DEVELOPMENTAL EFFECTS OF GONADAL STEROID HORMONES UPON NEUROPROTECTION OF THE NIGROSTRIATAL DOPAMINERGIC SYSTEM

By

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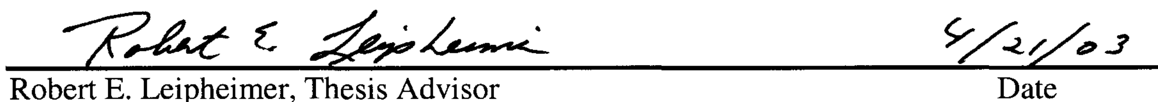

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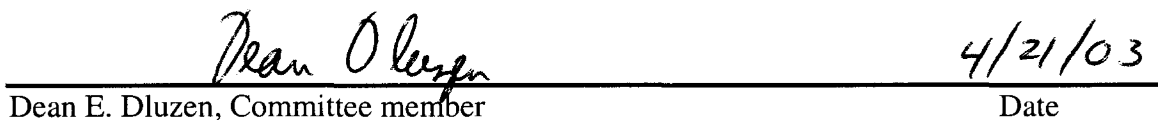

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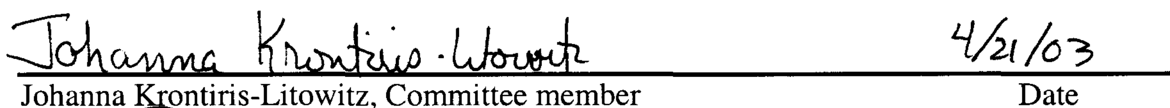

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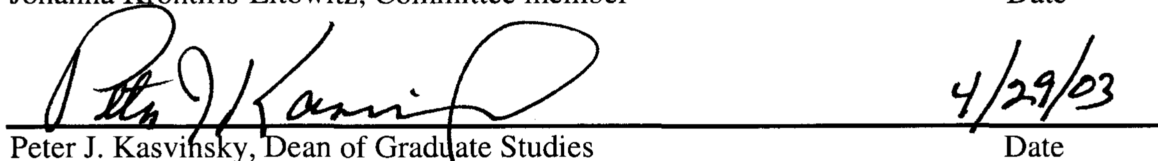

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ABSTRACT

DEVELOPMENTAL EFFECTS of GONADAL STEROID HORMONES UPON NEUROPROTECTION OF THE NIGROSTRIATAL DOPAMINERGIC SYSTEM

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Master of Science

Youngstown State University, 2003

The purpose of this report was to determine whether pre-pubertal gonadal steroid hormonal manipulation would alter estrogen's (E) ability to function as a neuroprotectant against methamphetamine (MA)-induced striatal dopamine (DA) depletion in the adult. Male and female CD-1 mice were gonadectomized (GNX) at 25 days of age and treated or not with testosterone (T). At 55 days of age mice were treated or not with E and at 62 days of age each of these 8 groups received either MA or vehicle. Striatal dopamine (DA) concentrations and release were measured to provide both a static and dynamic measure of neurotoxicity resulting from MA. As based upon striatal DA concentrations (pg of DA/mg of tissue) GNX at 25 days of age abolishes the capacity for E to function as a neuroprotectant since no differences are apparent between females treated or not with E as adults (4644±612 vs 3683±698) and pre-pubertal GNX failed to enable E to function as a nigrostriatal dopaminergic (NSDA) neuroprotectant in male mice (GNX+T+E = 2973±228: GNX+0+E = 1968±567). These data suggest a crucial role for E at the pre-pubertal period to enable E treatment to function as a neuroprotectant in the adult female. Also, the developmental effects exerted by T during the pre-pubertal period are not responsible for the inability of E to function as a NSDA neuroprotectant in the adult male. However, neuroprotection by E may be present in these same MA-treated males (P=0.612) and females (P=0.43) when compared with their respective vehicle treated controls as defined by potassium stimulated DA release. The divergent results in these data indicate the importance of the parameter used to measure neuroprotection. T and E have differential modulatory effects on DA function that are not different between P25 gonadectomized female and male mice.

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INTRODUCTION

Nigrostriatal Dopaminergic (NSDA) System

1.A. Anatomy of the Nigrostriatal Dopaminergic (NSDA) System

The NSDA system is comprised of a long loop pathway with cell bodies located within the substantia nigra that project to the striatum. Since the projections from the substantia nigra to the striatum primarily utilize the neurotransmitter dopamine (DA) the term nigrostriatal dopaminergic (NSDA) system has been applied to describe this pathway (Figure 1). The NSDA system is part of a larger system referred to as the basal ganglia. The basal ganglia is comprised of five large, extensively interconnected subcortical nuclei: the caudate nucleus, putamen, globus pallidus, subthalamic nucleus and substantia nigra. The caudate nucleus and the putamen develop from the same telencephalic structure, thus, they are composed of identical cell types and are fused anteriorly. They are collectively refered to as the striatum and serve as the input nuclei for the basal ganglia. The globus pallidus is derived from the diencephalon and lies medial to the putamen and lateral to the internal capsule. It is divided into internal and external segments. The subthalamic nucleus lies below the thalamus at its junction with the midbrain. The substantia nigra lies in the midbrain and has two zones. The pars reticulata is a ventral pale zone that cytologically resembles the globus pallidus. The pars compacta is a dorsal, darkly pigmented zone composed of dopaminergic neurons. This dark pigment is due to neuromelanin, a polymer derived from DA. The substantia nigra

pars reticulata and globus pallidus are often considered as a single structure due to similarities in cytology, function and neural connections. The substantia nigra pars reticulata and the globus pallidus comprise the major output nuclei of the basal ganglia. (Cote and Crutcher, 1991).

1.B. Functional Anatomy of the Nigrostriatal Dopaminergic System

Inputs - Together, the caudate nucleus, putamen, globus pallidus, subthalamic nucleus and substantia nigra function to integrate the control of movement. However, they do not make direct input or output connections with the spinal cord. The primary input is from the cerebral cortex and the output is directed through the thalamus back to the prefrontal, premotor, and motor cortex. Therefore, the frontal cortex orchestrates the motor functions of the basal ganglia. The striatum receives afferents from two major sources outside the basal ganglia. The corticostriate projection transmits motor, sensory, association, and limbic information from the entire cortex to specific areas of the striatum. The afferents from the intralaminar nuclei of the thalamus transmit information received from the motor cortex to the putamen. Cells from the striatum project topographically to specific parts of the globus pallidus and the substantia nigra. The external segment of the globus pallidus then projects to the subthalamic nucleus which in turn sends topographically specific projections to the substantia nigra pars reticulata and the globus pallidus. The motor cortex and the premotor cortex also have direct projections to the subthalamic nucleus. Lastly, the substantia nigra pars compacta sends a dopaminergic projection to the striatum. This nigrostriatal dopaminergic (NSDA) pathway is a long loop system containing 80% of the dopamine (DA) in the central

nervous system (Berlter and Rosengren, 1959). It is neurodegeneration within this NSDA pathway which is responsible for Parkinson's Disease (PD).

Output - The output from the striatum follows one of two major pathways. The direct pathway is composed of projections from the striatum to the globus pallidus interna, which then sends projections to the thalamus. The thalamus then sends this information back to the cortex. Stimulation of the direct pathway increases excitatory output from the thalamus to the cortex and thus facilitates movement. The neurotransmitter of the direct pathway is gamma-aminobutyric acid (GABA). Following the indirect pathway, the striatum projects to the globus pallidus externa and subthalamic nucleus which both relay information to the globus pallidus interna. GABA and glutamate are the neurotransmitters of the indirect pathway. Stimulation of the indirect pathway decreases excitatory output from the thalamus to the cortex thus inhibiting movement. The dopaminergic neurons of the substantia nigra innervate the striatum. The direct pathway is composed of excitatory DA D1 receptors in the striatum. The indirect pathway is mainly comprised of inhibitory DA D2 receptors. Thus, striatal dopamine release stimulates the direct pathway and facilitates movement (Cote and Crutcher, 1991). Counterbalance of these two pathways is responsible for the integration of sensorimotor function (Figure 2).

1.C. The Dopamine Neuron

The basic functional unit of the NSDA system is the dopamine (DA) neuron. At the cellular level, the synthesis of DA begins when tyrosine is transported across the blood brain barrier into the DA neuron. L-tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, the rate limiting step in DA synthesis. L-DOPA is

then converted to DA by L-aromatic amino acid decarboxylase. Under normal conditions rapid turnover during this step yields negligible DOPA levels in the brain (Roth and Elsworth,1995).

Vesicular monoamine transporter (VMAT) then moves the newly synthesized DA from the cytoplasm to storage vesicles, thus limiting the amount of free DA in the nerve terminal and protecting it from metabolism. An action potential in the DA nerve terminal produces a Ca^{2+}-dependent release of DA through vesicular fusion with the presynaptic membrane and expulsion into the synaptic cleft. The amount of DA released is a function of the rate and pattern of firing and presynaptic release-modulating autoreceptors (Cooper et al., 1996).

Once a DA nerve terminal has fired, the DA is subject to uptake, metabolism, or postsynaptic action. High affinity DA uptake sites terminate transmitter action and maintain transmitter homeostasis. Depending on the existent concentration gradient, a membrane carrier moves the DA in either direction. In addition, the DA transporter (DAT) actively pumps extracellular DA back into the nerve terminal, thus facilitating the recycling of DA. After reuptake, if the DA is not recycled it may be metabolized to DOPAC within the nerve terminal by monoamine oxidase (MAO). Also, released DA can be converted to homovanillic acid (HVA) through the actions of catechol-O-methyltransferase (COMT) and MAO. It is the remaining extracellular DA that activates postsynaptic DA receptors to exert its physiological function (Figure 3) (Cooper et al., 1996).

1.D. Clinical Significance of the NSDA System

Parkinson's Disease

a. Definition

The best appreciation of the NSDA system can be revealed with pathology of the system as occurs with Parkinson's disease (PD). In PD the counterbalance of the direct and indirect pathways is disrupted, with the result being severe disruptions in motor manifestations. PD was the first disease of the nervous system to be identified as a defect in transmitter metabolism (Calne, 2003).

b. Symptomology (Guyton,1981; Haavik and Toska,1998)

1-Rigidity of muscles may occur in a wide spread or isolated area. Excessive stimulation of the corticospinal system is believed to activate both afferent alpha motor fibers and the efferent gamma system as well. Thus, both the protagonist and antagonist muscles remain tightly contracted throughout movement.

2-Tremor at rest of the affected area, when present, is usually four to six cycles per second and may be temporarily blocked when voluntary movements are initiated. It is not really known what causes the tremor, but it is believed to result from hyperactivity of the cortico-basal ganglial-thalamic-cortical feedback circuit when DA is depleted in the basa ganglia.

3-Akinesia, an inability to initiate movement, is usully apparent in the final stages of Parkinson's disease. Extreme concentration by the patient is required to make even the

simplest movement. The cause of akinesia is unknown, but again it is believed to be due to the excess secretion of acetylcholine in the absence of DA inhibition in the striatum.

4-Bradykinesia, a slowness in ongoing movement, and

5-Loss of postural reflexes (Strange, 1992; Hallet, 2003) are other clinical characteristics of PD.

c. Etiology

The cause of PD is almost entirely unknown. Genetic (parkin), environmental factors (infectious agents, exogenous toxins, temperature, diet, residency) and various other metabolic disorders (excititory amino acid transmitters, endogenous reactive oxygen species (ROS), lack of neurotrophic hormones, or impaired DNA repair mechanisms) have all been linked with PD. (Mizuno, et al.,2001; Wszolek et al., 2001; Polymeropoulos et al., 1997; Takahashi and Yamada, 2001; McCormack et al., 2002; McLachlan, 2001; Schapira, 2001; Jenner,1992). Almost invariably PD results from the destruction of the substantia nigra, but may also result from damage to other related areas such as the globus pallidus. In any case, the symptoms result from a deficit in DA secretion from the nigrostriatal tract and possibly a decrease in the DA-biosynthetic capacity of the surviving cells. The DA secretion from the nigrostriatal tract normally inhibits or counters the acetylcholine secreting neurons that transmit excititory signals throughout the basal ganglia. Thus, the increase in excititory signals may be responsible for the characteristic movement deficits.

d. Gender differences

A literature review has reported a gender bias in the incidence of PD. Twenty-eight out of 34 studies (82%) in which the gender of Parkinson's patients was recorded

reported a greater occurrence in men (Dluzen et.al., 1998). More formal epidemiological studies have reported a two-fold higher incidence of PD in men (Baldereschi et al., 2000; Bower et al., 2000). These findings reveal an interesting and potentially important characteristic for understanding some of the bases for this condition since it raises the question of why there are gender differences in P.D. It is this question that, in part, serves as the foundation for this thesis.

In order to address this question it is necessary to use animal models of PD to: 1) determine whether a similar phenomenon (gender difference) exists and 2) perform controlled manipulations which can enable one to identify the mechanisms involved with these gender differences. Since the physiology and pathology of the NSDA system in an animal model are organized into ascending and descending pathways which are similar to those identified in the human (Lindvall and Bjorklund, 1978), the gender difference in nigrostriatal neurodegeneration can be replicated and investigated using an animal model (treated with a NSDA neurotoxin) of PD.

Figure 1. Sagittal view of the rodent brain. This view illustrates the major dopaminergic pathway from the substantia nigra to the striatum which comprises the nigrostriatal dopaminergic (NSDA) system (adapted from Cooper and Bloom, 1996).

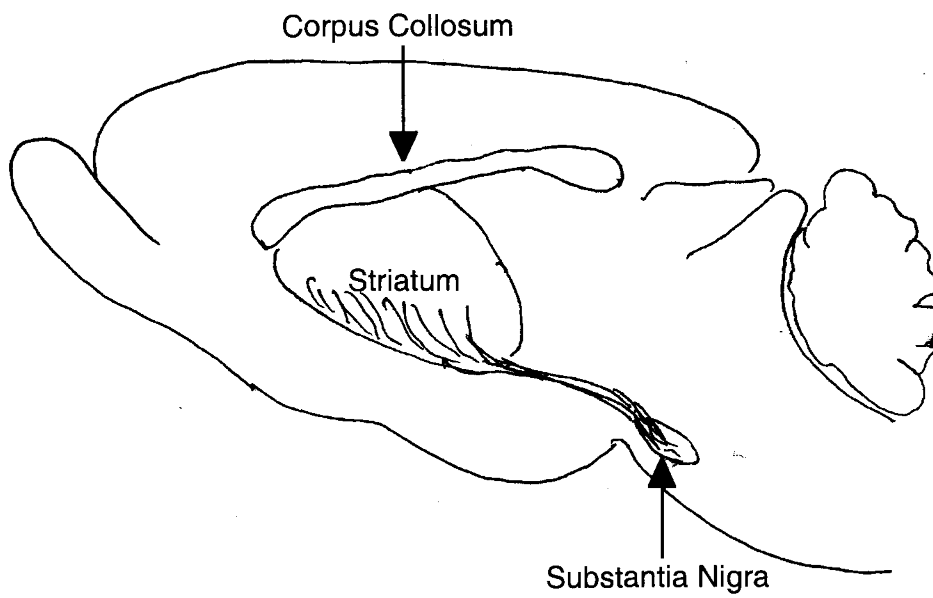

Corpus Collosum
Striatum
Substantia Nigra

Figure 2. Schematic diagram of the neural projections and neurotransmitters of the basal ganglia as described within the text (section 1B) (adapted from Cote and Crutcher, 1991).

DA receptor subtype 1 (D1)

DA receptor subtype 2 (D2)

gamma-aminobutyric acid (GABA).

lateral globus pallidus (LGP)

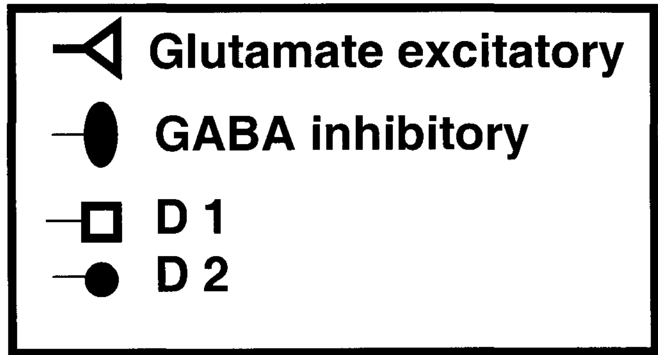
medial globus pallidus (MGP)

substantia nigra pars compacta (SNpc)

substantia nigra pars reticulata (SNpr)

subthalamic nucleus (STN)

ventroanterior and ventrolateral nuclei of the thalamus (VA/VL)


Glutamate excitatory
GABA inhibitory
D 1
D 2

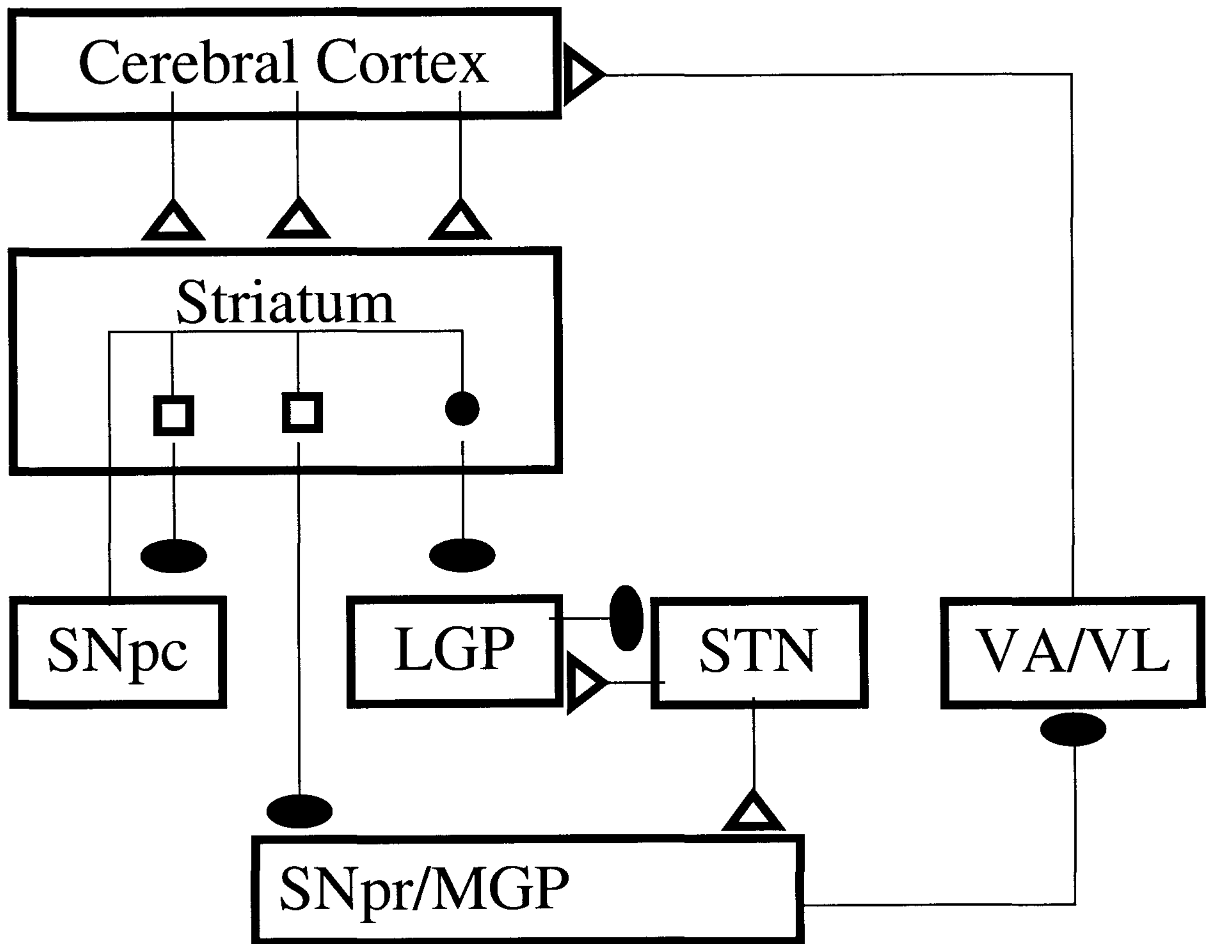
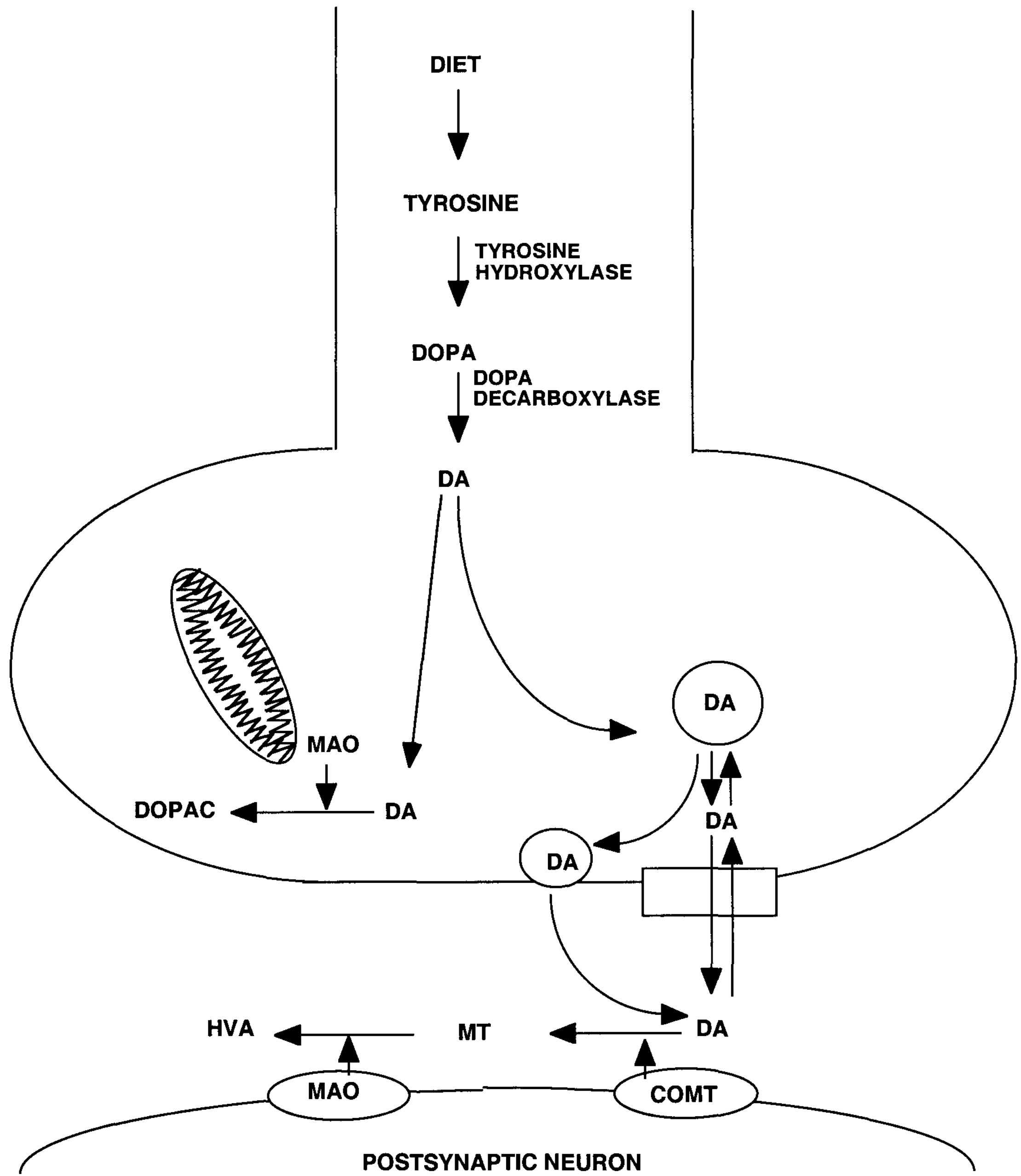

Cerebral Cortex
Striatum
SNpc
LGP
STN
VA/VL
SNpr/MGP

Figure 3. Model of Dopaminergic nerve terminal illlustrating some of the critical steps involved in dopamine (DA) synthesis and metabolism. (adapted from Cooper et al., 1996).


DIET
TYROSINE
TYROSINE
HYDROXYLASE
DOPA
DOPA
DECARBOXYLASE
DA
DA
MAO
DOPAC
DA
DA
DA
DA
HVA
MT
DA
MAO
COMT
POSTSYNAPTIC NEURON

NSDA System and Gender Differences – Animal Models

2.A. General Functional Differences

1. Behavioral

Spontaneous and amphetamine stimulated motor behaviors that are believed to reflect striatal dopaminergic activity exhibit gender differences. Female rats display increased motor activity when compared to males (Becker et al.,1982; Camp et al.,1986; Gordon et al., 1974). Female rats also display more intense amphetamine and apomorphine-stimulated stereotypies (Savageau et al., 1981). These increases in activity have been linked to increased striatal DA concentrations in the female when compared to the male (Gordon et al., 1974).

Not only are differences present between males and females, but within females differences are observed as a function of their estrous cycle stage. For example, performance on sensorimotor tasks (Becker et al., 1987) and amphetamine-induced behaviors (Joyce and Van Hartesveldt, 1984) vary within the female throughout the estrous cycle. These estrous cycle changes suggest that gender differences in behavior can involve modulation of NSDA function by gonadal steroid hormones and that these effects represent physiological actions of these gonadal steroids.

A. Neurochemical

In addition to the estrous cycle changes in behavioral responses involving the NSDA system, a number of other striatal dopaminergic indices vary with the estrous cycle. Accordingly, fluctuations in striatally mediated motor behaviors within the female may be orchestrated through changes in these parameters. For example, striatal DA (Xiao and

Becker, 1994) and its metabolite, homovanillic acid, (Fernandez-Ruiz et al., 1991), as well as L-DOPA evoked DA release (Dluzen and Ramirez, 1990) all vary as a function of the rat estrous cycle. These changes in striatal DA concentration and release may result from estrous cycle changes brought on by DA synthesis (Jori et al., 1976), uptake (Davis et al., 1977), and/or D_1 receptor density (Levesque et al., 1989), all of which show changes as a function of the estrous cycle.

2.B. Effects of Estrogen

In order to isolate the effects of estrogen from those of other hormones that fluctuate during the estrous cycle, studies have been conducted in which estrogen is administered to gonadectomized animals. The exogenous administration of estrogen causes changes in NSDA function when compared to non-estrogen treated gonadectomized controls. Estrogen has been reported to enhance striatal tyrosine hydroxylase activity (Pasqualini et al., 1995), which would indicate an increase in DA production. Increased DA metabolism in response to estrogen treatment has also been inferred from increased levels the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Di Paolo et al., 1985; Levesque and Di Paolo, 1988). In addition, *in vitro* superfusion studies have shown that estrogen acts synergistically to augment amphetamine- or potassium-stimulated DA release (McDermott et al., 1994b; Becker, 1990).

Although estrogen affects the dopamine uptake transporter (DAT), the effect on striatal DA uptake is not clear as there are conflicting reports. Morissette and Di Paolo have reported that acute and chronic estrogen administration increases DAT numbers in ovariectomized rats (Morissette et al., 1990; Morissette and Di Paolo, 1993), while others

indicate that DAT function is inhibited by estrogen (Arvin et al., 2000; Disshon et al., 1998; Disshon and Dluzen, 1999; Thompson, 1999). Such differences may be related to the specific conditions of estrogen administration, like those observed for DA receptors below.

The effects of estrogen on DA receptor number and affinity seem to be dependent on dose and duration of administration. Chronic treatment with estradiol tends to increase D1 (Levesque and Di Paolo, 1989; Hruska, 1986) and D2 (Di Paolo et al., 1982) receptor numbers, while a single dose of E seems to decrease D2 affinity (Levesque and Di Paolo, 1988). Thus, the dose and duration of estrogen exposure which result in changes in DA receptor numbers and binding affinities may be responsible for the differing reports on the effects of E treatments.

Results similar to those in animal studies have been observed in humans. For example, evidence suggests that DA turnover in humans is higher in females (Konradi et al,1992) while D_2 receptor affinity is higher in males (Pohjalainen et al, 1998). While a number of explanations are possible, it seems that E may play a role since overall E levels are greater in women, with values ranging between 100-400 pg/ml when compared to levels of less than 50 pg/ml in men (SmithKline Beecham Laboratories, 1999).

2.C. Neurotoxicity Differences

In addition to significant gender differences in the occurrence of PD (Dluzen et.al., 1998; Baldereschi et al., 2000; Bower et al., 2000), there are also gender differences in NSDA response to neurotoxins. Moreover, the incidence of methamphetamine (MA – a psychostimulant which activates the NSDA system) related deaths are relatively lower in

women (Hall and Broderick, 1991). The mechanisms responsible for sex differences in nigrostriatal responses to neurotoxins may also contribute to the pathology found in PD.

NSDA neurodegeneration has been modeled in laboratory animals to allow controlled hormonal manipulations and to identify the mechanisms involved in nigrostriatal neurodegeneration. These animal models involve the use of neurotoxic chemicals such as 1-methyl-4-phenyl-tetrahydropyridine (MPTP) or methamphetamine (MA) to produce nigrostriatal lesions (Gerlach and Riederer, 1996). Interestingly, in these animal studies there are also significant gender differences in neurodegeneration in response to neurotoxins that target the NSDA system.

2C1. Methamphetamine (MA)

MA is a psychostimulant with an oxidative component that, when administered in high doses, reduces striatal DA concentrations and DA uptake sites (Wagner et al., 1980; Gerlach and Riederer, 1996). It is thought that MA inhibits the activity of MAO which in turn leads to oxidative stress (Miller et al., 1998) and/ or the DA released by MA is converted to 6-hydroxydopamine (6-OHDA) which also leads to cell death (Seiden and Vosmer, 1984).

There exist significant male-female differences in the neurotoxic effects of MA, with male mice exhibiting more severe depletions of striatal DA concentrations (Miller et al., 1998; Wagner, et al., 1993; Yu and Liao, 2000a,b; Yu and Wagner, 1994; Heller et al., 2001). In addition, female mice show estrous cycle differences in NSDA neurotoxicity in response to MA (Yu and Liao, 2000a,b).

2C2. MPTP

MPTP was first discovered as a contaminant in synthetic heroin when patients receiving the drug developed the symptoms of PD (Davis et al., 1979). Since then, several MPTP – induced animal models of PD have been developed (Gerlach et al., 1991; Ali and Freyaldenhoven, 1999). The MPTP model of neurodegeneration is selective for NSDA depletion and more closely resembles the pathology of PD.

In order to exert its actions, MPTP must first be converted to its active metabolite, MPP^+, by MAO-B in the glia. This MPP^+ is then moved into the NSDA nerve terminals through the DA transporter. At this point, the neurotoxin may inhibit mitochondrial respiration and thus effect ATP depletion (Rollema et al., 1988), form free radicals that cause oxidative damage (Chiueh et al., 1994), or disrupt calcium homeostasis (Gerlach and Reiderer, 1996).

Similar to that observed in response to MA, male mice have greater nigrostriatal DA depletions following exposure to MPTP (Brooks et al., 1989; Freyaldenhoven, et al., 1996; Miller et al., 1998).

B. NSDA System and Neuroprotection by Gonadal Steroids-Activational

3.A. Estrogen

An important variable involved in the above mentioned gender differences is the gonadal steroid estrogen (E), which can function as a neuroprotectant of the NSDA system (Gao and Dluzen, 2001a; Dluzen et al., 1998; Dluzen, 2000; Dluzen et al., 2000a ; Dluzen et al., 2000b). Thus, events that alter E levels can influence NSDA neurodegeneration and may play a pivotal role in relation to gender differences and PD.

There is a clear gender difference in estrogen's ability to act as a neuroprotectant in response to MA (Wagner et al., 1993). In our lab we have found that E can serve as a neuroprotectant against MPTP (Dluzen et al., 1996), but not MA (Gao and Dluzen, 2001) in male mice. However, E supplements have been shown to preserve dopamine concentrations in ovariectomized female mice when treated with either MPTP (Dluzen et al., 1996; Miller et al., 1998) or MA (Gao and Dluzen, 2001a; Yu and Liao, 2000) when compared to non-E treated controls.

3.B. Progesterone-

The gonadal steroid, progesterone, can also modulate NSDA functioning (Dluzen and Ramirez, 1990b; Dluzen and Ramirez, 1990c).

Progesterone has also been shown to modulate MA-induced DA and serotonin depletions in C57BL/6J mice (Yu and Liao, 2000a). However, the increase in progesterone during estrus and diestrus is believed to increase neurotoxic effects of MA on striatal serotonergic neurons (Yu and Liao, 2000b). Therefore, in isolation progesterone may function as a neuroprotectant, but normally cycling progesterone does not protect NSDA neurons from the DA depletion induced by MA (Yu and Wagner, 1994).

3.C. Testosterone-

Like E, testosterone (T) modulates NSDA function. This is evidenced by increased *in vitro* DA release and locomotor activity following castration in male rats (Dluzen and Ramirez, 1989). However, neither testosterone (T) nor dihydrotestosterone (DHT) can act as a neuroprotectant against NSDA neurotoxins (Dluzen, 1996; Gao and Dluzen, 2001; Grandbois, et al., 1999). Not only does T not function as a neuroprotectant, but we

have observed that the presence of T tends to enhance MA-induced DA depletion in the corpus striatum (CS) (Gao, and Dluzen, 2001).

4. NSDA System and Neuroprotection by Gonadal Steroids-Developmental Definition/character

17ß-estradiol is a fundamental regulator of reproductive physiology and behaviors. In addition to this regulation of neuroendocrine function and sexual behaviors, E is also significantly involved in normal development and genderization of the central nervous system and has important neurotrophic and neuroprotective roles in the developing mammalian brain (Kelly, 1991).

The developing nervous system is sexually dimorphic. The female brain will develop in the presence or absence of E. Thus, the brain is by default female. In order for the male brain to develop, androgens must be present. Neonatal manipulations of gonadal steroid hormones can result in masculinized females or feminized males. Male rats that have been castrated at 5 days after birth behave like genetic females and will display lordosis when injected with E and progesterone (Crowley et al., 1978; Gorsky,1980). Gonadal steroid manipulations during the pre-pubertal developmental period have also been shown to disrupt reproductive capabilities in the adult animal (Gorsky, 1980; Kelly, 1991). Moreover, it may be the changes in the gonadal steroid hormones during puberty which are responsible for the gender differences seen in the adult NSDA system (Becker and Ramirez, 1981; Laviola, 2001).

Purpose of the Thesis

Gonadal steroid manipulation during the pre-pubertal developmental period disrupts reproductive capabilities in the adult animal. In addition, the gonadal steroid E has been shown to have activational affects that afford neuroprotection against a MA assault in the adult mouse (Dluzen and McDermott, 2000b; Dluzen, Disshon and McDermott, 1998; Dluzen, McDermott and Anderson, 2001; Gao and Dluzen, 2001a; Dluzen, Anderson and Pilati, 2002). Therefore, the purpose of the present thesis is to examine the developmental effects of gonadal steriod hormone manipulation during the pre-pubertal period upon neuroprotective capabilities of E in the adult.

The hypothesis tested was that gonadal steroid manipulation when performed at critical developmental periods can produce long term changes in central nervous system responsiveness to E with regard to its capacity to function as a neuroprotectant within the adult. Specifically, would the removal of steroid hormones during the pre-pubertal period abolish the neuroprotective ability for E to modulate the response of the NSDA system to a neurotoxin (MA) in the adult?

To accomplish this goal, in Experiment 1, pre-pubertal female CD-1 mice were gonadectomized to determine if the absence of E and/or the presence of T is the critical factor in changing responses to the NSDA neurotoxin MA. DA tissue content was measured and superfusion performed to evaluate changes in DA release and turnover. In Experiment 2 pre-pubertal male CD-1 mice were gonadectomized and subjected to hormonal manipulations identical to those in Experiment 1. These experiments addressed the question of whether E is capable of serving as a neuroprotectant regardless of the endocrine condition under which the brain has developed. The fact that gender

differences exist for responses to NSDA neurotoxins suggests that either brain organization is different between females and males and/or the acute presence of E enables a differential response. These experiments address this issue of the source for the differences observed in neurotoxins between females and males.

EXPERIMENT 1: Gonadectomized females at P25 ± T replacement ± E ± MA

INTRODUCTION

As presented in the general introduction, the gonadal steroid E has been shown to have activational affects that afford neuroprotection against a MA assault in the adult female mouse (Dluzen and McDermott, 2000b; Dluzen, Disshon and McDermott, 1998; Dluzen, McDermott and Anderson, 2001; Gao and Dluzen, 2001a; Dluzen, Anderson and Pilati, 2002). Since gonadal steroid manipulation during the pre-pubertal developmental period disrupts reproductive capabilities in the adult animal the question arises will these manipulations during the pre-pubertal period also affect the neuroprotective capability of E against a MA assault in the adult.

The purpose of Experiment 1 was to examine the developmental effects of gonadal steriod hormone manipulation during the pre-pubertal period upon neuroprotective capabilities of E in the adult female. To accomplish this goal, pre-pubertal female CD-1 mice were gonadectomized to determine if the absence of E and/or the presence of T is the critical factor in changing responses to the NSDA neurotoxin MA. DA tissue content was measured and superfusion performed to evaluate changes in DA release.

PROCEDURES

ANIMALS: Female CD-1 mice were used in this experiment. The mice were housed in the comparative medicine unit at NEOUCOM under a 12 hr light:dark cycle, with lights on at 0600 h. Water and standard laboratory chow were available *ad libitum* and room temperature was set at 21 - 23^0 C. All treatments of the animals adhered to the NIH guidelines for the care and use of animals and were approved by the IACUC committee at NEOUCOM.

GONADECTOMY: The mice were weaned at 21 days of age and at 25 days of age gonadectomized while anesthetized with a solution consisting of Ketamine (150mg): Acepromazine (5mg): Xylazine (30mg) (at a dose of 0.5-0.7 ml/kg body weight) administered i.m. as recommended in the procedures described by the Comparative Medicine Unit at NEOUCOM. At 25 days of age mice are in a pre-pubertal stage of development (AALAS, 1999) and therefore gonadectomies were performed prior to puberty in these mice.

GENERAL PROCEDURES: Eight separate treatment groups were generated. A timeline containing the treatment procedures and ages at treatments is presented in Figure 4.

At gonadectomy (25 days of age) the mice either received no hormonal treatment or a subcutaneous implant of a pellet containing 5 mg T, 60-day release (Innovative Research of America, Sarasota, FL) in the dorsal neck region. This dose was chosen since the 5 mg concentration has been indicated by the manufacturer to produce approximately physiological concentrations of serum T in male mice (Dr. Shaffre - Innovative Research of America, personal communication). The use of a 60-day release pellet assured that these T levels would be maintained into adulthood within these mice.

At 55 days of age the mice from each of the two groups generated (Gonadectomy ± T at 25 days) were implanted or not with an E pellet (17 ß-estradiol, 0.1 mg, 21-day release) in the dorsal neck region while under halothane anesthesia (ProVet). We have demonstrated previously that this dose of E produces serum concentrations of 266.3 ± 21.8 pg/ml within female mice (Gao and Dluzen, 2001), which are within the range of physiological levels. In addition, this dose of E has been shown to provide a

neuroprotective effect upon MA neurotoxicity of the nigrostriatal dopaminergic (NSDA) system within female mice (Dluzen and McDermott, 2000b; Dluzen, Disshon and McDermott, 1998; Dluzen, McDermott and Anderson, 2001; Gao and Dluzen, 2001a).

At 62 days of age the four groups generated thus far (gonadectomy ± testosterone at 25 days X ± estrogen at 55 days) were divided randomly into two groups and treated with methamphetamine (MA) (Sigma Chemical Company, St. Louis, MO) or its vehicle. The MA treatment consisted of 4 i.p. injections at a concentration of 20 mg/kg administered at 2 hour intervals. Vehicle treated mice received an identical regimen using saline. This dose of MA was used since previous work in our laboratory has shown it to produce a clear neurotoxicity upon the NSDA system, reducing striatal dopamine (DA) concentrations to 25% of that observed in controls (Gao and Dluzen, 2001). In intact female mice as well as female mice gonadectomized as adults, E can significantly reduce the amount of striatal DA depletion produced by MA (Gao and Dluzen, 2001a; Dluzen et al, 2000; Dluzen, 1997; Dluzen et al., 1996; Dluzen and McDermott, 2000b; Dluzen, Anderson and Pilati, 2002).

At 69 days of age, the mice were sacrificed by rapid decapitation. The bilateral corpus striatum was dissected with one side prepared for determination of DA concentration and the contralateral side prepared for determination of basal and potassium stimulated DA release using *in vitro* superfusion. In this way both a static and dynamic assessment of striatal dopaminergic function were achieved as a function of the treatments administered.

Figure 4. Timeline containing the treatment procedures and ages at treatments.

21 DAYS OLD — FEMALE CD-1 MICE WEANED

25 DAYS OLD — FEMALE CD-1 MICE GONADECTOMIZED

TESTOSTERONE | No TESTOSTERONE

55 DAYS OLD — ESTROGEN | No ESTROGEN | ESTROGEN | No ESTROGEN

62 DAYS OLD — MA | No MA | MA | No MA | MA | No MA | MA | No MA

69 DAYS OLD — SACRIFICE ANIMALS BY RAPID DECAPITATION

1/2 CS - DA CONCENTRATION

1/2 CS - DA RELEASE

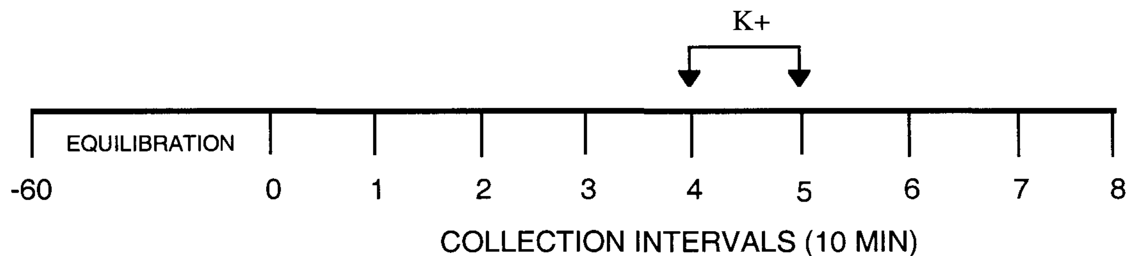
MA - Methamphetamine
DA - Dopamine

ENDOCRINE EVALUATION: All animals were inspected at sacrifice. Uterine, pituitary, and body weights were recorded from each animal to verify the effects of treatments administered (gonadectomy ± T or E).

TISSUE DISSECTIONS: Following a midline incision, the bisected brain was separated. The ventricles on the medial side of the brain were opened and the cortex cut away revealing the CS. The perimeter of the CS within the corpus callosum, anterior commisure and internal capsule/cortex was cut and the CS removed with curved scissors. One side of the CS was used for CS DA concentration determinations and the contralateral side was used to measure basal and potassium stimulated DA release using superfusion. The side used for each assay was alternated with each animal.

SUPERFUSION: The superfusion system (Figure 5) consisted of the barrel of a 1 ml plastic tuberculin syringe cut off at the 0.3 ml level and attached to a 22 guage stainless steel lumbar puncture needle. This assembly was placed in a temperature controlled water bath that maintained the tissue at 37^0 C. The superfusion medium consisted of a modified Kreb's Ringer Phosphate (KRP) buffer: 120 mM NaCl, 4.8 mM KCl, 0.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.8 mM NaH_2PO_4, 10.2 mM Na_2HPO_4 and 0.18% glucose at a pH of 7.4. The KRP medium was filtered (0.45 μm) prior to use. Superfusion medium was delivered into the bottom of the chamber through the 22 guage lumbar puncture needle at a flow rate of approximately 25 μl/min. The tissue from each individual animal was dissected into small tissue fragments (approximately 0.5 X 0.5 X 0.5 mm) prior to placement in the superfusion chamber. The tissue fragments were suspended within the chamber and maintained within 100 μl of medium volume by cellulose filters placed above and below the tissue fragments. The chambers were sealed with a stopper

containing two needles, one an entry port supplying filtered humidified air to oxygenate the tissue within the chamber and the other serving as an exit port for collection of effluent perfusate samples. The striatal tissue fragments were allowed to equilibrate for sixty minutes prior to collection of effluent samples. Effluent samples were collected at 10 minute intervals into 1.5 ml microcentrifuge tubes maintained on ice. Following a three interval basal collection period, medium containing a mild depolarizing concentration of potassium chloride (K^+ - 30mM) was infused during interval four. Superfusion medium containing the depolarizing concentration of potassium contained a corresponding reduction in NaCl to maintain an osmolarity at approximately 290 mOsm. With the start of collection interval five, the normal superfusion medium was resumed and maintained until the completion of the experiment at collection interval eight. At the end of the superfusion, tissue fragments were removed and weighed. The superfusion system for determination of *in vitro* catecholamine release has been extensively used and validated in our laboratory (Disshon and Dluzen,1997; Disshon and Dluzen, 1999; Dluzen, McDermott and Liu, 1996; Gao and Dluzen, 2001a; Gao and Dluzen, 2001b; Xu and Dluzen, 1996; Dluzen and McDermott, 2000).



TISSUE CONCENTRATION DETERMINATIONS: Tissue concentrations of DA were determined at sacrifice as well as after superfusion. For these determinations the

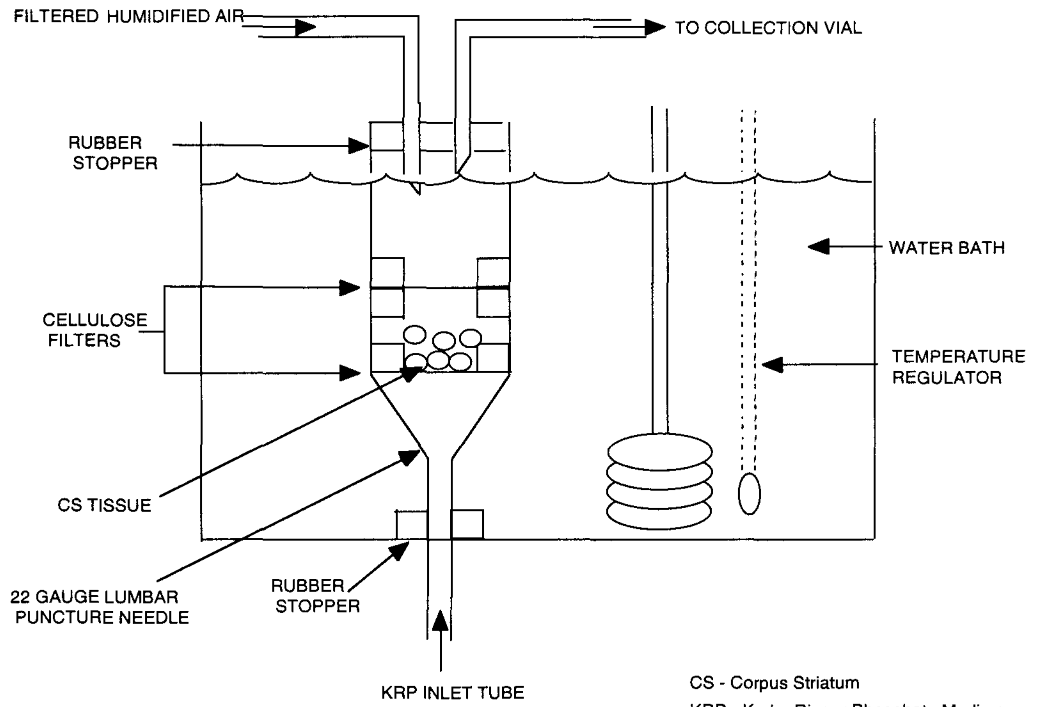
tissue was weighed and placed in vials containing 500 μl cold (4^0 C) 0.1 N perchloric acid. The tissue samples were sonicated 20 sec, centrifuged and a sample from the supernatant was removed for assay of neurotransmitter levels. These levels were expressed as pg/mg tissue weight.

NEUROCHEMICAL ASSAY: Measurements of DA from both superfusion and tissue samples were determined using HPLC-EC (ESA Inc.). Using a 100 mm X 3.0 mm, 5 μm Discovery C-18 reverse phase column (Sigma Chemical Company) with an isogradient mobile phase (27.4 mM citric acid, 50 mM sodium acetate, 10 mM sodium hydroxide, 0.1 mM sodium octyl sulfate, 0.1 mM EDTA in distilled water at a pH of 4.5). The mobile phase was filtered through a milipore filter (0.45 μm) and degassed prior to use. Standards for DA (Sigma Chemical Co.) stored in 0.1N perchloric acid were diluted in either perchloric acid or the KRP superfusion medium in doses of 6.25, 12.5, 25, 50, 100, 200, and 400 pg/20 μl and used to construct standard curves for tissue concentration or superfusate samples, respectively.

DATA ANALYSIS: The release rate data of the superfusions were blocked into two discrete segments of release - basal and stimulated. In this way, two different components of these data were analyzed and evaluated separately. To determine whether a difference in spontaneous (basal) release was present among the treatment conditions the mean output from collection interval 3 was calculated. This period of the superfusion typically displays the most stable basal release rate. Mean output from collection intervals 4-6 were used to calculate the potassium-stimulated release response since this represented the period of maximal responsiveness. ANOVAs were performed in mice

treated with MA or its vehicle at adulthood. A $p < 0.05$ was required for results to be considered statistically significant.

Figure 5. The superfusion system.


FILTERED HUMIDIFIED AIR
TO COLLECTION VIAL
RUBBER STOPPER
WATER BATH
CELLULOSE FILTERS
TEMPERATURE REGULATOR
CS TISSUE
22 GAUGE LUMBAR PUNCTURE NEEDLE
RUBBER STOPPER
KRP INLET TUBE
CS - Corpus Striatum
KRP - Krebs Ringer Phosphate Medium,

RESULTS

A summary of the striatal DA tissue content profile is shown in Figure 6. There were no statistically significant differences in DA concentration observed among the four vehicle treatment groups; therefore, these values were pooled into one vehicle control group (N=29). Although all of the MA treated groups had significant decreases in DA concentration when compared to the vehicle controls, there were no significant differences in DA concentration among the MA treatment groups (p=0.417). The inset represents the neuroprotection afforded by estrogen against a MA assault when the female mice were ovariectomized as adults (p=0.03). The disruption of a neuroprotective effect of E upon DA concentration in pre-pubertal gonadectomized females is indicated by three criteria. First, as shown in Figure 6, is the absence of a difference between the No T/No E and No T/E groups. The lack of a difference between these two critical groups illustrates an absence of any neuroprotectant capacity by E. Second, DA concentrations of E-treated females ovariectomized at 60 days of age (inset data) are significantly greater (p=0.011) than that obtained from pre-pubertally ovariectomized females treated or not with E as adults as presented in Figure 7. Finally, none of the females ovariectomized at 25 days and receiving hormonal manipulations were different from the females that were ovariectomized at 60 days and subjected to MA (p=0.435) (Figure 8). It is interesting to note in Figure 6 that DA concentrations of ovariectomized pre-pubertal mice treated with T were lower than those of the No T groups, although this decrease in DA concentration did not prove to be significant (p=0.19).

In vitro superfusion was performed to further characterize the effects of pre-pubertal hormonal manipulations through a dynamic measure of tissue responsiveness. The

overall DA release rate profiles for the four hormonal manipulations ± MA are presented in Figure 9. The superfusion data were blocked into discrete segments to analyze baseline (interval 3) and 30mM potassium stimulated (intervals 4-6) responses. Unpaired t-tests were used to compare vehicle vs MA treated release responses within each hormonal treatment group as summarized in Figures 10 – 13. For the P25 ovariectomized females receiving no hormones (0+0) both basal (p=0.009) and potassium stimulated (p=0.05) DA release rates of MA-treated mice were significantly decreased compared with vehicle treated controls (Figure10). Neither basal (p=0.13) nor potassium stimulated (p=0.43) DA release rates of E+MA-treated mice (Figure 11) or the of T+E+MA-treated mice (p=0.097 and p=0.083, respectively) (Figure 12) were significantly decreased compared with their respective vehicle treated controls (N.S.). Within the T+0 groups only the basal (p=0.023) DA release rates of T+MA-treated mice were significantly decreased compared with vehicle treated controls (Figure 13). It is interesting to note in Figure 9 that 30mM potassium stimulated (intervals 4-6) responses of ovariectomized pre-pubertal mice treated with T were lower than those of the 0 +E group. This decrease in DA concentration did not prove to be significant (p=0.177); however, there does seem to be a tendency, again suggesting that T may exacerbate MA-induced DA neurotoxicity.

Figure 6. Summary of the striatal DA tissue content (Mean±SEM in pg/mg) profile. Bars with different lettered superscripts indicate conditions which show statistically significant differences. The inset illustrates the typical neuroprotection observed in adult female mice treated with E.

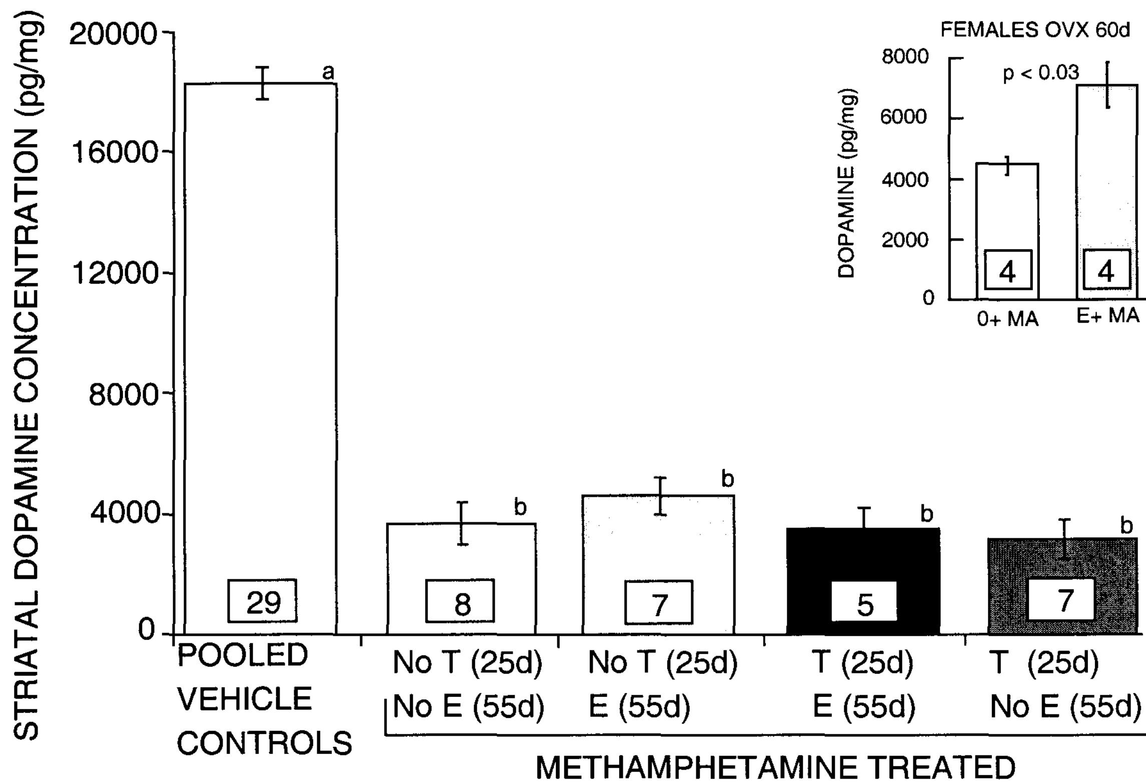

FEMALES
STRIATAL DOPAMINE CONCENTRATION (pg/mg)
20000
16000
12000
8000
4000
0
a
b
29
8
7
5
7
POOLED
VEHICLE
CONTROLS
No T (25d)
No E (55d)
No T (25d)
E (55d)
T (25d)
E (55d)
T (25d)
No E (55d)
METHAMPHETAMINE TREATED
FEMALES OVX 60d
DOPAMINE (pg/mg)
8000
6000
4000
2000
0
p < 0.03
4
4
0+ MA
E+ MA

Figure 7. Comparison of neuroprotection against MA among E-treated females ovariectomized at 60 days, a model of neuroprotection, (inset data) and females ovariectomized during the pre-pubertal period and receiving any hormonal manipulations. Bars with different lettered superscripts indicate conditions which show statistically significant differences.

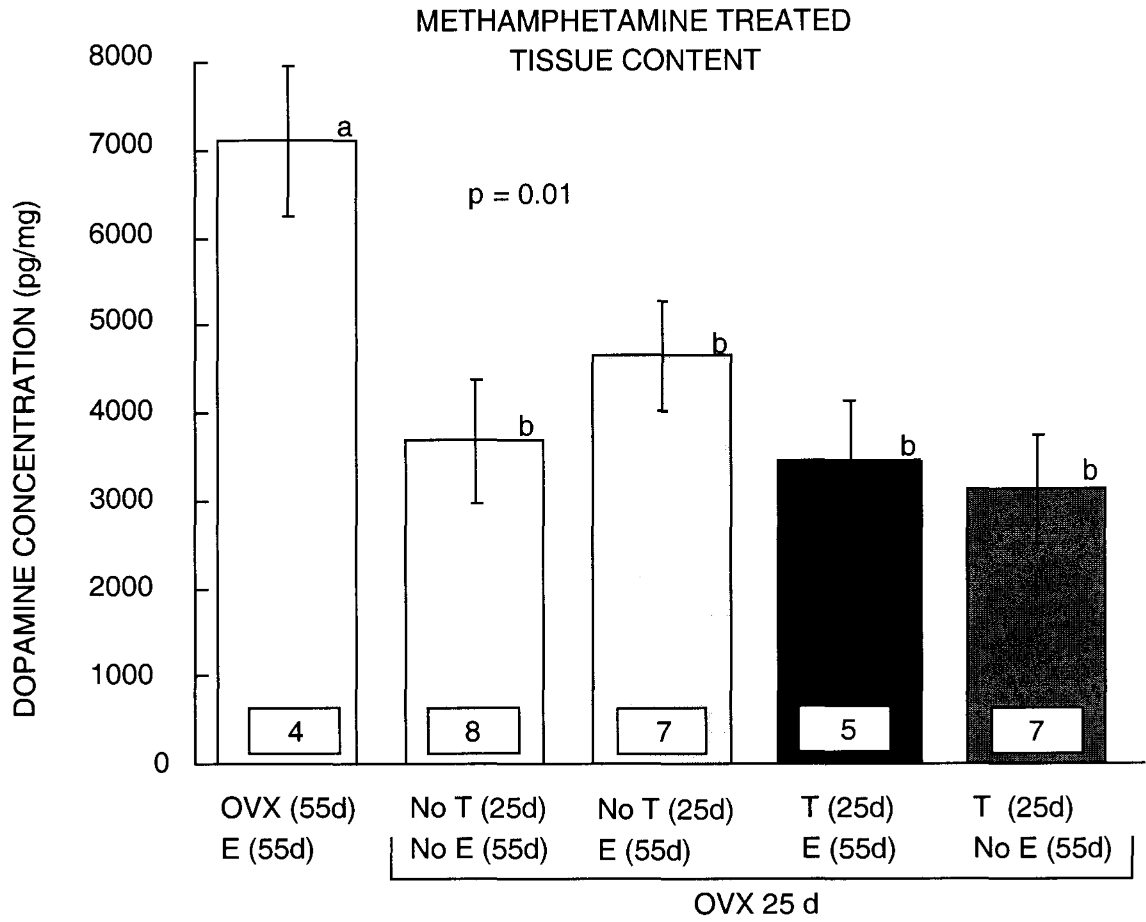

METHAMPHETAMINE TREATED
TISSUE CONTENT
DOPAMINE CONCENTRATION (pg/mg)
8000
7000
6000
5000
4000
3000
2000
1000
0
p = 0.01
a
b
b
b
b
4
8
7
5
7
OVX (55d)
E (55d)
No T (25d)
No E (55d)
No T (25d)
E (55d)
T (25d)
E (55d)
T (25d)
No E (55d)
OVX 25 d

Figure 8. Comparison of females ovariectomized at 60 days and subjected to MA (inset data) with females ovariectomized at 25 days and receiving hormonal manipulations and subjected to MA. No overall statistically significant differences were obtained among the groups (N.S.).

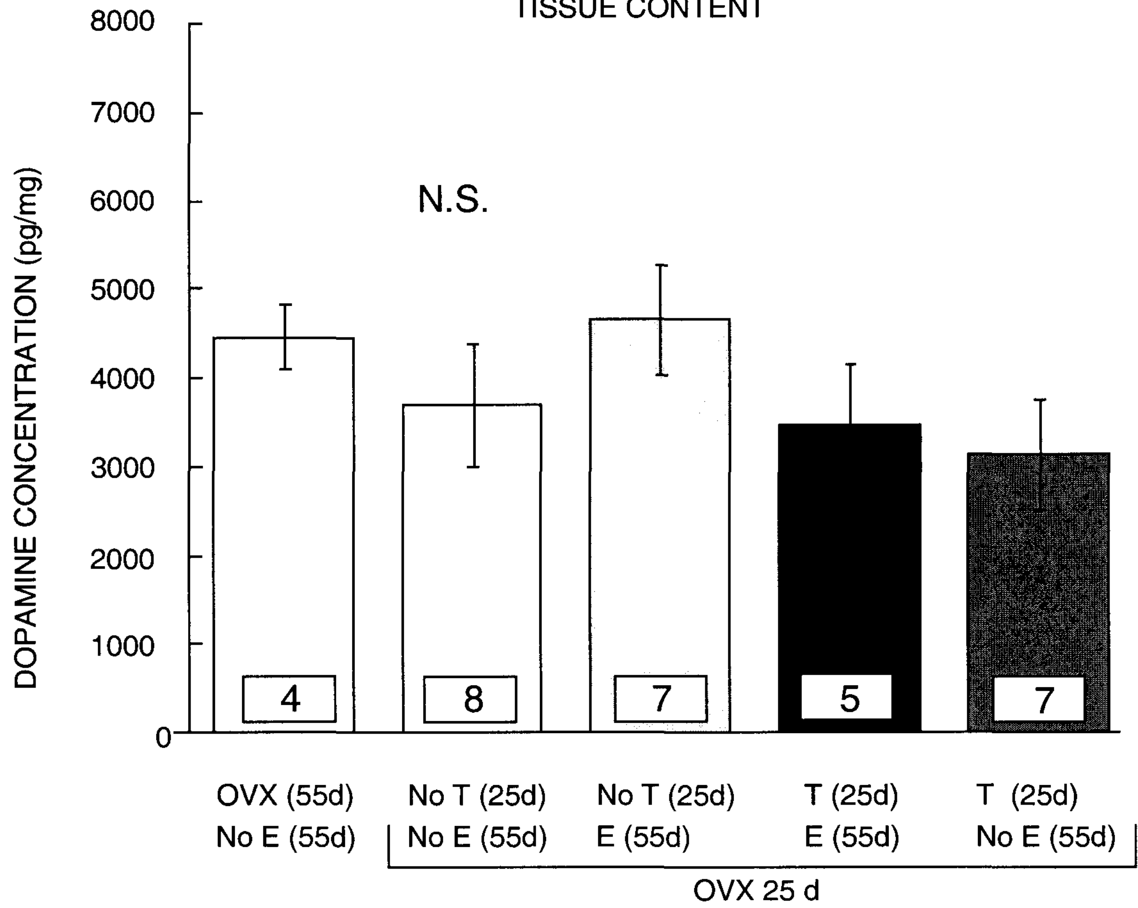

METHAMPHETAMINE TREATED
TISSUE CONTENT
DOPAMINE CONCENTRATION (pg/mg)
8000
7000
6000
5000
4000
3000
2000
1000
0
N.S.
4
8
7
5
7
OVX (55d)
No E (55d)
No T (25d)
No E (55d)
No T (25d)
E (55d)
T (25d)
E (55d)
T (25d)
No E (55d)
OVX 25 d

Figure 9. Overall summary of *in vitro* superfusion DA release rate profiles (pg/mg/min) for the four hormonal manipulations ± MA. The black bar on the X axis indicates the period of infusion of a depolarizing concentration of potassium (30mM) within the superfusion chambers. Bars for SEM were omitted from this figure for purpose of clarity. Means with SEM bars are contained within individual data analyses of each treatment group as presented in Figures 10 – 13.

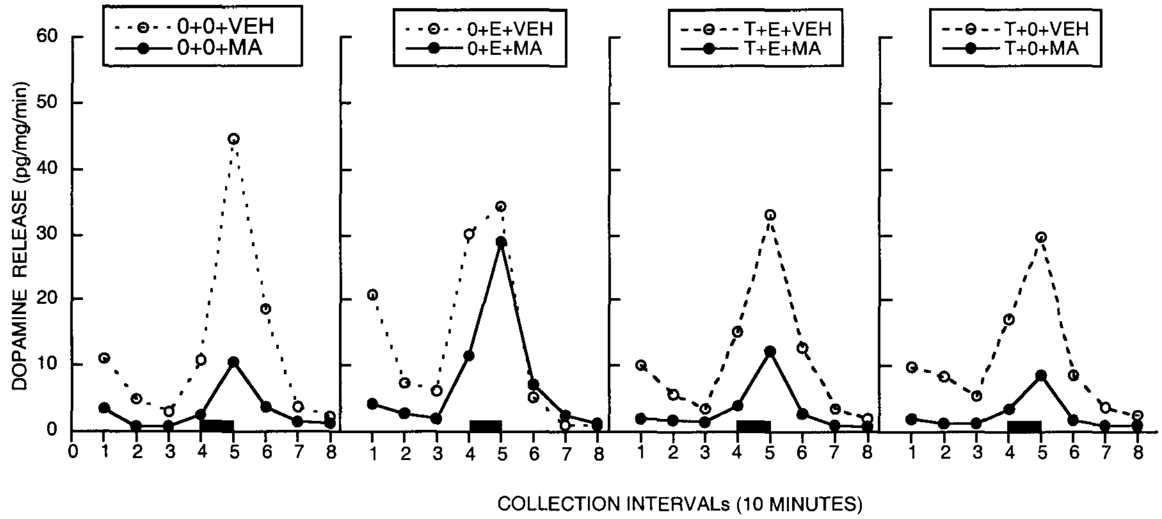

0+0+VEH
0+0+MA
0+E+VEH
0+E+MA
T+E+VEH
T+E+MA
T+0+VEH
T+0+MA
DOPAMINE RELEASE (pg/mg/min)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
COLLECTION INTERVALs (10 MINUTES)

Figure 10. Overall, mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) for P25 ovariectomized females receiving no hormones (0+0). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Both basal and potassium stimulated DA release rates of MA-treated mice were significantly decreased (p=.009 and .05, respectively) compared with vehicle treated controls.

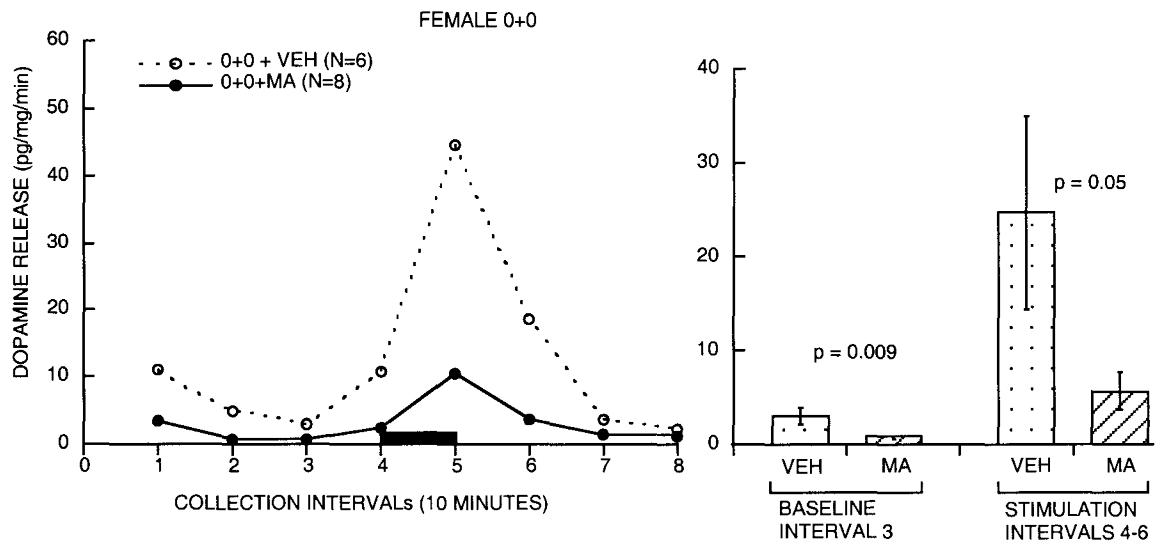

FEMALE 0+0
0+0 + VEH (N=6)
0+0+MA (N=8)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVALs (10 MINUTES)
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
p = 0.009
p = 0.05
VEH
MA
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Figure 11. Overall, mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) for P25 ovariectomized females receiving E (0+E). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Neither basal nor potassium stimulated DA release rates of E+MA-treated mice were significantly decreased compared with vehicle treated controls (N.S.).

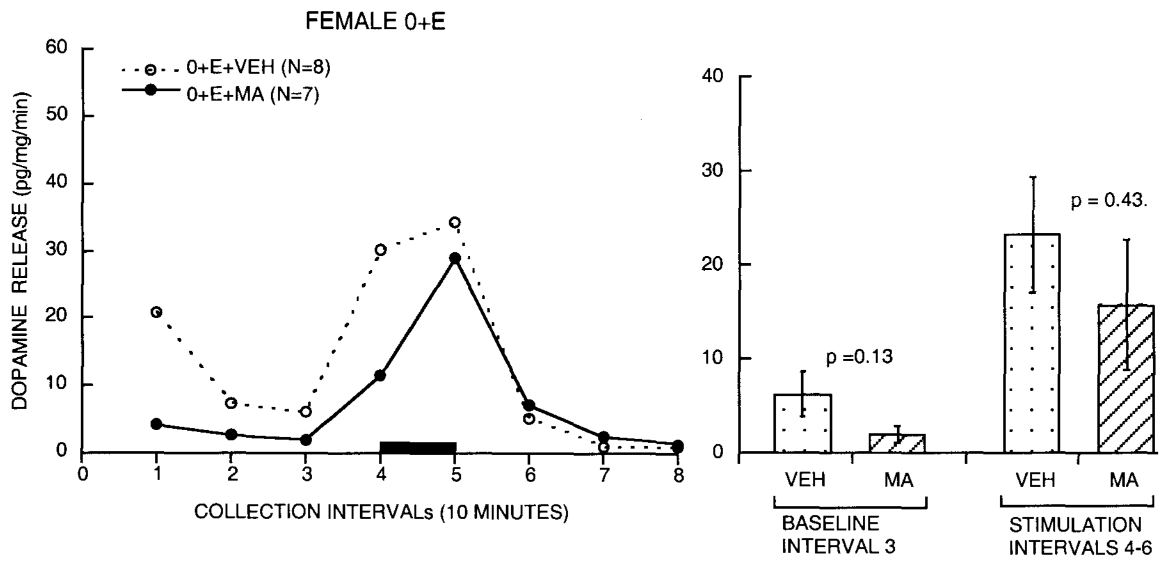

FEMALE 0+E
0+E+VEH (N=8)
0+E+MA (N=7)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVALs (10 MINUTES)
p =0.13
p = 0.43.
VEH
MA
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Figure 12. Overall, mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) for P25 ovariectomized females receiving testosterone and estrogen (T+E). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Although there seemed to be a decreased potassium stimulated release within the MA-treated group, neither basal nor potassium stimulated DA release rates of T+E+MA-treated mice were significantly decreased compared with vehicle treated controls (N.S.).

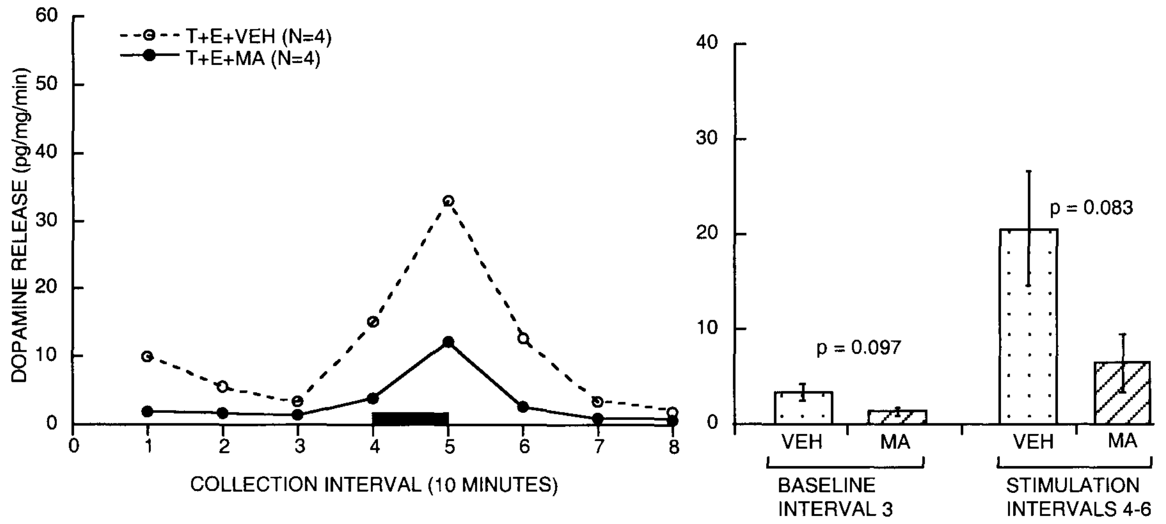
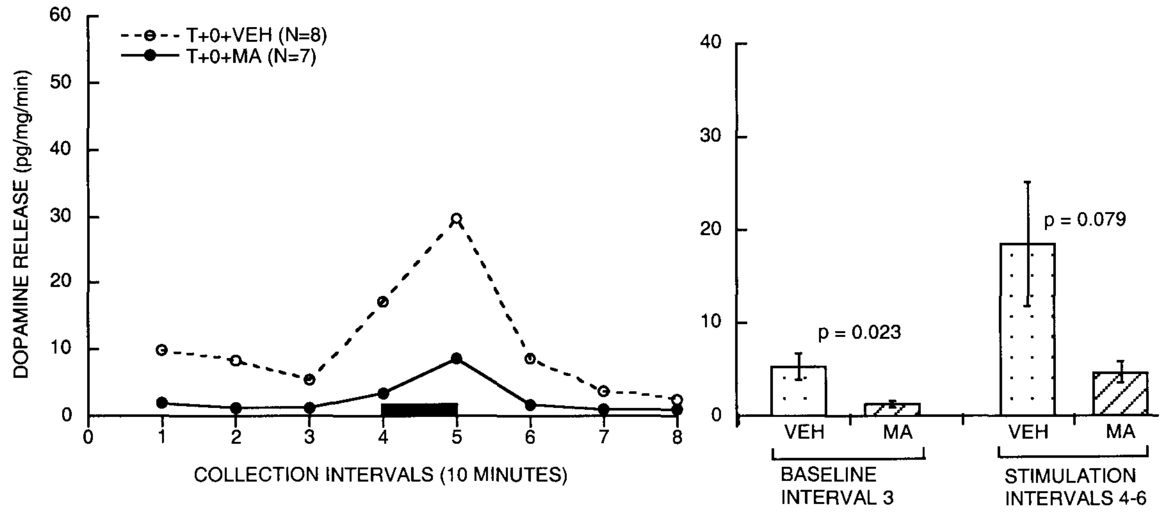

FEMALE T+E
T+E+VEH (N=4)
T+E+MA (N=4)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVAL (10 MINUTES)
p = 0.097
p = 0.083
VEH
MA
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Figure 13. Overall, mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) for P25 ovariectomized females receiving testosterone (T+0). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Even though there seemed to be a decreased potassium stimulated release within the MA-treated group, only the basal DA release rates of T+MA-treated mice were significantly decreased (p=.023) compared with vehicle treated controls.



FEMALE T+0
T+0+VEH (N=8)
T+0+MA (N=7)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVALS (10 MINUTES)
p = 0.023
p = 0.079
VEH
MA
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Uterine, pituitary, and body weights were recorded from each animal to verify the effects of treatments administered (gonadectomy ± T or E). Pituitary and uterine weights were measured in milligrams and divided by body weight in grams for each mouse. ANOVAS were performed and results recorded in Table 1 and Table 2. Different letters in parentheses indicate conditions which show statistically significant differences.

Table 1. ENDOCRINE EVALUATION: Pituitary/Body Weight ratios.

Pituitary (mg)/Body Weight(g)	Vehicle	Methamphetamine
0+0	0.073 ± 0.006 (a)	0.063 ± 0.007 (a)
0+E	0.108 ± 0.007 (b)	0.113 ± 0.005 (b)
T+E	0.100 ± 0.010 (b)	0.086 ± 0.008 (a,b)
T+0	0.063 ± 0.002 (a)	0.060 ± 0.006 (a)

Table 1 shows that E treatment alone or in combination with T significantly increased pituitary size. T alone was not able to increase pituitary size above that of non-hormonally treated animals. Further, when T was administered in conjunction with E the pituitary/body weight ratios were reduced.

Table 2. ENDOCRINE EVALUATION: Uterine/Body Weight ratios.

Uterus (mg)/Body Weight(g)	Vehicle	Methamphetamine
0+0	0.382 ± 0.039 (a)	0.338 ± 0.034 (a)
0+E	2.051 ± 0.184 (b,c)	2.156 ± 0.232 (b,c)
T+E	3.020 ± 0.330 (b)	2.254 ± 0.075 (b,c)
T+0	1.456 ± 0.128 (c)	1.700 ± 0.138 (c)

Table 2 shows that any hormonal treatment significantly increased uterine size above that of non-hormonally treated animals. T alone was not as effective in increasing uterine size as E. In contrast to Table 1, when T was administered in conjunction with E the uterine/body weight ratios were increased. These changes in ratios verify the effects of treatments administered (gonadectomy ± T or E).

DISCUSSION

The results from the static measure of striatal tissue DA content in Experiment 1 indicate that pre-pubertal hormonal manipulations did not effect changes in the striatal DA content as there were no significant differences in vehicle treated animals from all groups. The MA regimen used effectively produced a 75% reduction in striatal DA concentration within all hormonal treatment groups when compared to their vehicle controls. The lack of significant differences in DA concentration among the pre-pubertal gonadectomized female MA treatment groups ($p=0.417$), specifically the absence of a difference between the No T/No E and No T/E groups, demonstrates the loss of neuroprotection afforded by E against a MA assault when the female mice were ovariectomized as adults ($p=0.03$). In addition, the analyses which reveal that DA concentrations of E-treated females ovariectomized at 60 days of age are significantly greater ($p=0.011$) than that obtained from pre-pubertally ovariectomized females treated with E as adults along with the findings that none of the females ovariectomized at 25 days and receiving hormonal manipulations were different from the females that were ovariectomized at 60 days and subjected to MA ($p=0.435$) all suggest that pre-pubertal ovariectomy abolishes the capacity for E to function as a neuroprotectant in the adult female as defined by using the parameter of DA concentration.

It is interesting to note that DA concentrations of ovariectomized pre-pubertal mice treated with T were lower than those of the No T groups. This decrease in DA concentration did not prove to be significant ($p=0.19$). A similar trend for T to increase the degree of neurotoxicity to MA was observed previously (Gao, and Dluzen, 2001; Yu

and Lao, 2000). In this regard there does seem to be a tendency, suggesting that T may exacerbate MA-induced DA neurotoxicity.

The dynamic measure of tissue responsiveness, *in vitro* superfusion, was performed to further characterize the effects of pre-pubertal hormonal manipulations. In the P25 ovariectomized females receiving no hormones (0+0), the 30mM potassium stimulated DA release rates of MA-treated mice were significantly decreased compared with vehicle treated controls (p=0.05). Neither basal nor potassium stimulated DA release rates of E+MA-treated mice or the T+E+MA-treated mice were significantly decreased compared with their respective vehicle treated controls. Within the T+0 groups only the basal (p=0.023) DA release rates of T+MA-treated mice were significantly decreased compared with vehicle treated controls.

These results diverge from those obtained for DA concentration. When reviewing Figure 9 as well as the individual analyses figures of these data (Figures 10 – 13) one trend which seems to emerge is that the 0+E+MA females tend to be distinguished from the remaining treatment groups. For the remaining treatment groups, there exists a significant difference between potassium stimilated responses for vehicle versus MA groups in the case of 0+0 (p=0.05), or the difference closely approaches statistical significance T+E (p=0.083) or T+0 (p=0.079). The absence of a statistically significant difference for these latter two groups may be related to the greater degree of variability that results from a dynamic of measure of dopaminergic function. Nonetheless, the data of the 0+E group shows a salient departure from that of the other groups (p=0.43). If this trend is confirmed, as can be achieved with additional experiments, the data would

suggest that some hint of neuroprotection may be present in these pre-pubertal ovariectomized females using the criteria of a potassium stimulated DA release response.

Taken together, these data seem to indicate that E is not capable of serving as a neuroprotectant regardless of the endocrine condition under which the brain has developed as determined by DA concentration, but may indicate some hint of neuroprotection as determined by potassium stimulated release of DA. Overall, the absence of E and/or the presence of T during the pre-pubertal period seems to be a critical factor in changing responses of the female brain to the NSDA neurotoxin MA.

EXPERIMENT 2: Gonadectomized males at P25 ± T replacement ± E ± MA

INTRODUCTION

As has been noted in the general introduction, there is a clear gender difference in estrogen's ability to act as a neuroprotectant in response to MA (Wagner et al., 1993). Estrogen can serve as a neuroprotectant against MPTP (Dluzen et al.,1996), but not MA (Gao and Dluzen, 2001) in male mice. However, E supplements have been shown to preserve dopamine concentrations in ovariectomized female mice when treated with either MPTP (Dluzen et al., 1996a; Dluzen et al., 1996b; Miller et al., 1998) or MA (Gao and Dluzen, 2001a; Yu and Liao, 2000). Gonadal steroid manipulation during the pre-pubertal developmental period disrupts reproductive capabilities in the adult animal. Since the gonadal steroid E has been shown to have activational affects that afford neuroprotection against a MA assault in the adult mouse (Dluzen and McDermott, 2000b; Dluzen, Disshon and McDermott, 1998; Dluzen, McDermott and Anderson, 2001; Gao and Dluzen, 2001a; Dluzen, Anderson and Pilati, 2002), the question arises: will these manipulations during the pre-pubertal period also affect the neuroprotective capability of E against a MA assault in the adult male?

The purpose of Experiment 2 was to examine the developmental effects of gonadal steriod hormone manipulation during the pre-pubertal period upon neuroprotective capabilities of E in the adult male. To accomplish this goal, in Experiment 2 pre-pubertal male CD-1 mice were gonadectomized to determine if the absence of T and/or the presence of E is the critical factor in differing male/female responses to the NSDA neurotoxin MA. DA tissue content was measured and superfusion performed to evaluate changes in DA release.

PROCEDURES

In Experiment 2 pre-pubertal male CD-1 mice were gonadectomized and subjected to hormonal manipulations identical to those in Experiment 1 (Figure 14).

RESULTS

A summary of the striatal dopamine tissue content profile is shown in Figure 15. There were no statistically significant differences in DA concentration observed among the four vehicle treatment groups; therefore, these values were pooled into one vehicle control group (N=29). Although all of the MA treated groups had significant decreases in DA concentration when compared to the vehicle controls ($p=0.0001$), there were no significant differences in DA concentration among the MA treatment groups ($p=0.991$). Of particular note are the similarities in DA concentrations between the No T/No E and No T/E groups, which highlight the inability for any neuroprotection by E in these pre-pubertally gonadectomized male mice.

In vitro superfusion was performed to further characterize the effects of pre-pubertal hormonal manipulations. An overall summary of the DA release rate profiles for the four hormonal manipulations ± MA is presented in Figure 16. The superfusion data were blocked into discrete segments to analyze baseline (collection interval 3) and stimulated (collection intervals 4-6) responses. Unpaired t-tests were used to compare vehicle vs MA treated release responses within each hormonal treatment group and these results are summarized in Figures 17-20.

For P25 gonadectomized males receiving no hormones (0+0) basal release rates were similar (N.S.); however, potassium stimulated DA release rates of MA-treated mice were significantly decreased ($p=0.048$) compared with vehicle treated controls (Figure 17).

Both basal (p=0.118) and potassium stimulated (p=0.612) DA release rates for P25 gonadectomized males receiving E+MA-treated mice were not significantly different compared with vehicle treated controls (0+E) (Figure 18). As seen in Figure 19, for P25 gonadectomized males receiving T and E (T+E) both basal (p=0.417) and potassium stimulated (p=0.609) DA release rates of T+E+MA-treated mice were not significantly decreased (N.S.) compared with vehicle treated controls. Both basal (p=0.072) and potassium stimulated (p=0.062) DA release rates of T+0+MA-treated mice were not significantly decreased (N.S.) compared with vehicle treated controls (Figure 20).

Figure 14. Timeline containing the treatment procedures and ages at treatments.

Age								
21 DAYS OLD	MALE CD-1 MICE WEANED							
25 DAYS OLD	MALE CD-1 MICE GONADECTOMIZED							
	TESTOSTERONE				No TESTOSTERONE			
55 DAYS OLD	ESTROGEN		No ESTROGEN		ESTROGEN		No ESTROGEN	
62 DAYS OLD	MA	No MA	MA	No MA	MA	No MA	MA	No MA
69 DAYS OLD	SACRIFICE ANIMALS BY RAPID DECAPITATION							
	1/2 CS - DA CONCENTRATION							
	1/2 CS - DA RELEASE							

MA - Methamphetamine
DA - Dopamine

Figure 15. Summary of the striatal dopamine tissue content (Mean±SEM in pg/mg) profile. Bars with different lettered superscripts indicate conditions which show statistically significant differences.

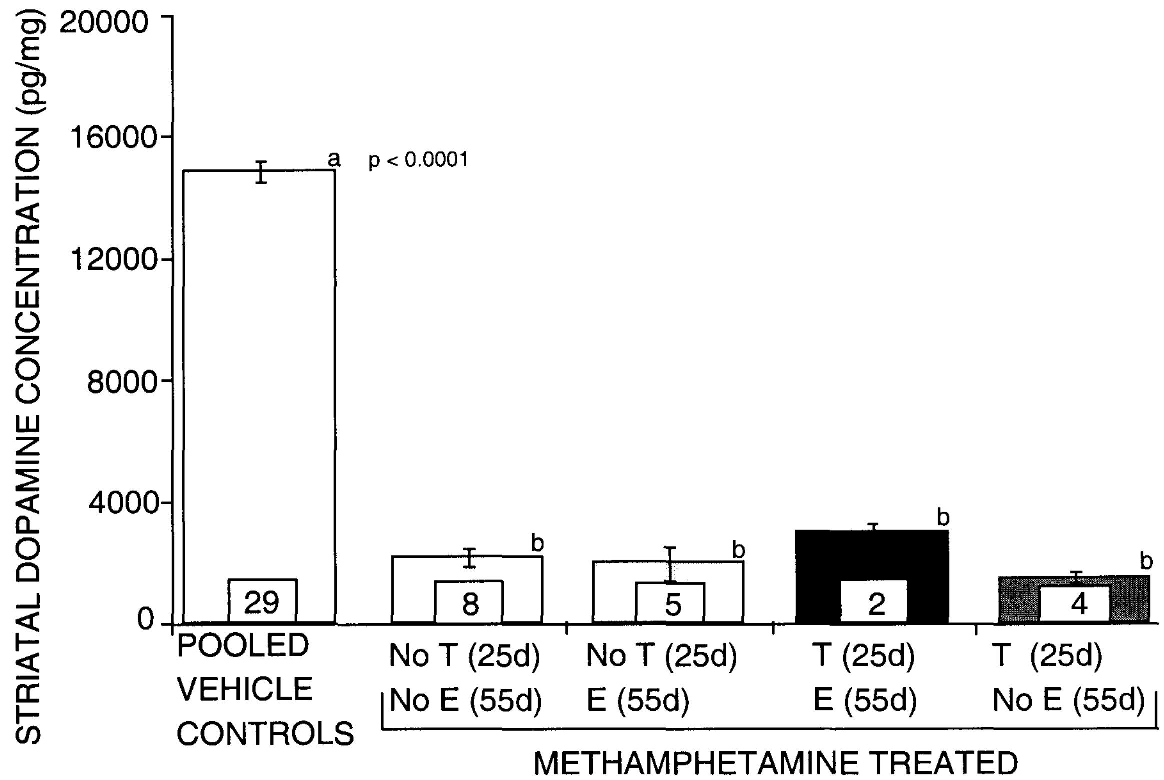
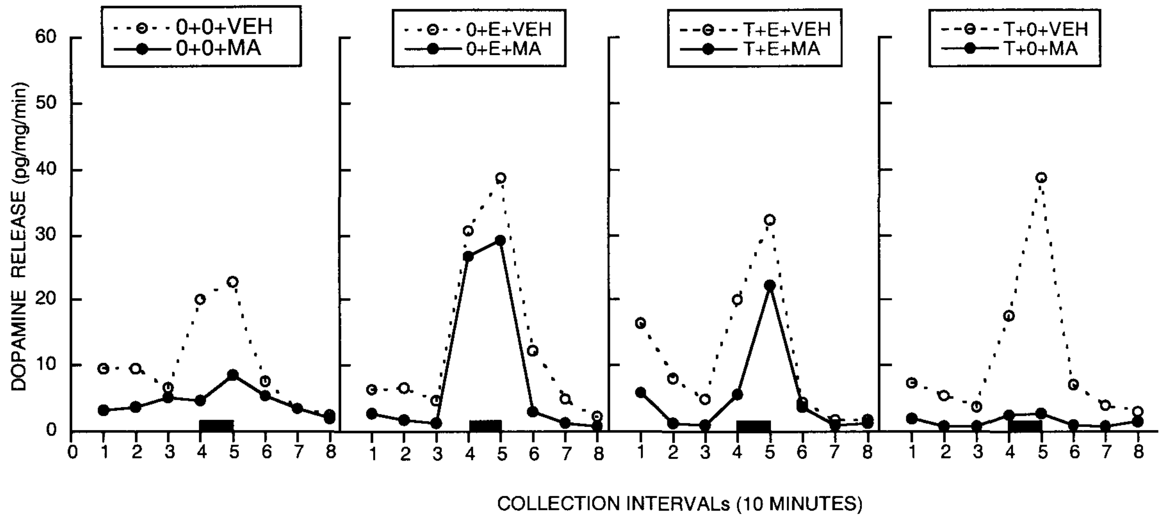

MALES
STRIATAL DOPAMINE CONCENTRATION (pg/mg)
20000
16000
12000
8000
4000
0
a
p < 0.0001
b
b
b
b
29
8
5
2
4
POOLED
VEHICLE
CONTROLS
No T (25d)
No E (55d)
No T (25d)
E (55d)
T (25d)
E (55d)
T (25d)
No E (55d)
METHAMPHETAMINE TREATED

Figure 16. Over all summary of *in vitro* superfusion DA release rate profiles (pg/mg/min) for the four hormonal manipulations ± MA. The black bar on the X axis indicates the period of infusion of a depolarizing concentration of potassium (30mM) within the superfusion chambers. Bars for SEM were omitted from this figure for purpose of clarity. Means with SEM bars are contained within individual data analyses of each treatment group as presented in Figures 17 – 20.



0+0+VEH
0+0+MA
0+E+VEH
0+E+MA
T+E+VEH
T+E+MA
T+0+VEH
T+0+MA
DOPAMINE RELEASE (pg/mg/min)
0
10
20
30
40
50
60
0 1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
COLLECTION INTERVALs (10 MINUTES)

Figure 17. Overall mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) responses for P25 gonadectomized males receiving no hormones (0+0). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Although basal release rates were similar (N.S.), potassium stimulated DA release rates of MA-treated mice were significantly decreased ($p=0.048$) compared with vehicle treated controls.

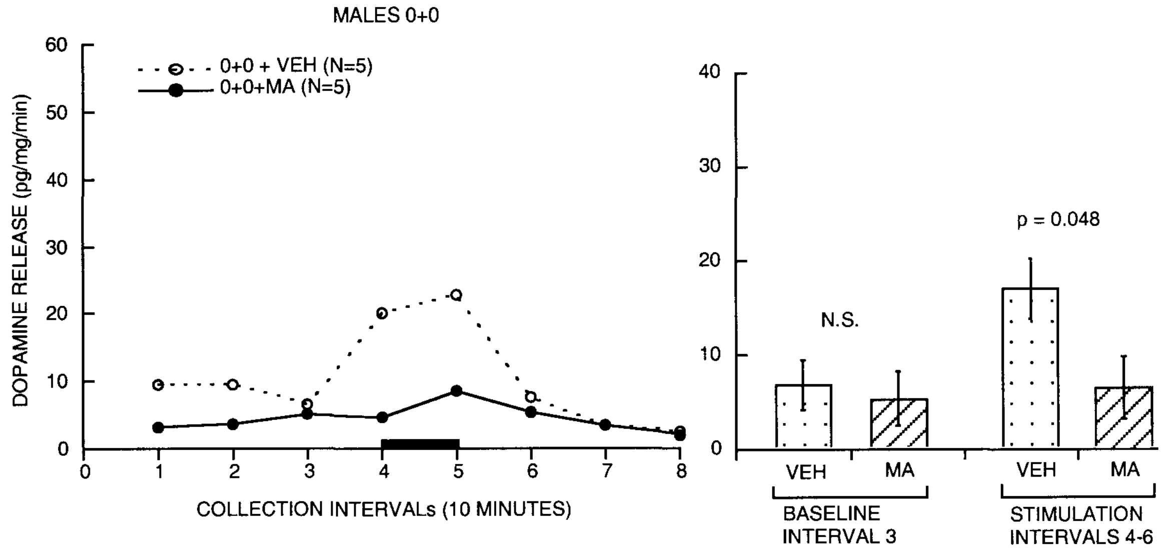

MALES 0+0
0+0 + VEH (N=5)
0+0+MA (N=5)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVALs (10 MINUTES)
N.S.
p = 0.048
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Figure 18. Overall mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) responses P25 gonadectomized males receiving E (0+E). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Both basal ($p=0.118$) and potassium stimulated ($p=0.612$) DA release rates of E+MA-treated mice were not significantly decreased compared with vehicle treated controls (N.S.).

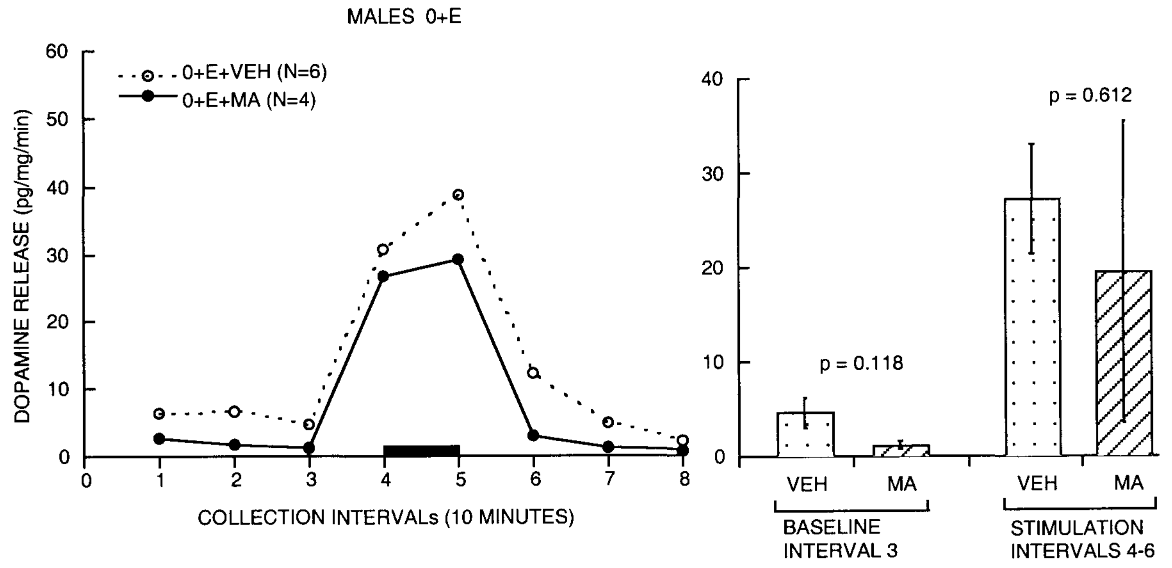

MALES 0+E
0+E+VEH (N=6)
0+E+MA (N=4)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVALs (10 MINUTES)
p = 0.118
p = 0.612
VEH
MA
VEH
MA
BASELINE INTERVAL 3
STIMULATION INTERVALS 4-6

Figure 19. Overall mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) responses for P25 gonadectomized males receiving testosterone and estrogen (T+E). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Both basal ($p=0.417$) and potassium stimulated ($p=0.609$) DA release rates of T+E+MA-treated mice were not significantly decreased (N.S.) compared with vehicle treated controls.

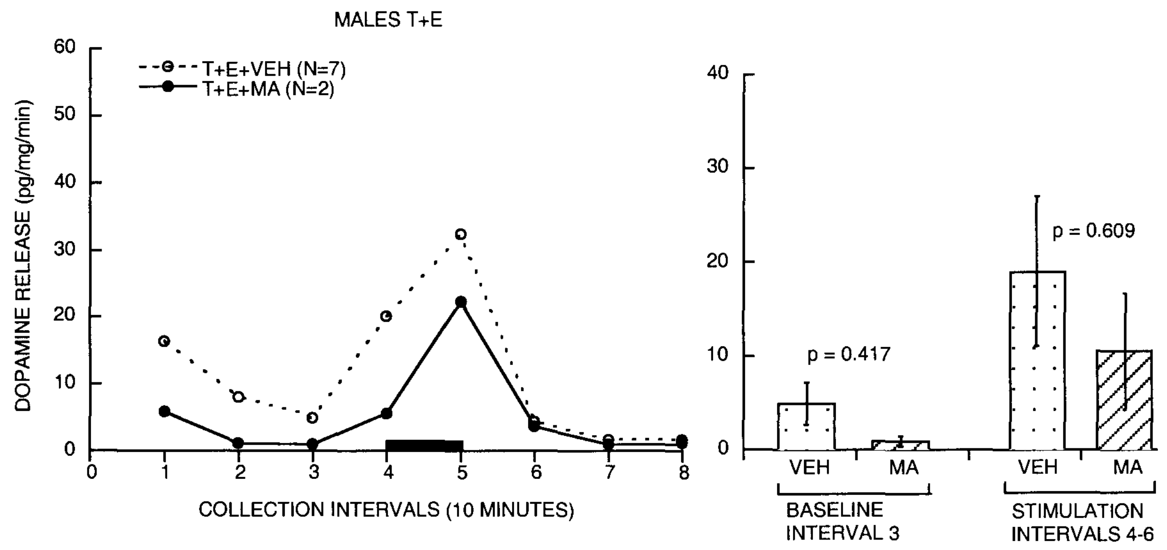
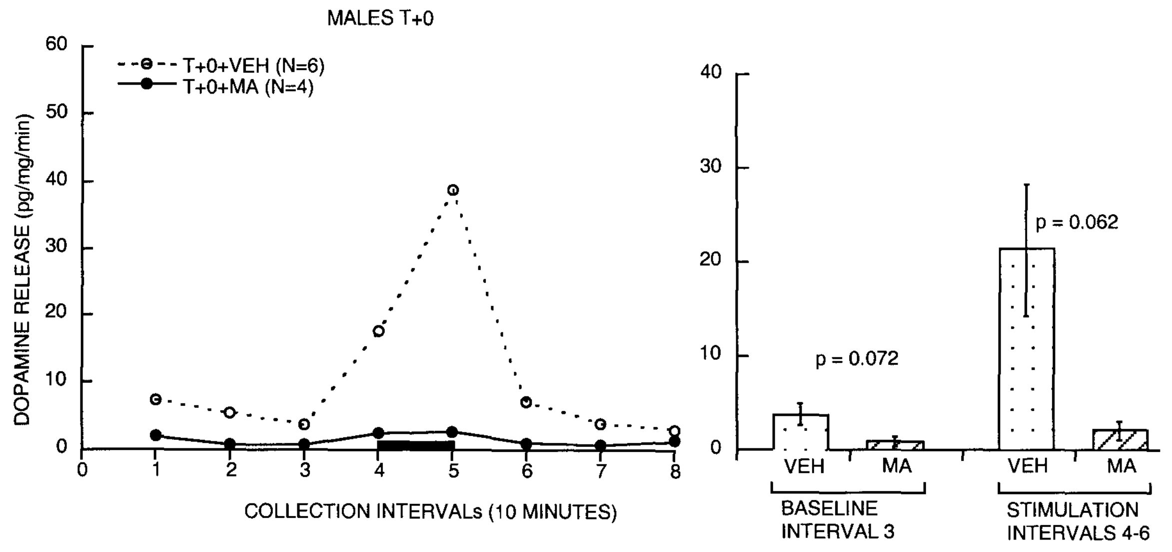

MALES T+E
T+E+VEH (N=7)
T+E+MA (N=2)
DOPAMINE RELEASE (pg/mg/min)
COLLECTION INTERVALS (10 MINUTES)
p = 0.417
p = 0.609
VEH
MA
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Figure 20. Overall mean release rate profiles (line graphs) along with summary of data analyses for basal (collection interval 3) and 30mM potassium stimulated (collection intervals 4-6) DA release (bar graphs) responses P25 gonadectomized males receiving testosterone (T+0). An unpaired t-test was used to compare responses between vehicle vs MA treated mice. Both basal (p=0.072) and potassium stimulated (p=0.062) DA release rates of T+MA-treated mice were not significantly decreased (N.S.) compared with vehicle treated controls.


MALES T+0
T+0+VEH (N=6)
T+0+MA (N=4)
DOPAMINE RELEASE (pg/mg/min)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
COLLECTION INTERVALs (10 MINUTES)
40
30
20
10
0
p = 0.072
p = 0.062
VEH
MA
VEH
MA
BASELINE
INTERVAL 3
STIMULATION
INTERVALS 4-6

Pituitary and body weights were recorded from each animal to verify the effects of treatments administered (gonadectomy ± T or E). Pituitary weights were measured in milligrams and divided by body weight in grams for each mouse. An ANOVA was performed and results recorded in Table 3. Different letters in parentheses indicate conditions which show statistically significant differences.

Table 3. ENDOCRINE EVALUATION: Pituitary/Body Weight ratios.

Pituitary (mg)/Body Weight(g)	Vehicle	Methamphetamine
0+0	0.073 ± 0.002 (a,d)	0.083 ± 0.003 (a,d)
0+E	0.114 ± 0.007 (b,c)	0.116 ± 0.007 (b,c)
T+E	0.087 ± 0.007 (a,c,d)	0.116 ± 0.002 (a,c)
T+0	0.061 ± 0.005 (d)	0.076 ± 0.004 (a,d)

Table 3 shows that estrogen treatment alone significantly increased pituitary size above that of non-hormonally treated males. When testosterone was administered in conjunction with estrogen the pituitary/body weight ratios were reduced. Further, testosterone alone was not able to increase pituitary size above that of non-hormonally treated animals.

DISCUSSION

The results from the static measure of striatal tissue DA content in Experiment 2 indicate that pre-pubertal hormonal manipulations in male CD-1 mice did not effect changes in the striatal DA content as there were no significant differences in vehicle treated animals from all groups. The MA regimen used effectively produced a 75-80% reduction in striatal DA concentration within all hormonal treatment groups when compared to their vehicle controls. The lack of significant differences in DA concentration among the pre-pubertal gonadectomized male MA treatment groups ($p=0.991$), specifically the absence of differences between the No T/No E and No T/E groups, highlights the inability for any neuroprotection by E in these pre-pubertally gonadectomized male mice. This is similar to findings that E cannot act as a neuroprotectant in male CD-1 mice that are castrated as adults (Gao, and Dluzen, 2001; McDermott et al., 1994).

The values for the males receiving T+E +MA are not very reliable as only two animals survived the MA injection regimen. Twelve animals were assigned to this group as we have found in the past that treatment with the gonadal steroids E + T produces severe acute toxicity to MA which is specific to male mice (Dluzen, Anderson and Pilati, 2002). The two values obtained from the surviving males were very different.

In vitro superfusion was performed to further characterize the effects of pre-pubertal hormonal manipulations. Only in P25 gonadectomized males receiving no hormones (0+0) were the potassium stimulated DA release rates of MA-treated mice significantly

decreased (p=0.048) compared with vehicle treated controls. Both basal and potassium stimulated DA release rates for P25 gonadectomized males receiving (0+E), (T+E), or (T+0) MA-treated mice were not significantly decreased compared with vehicle treated controls. Although the potassium stimulated (p=0.062) DA release rates of T+0+MA-treated mice were not significantly decreased compared with vehicle treated controls, the values tended to be lower.

These results diverge from those obtained for DA concentration. When reviewing Figure 16 as well as the individual analyses figures of these data (Figures 17 – 20) one trend which seems to emerge is that the 0+E+MA and the T+E+MA males tend to be distinguished from the remaining treatment groups. For the remaining treatment groups, there exists a significant difference between potassium stimilated responses for vehicle versus MA groups in the case of 0+0 (p=0.048), or the difference closely approaches statistical significance T+0 (p=0.062). The absence of a statistically significant difference for the T+E and T+0 groups may be related to the greater degree of variability that results from a dynamic of measure of dopaminergic function. Nonetheless, the data of the 0+E group shows a salient departure from that of the other groups (p=0.6115). If this trend is confirmed, as can be achieved with additional experiments, the data would suggest that some hint of neuroprotection may be present in these pre-pubertal orchadectomized males using the criteria of a potassium stimulated DA release response. However, if one stays faithful to a strict statistical measure of these responses then no such differences are apparent.

Taken together, these data seem to indicate that E is not capable of serving as a neuroprotectant regardless of the endocrine condition under which the brain has

developed as determined by DA concentration, but may indicate some hint of neuroprotection as determined by potassium stimulated release of DA. Overall, the absence of E and/or the presence of T during the pre-pubertal period seems to be a critical factor in changing responses of the male brain to the NSDA neurotoxin MA.

OVERVIEW

These experiments addressed a question of whether E is capable of serving as a neuroprotectant regardless of the endocrine condition under which the brain has developed during the pubertal period. The fact that gender differences exist for responses to NSDA neurotoxins suggests that either brain organization is different between females and males and/or the acute presence of E enables a differential response. These experiments addressed this issue to understand the source for the differences observed in neurotoxins between females and males.

Differences in onset, duration, and termination of critical developmental periods are major factors underlying the sexual differentiation of separate nuclei within the brain. During multiple critical periods the hormonal environment alters the morphology of the reproductive system in a permanent fashion (Chung et al., 2002). Further, hormonally influenced mechanisms are thought to produce structural sex differences in neural systems that underlie sexual behavior or reproductive function (Arai, et al., 1993; Davis, et al., 1995; Davis, et al., 1996; Suzuki and Nishiahara, 2002; Swaab, 2002; Tobet, 2002). The pubertal period was chosen because gonadal steroid hormonal changes that take place during the pubertal developmental period are believed responsible for the eventual gender differences observed in the NSDA system (Becker and Ramirez, 1981a; Laviola,

et al., 2001; Romeo, et al., 2002). These experiments were performed to determine if the pubertal gonadal steroid hormonal changes also alter another aspect of gonadal steroid modulation, neuroprotection.

To address this issue, female CD-1 mice were gonadectomized at 25 days and implanted or not with T to determine if the absence of E and/or the presence of T is the critical factor in changing responses to the NSDA neurotoxin MA. DA tissue content was measured and superfusion performed to evaluate changes in DA release. Neuroprotection by E as defined by tissue concentration of DA was abolished. These findings are similar to those of others (Yu, et al., 2002). However, neuroprotection by E may be present as defined by potassium stimulated DA release.

In Experiment 2, male CD-1 mice were gonadectomized at 25 days to determine if the absence of T and/or the presence of E is the critical factor in differing male/female responses to the NSDA neurotoxin MA. DA tissue content was measured and superfusion performed to evaluate changes in DA release. Neuroprotection by E as defined by tissue concentration of DA was not evident. However, neuroprotection by E may be present as defined by potassium stimulated DA release.

The divergent results in these data indicate the inferences made may depend on the parameter used to measure neuroprotection. The differences between results from the static measurement of DA content and the dynamic measure of potassium stimulated DA release may be due to the sensitivity of the latter measurement. These differences may indicate that one parameter is not sufficient for the determination of neuroprotection afforded by gonadal steroids.

A comparison of Figure 9 and Figure 16 reveals similar patterns of DA release for both sexes. The gender difference in estrogen's ability to act as a neuroprotectant in response to MA (Wagner et al., 1993; Gao and Dluzen, 2001a; Yu and Liao, 2000) is not apparent with regards to DA function. Similar to results from a previous study (Myers, et al., 2002), T and E exerted differential modulatory effects on potassium-stimulated DA output from superfused striatal tissue fragments. However, these modulatory effects are not different between P25 gonadectomized female and male mice. This implies that the gender differences in neuroprotection afforded by E may be due to different levels of DA concentration, with that of females being greater than that of males (Figure 6 vs Figure 15).

These data are of clinical significance since they show that the presence of endogenous estrogens in the developing nervous system may have far reaching effects on the neuroprotection naturally afforded against such neurodegenerative disorders as Parkinson's disease in the adult (Ferguson et al., 2000).

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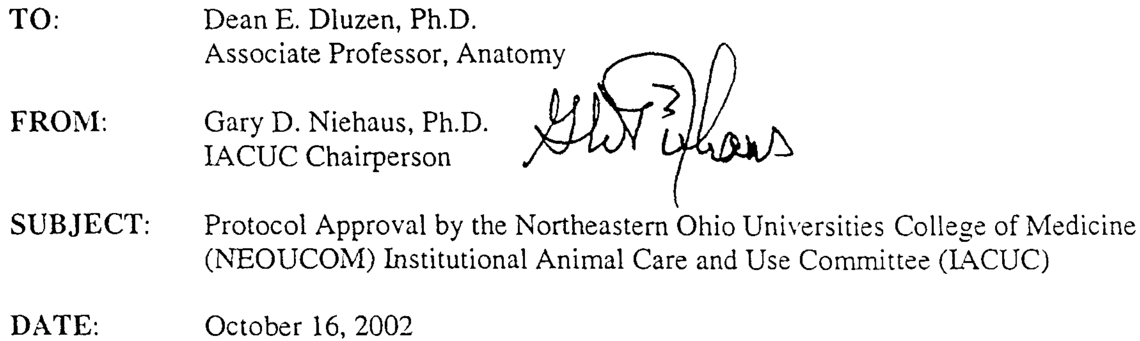
APPENDIX

IACUC Approval form

Northeastern Ohio Universities College of Medicine



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: October 16, 2002

The following NEOUCOM protocol was originally reviewed and approved by this Institution's Animal Care and Use Committee (IACUC) on February 12, 2002 and has subsequently been reviewed and approved annually. Modifications to this protocol were reviewed and approved by the IACUC on October 15, 2002.

NEOUCOM Protocol No.:	**02-001**
Title of Protocol:	**Sexual Differentiation and Methamphetamine Neurotoxicity**
Type of Vertebrate:	**Mice**
Funding Agency:	**National Institutes of Health**

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 M. Russell
Research & Sponsored Programs

File