia. An incision was made in the lower abdomen and the testes were pushed up toward the incision. After locating the testes, the ductus deferens was clamped using a hemostat at a point above the epididymis and the testes. The ductus deferens and the arteries and veins supplying the testes were tied off with a silk thread. Using scissors, the testes were removed and the tissue was checked for bleeding before

unclamping the hemostat. The remainder of the ductus deferens was put back carefully and the incision sutured. The animals were allowed to recover ten to fourteen days and until all the testosterone was absent (Dluzen, 2000).

Testosterone replacement

Testosterone was replaced in some GNX rats by implanting capsules containing testosterone. The capsules were made with silastic medical grade tubing (1.6 mm I.D., 3.2 mm O.D.), Dow Corning (Midland, MI). They were filled with testosterone to an active length of 40 mm. The tubing was sealed on the ends with 5 mm. wooden plugs and silastic medical adhesive silicone type-A, Dow Corning (Midland, MI). These capsules were made with modifications according to methods described by Smith et al. (1977) and are similar to capsules reported to maintain sexual reflexes in castrated male rats (Leipheimer and Sachs, 1993). The capsule was inserted subcutaneously in the mid dorsal thoracic region under light Halothane anesthesia. The animals were given a week to allow testosterone levels to return to normal levels (Dluzen, 2000).

Solutions

Kreb Ringer Phosphate Buffer (KRP)

The superfusion medium (KRP) consisted of: 120 mM NaCl, 4.8 mM KCL, 0.8 mM of $CaCl_2$, 1.2 mM of $MgSO_4$, 10.2 mM Na_2HPO_4, 1.8 mM NaH_2PO_4 and $3.25x10^4$ mM glucose adjusted to a pH of 7.4 (Dluzen, 2000).

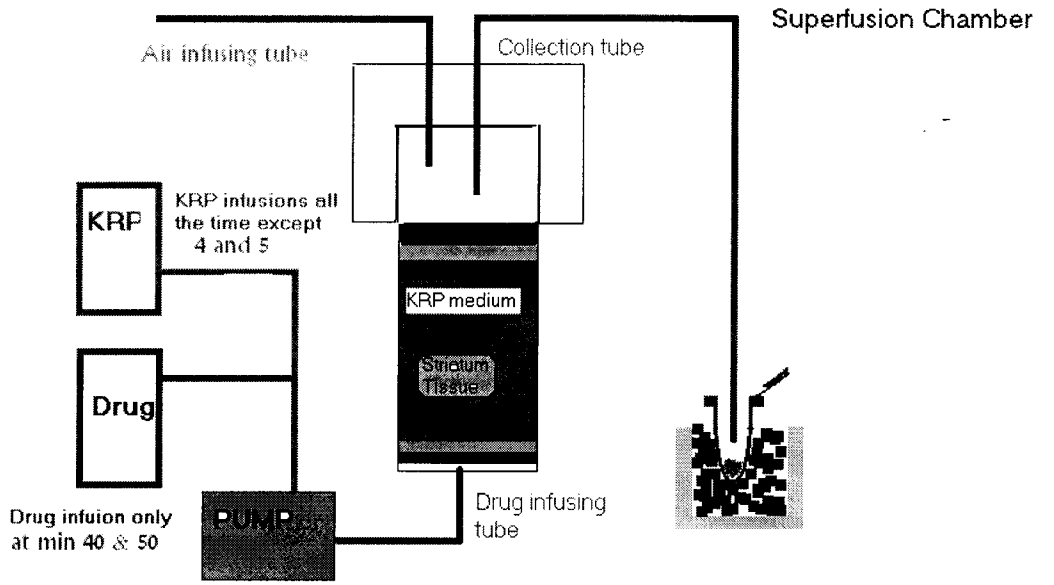
Pharmacological Agents

We used three different drugs for the purpose of this thesis: First, MPP^+ (10μM, Research Biochemical International) to cause dopamine release, second, Dopamine (1μM, Sigma Chemical Company), and third, Nomifensine (1mM, Sigma Chemical Company) was used to block the DAT.

Superfusion Chamber

The superfusion chamber consists of a 1cc syringe with a 21-gauge needle at the end. The chamber must house the tissue for the whole experiment and must prevent the tissue from leaving the chamber. This was accomplished using filter paper and corks with

the corks and the filter papers positioned in the chamber as follows. The filter is first inserted into the syringe and pushed down until it reaches the end of the syringe (chamber). The cork is then inserted and also pushed all the way in. Striatal tissue is put into each chamber, followed by a second filter and cork. Caps with an attached air hose were placed into each chamber to guarantee adequate delivery of oxygen to the tissue at all times. Prior reaching the tissue chamber air was passed through distilled water to moisten the air and collect unwanted particles. The tissue chambers were submerged in a water bath at a temperature of 37 °C. Samples were collected in microcentrifuge tubes on



ice every 10 minutes at a flow rate of 25µl/min [see illustration (2)].

Illustration (2)

Experimental Procedure

On the day of the experiment, three rats from the assigned groups were obtained (control, castrated plus testosterone treated, and castrated with no treatment) and decapitated using a sharp guiotine. Decapitation was done with no prior anesthetesia, because anesthesia interferes with catecholamine concentrations in the brain. The brain was harvested and the striatal tissue was removed from the brain by making small

incisions on the posterior and anterior sides of the lateral ventricles. Once the incisions were made, the lateral ventricles were pushed aside exposing the corpus striatum. The striatum is characterized by having striations from side to side that can be seen with the naked eye.

The striatal tissue is then collected in small beakers containing KRP buffer and put on ice to preserve the tissue. The corpus striatum from each animal was cut into approximately three equal parts and then minced prior to treatment with the three drugs (see illustration 3).

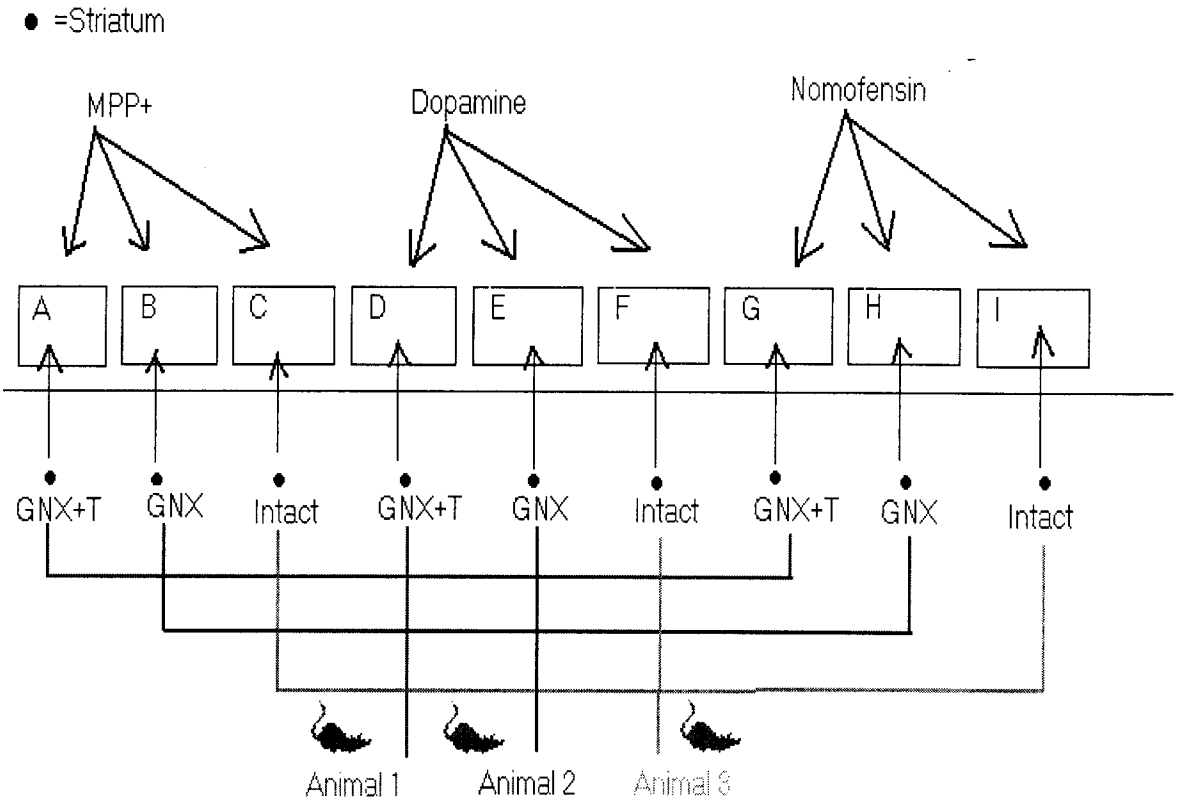


Illustration (3)

Tissue was placed into a superfusion chamber with KRP buffer being infused at 25ul/min. The tissue was placed allowed to equilibrate for one hour before samples were

collected. KRP saline buffer from the tissue chamber was collected for 10min and pooled as a single sample. Sample 1-3 were collected during the infusion of control KRP buffer. The experimental drugs were infused during the collection of sample 4-5. Control buffer was again infused during collection of the remaining samples (6-10). Ten samples were collected from each chamber every ten minutes for a total of ninety samples for each experiment (see illustration 4).

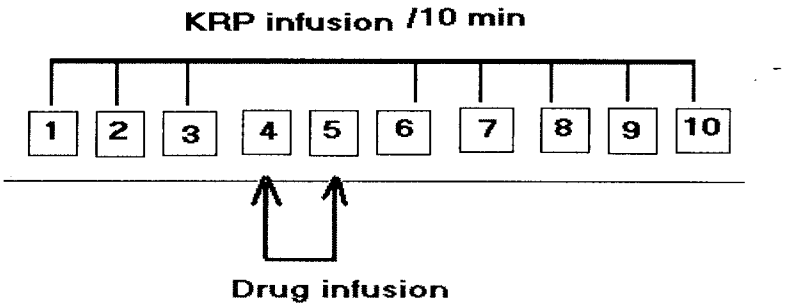


Illustration (4)

Prior to their use, the centrifuge tubes for sample collections were weighed and recorded. After the samples were collected, the tubes were weighed again, which allowed the determination of the final volume in the tubes. The samples were analyzed by a high-pressure liquid chromatography (HPLC) for dopamine and DOPAC concentrations (Dluzen, 2000).

HPLC separates complex mixtures using high pressure to force a sample that has been dissolved in a solvent (the mobile phase), through a narrowly packed column (the stationary phase). As the solute is being infiltrated through the column, the molecules become separated from each other due to different chemical and physical interactions with the packing material. Because of these interactions, the components are retained by the stationary phase and move at different rates through the column. As the separated

components elute from the column, they pass through a electrochemical detector which detects the amounts of neurotransmitters present (Pokrasen et. al, 1997).

Tissue Contents

Following sample collection, the striatal tissue was recovered from the tissue chambers, placed on a paper towel to dry, weighed, and put into 500 µl of 0.1N perchloric acid ($HClO_4$) to preserve it. The tissue was then sonicated for twenty seconds, centrifuged for fifteen minutes, and the supernatant removed and stored in 500 µl of 0.1N of $HClO_4$. Tissue concentrations of dopamine and DOPAC were analyzed from the HPLC instrument in pg and then expressed as pg/mg of tissue. The tissues were analyzed for dopamine and DOPAC content at the end of the superfusion experiments to verify that these experiments did not result in the complete depletion of these catecholamines in the tissue. There were no significant differences in the tissue content between treatment groups in this study and therefore the data is not presented in this thesis.

Data Analysis

Data was analyzed using the SPSS software program (SPSS Inc.). Dopamine and DOPAC releases from all three groups (control, castrated with testosterone replacement treatment, and castrated with no treatment) were analyzed for variation between the groups. Oneway ANOVA was utilized, followed by LSD Post Hoc tests and significant differences between groups were noted when $p \leq 0.05$.

Percent change for each group was determined by taking the stimulated release value minus the baseline value, divided by the baseline value, multiplied by 100. The percent change numbers were then converted to arcsine numbers for statistical analysis.

Criteria of Data Collection

This thesis is based on six experiments, where we collected 540 released samples and 54 tissue samples. Each released sample was plotted in Excel and visually assessed. We considered data to be valid if the baseline equilibrated between sample one and four (where the baseline concentration of dopamine and DOPAC was very low), followed by a

response to drug stimulation (an increase from baseline) followed by a decline from the peak response to baseline after wash out with KRP buffer. Tissue responses that did not fit these criteria were eliminated.

Baseline Collection

The lowest concentration in pg/mg/min of dopamine or DOPAC between sample one and four was taken to be the baseline reading for that particular release. Baseline data were grouped together based on the animal model and what kind of treatment the group received. For example, data from GNX animals treated with dopamine infusions were grouped together and the average of the six runs (experiments) was collected for analysis with SPSS.

Release Data Collection

For the release study, the data were examined and the average values were determined for the release of dopamine and DOPAC. Release data was grouped together based on the animal model the treatment received. For example, data from GNX animals treated with dopamine infusions were grouped together and the average of the six runs (experiments) was collected and analyzed with SPSS.

Baseline and Release Data Calculation

Baseline and release values were determined using the following formulas: Baseline Concentration = [(1/tissue weight) x 0.005] x volume in the centrifuge tube x concentration in pg collected from HPLC machine. The final concentration is given as pg/mg of tissue/min.

Release Concentration = [(1/tissue weight) x 0.005] x volume in the centrifuge tube x concentration in pg collected from HPLC machine. The final concentration is given as pg/mg of tissue/min. Our hypothesis is that testosterone acts to increase DAT activity. By removing testosterone, we predict that castration will reduce DAT activity. To test this hypothesis, striatal tissue was obtained from intact (control), castrated (GNX), and castrated plus testosterone (GNX+T) male rats. The striatal tissues were infused with KRP buffer plus either dopamine (1μM), NMF (1mM), and MPP^+ (10μm). The amount of dopamine recovered from the tissue was then determined.

Results

Experiment (1). The effects of infused dopamine on the release of dopamine and DOPAC in corpus striatum tissue.

Dopamine Recovery

Infusion of control KRP buffer provided baseline data for dopamine release from the striatal tissue. After 30 min of control infusion, the saline was changed to KRP buffer plus dopamine (1μM) for 20 min. Control buffer was then infused for the reminder of the experiment.

There were no significant differences in the levels of baseline dopamine recovery from striatal tissue obtained from the three animal groups (Fig. 1). Baseline values were 0.06 ± 0.16 pg/mg/min for control animals (n = 6), 0.22 ± 0.20 pg/mg/min for GNX animals (n = 6), and 0.25 ± 0.10 pg/mg/min for GNX+T animals (n = 6).

As expected, infusion of dopamine increased the amount of dopamine recovered for all groups (Fig.1 infusion section of the graph). There were no significant differences between the control group (1.11 ± 0.55 pg/mg/min; n = 6) and the GNX+T group (0.60 ± 0.14 pg/mg/min; n =6). However, the amount of dopamine recovered from the GNX group was greatly elevated to 9.08 ± 3.21 pg/mg/min (n = 6), which was significantly greater than the control group (p=0.0009) and the GNX+T group (p=0.006).

Figure 2 shows the percent change in dopamine recovery between the baseline and stimulated response for the three groups. Dopamine infusion increased dopamine recovery by 23.9 ± 8.4% for the control group, 38.8 ± 8.3% for the GNX group and 13.9 ± 4.9% for the GNX+T group. The observed increase for the GNX group was significantly greater than GNX+T group (p = 0.031). Due to variability in control tissue responses, there were no significant differences between the GNX and intact groups.

DOPAC Recovery

Examination of DOPAC, a metabolite of dopamine, released from the striatal tissue showed a similar response to the infusion of dopamine (Fig. 3). Baseline values were 0.83 ± 0.27 pg/mg/min for control animals (n = 6), 0.90 ± 0.47 pg/mg/min for the

GNX group (n = 6), and 0.067 ± 0.19 pg/mg/min for the GNX+T (n = 6) group. There were no significant differences in the DOPAC recovery level between the control and the GNX+T groups. However, the GNX group was significantly higher than GNX+T group (p = 0.050), but not significantly different from the intact group.

The percent change in DOPAC recovery was also determined. Dopamine infusion increased recovery by 12.4 ± 4.30% for the control group, 11.8 ± 5.76% for the GNX group, and 5.83 ± 1.99 for the GNX+T group. There were no significant differences between groups.

Experiment (2). The effects of infused MPP^+ on the release of dopamine and DOPAC from the corpus striatum tissue.

Dopamine Recovery

There was no significant difference in the levels of baseline dopamine recovery from striatal tissue obtained from the three animal groups (Fig. 5). Baseline values were 0.95 ± 0.16 pg/mg/min for control animals (n = 6), 1.19 ± 0.60 pg/mg/min for GNX animals (n = 6), and 2.23 ± 1.08 pg/mg/min for GNX+T animals (n = 6).

Following MPP+ infusion, there also were no significant differences in dopamine recovery between groups. Average dopamine recovery for the intact group was 4.45 ± 1.75 pg/mg/min (n=6), 10.1 ± 7.06 pg/mg/min (n=6) for the GNX animals, and 10.5 ± 6.63 pg/mg/min (n=6) for the GNX+T group.

Figure 6 shows the percent change in dopamine recovery for each group between the baseline and stimulated responses. MPP^+ infusions increased dopamine recovery by 11.37 ± 1.29% for the control group, 14.75 ± 2.13% for the GNX group, and 10.16 ± 2.06% for the GNX+T group. The observed increases were not significantly different in between groups.

DOPAC Recovery

Baseline analysis for the DOPAC release showed some variation. Baseline values were 0.84 ± 0.15 pg/mg/min for the intact group (n = 6), 1.33 ± 0.41 pg/mg/min for GNX group (n = 6), and 3.38 ± 1.25 pg/mg/min for the GNX+T (n = 6) animals. There was a

significant difference between the GNX+T and intact groups ($p = 0.03$), however, there were no significant differences between other groups (Fig. 7).

After MPP^+ infusion, there were no significant differences in DOPAC recovery between groups (Fig. 7). Average DOPAC recovery for the intact group was 1.64 ± 0.38 pg/mg/min ($n = 6$), 0.96 ± 1.28 pg/mg/min ($n = 6$) for the GNX group, and 3.16 ± 1.76 pg/mg/min for the GNX+T ($n = 6$).

Figure 8 shows the percent change in DOPAC recovery between the baseline and stimulated response for the three groups. MPP^+ infusions increased DOPAC recovery by $6.52 \pm 1.29\%$ for the control group, $8.78 \pm 1.75\%$ for the GNX group, and $5.94 \pm 2.95\%$ for the GNX+T group. The observed increases were not significantly different in between any group.

Experiment (3). The effects of infused NMF on the release of dopamine and DOPAC from the corpus striatum tissue.

Dopamine Recovery

Infusion of control KRP buffer provided baseline data for dopamine release from the striatal tissue. After 30 min of control infusion, the saline was changed to KRP buffer plus NMF (1mM) for 20 min. Control buffer was then infused for the reminder of the experiment.

There were no significant differences in the levels of baseline dopamine recovery from the striatal tissue obtained from the three animal groups (Fig. 9). Baseline values were 0.34 pg/mg/min $\pm$ 0.17 for control group ($n = 6$), 0.10 ± 0.04 pg/mg/min for GNX group ($n = 6$), and 0.03 ± 0.04 pg/mg/min GNX+T ($n = 6$) group.

There were also no significant differences in the levels of stimulated dopamine recovery from striatal tissue obtained from the three animal groups (Fig. 9). The mean values were 1.18 ± 0.81 pg/mg/min for the control group ($n = 6$), 2.52 ± 0.62 pg/mg/min for the GNX group, and 0.90 ± 0.52 pg/mg/min for the GNX+T group ($n = 6$).

In contrast, evaluation of the percent change data for dopamine recovery demonstrated significant effects following NMF infusion (Fig.10). Gonadectomy resulted in a significant increase in average recovery following NMF infusion when compared to

the control group (p=0.0032). There were no significant difference between the other groups 6.78 ± 3.97% for the control group, 30.99 ± 5.24% for the GNX group and 20.13 ± 13.45% for the GNX+T group.

DOPAC Recovery

Baseline analysis for DOPAC release showed some variation, however there were no significant differences between groups. Baseline values were 0.93 ± 0.32 pg/mg/min for intact group (n = 6), 0.53 ± 0.17 pg/mg/min for GNX group (n = 6), and 0.60 ± 0.24 for the GNX+T pg/mg/min (n = 6) (Fig. 11).

The NMF DOPAC stimulated recovery levels also showed no significant differences between groups. The mean values were 3.50 ± 1.37 pg/mg/min for the control group (n = 6), 2.73 ± 0.75 pg/mg/min for the GNX group (n = 6) and 1.28 ± 0.79 pg/mg/min for the GNX+T group (n = 6) (Fig. 11).

Figure 12 illustrates percent change in DOPAC recovery after NMF infusion. Average DOPAC recovery for the groups was 7.26 ± 1.17% for the control group (n = 6) animals, 14.65 ± 2.85% for the GNX group (n = 6), and 12.47 ± 3.24% for the GNX+T (n = 6). There was a significant increase in DOPAC recovery between GNX and intact groups (p=0.005). There were no significant differences found between the other groups.

Experiment 4. Content Study.

Dopamine and DOPAC Tissue Content

Statistical analysis of the dopamine and DOPAC tissue content did not show any significant differences between groups; GNX, GNX+T or intact. This statement applies to all of the superfusion experiments done in this thesis (data not shown).

Figure 1. Dopamine baseline and recovery (pg/mg/min) after dopamine infusions. Values given are the mean ± SEM for all figures. There were no significant differences in baseline between groups. *Dopamine recovery during infusion was significantly greater in the GNX group when compared to intact [p=0.0009] or GNX+T [p=0.006] animals (n=6 for each group).

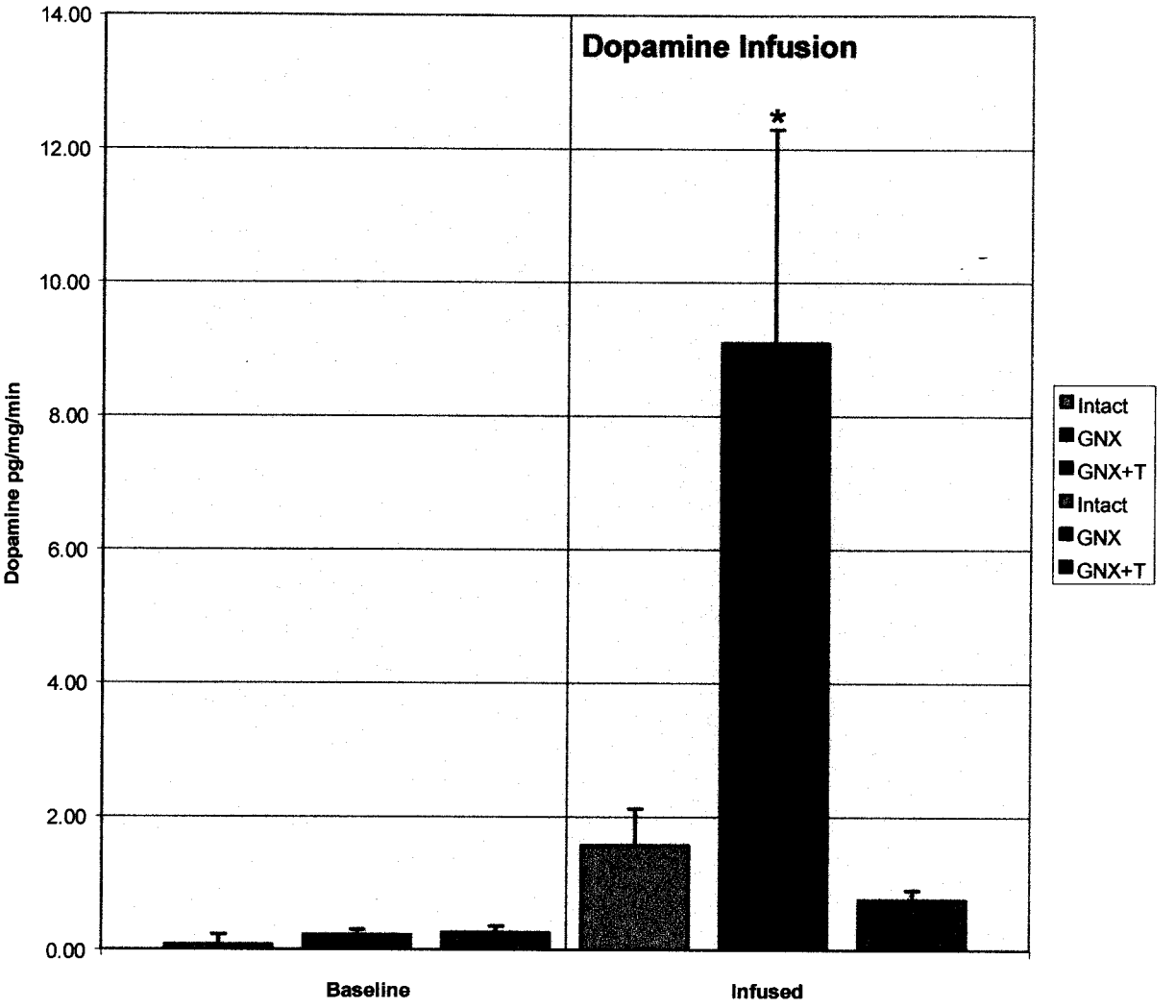
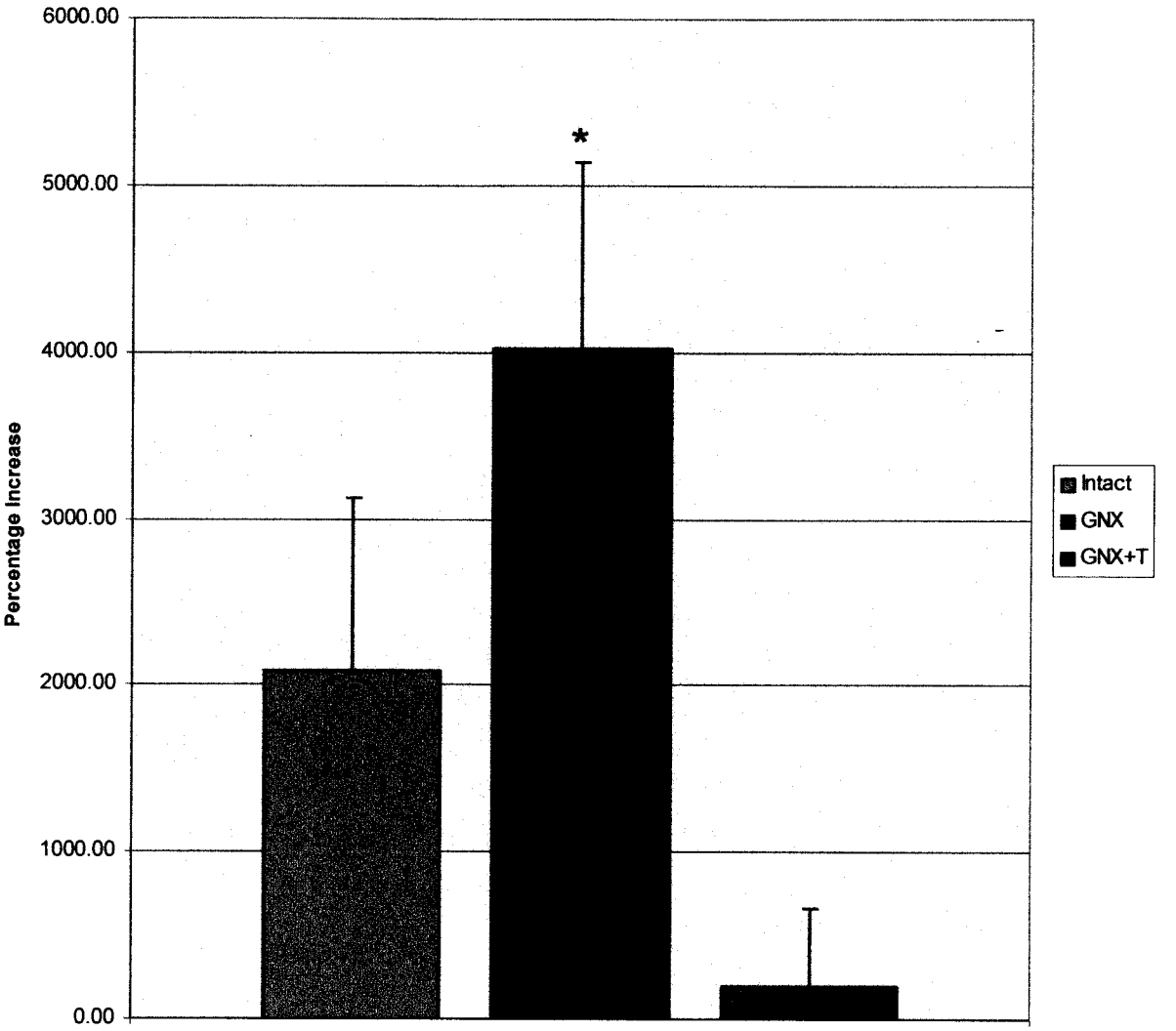

Dopamine Infusion
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Dopamine pg/mg/min
*
Baseline
Infused
Intact
GNX
GNX+T
Intact
GNX
GNX+T

Figure 2. Percent change between dopamine baseline and dopamine recovery. *Dopamine percent increase was significantly greater in the GNX group when compared with GNX+T group [p=0.031] (n=5 for each group).


6000.00
5000.00
4000.00
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Percentage Increase
*
Intact
GNX
GNX+T

Figure 3. DOPAC baseline and DOPAC recovery (pg/mg/min) after dopamine infusions. There were no significant differences in dopamine baseline between groups. *DOPAC recovery was significantly greater in the GNX group when compared to GNX+T [p=0.05] animals (n=6 for each group)

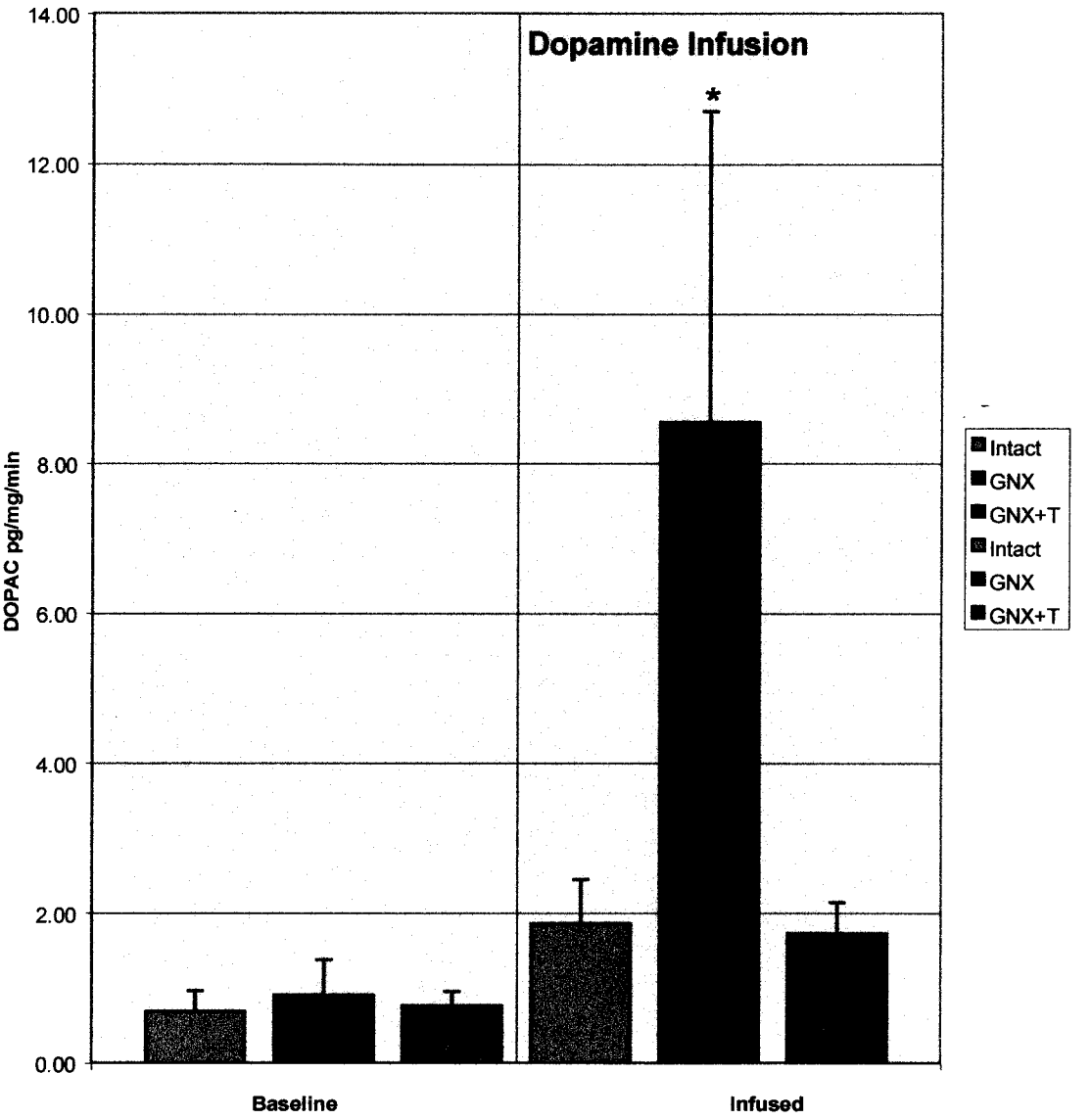

Dopamine Infusion
*
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
DOPAC pg/mg/min
Baseline
Infused
Intact
GNX
GNX+T
Intact
GNX
GNX+T

Figure 4. Percent change between DOPAC baseline and DOPAC recovery after dopamine infusions (n=5 for each group) There were no significant differences between groups.

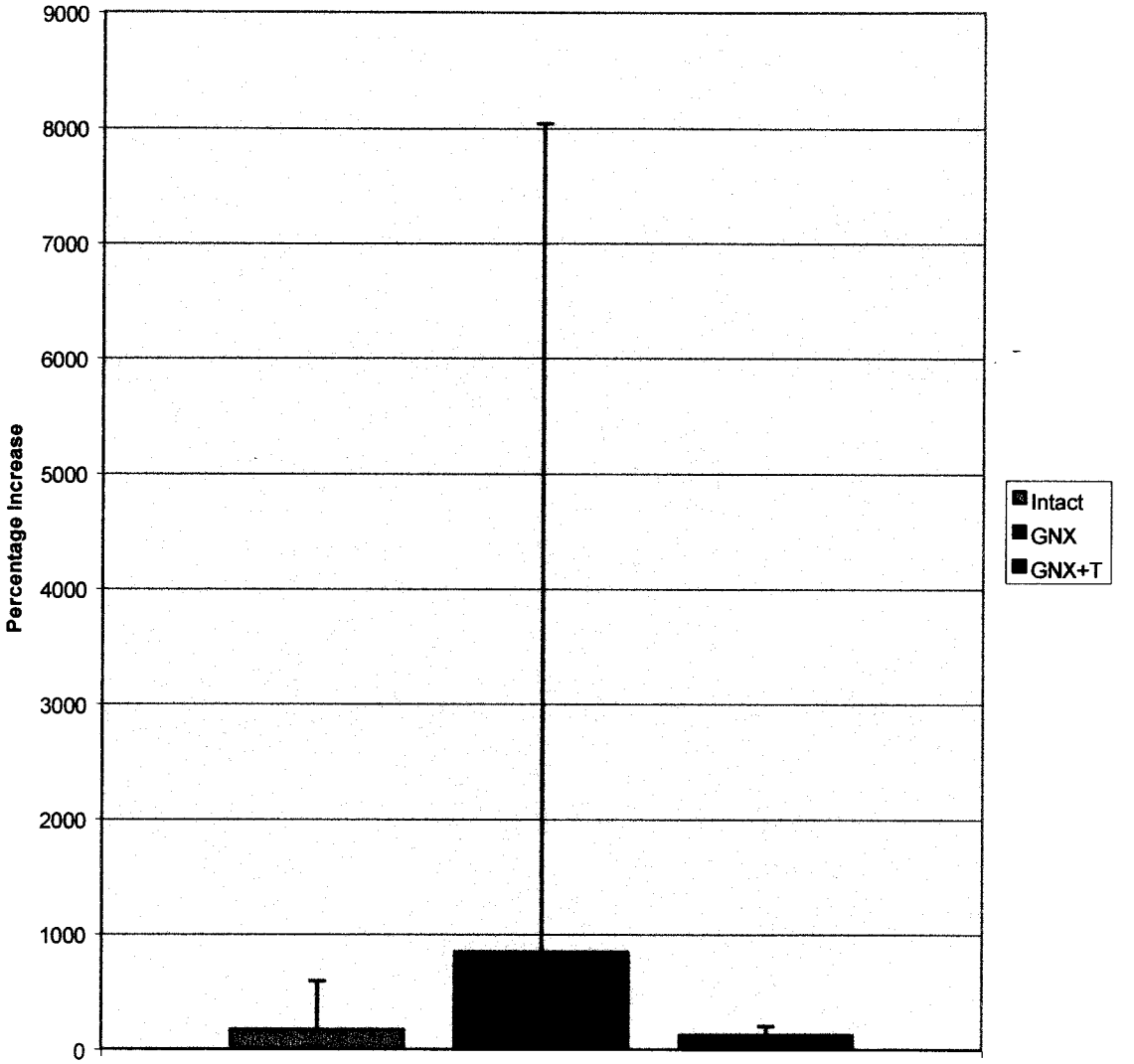

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Percentage Increase
Intact
GNX
GNX+T

Figure 5. Dopamine baseline and dopamine recovery (pg/mg/min) after MPP^+ Infusions. There were no significant differences between groups in either the baseline or during drug infusion periods. (n=6 for each group).

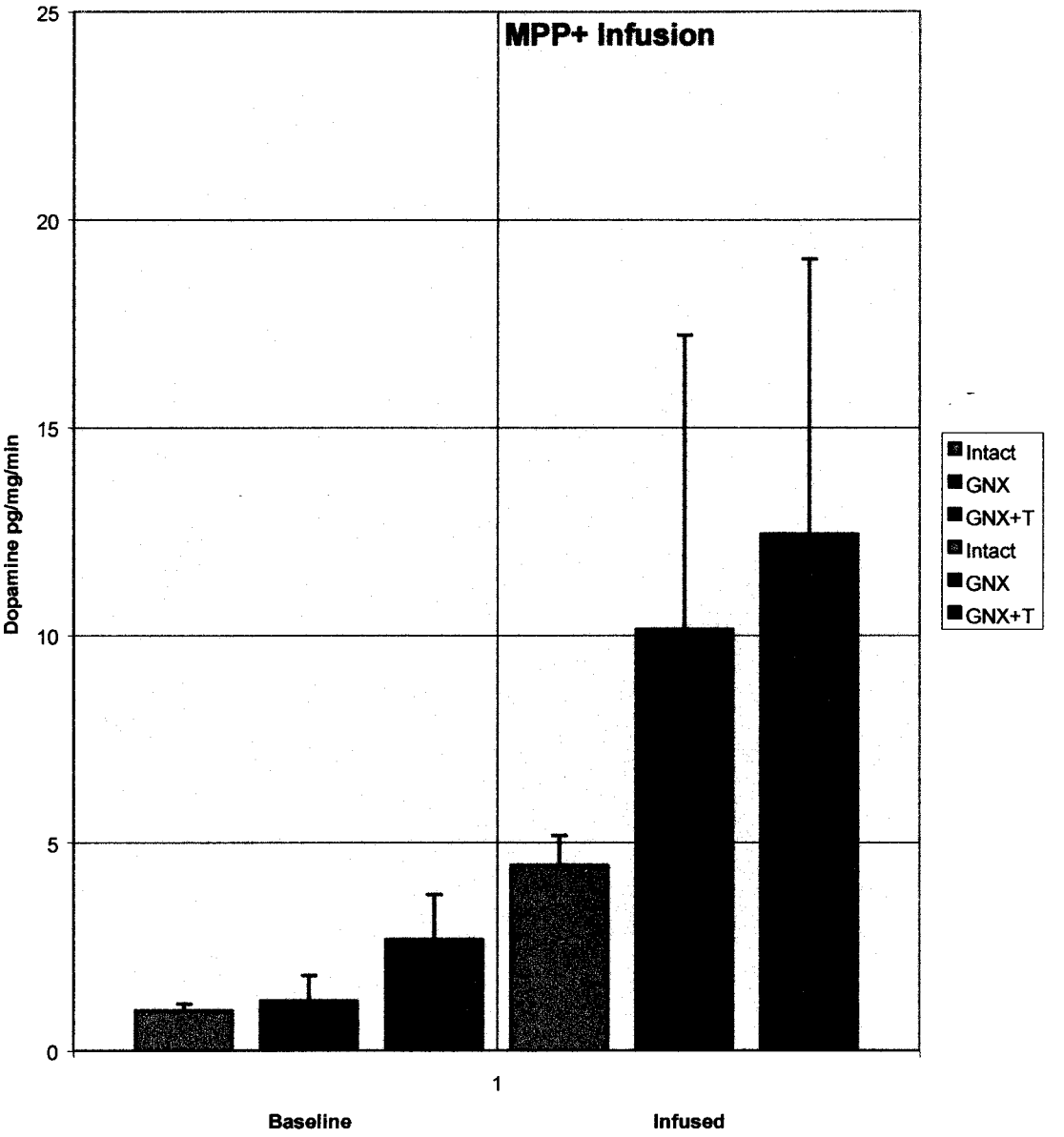

MPP+ Infusion
Dopamine pg/mg/min
25
20
15
10
5
0
1
Baseline
Infused
Intact
GNX
GNX+T
Intact
GNX
GNX+T

Figure 6. Percent change between dopamine baseline and dopamine recovery after MPP^+ infusions (n=4 for each group). There were no significant differences between groups.

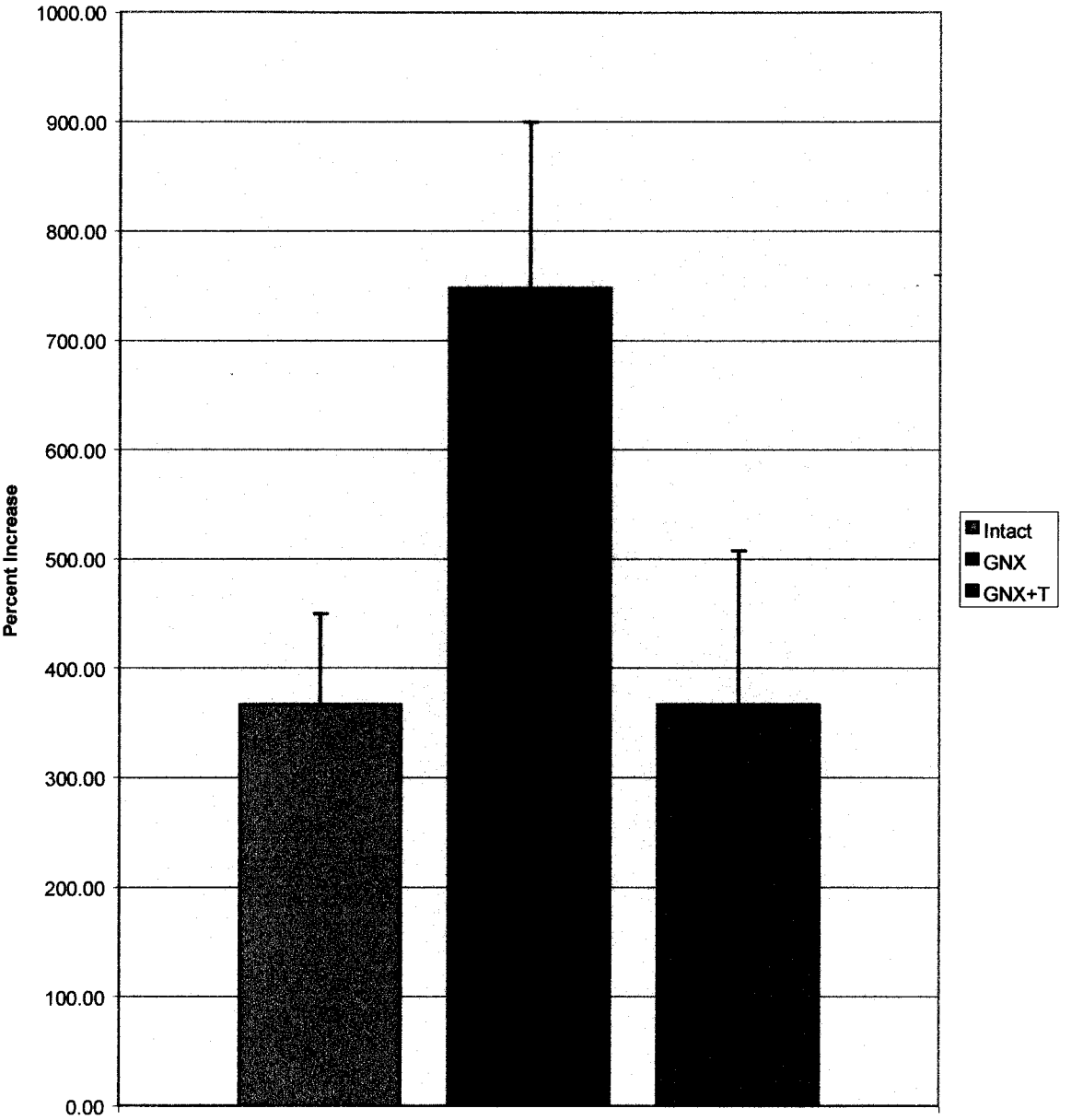

1000.00
900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Percent Increase
Intact
GNX
GNX+T

Figure 7. DOPAC baseline and DOPAC recovery (pg/mg/min) after MPP^+ infusions (n=6 for each group). *DOPAC baseline was significantly greater in the GNX+T group when compared to the intact [p=0.03] group. There were no significant differences in baseline between groups during the drug infusion period.

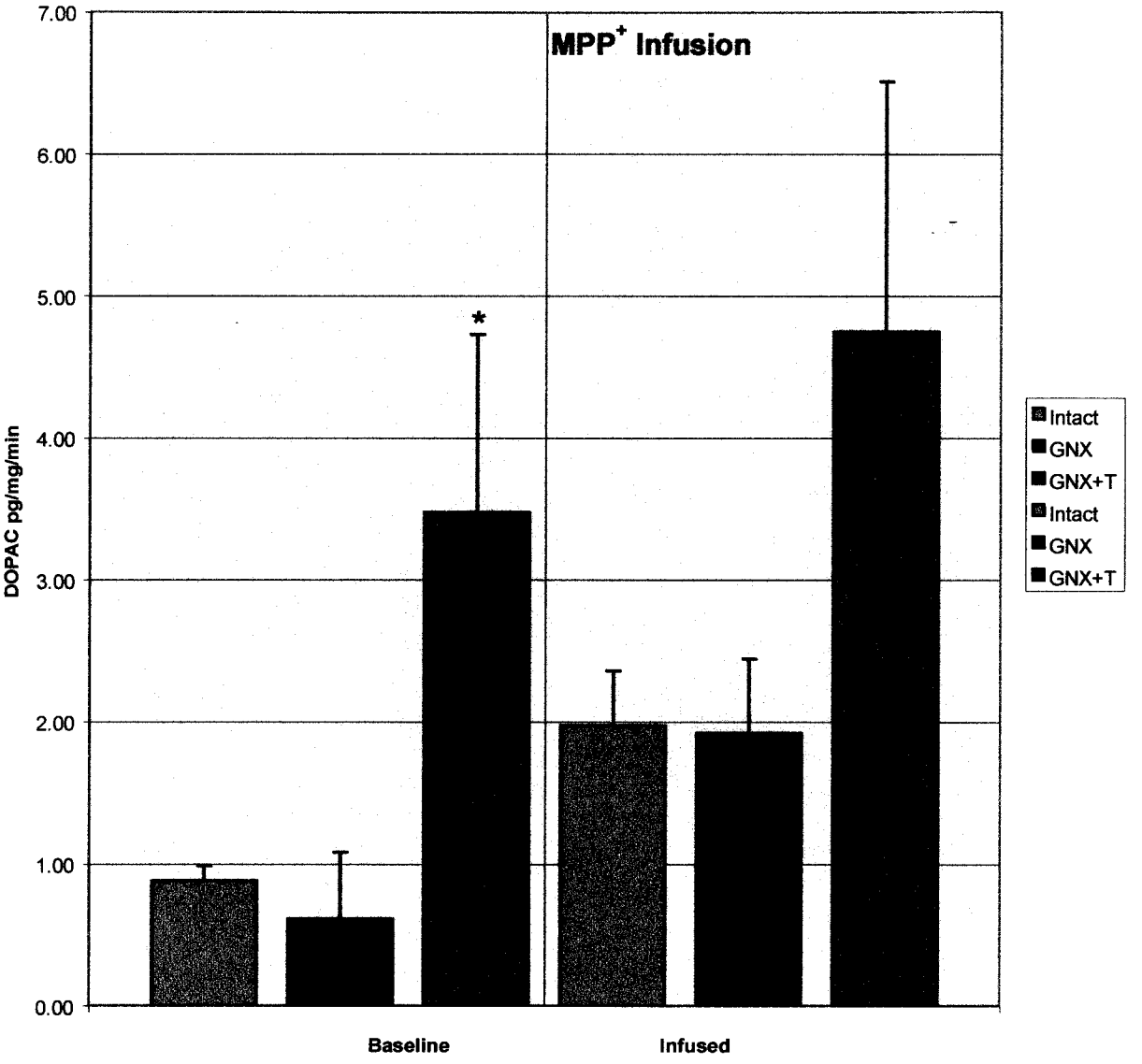

MPP+ Infusion
DOPAC pg/mg/min
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
*
Baseline
Infused
Intact
GNX
GNX+T
Intact
GNX
GNX+T

Figure 8. Percent change between dopamine baseline and dopamine recovery After MPP^+ infusions (N=4 for each group). There were no significant differences between groups.

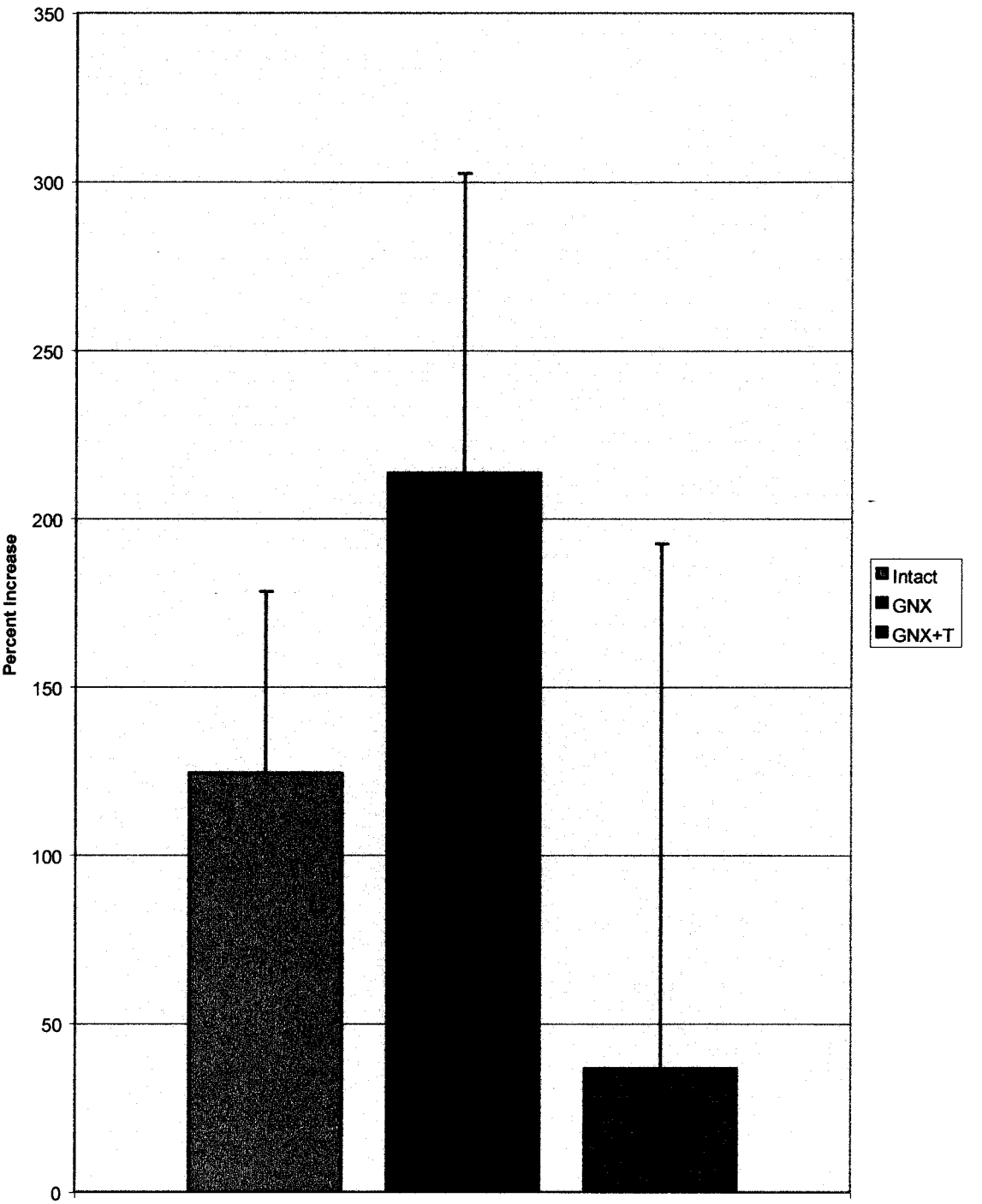

350
300
250
200
150
100
50
0
Percent Increase
Intact
GNX
GNX+T

Figure 9. Dopamine baseline and dopamine recovery (pg/mg/min) after NMF infusions (n=6 for each group). There were no significant differences between groups in either baseline or during drug infusion periods.

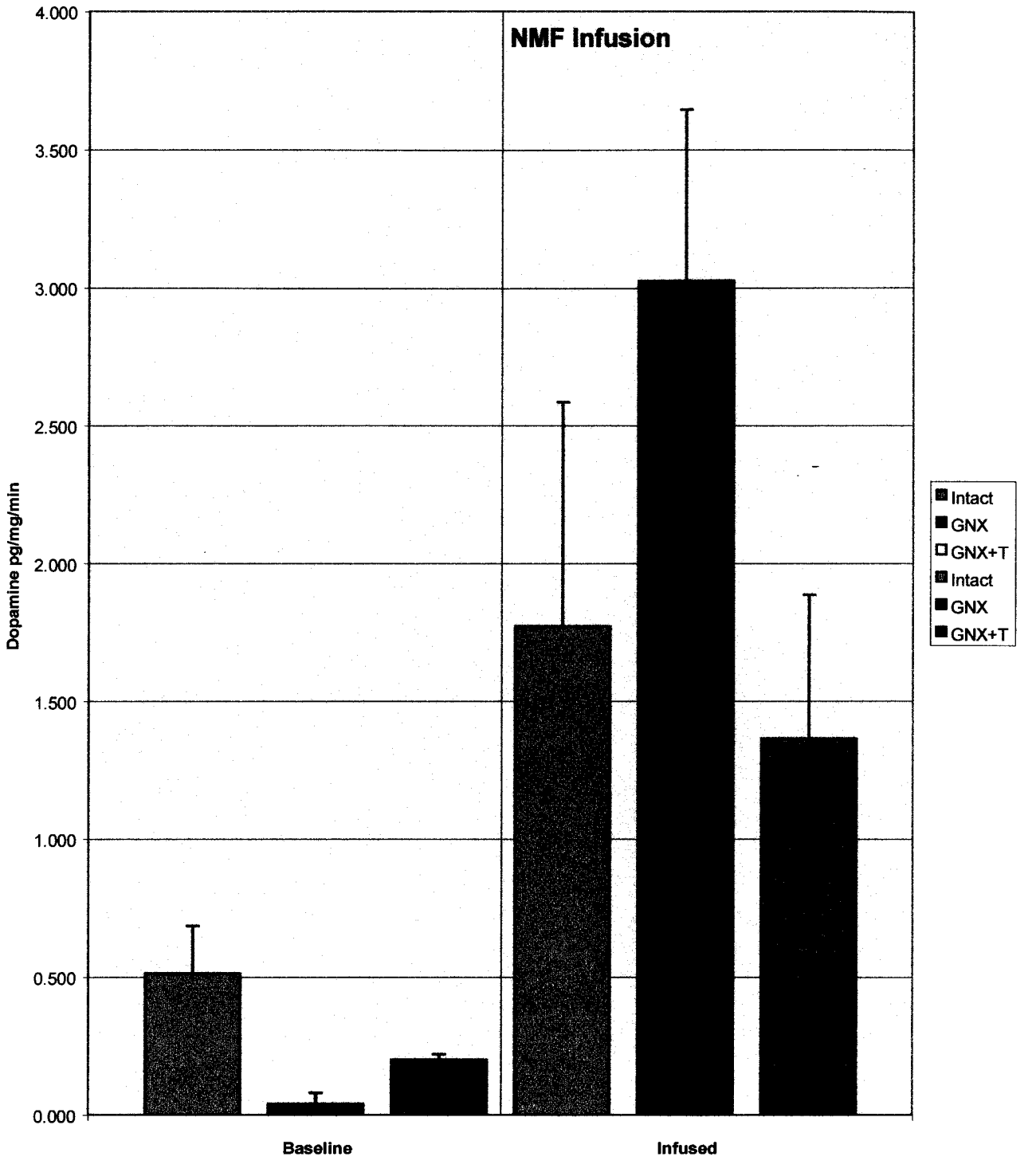

NMF Infusion
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
Dopamine pg/mg/min
Baseline
Infused
Intact
GNX
GNX+T
Intact
GNX
GNX+T

Figure 10. Percent change between dopamine baseline and dopamine recovery after NMF infusions. *Dopamine recovery was significantly greater in the GNX group when compared to intact group [p=0.032] (n=4 for each group).

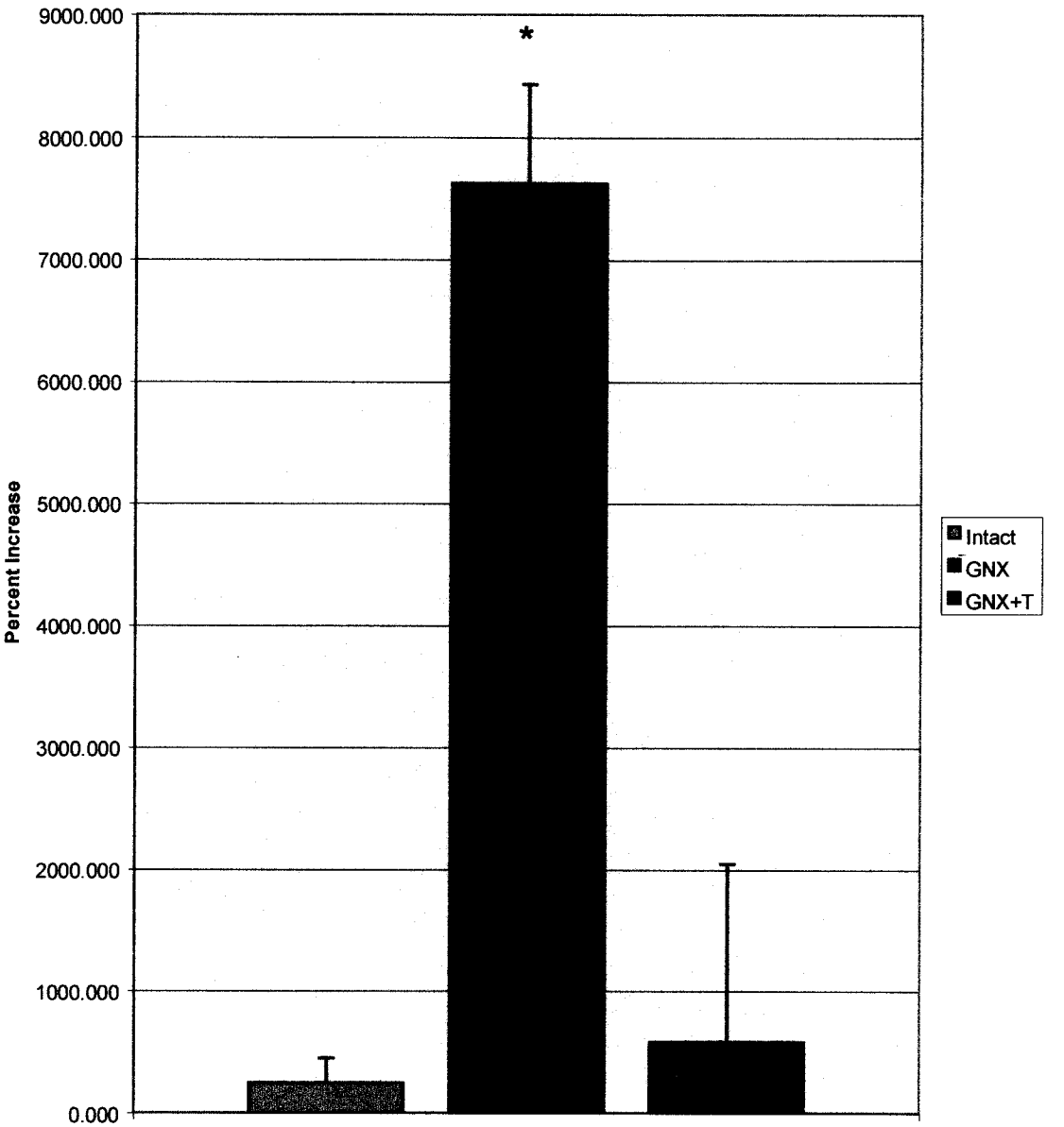

9000.000
8000.000
7000.000
6000.000
5000.000
4000.000
3000.000
2000.000
1000.000
0.000
Percent Increase
*
Intact
GNX
GNX+T

Figure 11. DOPAC baseline and DOPAC recovery (pg/mg/min) after NMF infusions (n=6 for each group). There were no significant differences between groups.

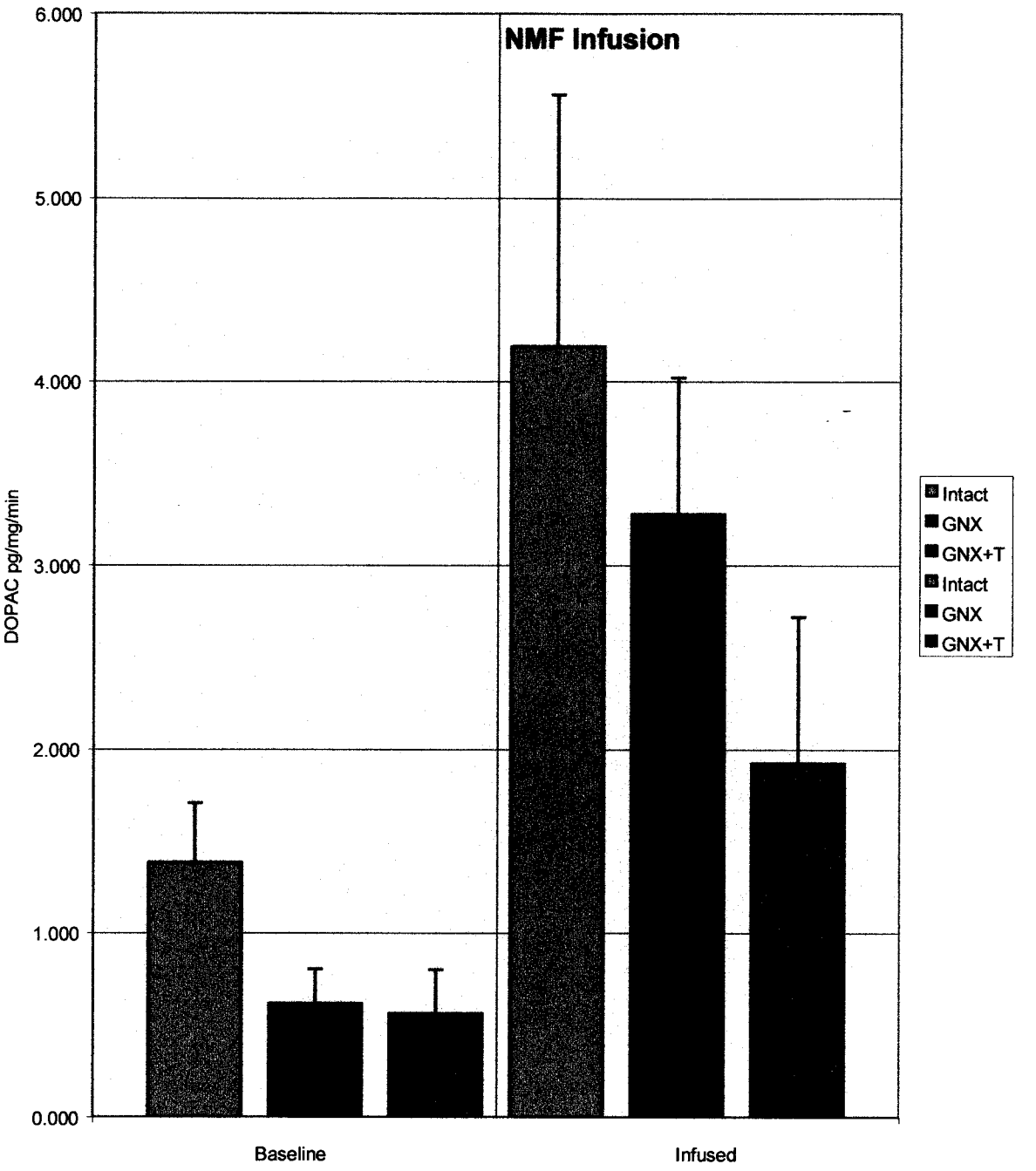
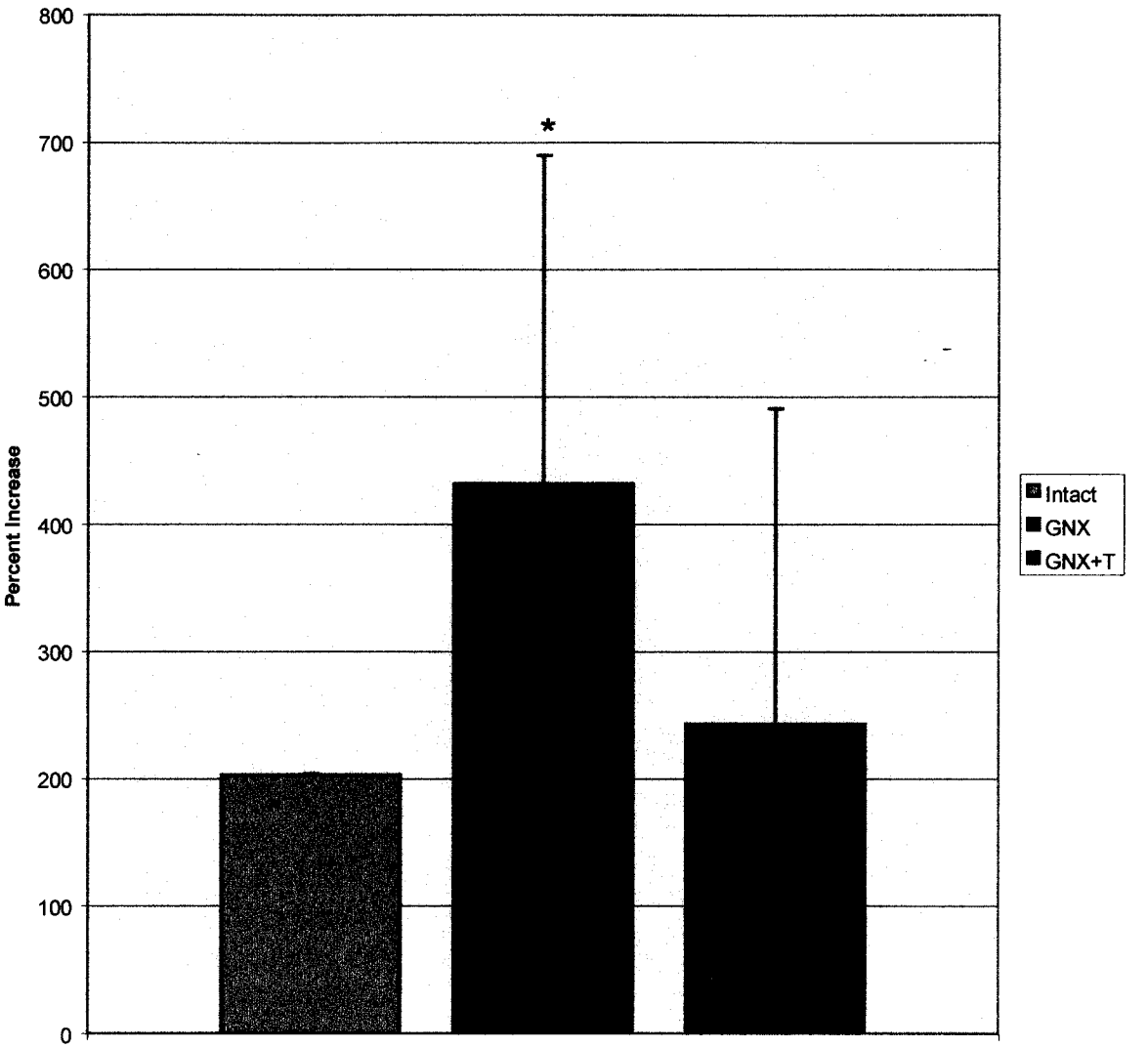

NMF Infusion
6.000
5.000
4.000
3.000
2.000
1.000
0.000
DOPAC pg/mg/min
Baseline
Infused
Intact
GNX
GNX+T
Intact
GNX
GNX+T

Figure 12. Percent change between DOPAC baseline and DOPAC recovery after NMF infusions. *DOPAC recovery was significantly greater in the GNX group when compared to intact group [p=0.050] (n=4 for each groups).


800
700
600
500
400
300
200
100
0
Percent Increase
*
Intact
GNX
GNX+T

Discussion

An exploration of the literature suggests a greater occurrence of Parkinson's disease in males versus females. Clinical observations in geriatric medicine revealed the first gender differences in relation to Parkinson's disease. This literature supports the notion that men are more at risk of acquiring the disease than women (Dluzen and McDermott, 2000).

Substantial amounts of data from experiments in animal models suggest a reason for this gender difference. Estrogen has been reported to act as a neuroprotectant of the nigrostriatal dopaminergic system by inhibiting DAT function and thereby reducing the uptake of specific neurotoxins that in turn promote the degeneration of neurons within substantia nigra neurons (Arvin et. al. 2000, Dluzen 2000, Disshon and Dluzeñ 1997).

The results for the dopamine infusion experiment show higher concentrations of dopamine and DOPAC recovery in the GNX animals versus the intact and the GNX+T rats. As indicated by the results of experiment one, the loss of testosterone appears to slow the DAT activity and lead to an increase in the recovery in the superfusion fluid. During dopamine infusion, less dopamine and DOPAC were recovered from tissue taken from animals having testosterone (either intact or GNX+T). In contrast, data acquired from experiments infusing estrogen into ovariectomized rats have demonstrated that estrogen blocks the DAT (Arvin et. al. 2000). When methamphetamine was infused into striatal tissue, it significantly increased dopamine release, but this was prevented in the presence of estrogen. This indicates that estrogen acts to inhibit DAT function (Myers, 2003), and therefore the striatal neurons were unable to recover the neurotoxin, which remained in the synaptic cleft (Dluzen, 2000).

The neurotoxin MPP^+ causes a selective destruction of dopaminergic neurons of the nigrostriatal pathway in humans, rats and mice and therefore has been used as a model for investigating PD. It is taken into the neuron terminal via the DAT and acts by displacing dopamine from its storage sites in synaptic vesicles, causing an increase in dopamine concentration gradient in the cyotsol. This reverses the direction of the DAT and eventually causes the release of dopamine into the extracellular space (Dluzen, 2000). Examination of figures 6 and 8 indicates a similar trend in the data following

MPP^+ infusions. We consistently found that the greatest percent increase in dopamine or DOPAC recovery was from the gonadectomized rats. The dopamine and DOPAC recovery from intact or GNX+T rats was reduced. These results support the hypothesis that DAT activity is reduced in the absence of testosterone. A study done by Dluzen and Disshon (1998) indicated that when estrogen was present and mice were treated with MPP^+, the outcome was increased dopamine released into the superfusion fluid. However, when they blocked the effect of estrogen with Tamoxifen (an estrogen receptor antagonist), the dopamine concentration was significantly less. This finding suggests that estrogen acts to inhibit DAT activity. In contrast, the results of the present study suggest that the presence of testosterone serves to enhance dopamine transporter function within the nigrostriatal dopaminergic system in the male brain. The increased dopamine released from corpus striatal tissue in castrated male animals in this study indicates that DAT function is decreased in the chronic absence of testosterone. These findings then suggest that in the presence of testosterone, DAT activity is increased. This elevated activity would enhance the ability of the dopaminergic neurons to take up neurotoxins, resulting in the death of these neurons and the eventual onset of PD.

This study confirms that nomifensine is working as a DAT blocker, as increased concentrations of dopamine and DOPAC were recovered during nomifensine treatment (figure 9 and 11). Analysis of the percent change data indicates that GNX animals had eight times more dopamine recovered in the superfusion fluid than either the GNX+T or intact groups (figure 10). These results for nomifensine treatment support the results obtained for dopamine infusion. Both studies confirmed that dopamine and DOPAC recovery rates were greatest from GNX animals when compared to GNX+T and intact rats. These results suggest that DAT activity is significantly inhibited in the absence of testosterone. In contrast, DAT activity is increased in the presence of testosterone suggesting a role for testosterone in the physiological modulation of the efficiency and efficacy of the DAT. In contrast, a study done by Disshon and Dluzen (1999) shows results of an in vitro superfusion study using estradiol, which like nomifensine increased the recovery of infused dopamine from the striatal tissue of ovariectomized female rats.

The experiments presented in this thesis suggest that androgens may function to increase the activity or efficiency of the DAT. In contrast, many experiments have reported that estrogen inhibits the activity of the DAT (Arvin, 1998; Dluzen, 2000; Disshon and Dluzen 1999; McDermott and Anderson 1999). Therefore, with respect to neurotoxins such as MPP+ that are known to gain entry to dopamine neurons via the DAT and lead to symptoms of classic PD it appears that men (higher levels of testosterone) maybe at greater risk of accruing the disease than women (higher levels of estrogen). The mechanism(s) by which the presence or absence of testosterone (or estrogen) facilitates or inhibits the DAT remain unknown. However, it has been reported that the activation of protein Kinase C (PKC) results in the phosphorylation of the DAT. This phosphorylation is linked to the internalization and subsequent down regulation of the DAT, which inhibits the reuptake of dopamine (Drew and Werling, 2001). Thus it is interesting to speculate that steroid hormone may alter DAT activity through mechanisms that involve the regulation of PKC.

In conclusion, testosterone is an important steroid that appears to modulate the function of the DAT. Results of our experiments demonstrated that dopamine and DOPAC recovery were greatest in the GNX rats and were decreased in the intact or the GNX+T animals. These findings suggest that testosterone acts to enhance DAT activity while the loss of testosterone reduces DAT function. With respect to activity of neurotoxins (such as MPP+) to cause symptoms of PD, our results suggest that men may be more prone to this disease due to the facilitatory actions of testosterone on DAT activity. Further experiments are needed to examine in detail the mechanisms of action of testosterone and on DAT function and to explore the relationship between DAT activity and neurodegenerative diseases.

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TO: Dean E. Dluzen, Ph.D.
Associate Professor, Anatomy

FROM: Gary D. Niehaus, Ph.D.
IACUC Chairperson

SUBJECT: Protocol Approval by the Northeastern Ohio Universities College of Medicine (NEOUCOM) Institutional Animal Care and Use Committee (IACUC)

DATE: December 10, 2002

The following NEOUCOM protocol was reviewed and approved by this Institution's Animal Care and Use Committee (IACUC) on December 10, 2002. Protocols involving the use of human tissues require Institutional Review Board (IRB) approval.

NEOUCOM Protocol No.:	02-033
Title of Protocol:	Gonadal Steroid Hormonal Modulation of Dopamine Transporter Function Within Male Rats
Type of Vertebrate:	Rats
Funding Agency:	Internal Funds

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW). The Assurance number is A3474-01. This institution is also registered with the United States Department of Agriculture (USDA). The USDA registration number is 31-R-0092.

The Comparative Medicine Unit (CMU) at the Northeastern Ohio Universities College of Medicine (NEOUCOM) has been accredited with the Association for Assessment for Accreditation of Laboratory Animal Care (AAALAC) International since June 8, 1982. Full accreditation was last renewed on July 8, 2002.

Thank you.

GDN:lkn

Cc: Gary B. Schneider, Ph.D.
Associate Dean of Basic Medical Sciences
Associate Dean for Research
NEOUCOM Institutional Official

Shannon Russell
Research & Sponsored Programs

File