The Role of Gonadal Hormones in Mesencephalic Dopaminergic Systems

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University
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ABSTRACT

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Abstract

Dopamine regulates movement, cognition and the rewarding effects of addictive drugs. Sex differences mediated by gonadal hormones affect each of these processes. An extensive literature suggests that estrogen augments dopaminergic function. Our laboratory found that female rats exhibit increased locomotor stimulation in response to cocaine and greater cocaine-induced dopamine overflow compared to males, sex differences that emerge in early adulthood. Currently, the underlying mechanisms for these differences are poorly understood. I hypothesized that female rats would have more dopamine neurons in midbrain regions and that ovarian hormones would exert trophic effects on dopamine neurons. Immunohistochemical and stereological techniques were used to quantitate the number of cells in the SNpc and VTA of male and female rats and mice to assess: (1) if sex differences in dopamine neuron number exist and when they emerge, (2) how gonadal hormones influence dopaminergic cell number and dopamine-mediated behaviors (3) the role of specific hormone receptors in the effects on cell number (4) the possibility that dopamine neuron number is directly linked to cocaine-stimulated behavior and electrically-stimulated dopamine release and that these responses to cocaine are mediated through gonadal hormone modulation of midbrain dopamine neuron number. I discovered sex differences in midbrain dopamine neuron number; adult female rodents have more neurons in the SNpc and VTA. We also

found that gonadectomy in adulthood reduced midbrain dopamine neuron number in females and increased neuron number in males, establishing the trophic effects of estrogen in the intact midbrain and possible suppressive effects of androgens. Treatment with agonists for estrogen receptor subtypes alpha and beta and androgen receptor reversed the effects of gonadectomy on cell number in females and males, respectively. In an effort to bridge cocaine-stimulated behavior and cell number in sham ovariectomized and ovariectomized females, we discovered cocaine-stimulated behavior, dopamine release and SNpc cell density were positively correlated in intact female rats, an effect that is lost with ovariectomy. This dissertation demonstrates that estrogen is critical for the maintenance of dopaminergic cell populations that enhance behavioral responses to psychostimulants in females, thereby contributing to the observed sex differences.

Acknowledgements

Earlier this year, I received a fortune cookie, and the slip of paper on the inside read: "Hard work will bring you much happiness." While I am not sure that this is the case, I am sure that it does pay off. I have learned and accomplished many things through this experience. Most importantly, I've learned how to focus and persevere through not so pleasant times in my life to reach a goal. Graduate school has taught me that I can rely on myself and for that I am most grateful. I am also grateful for all of the people who I have had the opportunity to work with. Each of whom has helped to shape my graduate school experience and life for the better.

I would first like to thank my advisor Cindy Kuhn for giving me the opportunity to do research in an area that I found exciting and for giving me the opportunity to have creative freedom with my project. I would also like to thank the members of my committee Dona Chikaraishi, Donald McDonnell, Victor Nadler, Shelly Schwartz-Bloom and Ted Slotkin for their assistance and input over the years. I would like to especially thank Dona for her help with the antigen retrieval, which was a great help to my project, and for opening up her lab to me in times of need. I would also like to thank Dr. Nadler for his help and support with immunostaining and stereology.

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like to thank God for giving me this opportunity, because it is by His grace that I made it through.

All in all, this has been an interesting ride. Like the Oscars, I hope I haven't left anyone out, and if I have just know that your help means more to me than you'll ever know. As I move on to the next stage of my life, I will look fondly upon this experience. Like any great TV show or movie, it must end. I'm looking forward to a decent spinoff….but not a sequel. In the great words of the Shed Seven song *Some People*, "Feet don't fail me now…."

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List of Abbreviations

6-OHDA 6-hydroxydopamine

αERKO.. Estrogen receptor alpha knockout

ADHD.. Attention Deficit Hyperactivity Disorder

AF-1... activation function - 1

ANOVA.. analysis of variance

ANCOVA .... analysis of covariance

βERKO ....Estrogen receptor beta knockout

BDNF ....brain-derived neurotrophic factor

BrdU ....bromodeoxyuridine

BSA.. bovine serum albumin

bw ....body weight

BrdU ....bromodeoxyuridine

CNS .... central nervous system

DAB...3, 3'-diaminobenzidine

DAT.. dopamine transporter

DHT...5α-dihydrotestosterone

DMSO ....dimethyl sulfoxide

DNA .... deoxyribonucleic acid

DPN ... diarylpropionitrile

E2 ... 17β-estradiol

ER ... estrogen receptor

ERα ... estrogen receptor alpha

ERβ ... estrogen receptor beta

FSCV ... fast scan cyclic voltammetry

Fig ... Figure

g ... gram

GFAP ... glial fibrillary acidic protein

HCl ... hydrochloric acid

HMAR ... heat-mediated antigen retrieval

IACUC ... Institutional Animal Care and Use Committee

IN ... immnonegative

i.p. ... intraperitoneal

IR ... immunoreactive

kg ... kilogram

LC ... locus coeruleus

MAPK ... mitogen-activated protein kinase

MCL ... mesocorticolimbic

µm ... microgram

mg....................milligram

μm....................micron

MPP+....................1-methyl-4-phenyl-2,3-didydropyridium ion

MPTP....................1-methyl-1,2,3,6-phenyltetrahydropyridine

mRNA....................messenger ribonucleic acid

NIEHS....................National Institute of Environmental Health Sciences

NR....................nuclear receptor

NS....................nigrostriatal

ovx....................ovariectomized

PBS....................phosphate-buffered saline

PD....................Parkinson's disease

PFA....................paraformaldehyde

PI3K....................phosphotidylinositol-3 kinase

PN....................postnatal day

PPT....................propyl pyrazole triol

RRF....................retrorubral field

RT-PCR....................reverse transcription polymerase chain reaction

SEM....................standard error of the mean

SNpc....................substantia nigra pars compacta

SP-1 .... stimulating protein-1

TBS .... tris-buffered saline

TH .... tyrosine hydroxylase

VTA .... ventral tegmental area

WT .... wild type

1. General Introduction

1.1 Summary

Gonadal hormones are classically known to regulate development of reproductive behaviors and functions. In the brain, gonadal hormones influence the development of neural circuits and pathways responsible for sexual differentiation and sex-specific behavior. The effects of gonadal hormones in mesencephalic dopaminergic pathways that influence motor function, cognition and reward have been investigated in recent years due to their contribution to gender differences in the development of neurodegenerative and psychiatric disorders affecting these pathways.

Clinical studies have shown that while men have an increased risk in the development of drug addiction, women have been shown to be more vulnerable to certain aspects of psychostimulant drug abuse [1]. In contrast, women are more protected from the development of Parkinson's disease (PD) compared to men [2-4]. There is a substantial literature suggesting that ovarian hormones, specifically estrogen, augment dopaminergic function and have neuroprotective effects in midbrain dopaminergic pathways.

Studies from our laboratory show that female rats are more sensitive to the effects of the psychostimulant drug cocaine and experience greater electrically-stimulated dopamine release than males [5-7]. Animal models of neurotoxin-induce damage, which model PD, suggest that estrogen protects cells in the substantia nigra pars compacts

(SNpc) and their projections to frontal brain regions from degeneration. Currently the mechanisms underlying these sex differences observed in humans and animals are unknown. Many of these studies have focused on areas of dopaminergic function such as postsynaptic receptor binding and transporter binding. However, few have investigated whether these sex differences are due to differences in neuron number in males and females. One study in primates suggests that estrogen influences the maintenance of cells in the SNpc [8]. This study not only suggests that hormones can regulate the maintenance of dopamine neurons, but that these sex differences may be due to under-investigated anatomical mechanisms. Currently, little is known about sex differences in dopamine neuron number in the rodent midbrain and the mechanisms by which gonadal hormones influence dopamine neuron number. Also, it is currently unknown whether gonadal hormone regulation of dopamine neuron number plays a role in the control of both behavioral and functional dopaminergic responses to psychostimulant drugs.

The purpose of this thesis was to investigate potential sex differences in dopamine neuron number in midbrain regions, determine a potential mechanism by which gonadal hormones influence the survival or degeneration of neurons in these brain regions and determine if these gonadal hormone-regulated cell number impacts cocaine-stimulated behavior and dopaminergic function.

1.2 Dopaminergic Systems

Dopamine is a monoamine neurotransmitter that is widely distributed throughout the mammalian brain. Dopamine is classified as a catecholamine, which is used to describe structurally similar compounds containing a catechol nucleus, which consists of a benzene ring with two adjacent hydroxyl substituents and an amine group [9, 10] (*Fig. 1-1*). Dopamine serves a precursor in the synthesis of two other catecholamines, norepinephrine and epinephrine. This biosynthetic pathway begins with the conversion of the amino acid tyrosine, which is obtained from the diet, to 3,4-dihydrophenylalanine (DOPA) by tyrosine hydroxylase (TH). TH is the rate limiting enzyme in catecholamine biosynthesis in the brain. DOPA is converted to dopamine by the enzyme aromatic amino acid decarboxylase. Dopamine can be converted to norepinephrine by dopamine β-hydroxylase (*Fig. 1-1)* [10-12].

Dopamine-synthesizing neurons and their axonal projections form systems that regulate several functions which include movement, cognition and endocrine function. There are three major groupings of dopamine neurons. The first is the telencephalon which is comprised of neurons in Group A16, the olfactory bulb, and retina (A17). These cells make localized connections and are referred to as ultrashort systems[9, 13]. Dopamine neurons contained in the diencephalon and comprise Groups A11-15 are found in the hypothalamic region of the brain and project to the pituitary and spinal cord [13, 14].

The majority of dopamine neurons in the brain are found in the mesencephalon or midbrain. There are three major midbrain structures: the retrorubral field (RRF, Group A8), the SNpc (Group A9) and the VTA (Group A10) (*Fig. 1-2*) [15, 16]. Dopamine neurons originating in the VTA and RRF and projecting to the nucleus accumbens, prefrontal cortex, olfactory tubercle, amygdala and lateral septum form the mesocorticolimbic (MCL) pathway which regulates the rewarding effects of drugs, motivation, cognitive function and reinforcement [17, 18]. The nigrostriatal pathway (NS) is comprised of neurons that arise in the SNpc and project to the dorsal striatum and regulates voluntary movement as well as the habit learning component of drug addiction [19, 20]. The NS pathway is implicated in Parkinson's disease which is caused by the selective degeneration of dopamine neurons in the SNpc. Because of their role in the control of cognitive and behavioral function, dysregulation of midbrain doapminergic pathways is implicated in several neurological and psychiatric disorders which include Parkinson's disease (PD) [21, 22], schizophrenia [23], attention deficit hyperactivity disorder (ADHD)[24] and substance abuse[25].

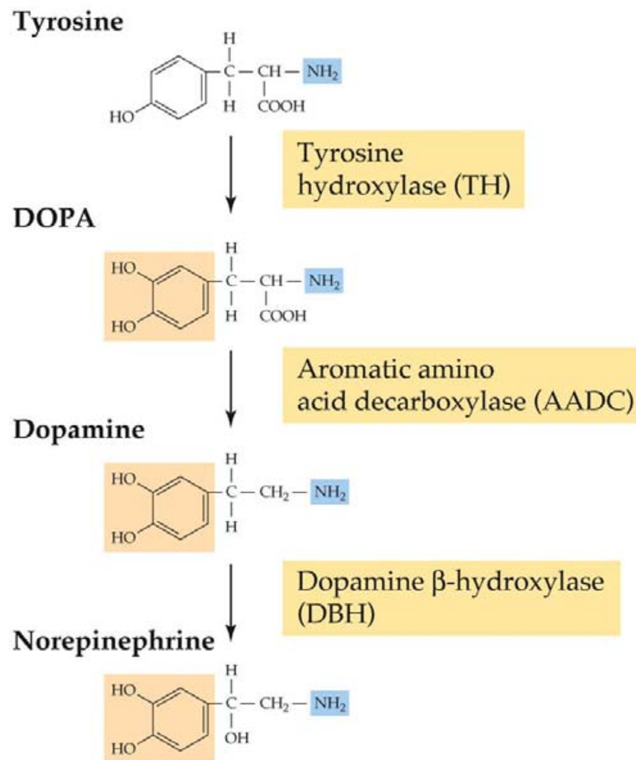


Fig. 1-1. Catecholamine biosynthesis. Tyrosine is converted to dopamine and other catecholamines through enzymatic conversions. TH is the rate-limiting enzyme in this process. *Adapted from Psychpharmacology: Drugs, the Brain and Behavior.*

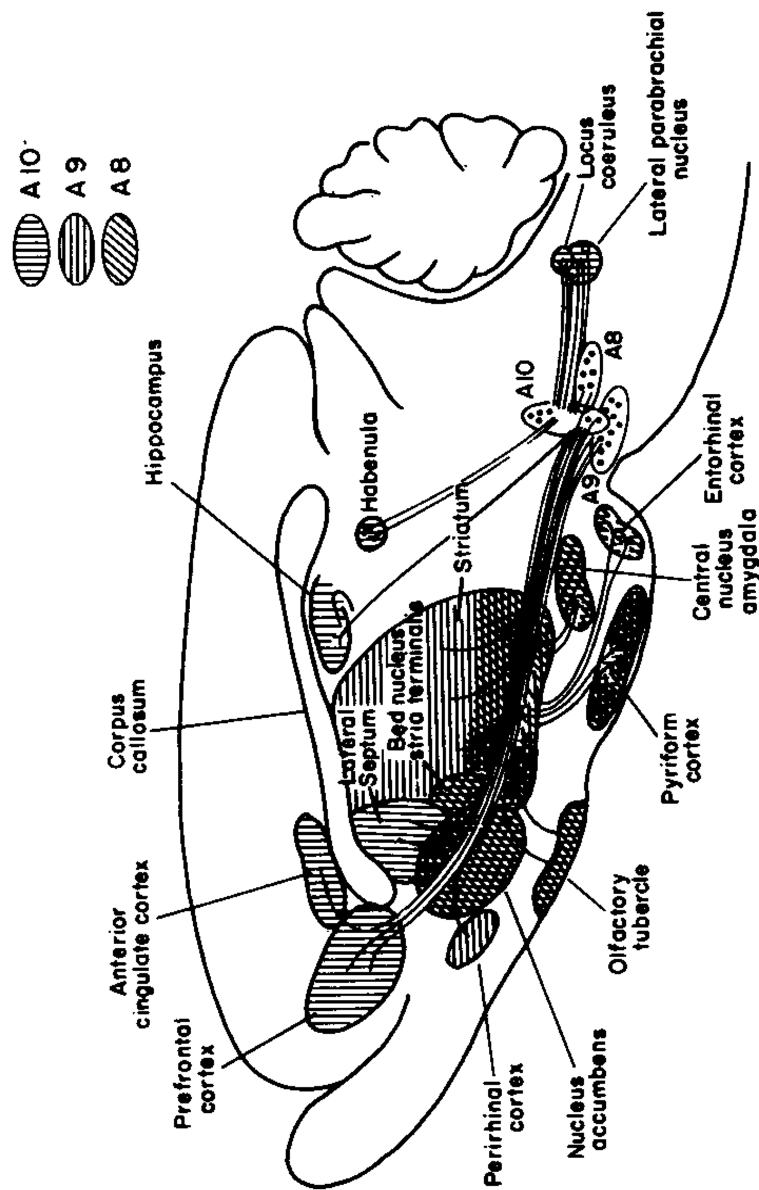


Fig. 1-2. Mesencephalic Dopaminergic Pathways. Dopamine Neurons originating in the SNpc (A9) and projecting to the dorsal striatum comprise the NS pathway. A10 (and A8) neurons project to cortical regions as well as the nucleus accumbens, amygdala, olfactory tubercle and lateral septum to form the MCL pathway. From the *Biochemical Basis of Neuropharmacology*, 8th Ed.

1.3 Gonadal Hormones

Gonadal hormones are members of the steroid hormone family which include glucocorticoids, mineralcorticoids, progestins, androgens and estrogens. All steroid hormones are biosynthetically produced from cholesterol through a series of enzymatic conversions [26, 27]. Steroid hormones are secreted by the gonads, adrenal gland, brain and adipose tissue [26]. The focus of this thesis is on gonadal hormones which are primarily derived from the testes and ovaries and include androgens, progestins and estrogens. Testosterone is the primary androgen and, it is converted to two metabolites to exert its effects. The first, 5α-dihydrotestosterone (DHT), is the product of enzymatic conversion of testosterone by 5α-reductase. DHT is more potent and has greater affinity for androgen receptor (AR) than testosterone [27]. Testosterone can also be converted to 17β-estradiol (E2), which is the primary estrogen in premenopausal women. Along with progesterone, E2 is secreted by the ovary to regulate female reproduction and sexual behaviors. Both androgens and estrogens influence sexual differentiation in the brain and the development of sex-specific reproductive behaviors [28].

Gonadal hormones influence the development, maintenance and function of the central nervous system (CNS). Gonadal hormones contribute to CNS formation both developmentally and acutely during adulthood, through organizational and activational effects. Organizational effects occur during critical time points in development, gonadal hormones exert permanent effects that influence the formation of sexual dimorphisms in

reproductive function and behavior, brain structure volume and neural cell populations [27, 29, 30]. Unlike the organizational effects of gonadal hormones, activational effects are transient and reversible and are dependent upon the presence or absence of circulating hormones [31]. Activational effects include responses that vary across the estrous cycle in females as well as responses from gonadectomy that can be restored with hormone replacement [29, 31].

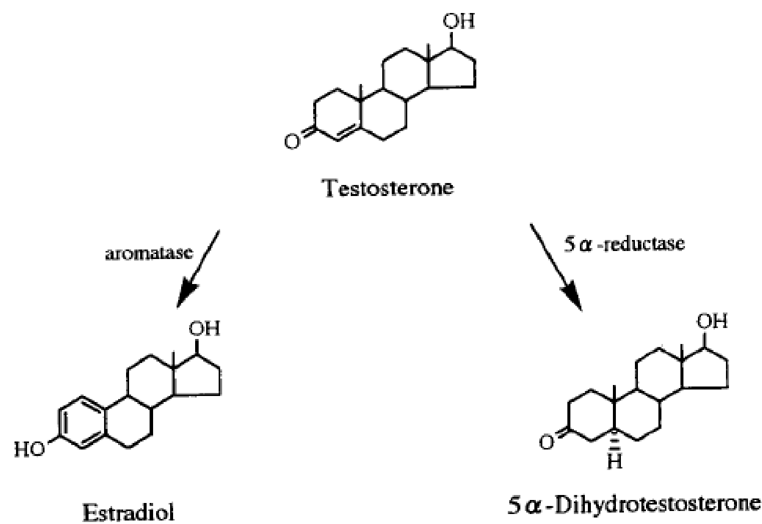


Fig. 1-3. Conversion of Testosterone to DHT and Estradiol. *Adapted from Kawata Neurosci. Res. 24 1995.*

1.4 Nuclear Hormone Receptors and Structure

Gonadal hormones exert many of their organizational and activational effects via hormone receptors. Androgen and estrogen receptors (ARs and ERs, respectively) are Class I members of the nuclear receptor (NR) superfamily [32, 33]. Hormone receptors are classified in this category due to their ability to bind deoxyribonucleic acid (DNA) as well as dimerization properties. Structurally, NRs contain five functional domains labeled A-F. The A/B domain, located at the N-terminus, contains the ligand-independent activation function-1 (AF-1) site which plays a role in the transcription of target genes and protein-protein interactions. The centrally located C domain is the site of DNA-binding, which is highly conserved among members of the NR superfamily. ER subtypes alpha and beta (ERα and ERβ, respectively) are at least 95% homologous in this region (*Fig. 1-4*) [34]. The D domain is known as the "hinge" region and plays a role in the conformational change that occurs in the hormone-receptor complex upon ligand binding. The E domain is known as the ligand-binding domain and contains a second, ligand-dependent activation function site (AF-2), which also plays a role in transactivation. The ERs only contain between 55-60% homology in this domain [32, 33, 35]. The F domain, located at the C-terminus, is not well characterized. The F domain is only present in some of the NRs, specifically ERs. The F domain is highly variable in members of the NR superfamily and may contribute to the affinities of each receptor for specific ligand [36].

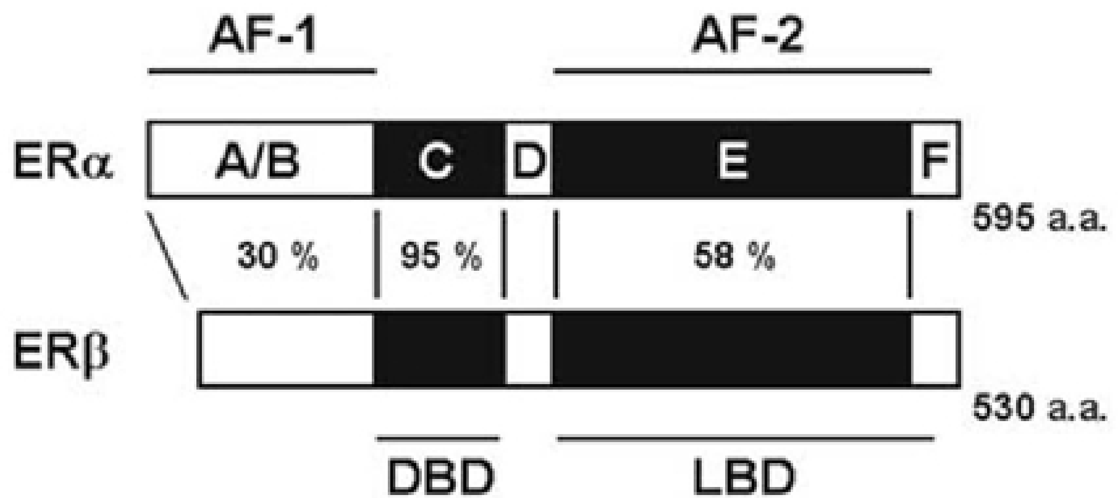


Fig. 1-4. Nuclear Hormone Receptor Functional Domains. Shows homology between ER subtypes in the ligand-independent transactivation region (A/B domain), DNA-binding (C) and ligand-binding domain (E). *Adapted from Marino et al., Current genomics 7: 497-508 (2007)*

1.5 Mechanisms of Hormone Action

Classically, testosterone and E2 exert their effects through binding to a nuclear hormone receptor to induce gene transcription. Prior to ligand (hormone) binding, receptors exist in an inactive state in complex with heat shock proteins. Upon ligand binding, the receptor becomes activated and undergoes a conformational change which results in dissociation of heat shock proteins, dimerization and translocation to the nucleus. Upon translocation, the newly formed hormone-receptor complex binds to specific DNA sequences, known as hormone response elements (HREs) in the promoter region of target genes [33]. Coregulatory proteins, coactivators and corepressors, can be recruited to enhance or suppress transcription [37]. These effects are known as genomic effects, and take hours to days to occur.

Hormones receptors can also exert effects through two other mechanisms which deviate from the classical model. The first is ligand-independent gene transcription in which the receptors interact with other transcription factors such as activator protein-1 (AP-1) [38, 39] and stimulating protein-1 (SP-1) to bind DNA and induce transcription of target genes [40]. Hormone receptors also exert nongenomic or rapid effects, occurring in seconds to minutes, that may also result in gene transcription. Instead, hormones bind to membrane-bound receptor (hormone or G-protein coupled receptors) to activate various signaling pathways such as mitogen- activated protein kinase (MAPK) and phophotidylinositol-3 kinase (PI3K)/Akt [41, 42].

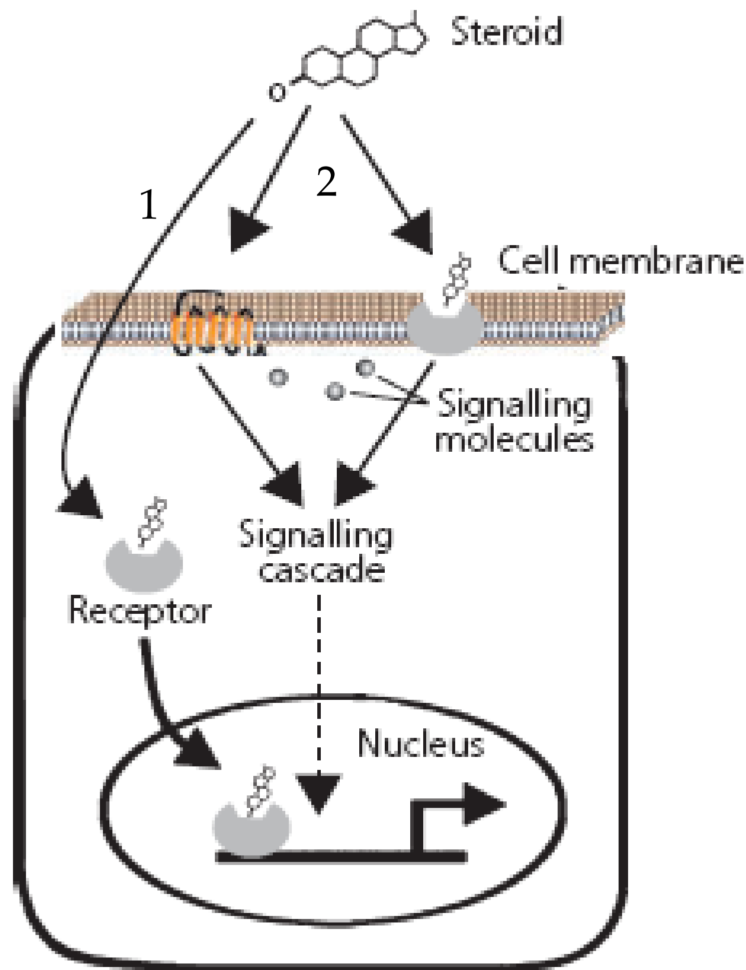


Fig. 1-5. Mechanisms of Hormone Action. Schematic shows both genomic and nongenomic actions of hormones. Classical effects (1) occur when ligand (hormone) binds to a hormone receptor to form a complex that is translocated into the nucleus to induce transcription of target genes. Nonclassical effects (2) occur when ligand binds to a membrane-bound hormone receptor or G-protein coupled receptor to activate signaling pathways may or may not regulate gene transcription. *Adapted from Kawata et al.,J. Neuroendocrinol. 20: 676 (2008).*

1.6 Hormone Receptor Expression and Action

ERα

ERα is widely expressed in many tissues throughout the body. Reverse-transcription polymerase chain reaction (RT-PCR) experiments have shown expression of ERα in the reproductive systems of males and females. ERα messenger ribonucleic acid (mRNA) is detected in the mammary gland and uterus of females as well as the testis and epididymis of males [43, 44]. ERα plays a significant role in reproduction as both male and female mice lacking the gene for ERα (αERKO) are infertile. ERα is also expressed in pituitary, liver, heart, adrenals, bone, thyroid and prostate [43, 44].

ERα is expressed in the brain and mediates many estrogenic effects in the CNS. In situ hybridization and immunohistochemical studies have revealed that ERα is highly expressed in the hypothalamic regions and amygdala of rat [45]. Through ERα binding, estrogens can regulate the transcription of genes that play a role in reproduction and lordosis behaviors such as the progesterone receptor [46], oxytocin [47, 48] and preproenkephalin genes [49-51]. In the brain, many of the protective effects of estrogen are mediated through ERα. Transcription of genes for neurotrophic factors such as brain derived neurotrophic factor (BDNF) [49, 52-54] and anti-apoptotic proteins such as Bcl-2 [49, 55, 56] is also ERα-mediated. In rodent ischemia models, ERα has been shown to mediate the neuroprotective effects of estrogen [57-59]. αERKO mice were less protected from cerebral cortex injury than WT mice in a model of focal ischemia [60]. An increase

in ERα mRNA levels occurs in the adult cortex after ischemic brain injury, despite being absent in the adult WT animals [60].

Low to no ERα has been detected in the rodent midbrain [45, 61, 62]. Studies in both rats and mice show that no ERα is expressed in the VTA, and low to no expression is detected in the SNpc [45, 62, 63]. However, studies show that in the midbrain, estrogen may protect dopamine neurons from neurotoxin-induced damage via ERα. αERKO mice are more sensitive to the effects of the dopamine transporter (DAT)-selective toxin, 1,4-methyl-1,2,3,6-phenyltetrahydropyridine (MPTP) [64] than ERβ-knockout (βERKO) and wild type (WT) mice [65, 66]. The ERα-selective agonist propyl pyrazole triol (PPT) protects mice from MPTP-induced depletion of striatal dopamine, suggesting that ERα is required for the maintenance of the NS pathway [67].

<u>ERβ</u>

RT-PCR experiments show that ERβ is expressed in the prostate and ovary. Low levels of this subtype have also been detected in the uterus. Like ERα, ERβ is expressed in the adrenals, epididymis, thyroid, bone and brain [43, 44]. Immunohistochemical and *in situ* hybridization studies have shown that ERβ is highly expressed in the hypothalamus, diencephalon, amygdala and bed nucleus of the stria terminalis [68, 69]. In the SNpc and VTA, low to moderate expression of ERβ has been detected, with conflicting results on the localization of ERβ expression [68, 69] and the cell types in which it is expressed. Studies have shown that a small subset of neurons in the SNpc

and VTA express ERβ [70]. However, a more recent study suggests that up to 40% of neurons and astrocytes in the SNpc express ERβ [71].

Considerably less is known about the role of ERβ in the brain. Currently, the role of ERβ in the midbrain is unclear. In animal models of neurotoxin-induced damage, the ERβ-selective agonist, diarylpropionitrile (DPN), is ineffective in the prevention of MPTP-induced striatal dopamine depletion [67]. In addition to a lack of protection with DPN, βERKO mice are more protected from MPTP depletion of striatal dopamine content [65, 66]. In contrast, neuron number in the SNpc of βERKO mice is reduced relative to WT at two years of age [72]. βERKO mice also experience differences in brain morphology compared to WT as the cerebral cortices are reduced in size [72, 73]. In studies assessing the role of gonadal hormones on dopaminergic function, ovariectomy of adult rats reduced dopamine D2 receptor and DAT binding [74, 75]. Treatment with estrogen and DPN reversed these effects. PPT had no effect, suggesting that there may distinct roles for each ER subtype. These findings suggest that ERβ may regulate protein expression but may not play a trophic role in dopaminergic systems.

AR

Androgen receptor is highly expressed in the male reproductive tissues as well as various brain regions [45, 76]. In the brain, AR is highly expressed in the amygdala and the bed nucleus of the stria terminalis [45]. In the midbrain, AR mRNA is moderately

expressed in the SNpc and VTA, Immunohistochemical studies have shown that like ERβ, a small subset of dopamine neurons in the SNpc and VTA express AR [77]. AR knockout mice exist, but the role of AR in the midbrain using such animals is not well characterized. Testosterone is known to exert suppressive effects in psychostimulant-induced behavior in animal models [1, 78-80] and provide no neuroprotection in models of neurotoxin-induced damage [81, 82]. Aside from these studies, little is known about the role of AR in the midbrain.

1.7 Sex Differences in Midbrain Dopaminergic Pathways

Clinical studies show sex differences in diseases in which dopaminergic pathways play a role such as drug addiction [1, 83, 84] and PD [2-4]. Studies reveal that while a majority drug addicts are men, women are more vulnerable to certain aspects of addiction. Women increase their rate of cocaine consumption more rapidly and are more likely to progress to addiction of psychostimulant drugs faster than men [83-86]. Some studies report that women experience more euphoria and nervousness after taking cocaine than men despite having lower blood cocaine levels [87-90]. Women also experience increased cardiovascular responses compared to men after smoking crack or intranasal cocaine administration [89]. Drug-dependent women were reported to have stronger cravings for cocaine than men when shown drug-related stimuli [91]. Ovarian hormones play a role in the sex differences in response to psychostimulant drugs as studies have shown that women are more sensitive to the subjective effects of cocaine during the follicular phase of the menstrual cycle, in which levels of both estradiol and progesterone are low compared to the luteal phase [1]. In contrast to drug addiction, women are less vulnerable in the development of PD, as the disorder has a higher incidence and earlier age of onset in men [2, 4, 92]. Currently, the mechanisms underlying these sex differences are poorly understood.

Behavioral studies in animals support the sex differences in response to psychostimulants observed in humans. Our laboratory and others have shown that

adult female rats are more active than males [6, 79]. This sex difference is exaggerated when animals are given acute doses of cocaine and amphetamine [1, 6, 79, 80]. Females experience greater sensitization to psychostimulants than males [93]. Some studies show that female rats also acquire self-administration of cocaine more rapidly than males at low doses [1, 79, 94]. Female rats have greater conditioned place preference for cocaine than males [93, 94].

Sex differences have also been observed in animal models of neurotoxin-induced damage, which are used to model PD. In adult rats receiving a unilateral lesion with the neurotoxin 6-hydroxydopamine (6-OHDA), which selectively taken up by DAT in dopamine neurons to induce damage [95], females were more protected from the behavioral deficits and cell loss in the SNpc associated with the lesion [96-98]. In mice treated with MPTP, more striatal dopamine damage was observed in males than females [99]. These studies suggest that sex differences in dopaminergic function may be due to gonadal hormone modulation of these systems.

Sex differences in dopaminergic function observed in animal models may mediate these differences. Studies from our lab have shown that female rats exhibit more electrically-stimulated dopamine uptake and release than intact males [5]. Female rats also exhibit greater DAT mRNA expression in the striatum than males. In contrast, male rats have been shown to have more dopamine D1 receptors in the striatum than

females. Studies report either no sex differences in dopamine D2 receptor or that female rats have fewer striatal dopamine D2 receptors than males [1].

1.8 Gonadal Hormone Effects in Midbrain Dopaminergic Pathways

Ovarian Hormone Effects in the Dopaminergic Systems

In the previous section, many of the sex differences described showed increased responses to psychostimulant drugs and dopaminergic function in females compared to males, suggesting that these sex differences may be ovarian hormone-mediated. Responses to psychostimulant drugs have been shown to vary across the estrous cycle in rats, with behavioral responses being greatest during estrous and proestrous, points in the cycle when estrogen levels rise and are highest, respectively [80]. Ovariectomy attenuates the locomotor responses to chronic and acute treatment with psychostimulant drugs as well as sensitization, conditioned place preference and drug self-administration [100]. All of these responses are restored with estrogen replacement [6, 101]. While estrogen has stimulatory effects on the behavioral and neurochemical responses to psychostimulants [1, 79], progesterone has been shown to have suppressive effects on psychostimulant-induced behavior, sensitization and dopamine release. [1, 79, 80, 102]

The effects of ovarian hormones are not limited to animal models of addiction. In animal models of neurotoxin-induced damage, both estrogen and progesterone have protective effects against MPTP in mice [65]. Ovariectomy results in a lack of protection against 6-OHDA lesioning [97, 98]. Estradiol replacement in ovariectomized rats results in a protection of striatal dopamine content against 6-OHDA [97, 98].

The effects of ovarian hormones also extend to dopaminergic function. Ovariectomy reduces DAT and dopamine D2 receptor number and binding as well as psychostimulant-induced dopamine release. Replacement with estrogen and estrogenic compounds restores these responses [66, 74, 75]. Estrogen replacement in ovariectomized rats increases neuronal firing in the SNpc [103].

Testicular Hormone Effects in Midbrain Dopaminergic Systems

While the effects of ovarian hormones have been investigated, considerably less is known about the role of androgens in dopaminergic function. Currently, animal models of addiction provide the best insight for testosterone's effect in dopaminergic pathways. Removal of the testes results in an increase in amphetamine and apomorphine-stimulated behavior [78]. Castration increases locomotor behavior and sensitization to pychostimulant drugs compared to intact animals, while testosterone treatment in castration suppresses these responses [78, 104, 105]. The suppressive effects of testosterone on behavior can also be organizational. Testosterone treatment in neonatal female rats permanently decreases responses to amphetamine, despite the presence of estrogen. In addition to the effects in females, prepubertally castrated male rats also experience a permanent increase in locomotor behavior [106, 107].

In animal models of neurotoxin-induced damage, castration of adult male rats results in protection against 6-OHDA relative to sham castrated controls [97, 98]. In

castrated male mice, testosterone and DHT replacement failed to protect against MPTP-induced loss of striatal dopamine content [81, 82]. Studies in rodents demonstrate the suppressive effects of testosterone on dopaminergic function. In mice, testosterone administration to castrated adult males reduces potassium-stimulated dopamine release [108]. Taken together, these studies suggest that androgens have suppressive or limited effects in midbrain dopaminergic systems.

1.9 Rationale and Specific Aims

Previous findings in this laboratory suggest that adult female rats exhibit greater neurochemical and behavioral responses to cocaine than males [5, 6]. Unpublished findings in our laboratory revealed that these sex differences were not due to differences in measures such as dopamine content, transporter number or vesicle number. A study in primates revealed that females have more dopamine neurons in the SNpc than males [8]. Ovariectomy of these animals reduced cell number, while estrogen prevented this loss [8], suggesting that perhaps sex differences in dopamine neuron number could mediate the behavioral and neurochemical sex differences observed in rat and that gonadal hormones contribute to the regulation of cell number.

There is also an extensive literature concerning the protective effects of estrogens after dopamine neurotoxins and a higher incidence of PD in men than women [2-4]. Much of the work in the field has focused on the neuroprotective effects of gonadal hormones in response to a neurotoxin mimicking the effects of PD. Only a handful of studies have investigated the effects of estrogens on midbrain dopamine cell populations under basal conditions. The purpose of this study was to investigate the role of gonadal hormones in midbrain cell populations using the following specific aims.

Specific Aim 1: ***Determine if sex differences exist in midbrain dopaminergic neuron number and when they appear developmentally.*** The number of dopamine neurons was quantitated using unbiased stereological techniques in the SNpc and VTA throughout postnatal development in male and female rats. Cell counts were validated by incorporating heat-mediated antigen retrieval into the staining method.

Specific Aim 2: ***Determine the effect of estrogen on dopamine neuron number in the SNpc and VTA of adult rats and mice, how replacement with estrogenic compounds influences neuron number and determine which estrogen receptor subtype is involved in these effects.*** The number of neurons was quantitated in adult female rats and mice gonadectomized at PN60. A second experiment was conducted to assess the effects of estrogenic compounds on dopamine neuron number. Rats and mice were gonadectomized at PN60 and replaced with estrogen, an ERα-selective agonist, PPT, and an ERβ-selective agonist, DPN. In a final study, cell number was quantitated in ERKO, βERKO and WT mice to determine which ER subtype is involved in the maintenance of dopamine neuron number.

Specific Aim 3: ***Investigate the role of ovarian hormones in the relationship between dopamine neuron number, dopamine release and cocaine-stimulated behavior.*** All three measures were assessed in individual animals that were either sham ovariectomized

and ovariectomized on PN55 and housed until PN90 for testing. Correlational analyses were performed to determine functional relationships between cell number, dopamine release and cocaine-stimulated behavior.

<u>*Specific Aim 4:*</u> ***Investigate the role of androgens on dopamine neuron number in the SNpc and VTA.*** Cell number was quantitated in male rats sham castrated and castrated at PN60. In a second study, the effects of androgenic drugs were assessed as cell number was quantitated in castrated animals receiving testosterone and DHT.

2. Determination of Sex Differences in Mesencephalic Dopaminergic Systems Using Immunhistochemical and Stereological Analysis

2.1 Introduction

An extensive literature suggests that there are sex differences in dopaminergic function. Previous findings in this laboratory revealed that adult female rats exhibit greater baseline dopamine uptake and release and increased sensitivity to the behavioral stimulation caused by the psychostimulant drug cocaine compared to their male counterparts [6, 79]. Previous investigations by our laboratory revealed no sex differences in measures such as dopamine content, transporter number or vesicle number (unpublished findings). However, studies from other laboratories have shown that these sex dimorphisms may result in part from gonadal hormone effects on dopamine neuron survival and that estrogen plays a role in the protection of mesencephalic dopamine neurons and their projections. A study performed in primates showed female African Green monkeys have a greater density of tyrosine hydroxylase immunoreactive (TH-IR) cells in the SNpc than males [8]. This study also revealed that estrogen is required for the maintenance of dopamine neuron number as ovariectomy reduced the number of TH-IR cells in the SNpc, and estrogen replacement restored TH-IR cell number. Estrogen also maintains terminal density in primates as ovariectomy results in a reduction of TH-IR fibers in the dorsal striatum, suggesting that gonadal

hormones can modulate anatomical measures [109]. These findings in primates also suggest that the sex differences observed in cocaine-stimulated behavior and dopamine release of rats may be due to anatomical differences in males and females. Currently little is known about the existence of sex differences in dopamine neuron number in the SNpc and VTA of rodents. Therefore, we investigated if sex differences existed in dopamine neuron number and when they emerged. We hypothesized that female rats would have more dopaminergic cell bodies than males in the midbrain regions and that this sex difference would emerge during late adolescence based on our previous findings with sex differences in psychostimulant-induced behavior and dopamine uptake and release. To determine if these sex differences emerging in late adolescence were dependent upon neuron number in midbrain dopaminergic regions (SNpc and VTA), the number of cell bodies immunoreactive for TH, the rate-limiting enzyme in dopamine biosynthesis, was quantitated using unbiased stereological techniques. In this study, sex differences in neuron number were investigated in weanling to adult male and female rats. The results of these experiments suggest that TH-IR neuron number decreases across adolescence in the SNpc and VTA and that a sex difference, in which females have more TH-IR cell bodies in the SNpc and VTA emerges in adulthood.

2.2 Materials and Methods

Animals

Male and female Sprague Dawley rats ages PN21 (post-weaning), PN28 (early adolescent), PN42 (mid-adolescent), PN65 (late adolescent/early adult) and PN90 (adult) (Charles River Laboratories, Raleigh, NC) were used for this study. Animals were housed under a 12:12 hour light-dark cycle in a temperature and humidity controlled environment with *ad libitum* access to food and water.

In a separate study incorporating heat-mediated antigen retrieval (HMAR) to validate the sex differences in found in adult rats, male and female rats at PN60 and PN90 were subjected to the previously described housing and care conditions. Animal care and housing were in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 865-23 Bethesda, MD) and approved by the Institutional Animal Care and Use Committee (IACUC).

Tissue Preparation and TH Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA). Brains were extracted, postfixed overnight in PFA, and cryoprotected with 30% sucrose solution. After sinking in cryoprotectant, brains were cut on a cryostat at 30 μm thickness and allowed to dry overnight. Tissue

was permeabilized with 0.3% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) for 10 minutes at room temperature. Sections were then incubated in 0.5% hydrogen peroxide in PBS for 30 minutes to block endogenous peroxidase. Sections were rinsed for 10 minutes in PBS and incubated in blocking buffer (0.5% BSA + 0.3% Triton X-100 in PBS) for 30 minutes at room temperature. Sections were then incubated in primary antibody (TH) diluted in blocking buffer (1:3500, Immunostar Inc., Hudson, WI) overnight at room temperature. The following day, sections were rinsed in PBS and incubated in biotinylated secondary antibody (1:1000, Vector Labs, Burlingame, CA) at room temperature for one hour. Sections were rinsed and labeled with avidin-biotin complex (Vector Elite Standard Kit, Vector Labs) for one hour at room temperature. After a final wash in PBS, sections were stained with 3, 3′-diaminobenzidine (DAB, Vector Labs), rinsed in deionized water, dehydrated through graded alcohols, mounted and coverslipped.

Tissue Preparation and TH Immunohistochemistry Using HMAR

Animals were deeply anesthetized and transcardially perfused with 10% neutral buffered formalin. After perfusion, the brains were extracted and post-fixed overnight in 10% formalin. Brains were then equilibrated in a 30% sucrose cryoprotectant solution and stored at 4°C. Serial coronal sections (30 µm thick, every 3rd) were cut on a cryostat and thaw-mounted to slides. Sections were allowed to dry overnight at 37°C. Heat

mediated antigen retrieval [110-112] was performed to increase immunoreactivity of the tissue for TH. Sections were pressure cooked (Deni electric pressure cooker, Keystone Manufacturing, Buffalo, NY) at high pressure for 1 minute and 30 seconds in citrate buffer (pH = 6.0) [110]. This length of time allowed for optimal staining without compromising cell morphology. Sections were rinsed in PBS and incubated in 0.3% hydrogen peroxide-methanol for 30 minutes to quench endogenous peroxidase. Sections were rinsed and blocked in 0.5% BSA + 0.3% Triton X-100 for 15 minutes at room temperature. After blocking, sections were incubated in primary antibody diluted in blocking buffer (1:10000, Immunostar, Inc.) overnight at 4°C. The next day, sections were rinsed and incubated in a biotinylated horse anti-mouse secondary antibody (1:1000, Vector Labs) for one hour at room temperature. The sections were then rinsed and incubated in avidin-biotin complex for one hour at room temperature. The sections were then rinsed and stained with DAB (Vector Labs). Sections were rinsed, dehydrated through graded alcohols, mounted and coverslipped.

Stereology

Unbiased stereological estimation of the total number of TH-IR cell bodies in the SNpc and VTA was performed using the optical fractionator method [113]. For the standard method every third section was analyzed, resulting in a total of 11-14 sections sampled for each side of the brain. A computerized counting system containing a Nikon

Optiphot-2 microscope, a digital camera (Dage-MTI, Michigan City, IN) and motorized stage (Ludl Electronic Products, Hawthorne, NY) was used to estimate the total number of cells. Each region of interest was projected onto a monitor, traced at low (4x) magnification and a sampling grid was superimposed on the traced region by the StereoInvestigator software (MicroBrightField, Inc., Williston, VT). After shrinkage, final thickness of the sections used averaged 12 μm. Therefore, a 40 x 40 μm counting frame with a dissector height of 8 μm was used. Each counting frame was randomly spaced 80 μm apart and guard zones of 2 μm from the top and bottom of the section were used. Individual cell bodies were visualized at with a 100x oil immersion lens (numerical aperture = 1.3). Enough cells were counted to achieve a coefficient of error that was ≤0.10. All sections were analyzed blindly.

After the incorporation of HMAR, the number of TH-IR cell bodies in the SNpc and VTA was estimated using the optical fractionator method. Every 6th section through the extent of the midbrain on both sides of the brain was analyzed. Due to improvement of staining, fewer cells needed to be counted to achieve an appropriate coefficient of error. Starting sections were selected at random, and all brains were coded and analyzed blindly. Midbrain regions were manually traced at low magnification (4x). Individual cells were visualized for counting with a 100x oil immersion objective (numerical aperture = 1.3). Sections were systematically sampled with counting frames measuring 40 x 40 μm (1600 $μm^2$ area) spaced randomly 80 μm apart along the x and y

axes (sampling grid area = 6400 μm^2). Due to extensive staining methods, some tissue shrinkage occurred resulting in a mounted thickness of 12 μm. Therefore, a disector height of 8 μm was used, with top and bottom guard zones of 2 μm. Only cells that came into focus at the fixed height were counted. Using these parameters, we were able to count enough cells to result in a coefficient of error for each estimate that ranged between 0.05 – 0.10.

Statistics

All statistical analyses were performed using a two-way analysis of variance (ANOVA) (NCSS) with a significance level of $p<.05$. Post hoc analysis was performed using the Newman-Keuls Multiple Comparison and the Fisher's Least Significant Difference tests.

2.3 Results

Midbrain TH-IR Cell Number Decreases with Age and a Sex Difference in TH-IR Emerges in Early Adulthood

The number of TH-IR cell bodies throughout normal postnatal development in male and female rats was quantitated. In the SNpc, neuron number decreased with age, but stabilized during adolescence (PN42) in females and during adulthood (PN65) in males (*Fig. 2-1*). ANOVA indicated a main effect of age [$F(4,58) = 115.1$, $p<.0001$] and an interaction of sex and age [$F(4,58) = 4.2$, $p<.01$]. In the VTA, a similar decrease in cell number throughout puberty was observed (*Fig. 2-2*). Post hoc analysis revealed that the number of TH-IR cells in the VTA was significantly reduced in prepubertal (PN21) and early adolescent female (PN28) rats compared to age-matched males. In both males and females, the number of TH-IR cells was not different between PN42 and PN65 but continued to decrease at PN90. ANOVA indicated a main effect of sex [$F(1,56) = 9.7$, $p<.01$)], a main effect of age [$F(4,56) = 63.5$, $p<.0001$] and a sex by age interaction [$F(4,56) = 4.8$, $p<.01$]. Fig. 2-3 and 2-4 show the differences between age groups for males and females, respectively.

Previous findings in our laboratory revealed that sex differences in dopamine release and cocaine-stimulated locomotor behavior emerge at PN65 and persist into adulthood. Based these findings and there being a significant interaction of sex and age in the SNpc and VTA, sex differences in TH-IR cell number in adulthood were investigated. Fig. 2-5 shows a reanalysis of TH-IR cell counts in the adult (PN65-PN90)

rat SNpc. ANOVA indicated a main effect sex [$F(1, 24) = 14.1$, $p<.01$] and a main effect of age [$F(1,24) = 5.1$, $p<.05$]. Females were found to have more nigral TH-IR cell bodies at PN65 and had a trend of higher cell number at PN90. In the VTA, ANOVA revealed a main effect of sex [$F(1,24) = 5.9$, $p<.05$] and a main effect of age [$F(1,24) = 41.7$, $p<.0001$] (*Fig. 2-6*). Post hoc tests showed that both male and female rats had fewer cells at PN90 than their PN65 counterparts.

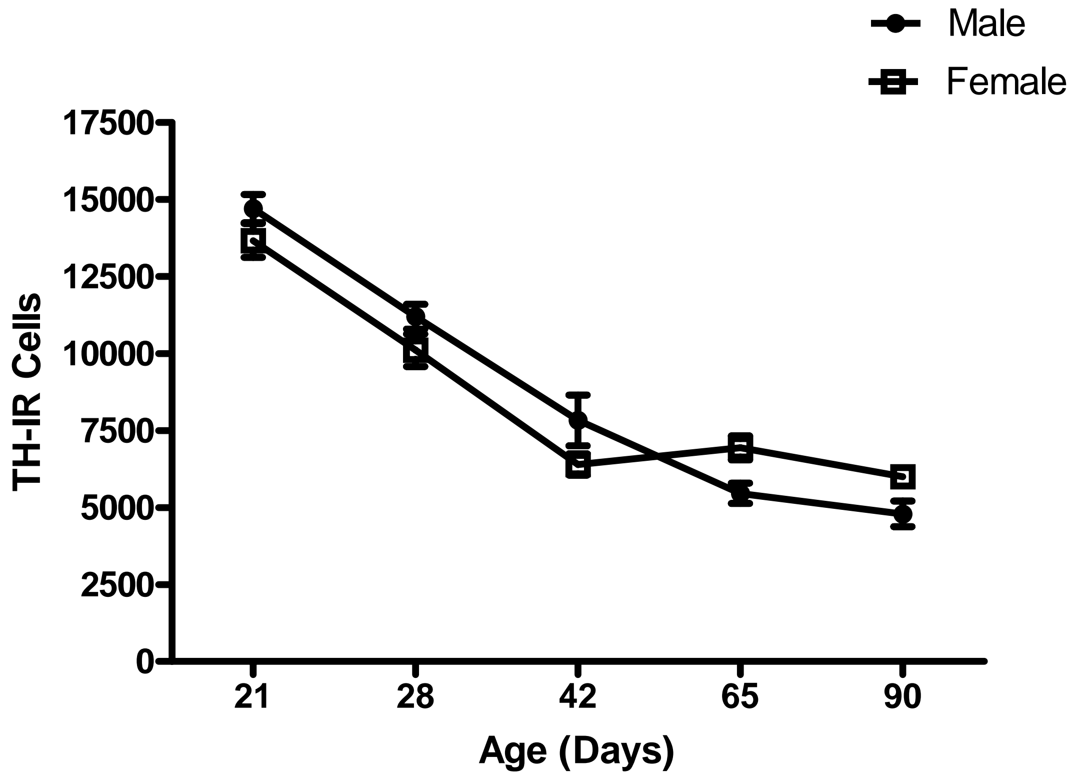


Fig. 2-1. TH-IR cell number throughout postnatal development in the SNpc. Cell number decreases with age in both male and female rats. Males are presented as closed circles and females are presented as open squares. TH-IR neuron number stabilizes in females at PN42 while neuron number stabilizes at PN65 in males. Data are expressed as means ± SEM. (n = 5-7)

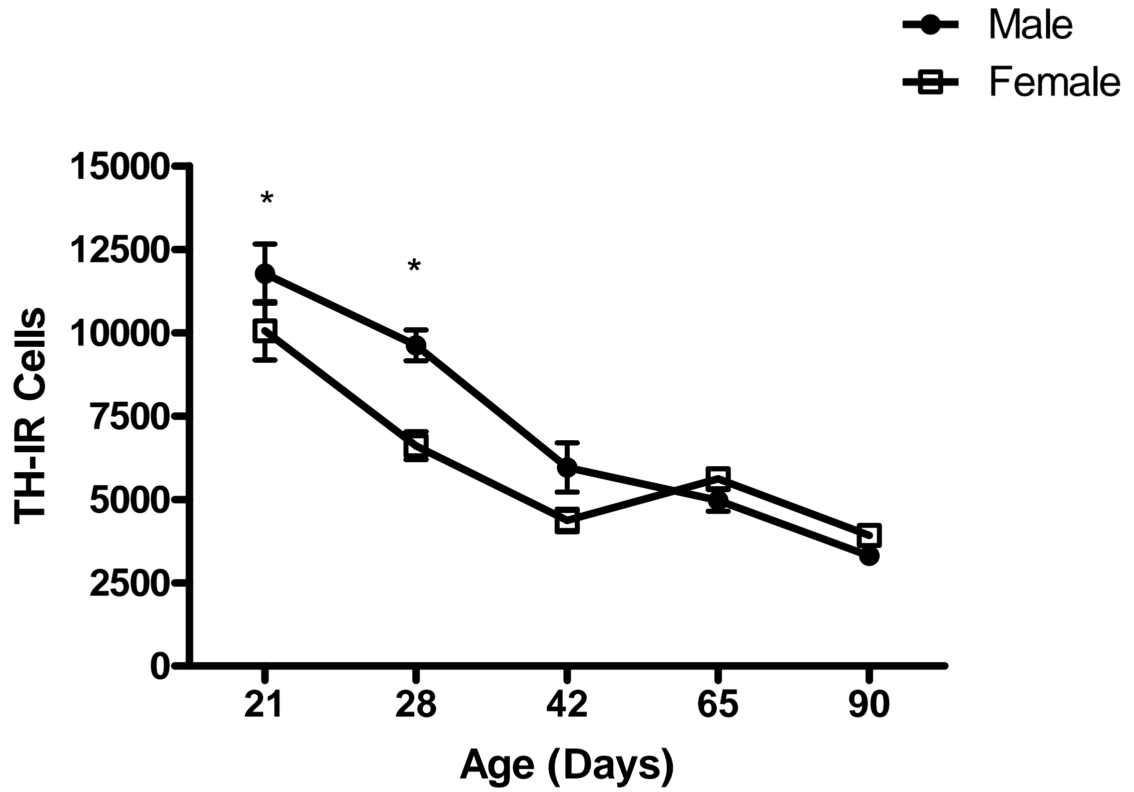
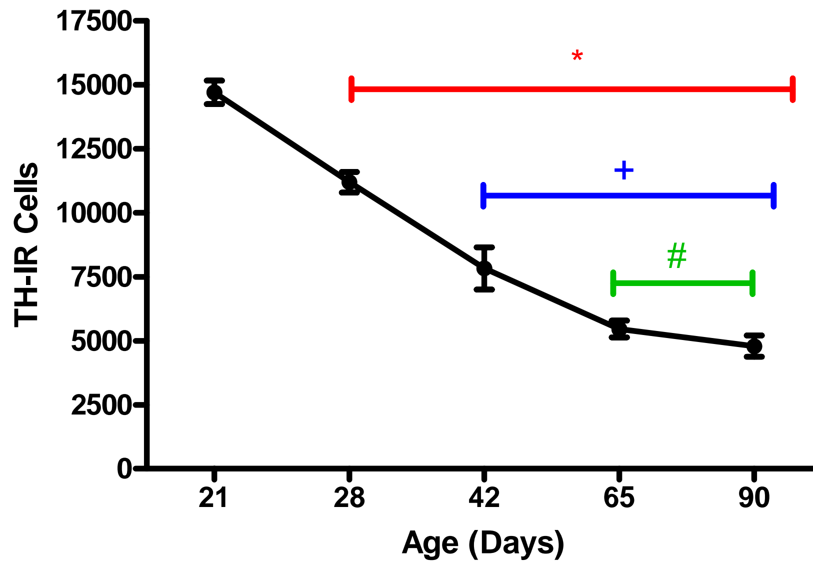


Fig. 2-2. TH-IR cell number throughout postnatal development in the VTA. Cell number decreases with age in both male and female rats. Males are presented as closed circles and females are presented as open squares. TH-IR number continues to decrease throughout postnatal development in males while cell number stabilizes in females at PN42. Males have a greater number of TH-IR cells at PN21 and PN28 than females. Data are expressed as means ± SEM. * indicates sex difference, $p<.01$. ($n = 5\text{-}7$)



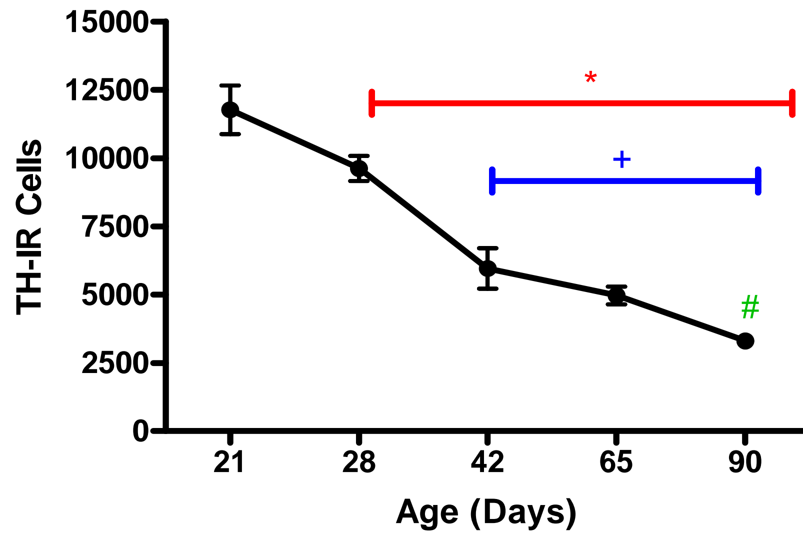
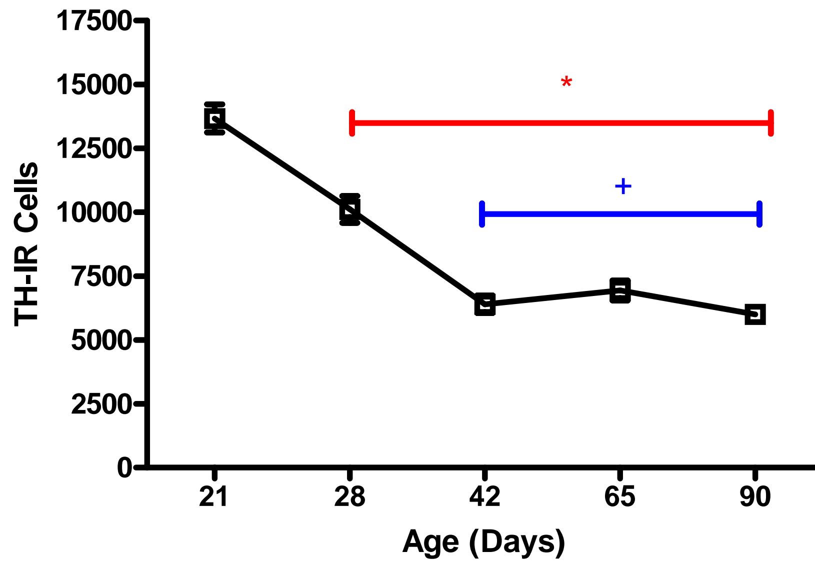


Fig.2-3. Effect of age on TH-IR cell number throughout postnatal development of male rats. TH-IR cell number decreases with age in the SNpc (top panel) and VTA (bottom panel). Data are expressed as means ± SEM. * indicates fewer cells than PN21, + indicates fewer than PN28 and # indicates fewer than PN42, p<.0001. (n = 5-7)



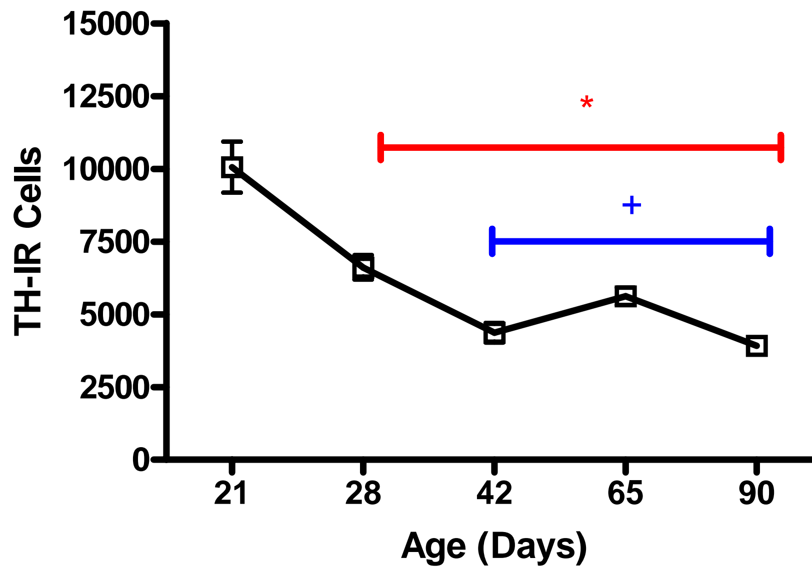


Fig.2-4. Effect of age on TH-IR cell number throughout postnatal development of female rats. TH-IR cell number decreases with age in the SNpc (top panel) and VTA (bottom panel). Data are expressed as means ± SEM. * indicates fewer cells than PN21 and + indicates fewer than PN28, $p<.0001$. ($n = 5$-6)

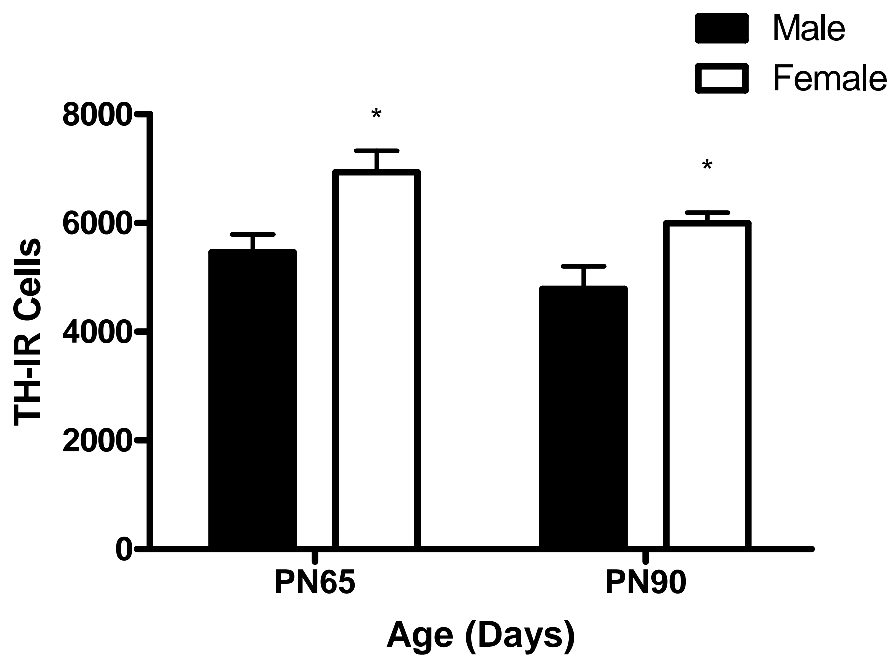


Fig. 2-5. TH-IR cell number in the SNpc in adulthood. Female rats have more TH-IR cells in the SNpc than males at both PN65 and PN90. Data are expressed as means ± SEM. * indicates different from male, $p<.01$. ($n = 5\text{-}7$)

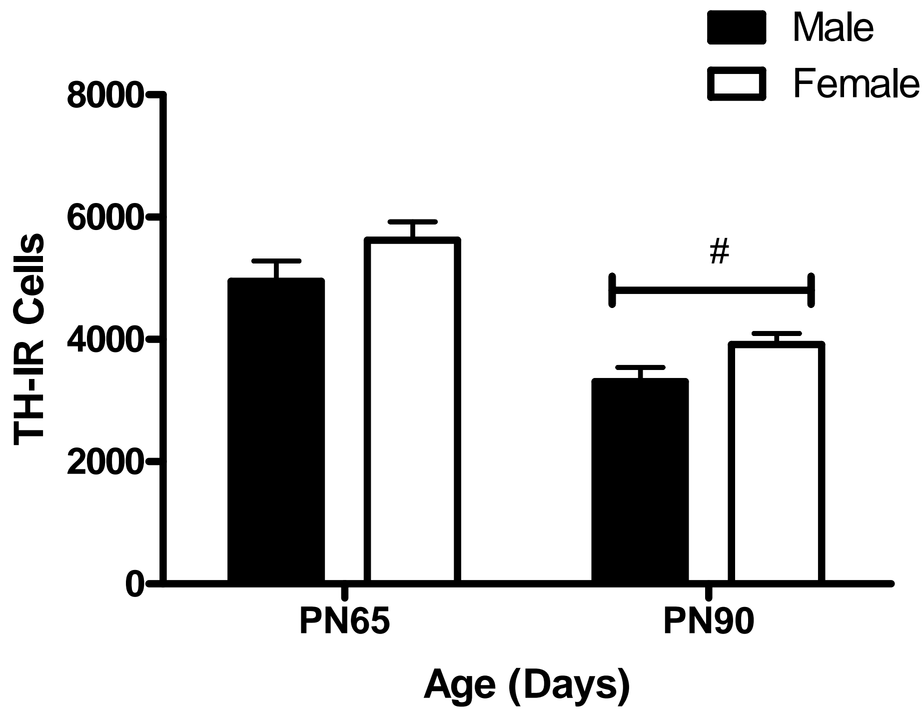


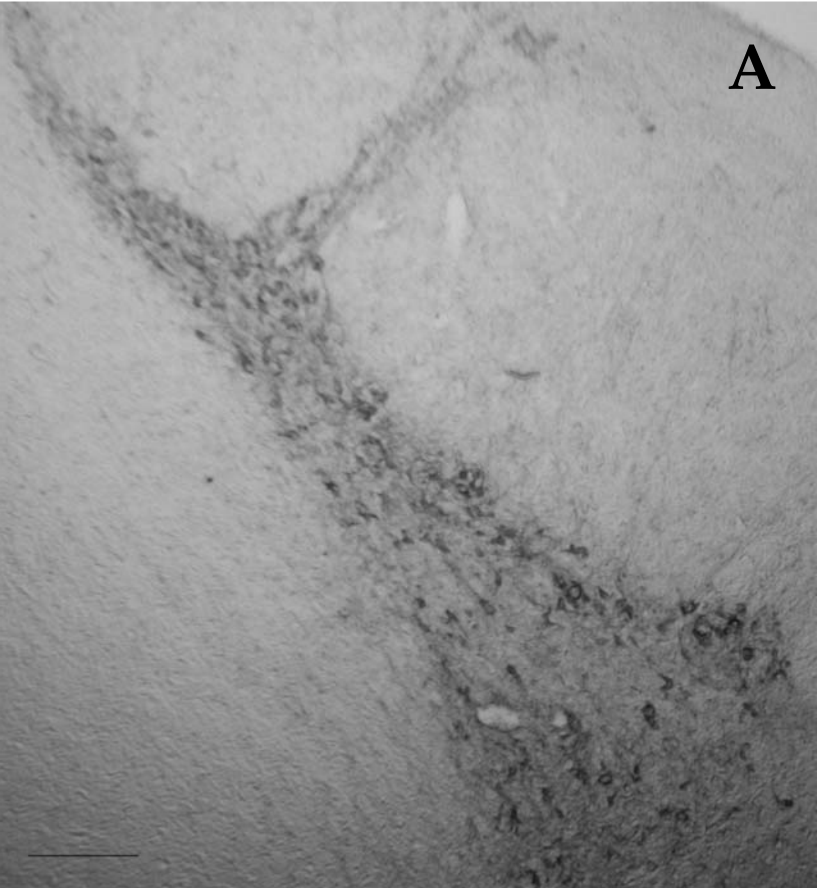
Fig. 2-6. TH-IR cell number in the VTA in adulthood. No sex differences in TH-IR cell number were observed at both ages. However, an age difference was observed as PN90 males and females had fewer cells than their PN65 counterparts. Data are expressed as means ± SEM. # indicates different from PN65 animal of same sex, $p<.0001$. ($n = 5$-7)

Confirmation of Sex Differences Using HMAR

The stereological estimates for cell number in these regions were significantly lower than those reported in the literature. To confirm that the previously discovered differences were not the result of insufficient TH staining or decreasing TH levels throughout normal ontogeny, HMAR [110-112] was incorporated into the previous TH staining method to increase TH immunoreactivity in sections. Using this method greatly improved TH staining and increased cell number by approximately two-fold. However, this had no effect on our previous findings that females have more TH-IR cell bodies in midbrain regions. The incorporation of HMAR also increased the visibility of TH-IR projections in midbrain regions (*Figs. 2-7 and 2-8)*. The sex difference found in early adulthood in the SNpc was replicated (*Fig. 2-9)*. With the incorporation of antigen retrieval into the TH staining protocol, the magnitude of sex difference was larger. The results of this study were consistent with the previous findings that females had more TH-IR cell bodies in the SNpc. ANOVA indicated a main effect of sex [$F(1,17) = 57.1$, $p<.001$]. Post hoc tests showed that adult males had fewer TH-IR cells than females and that neuron number does not change between PN60 and PN90 in both sexes. In the VTA, we previously observed an age difference between PN65 males and females and their PN90 counterparts. After HMAR, male rats at PN90 had fewer cells than males at PN60, suggesting that TH-IR cell number may still be decreasing in the male VTA (*Fig.2-10*). Females also have more TH-IR cells in the VTA at PN90 compared to age-matched

males. The fall in cell number between early adulthood and PN90 was not replicated in females. ANOVA indicated a main effect of sex [$F(1,18) = 8.7$, $p<.05$] and an interaction of sex and age [$F(1,18) = 4.8$, $p<.05$].

Fig 2-7. Impact of HMAR on TH staining. Representative light photomicrographs of SNpc taken in PN65 female rats without (*Panel A*) and with (*Panel B*) HMAR staining. Both cell bodies and projections are more intensely stained in the section receiving HMAR. Scale Bars = 250 μm


A

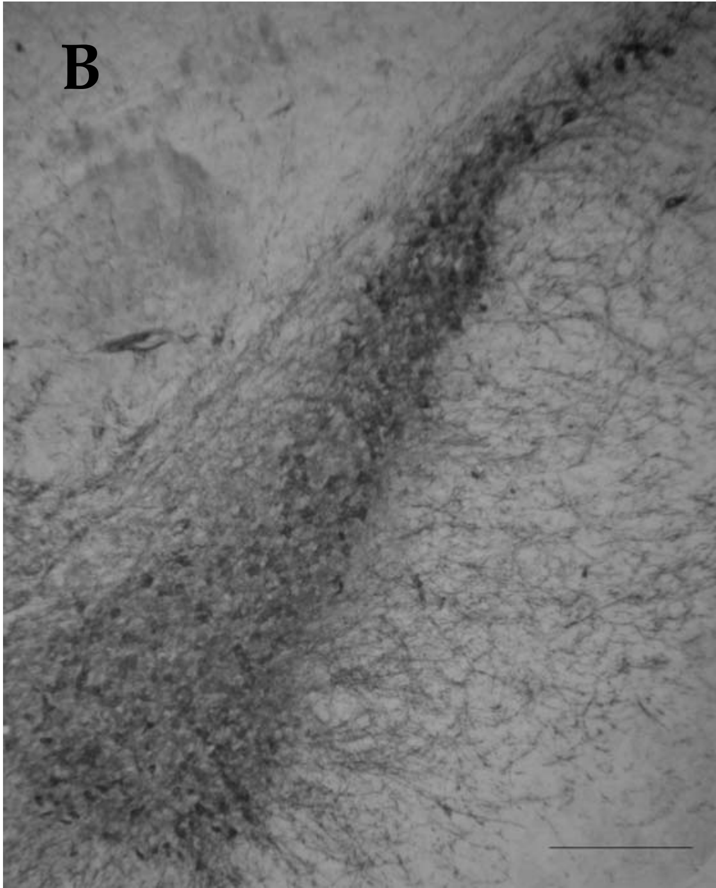
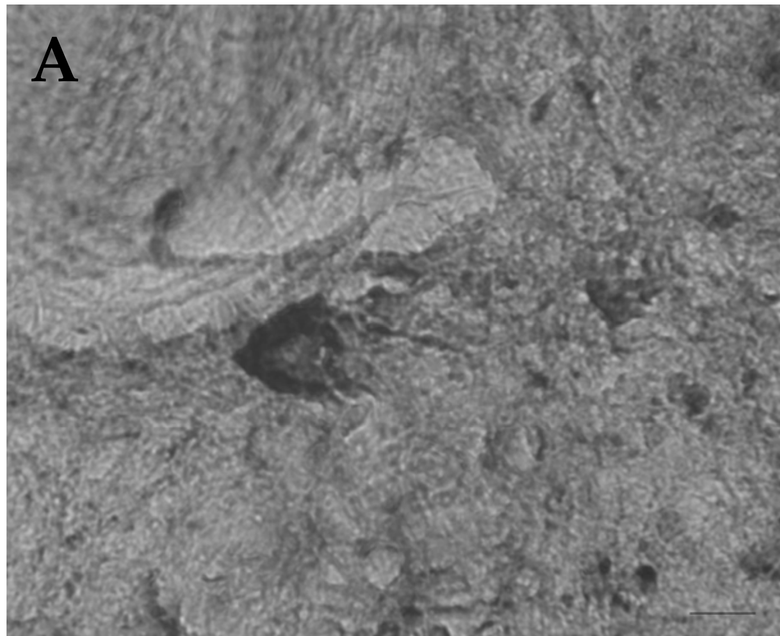
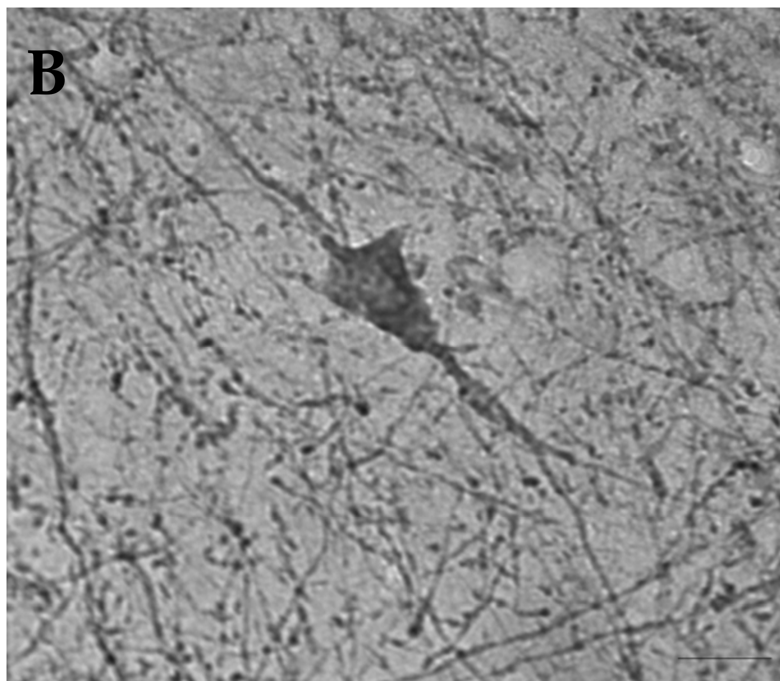

B

Fig 2-8. HMAR improves the ability to count individual cell bodies. Representative light photomicrographs of SNpc taken in TH-IR stained cell bodies. Sections are taken from PN65 female without (*Panel A*) and with (*Panel B*) HMAR staining. While TH-IR cell bodies are intensely stained using both methods. TH-IR fibers and varicosities are visible after incorporation of HMAR. Photomicrographs taken at 25x magnification. Scale Bars = 20 μm





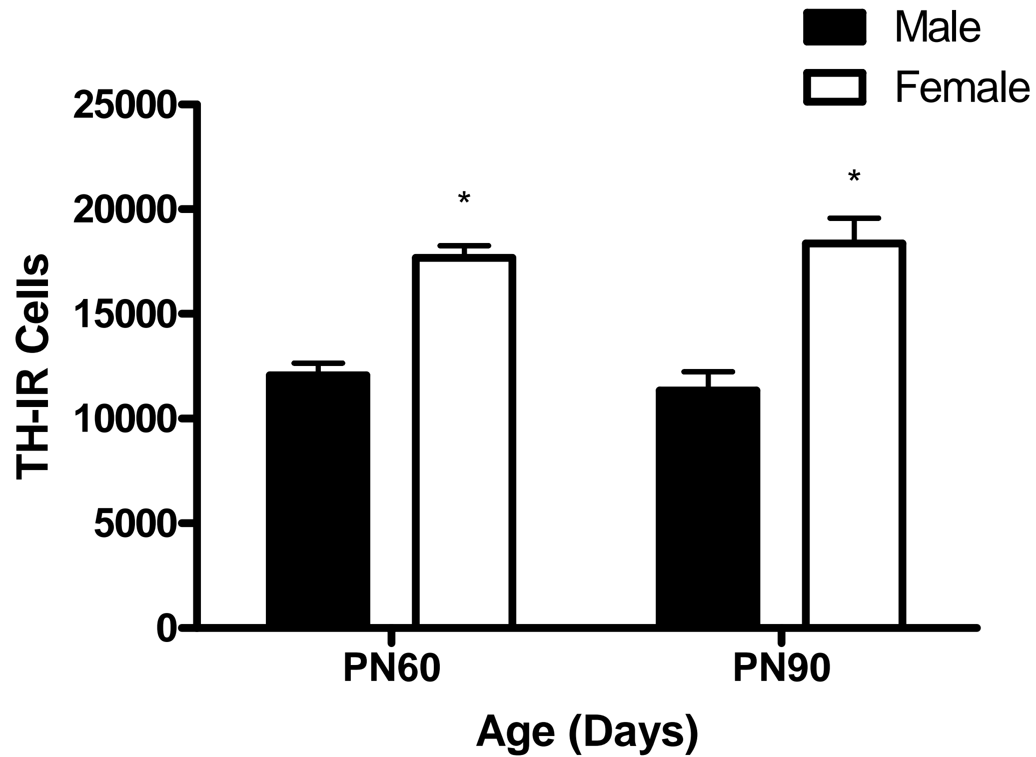


Fig. 2-9. TH-IR cell number in the adult SNpc after HMAR. The total number of detectable cells increased with the incorporation of antigen retrieval. Females have a greater number of TH-IR cells than males. Data are expressed as means ± SEM. * indicates different from male, p<.001. (n = 4-5)

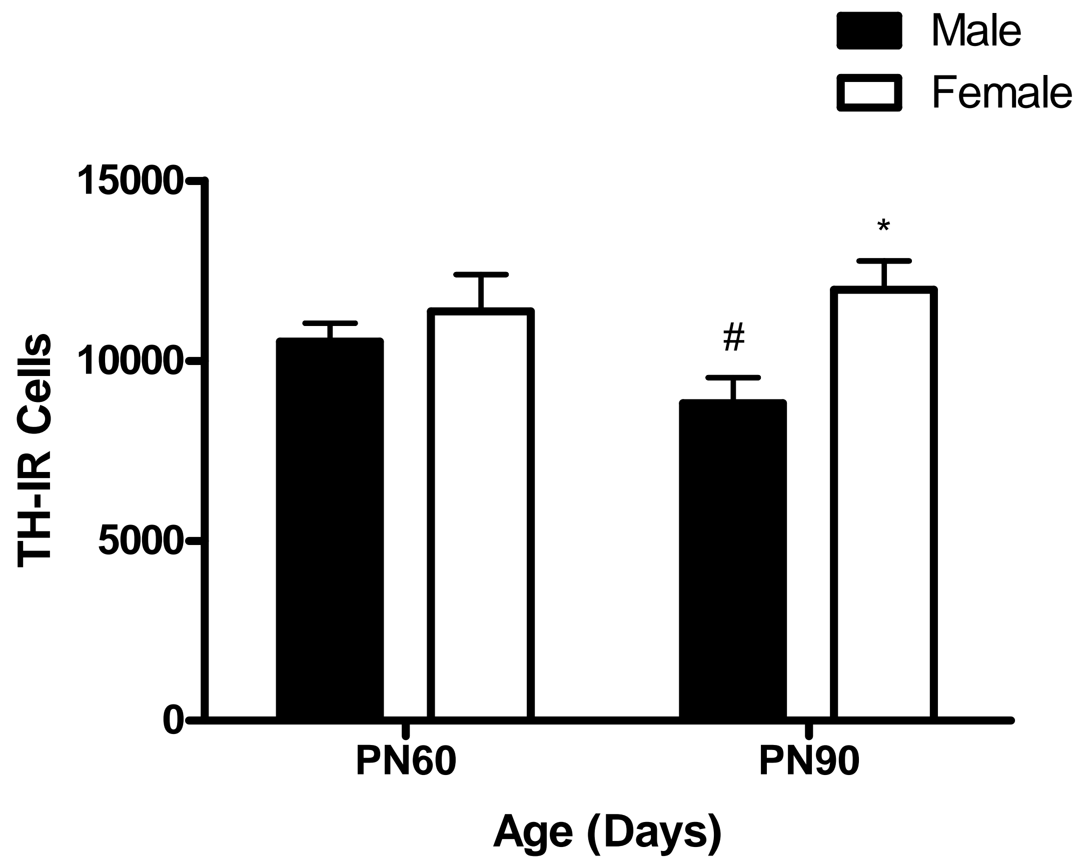


Fig. 2-10. TH-IR cell number in the adult VTA after HMAR. The total number of detectable cells increased with the incorporation of antigen retrieval. A sex difference emerges at PN90. Data are expressed as means ± SEM. * indicates different from PN90 male, $p<.05$ and # indicates different from PN60 male, $p<.05$. (n = 4-5)

2.4 Discussion

In this study, the number of cells immunoreactive for TH was quantitated throughout postnatal development to determine if and when sex differences in TH-IR neuron number emerge in midbrain regions. In the first series of experiments which did not incorporate HMAR into the staining method, we observed that TH-IR cell number decreases with age in the SNpc and VTA of male and female rats. In the SNpc, TH-IR cell number stabilized during mid-adolescence (PN42) in females but did not stabilize until PN65 in males. A sex difference emerges in adulthood as females have more cells than males at PN65. In the VTA, a similar decrease in TH-IR cell number was observed with a stabilization patterns for males and females comparable to the SNpc. However, a sex difference was observed at PN21 and PN28 with males having a greater number of TH-IR cells. This sex difference did not persist into adulthood. These data are the first findings to suggest that there is a decrease in TH-IR cell number throughout postnatal development and a sex difference emerges in TH-IR emerge in the prepubertal, adolescent and adult midbrain. One potential mechanism for this reduction could be cell loss due to apoptosis or programmed cell death. It is established that two natural waves of apoptosis occur in the rat SNpc at birth and during the second week of postnatal life [114-116]. No other studies have reported the detection of cell death past PN14. Many other studies have focused on dopaminergic cell death in response to brain trauma or cell damage, however, little is known about the possibility of naturally

occurring cell death in the adult rat midbrain. Another possible explanation for the decrease in TH-IR cell number with age could be that TH-levels are decreasing with age, making the detection of cells using traditional staining methods more difficult. However, studies in the rat SNpc revealed no difference in TH expression in cells of males and females throughout ontogeny [117]. In an additional study, more TH mRNA expression was detected early in postnatal development in male and female mice, but showed there were no differences in expression during adolescence and adulthood [118]. These findings suggest that differences in TH levels may not be the reason for the decrease in TH-IR cell number observed.

The sex differences that emerged in adulthood in the SNpc were concordant with findings in our laboratory that sex differences in cocaine-stimulated behavior and electrically-stimulated dopamine release emerge in early adulthood (PN65) [101]. However, in the initial study, the total cell number for the SNpc and VTA was lower than totals reported in published studies. Anatomical studies in adult and adolescent rats report that there are between 5000 and 15,000 TH-IR neurons on one side of the SNpc and between 5000 and 20,000 on one side of the VTA [119-122]. Therefore, a validation study incorporating HMAR into the staining method to improve TH immunoreactivity was performed. The incorporation of HMAR improved the intensity and clarity of TH staining and increased total cell number by approximately two-fold, placing cell counts within the previously described cell count ranges in published

studies. This study was only performed in adult animals (PN60 and PN90). The sex difference observed in adulthood was replicated in the SNpc. In the VTA, the initial study without HMAR treatment of sections revealed that a sex difference was not observed in adulthood, although cell number decreased with age in both males in females. After the incorporation of HMAR into the staining method, a sex difference was not observed at PN60, which is a replication of the previous study. However, a sex difference with females having a greater number of TH-IR cells emerged at PN90. A decrease in TH-IR cell number between PN60 and PN90 males was observed, suggesting that VTA TH-IR cell number may still be decreasing in adult males.

The finding that females have a greater number of TH-IR cells in midbrain regions is concordant with an extensive literature suggesting that estrogen has protective and stimulatory effects in the midbrain. *In vivo* studies in primates show that estrogen is required for the maintenance of TH-IR cell number in the SNpc as ovariectomy resulted in a reduction in TH-IR cell number which was revered with estrogen replacement. Female primates were also found to a greater density of TH-IR cell bodies in the SNpc than males [8]. Our observation of a sex difference in which females have a greater number of cells in the SNpc is consistent with this study.

Few studies have investigated sex differences in dopamine neuron number in the rodent midbrain. A study in mice found no sex differences in the SNpc and VTA [123]. Gillies and colleagues published the only study in rat showing sex differences in both

the SNpc and VTA [124]. However, the findings of this study are discordant with our observations in the SNpc. In the Gillies study, males were found to have a greater number of TH-IR cells than females in the SNpc. Their findings in the VTA were concordant with ours as they found that females have a greater number of TH-IR cells than males. The sex differences in the Gillies study were modest and total cell number lower than that described in other studies employing stereologic counting methods. The primate study along with our study utilized sterologic techniques. In our study, the optical fractionator method was employed. This stereologic probe takes into account the section thickness and accounts for shrinkage after staining [113, 125]. Through the use of a microscope equipped with a motorized stage that can scan through the depth of a section (z-axis), we are able to count cells that may be unseen below the section surface and cells that are superimposed can be distinguished. Stereology in combination with HMAR to ensure that many more cells are stained makes the methods used in this study more accurate. Taken together, these considerations suggest that gonadal hormones play a role in mediating midbrain sex differences in TH-IR cells number.

In conclusion, the results of this study reveal possible natural cell loss occurring in late adolescence. These observations demonstrate that midbrain regions are sexually dimorphic as females are spared from this loss and have more TH-IR cell bodies in adulthood. These findings suggest that gonadal hormones influence midbrain cell populations and that estrogen may play a protective role in both the SNpc and VTA.

3. The Role of Estrogenic Compounds in the Maintenance of Tyrosine Hydroxylase Immunoreactive Cell Number in Mesencephalic Regions

3.1 Introduction

Midbrain dopaminergic neurons that arise in the SNpc and the VTA and project to forebrain regions form systems that regulate behavior and cognitive function [17, 19]. Dysregulation of these pathways is implicated in a number of pathological disorders such as drug addiction and Parkinson's disease (PD) [21, 25]. Women are more likely to progress more rapidly in drug taking and are more likely to experience relapse than men [83, 84]. In contrast, studies show a lower incidence of PD in women than men [2, 4]. Sex differences in dopaminergic function represent a potential mediator for these differences.

Our laboratory and others have shown that adult female rats exhibit greater locomotor responses to psychostimulant drugs such as cocaine or amphetamine or than males [1, 6, 79]. Some studies show that female rats self-administer psychostimulant drugs more than males and show enhanced place preference [93, 126]. Females rats also show greater electrically-stimulated striatal dopamine uptake and release than males [5, 7].

Estrogen influences on dopaminergic function may mediate these sex differences in behavior. In rodent behavioral studies, ovariectomy results in the attenuation of locomotor responses to psychostimulants, which can be restored with estrogen

replacement [6, 93, 94, 127]. In contrast, replacement with progesterone in these models resulted in a suppression of behavior, suggesting that estrogen has stimulatory effects in the dopaminergic pathways influencing behavior [94, 127].

Studies in humans and animals provide evidence that estrogen not only mediates sex differences in dopaminergic function, but plays a role in dopaminergic cell survival and protection. Estrogen replacement therapy in postmenopausal women reduces PD risk and reduces PD-related symptoms in postmenopausal early onset patients [92, 128]. Gonadectomy of female primates results in loss of neurons which is prevented by estrogen replacement [8]. Estrogen also can protect from neurotoxin-induced damage to the nigrostriatal pathway. Adult female rats are less susceptible to the behavioral deficits and dopamine cell loss after lesion with the neurotoxin 6-OHDA [96]. Replacement with E2 in ovariectomized rodents prevents the loss of striatal dopamine content in neurotoxicity induced animal models [67, 108, 129]. Ovariectomy of female rats results in a decrease in dopamine receptor and dopamine transporter number. Estrogen replacement can restore these functional responses [65, 74, 75].

The effects of estrogen on normal dopamine function are mediated through binding to ERs, ERα and ERβ, which are ligand-dependent transcription factors [130]. *In vivo* studies involving ER-selective agonists and transgenic mice reveal that both subtypes may contribute to the trophic effects of estrogen on dopamine neurons. The ERα-selective agonist, propyl pyrazole triol (PPT), and E2 protect striatal dopamine in a

mouse model of PD while the ERβ-selective agonist diarylpropionitrile (DPN) has no effect [67]. ERα-knockout mice (αERKO) are more vulnerable to the MPTP toxicity than ERβ-knockout (βERKO) mice, suggesting that ERα plays a significant role in the protection of dopaminergic pathways. In contrast, studies in rat reveal that maintenance of dopamine D_2 receptor and dopamine transporter (DAT) binding is ERβ-dependent [74, 75]. Studies in βERKO mice reveal a reduction of neurons in the SNpc, suggesting that both subtypes may contribute to the maintenance of these pathways.

Despite the current findings on the role of estrogen in animal models of dopamine neuron degeneration and regulation of dopaminergic function, little is known about the role of estrogen in the maintenance of dopamine neuron number in midbrain regions in rodent models and if estrogen regulation of neuron number could mediate the sex differences observed in psychostimulant-induced behavior. The aims of the present study were to investigate how the loss of estrogen affects TH-IR cell number in midbrain regions in rat and mice and to determine which ER subtype contributes to the trophic effects of estrogen on dopamine neurons. We quantitated TH-IR cell number in ovariectomized rats and mice replaced with E2, PPT and DPN and quantitated cell number in adult female αERKO and βERKO mice.

3.2 Materials and Methods

Animals and Staining Procedures for Preliminary Gonadectomy Study

Male and female Sprague Dawley rats sham gonadectomized and gonadectomized at PN55 were purchased from Charles River Laboratories (Raleigh, NC). Animals were segregated by sex and surgical condition and housed in plastic cages under a light-dark cycle (12:12 hour) with *ad libitum* access to food and water. Animals were housed until PN90. On PN90, animals were transcardially perfused with 4% paraformaldehyde and postfixed over night at 4°C. The following day, brains were equilibrated in a 30% sucrose cryoprotectant solution. Brains were sectioned, stained and counted according to the procedures described in Chapter 2 for non-HMAR staining.

Animals, Surgery, Hormone Replacment and Housing

In Experiment 1, female Sprague Dawley rats ovariectomized or sham ovariectomized on postnatal day (PN) 60±5 were purchased from Charles River Laboratories (Raleigh, NC). Animals were segregated by surgical condition and housed in plastic cages under a light-dark cycle (12:12 hour) with *ad libitum* access to food and water. In a parallel study, female C57Bl/6 mice ovariectomized at PN60±5 (Charles River Laboratories, Raleigh, NC) were separated by surgical condition and housed in plastic cages under a light-dark cycle (12:12 hour) with *ad libitum* access to food and

water. Rats were transcardially perfused with 10% neutral buffered formalin (VWR) on PN90. In Experiment 2, bilateral ovariectomy and sham surgery was performed on adult rats (PN60) while animals were under ketamine (60 mg/kg) and xylazine (8 mg/kg) anesthesia. While under anesthesia for ovariectomy, a small incision was made at the nape of the neck above the right shoulder and an osmotic minipump was implanted (Alzet, Model 2004, 0.25 μl/hour). Minipumps contained vehicle (50% DMSO/normal saline), E2 (6 μg/day), ERα–selective agonist PPT (2 mg/kg/day) and ERβ-selective agonist DPN (8 mg/kg/day) [131-133]. Due to inadequate replacement of PPT by the minipump, an additional study was conducted in which PPT dissolved in sesame oil vehicle was administered daily via subcutaneous injection. PPT replacement animals were implanted with a minipump-containing vehicle to make them surgically comparable to the minipump-replaced animals. A subset of controls received vehicle pumps and a daily subcutaneous injection of sesame oil vehicle. Following surgery animals were housed under previously described conditions until PN90 for perfusion. In a parallel study, female mice were sham or ovariectomized at PN60 by the supplier (Charles River Laboratories, Raleigh, NC) and shipped the following morning. Animals were administered daily subcutaneous injections of sesame oil vehicle, E2 (3 μg), PPT (2 mg/kg) and DPN (8 mg/kg) starting the day after receipt. Animals in both studies were housed in plastic cages on ventilated racks under a 12:12 light:dark cycle. Food and

water were provided *ad libitum*. Both rats and mice in the hormone replacement study were housed until PN90 for perfusion.

In Experiment 3, female ERKO and βERKO mice were a generous gift from Dr. Ken Korach at the NIEHS (Research Triangle Park, NC). Animals were perfused at PN85 with 10% neutral buffered formalin by NIEHS personnel. Animal care and housing were in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 865-23 Bethesda, MD) and approved by the Institutional Animal Care and Use Committee (IACUC).

Tissue Preparation and Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused with 10% neutral buffered formalin. After perfusion, the brains were extracted and post-fixed overnight in 10% formalin. Brains were then equilibrated in a 30% sucrose cryoprotectant solution and stored at 4°C. Serial coronal sections (30 μm thick) were cut on a cryostat and thaw-mounted to slides. For rats, every 3rd section was collected and for mice every 2nd section was collected. Sections were allowed to dry overnight at 37°C. Heat mediated antigen retrieval [24, 25] was performed to increase immunoreactivity of the tissue for TH. Sections were pressure cooked (Deni electric pressure cooker) at high pressure for 1 minute and 30 seconds in citrate buffer (pH=6.0) [26]. This length of time allowed for optimal staining without compromising cell morphology. Sections were rinsed in PBS

and incubated in 0.3% hydrogen peroxide-methanol for 30 minutes to quench endogenous peroxidase. Sections were rinsed and blocked in 0.5% BSA + 0.3% Triton X-100 for 15 minutes at room temperature. After blocking, sections were incubated in primary antibody diluted in blocking buffer (1:10000, Immunostar, Inc., Hudson, WI) overnight at 4°C. The next day, sections were rinsed and incubated in a biotinylated horse anti-mouse secondary antibody (1:1000, Vector Labs, Burlingame, CA) for 1 hour at room temperature. The sections were then rinsed and incubated in avidin-biotin complex for 1 hour at room temperature. The sections were then rinsed and stained with DAB (Vector Labs). Sections were rinsed, dehydrated through graded alcohols, mounted and coverslipped. To quantitate the number of cells that were TH immunonegative (TH-IR) or lacking the DAB stain, sections in hormone replaced animals were counterstained with 0.5% cresyl violet after DAB staining and coverslipped.

Unbiased Stereology

Unbiased stereological estimation of the total number of TH-IR cell bodies in the SNpc and VTA was performed using the optical fractionator method [27]. In rats, every third section was collected through the extent of the midbrain, and every 6th was section analyzed for cell counting in rostral-caudal fashion through the extent of the midbrain, resulting in a total of 6-8 sections sampled for both the right and left sides of the brain.

For mice, every 2nd section was collected through the extent of the midbrain and every 4th was analyzed for stereology, resulting in 6-7 sections per animal. A computerized counting system containing a Nikon Optiphot-2 microscope, a camera (Dage) and motorized stage (Ludl) was used to estimate the total number of cells. Each region of interest was projected onto a monitor, traced at low (4x) magnification and a sampling grid was superimposed on the traced region by the StereoInvestigator software (MicroBrightField). After shrinkage, final thickness of the sections used averaged 12 μm. Therefore, a 40 x 40 μm counting frame with a dissector height of 8 μm was used. Each counting frame was randomly spaced 80 μm apart and guard zones of 2 μm from the top and bottom of the section were used. Individual cell bodies were visualized with a 100x oil immersion lens (numerical aperture = 1.3). Enough cells were counted to achieve a coefficient of error that was ≤ 0.10. The stereologer was blinded to all surgical and treatment groups for each experiment.

Topographical Analyses

In addition to counting the total number of cells, the distribution of TH-IR cells through the extent of the midbrain was also assessed. The actual number of counted TH-IR cells from the left and right side through the extent of the SNpc and VTA was averaged to obtain a number for the cells counted at a given location in the midbrain. Every 6th section in rat and every 4th section in mouse was analyzed. Therefore, a total of at least

six sections (left and right), spaced 180 μm and 120 μm apart, respectively, were analyzed. The first (most rostral) section counted at the start of each region was considered 0 μm and successive sections were measured from this reference point. Data from the ovariectomy study and the hormone replacement controls for both rats and mice were combined to obtain complete data sets.

Uterine Weights

Uterine weights were collected as a measure of successful replacement of ERα active compounds in comparison with intact controls in both rats and mice. After fixation of tissues by transcardial perfusion, uteri were removed. All fat and connective tissues associated with the uteri were removed prior to weighing.

Statistical Analysis

All statistical analyses were performed using one and two-way ANOVA (NCSS) with a significance level of $p<.05$. Post hoc analysis was performed using the Newman-Keuls multiple comparison and Fisher's least significant difference tests to determine group differences.

** All mouse data presented in this thesis were contributed by Cheryl Ho, an undergraduate student under my supervision.

3.3 Results

Effects of Gonadectomy on TH-IR cell number in the Midbrain

As discussed in Chapter 2, the number of TH-IR cells was determined in sections stained with and without HMAR. Figures 3-1A and B show the effects of ovariectomy during adulthood on TH-IR cell number in male and female rats. The data presented in these figures were obtained from sections that did not receive HMAR treatment. In the untreated sections, ANOVA indicated a significant interaction of sex and treatment in both the SNpc [$F(1,12) = 19.1$, $p<.01$] and VTA [$F(1,11) = 9.0$, $p<.05$]. Post hoc analysis revealed that ovariectomy in adulthood resulted in a decrease in TH-IR cell number in the SNpc and VTA.

These previous results were replicated after the incorporation of HMAR into the staining method in separate cohort of animals. As observed in Chapter 2, HMAR resulted in a two-fold increase in TH-IR cell number in both regions. Ovariectomy at PN60 decreased TH-IR cell number in the SNpc and VTA of female rats (Fig. 3-2A and B) and mice (Fig. 3-3A and B) at PN90 relative to the sham surgery controls. In rats, ANOVA revealed a significant effect surgery [$F(1,12) = 10.3$, $p<.01$] in the SNpc and VTA [$F(1,12) = 5.8$, $p<.05$]. In mice, ANOVA also revealed a significant effect of surgery in the SNpc [$F(1,10) = 19.8$, $p<.01$] and VTA [$F(1,10) = 6.8$, $p<.05$].

A

B

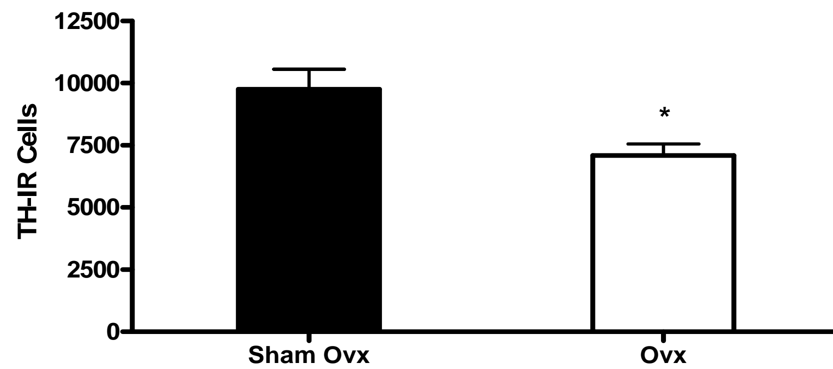
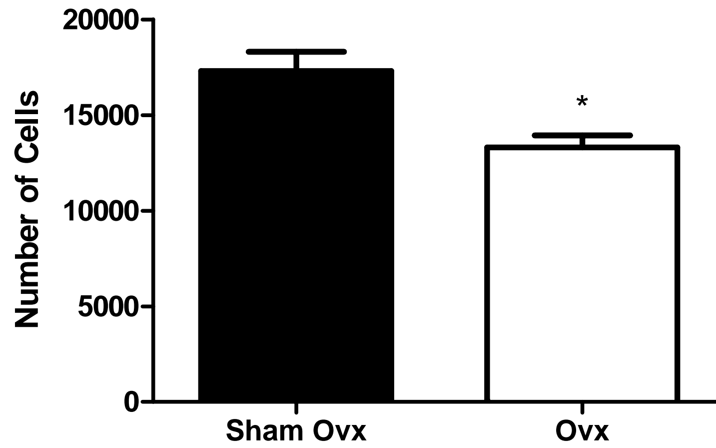


Fig. 3-1. Effect of ovariectomy on midbrain TH-IR cell number without HMAR staining method**.** The total number of TH-IR cells in the SNpc (A) and VTA (B) of rats sham ovariectomized (sham ovx) and ovariectomized (ovx) at PN55 was determined one month post-surgery. * indicates different from sham ovx, for A: $p<.01$ and for b: $p<.05$ *Data are expressed as means ± SEM, (n = 5-7/group).

A



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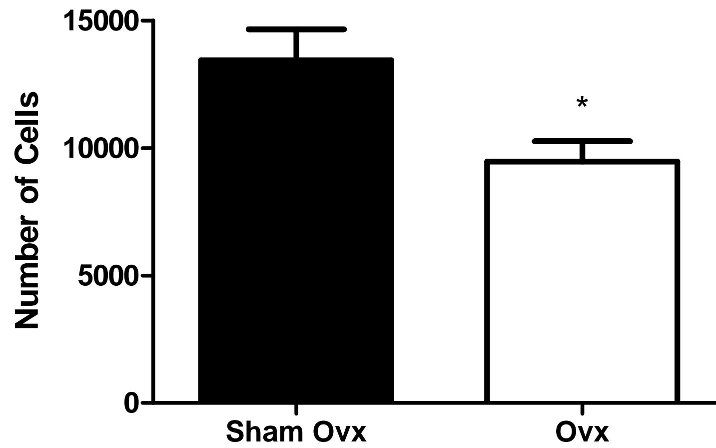
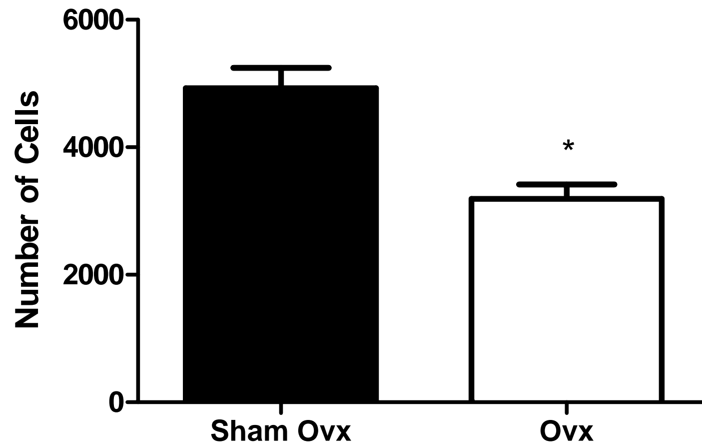


Fig. 3-2. Effect of ovariectomy on midbrain TH-IR cell number with HMAR staining method. The total number of TH-IR cells in the SNpc (A) and VTA (B) of rats sham ovx and ovx at PN55 was determined one month post-surgery. * indicates different from sham ovx, for A: $p<.01$ and for b: $p<.05$ *Data are expressed as means ± SEM, (n = 6/group).

A



B

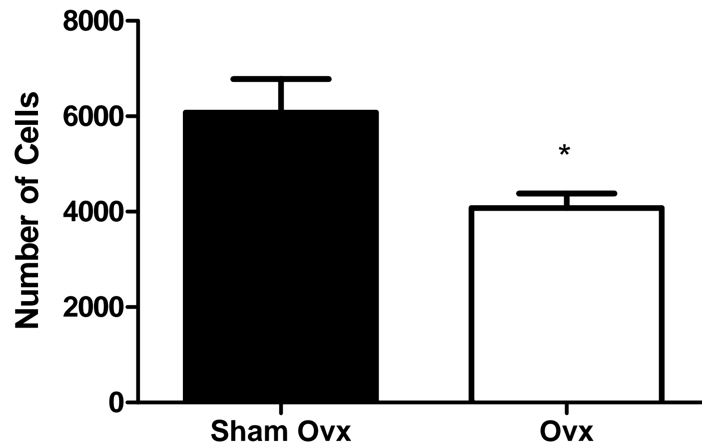


Fig. 3-3. Effect of ovariectomy on midbrain TH-IR cell number with HMAR staining method**.** The total number of TH-IR cells in the SNpc (A) and VTA (B) of mice sham ovx and ovx at PN55 was determined one month post-surgery. * indicates different from sham ovx, p<.01. Data are expressed as means ± SEM, (n = 5/group).

Role of Estrogen and ER-selective agonists in the Midbrain on TH-IR Cell Number

In the first replacement study, all hormones including the ERα-selective agonist PPT were administered via minipump. The effectiveness of hormone replacements over the one month period was confirmed by monitoring body weight gain during the study and collecting fixed (wet) uterine weights post-perfusion. ANOVA indicated a significant effect of treatment for uterine weight [$F(4,48) = 38.3$, $p<.0001$] and body weight gain [$F(4,53) = 17.3$, $p<.0001$]. Post hoc analysis revealed that ovariectomy significantly reduced uterine weight (*Fig. 3-4A*) and resulted in significant body weight gain over the one month period (*Fig. 3-4B*) relative to the sham controls. E2 replacement increased uterine mass relative to the ovariectomized animals that received vehicle and prevented body weight gain. As expected, DPN had little uterotrophic effect and did not prevent weight gain. PPT was weakly uterotrophic when administered via minipump and but did prevent weight gain relative to the ovariectomized animals.

In the cell counting experiments, we replicated our finding that ovariectomy reduces TH-IR cell number in the SNpc. E2 and DPN maintained TH-IR cell number in the SNpc to levels comparable to the sham controls (*Fig. 3-5A*). ANOVA indicated a main effect of treatment [$F(4,30) = 8.8$, $p<.0001$]. Post hoc tests showed that ovariectomized and PPT groups were different from the sham controls and that sham, E2 and DPN were different from the ovariectomized animals. In this study, PPT replacement was ineffective. In the VTA, ANOVA revealed there was no main effect of

treatment (*Fig. 3-5B*). The results of the SNpc cell counts suggested that ERβ possibly played a more significant role in the maintenance of TH-IR cells number in the SNpc than ERα.

An ERα selective agonist, PPT should have had greater uterotrophic effects at the dose given than shown in this experiment. The lack of uterine protection by PPT is inconsistent with published studies, all of which administer PPT via subcutaneous injection [131, 134]. It is possible that PPT precipitated while in the pump, as it is a very difficult drug to dissolve. Therefore, we conducted an additional experiment in which PPT was administered daily via subcutaneous injection. PPT was dissolved in sesame oil. Control animals (sham and ovariectomized) received a daily injection of vehicle. All animals received a minipump containing the original vehicle (50% DMSO/normal saline) to make them surgically comparable to the initial group of replacement animals. When administered via subcutaneous injection, PPT significantly increased uterine weight and prevented weight gain relative to ovariectomized controls and PPT-treated rats were comparable to the sham surgery animals providing evidence that the hormone replacements were effective (*Figs. 3-6A and B, respectively*). For uterine weight, ANOVA indicated a significant effect of treatment [$F(2,12) = 16.5$, $p<.001$]. Post hoc analysis revealed that the PPT treated animals were not different from the sham controls and that both intact and PPT-treated animal uteri were significantly greater than the ovariectomized controls. For body weight gain, ANOVA indicated a significant effect of

treatment [$F(2,17) = 62.6$, $p<.0001$]. Post hoc tests revealed that the ovariectomized controls gained more weight over the one month period than both the sham controls and PPT replaced groups. However, PPT replaced animals gained the least amount of weight as they were significantly lower than the sham controls. These results confirmed that PPT replacement was successful. These uterine weights and midbrain cell counts were combined with data from the previous study.

The new PPT data replaced the data from ineffective replacement in the previous study. For the combined data, ANOVA indicated a significant effect of treatment for uterine weight [$F(4,66) = 52.6$, $p<.0001$] (*Fig. 3-7A*). Post hoc analysis revealed that both E2 and PPT replaced animals were significantly greater than the ovariectomized and DPN treated rats but significantly lower than the sham controls. The uteri of the intact animals weighed more than the uteri in all other treatment groups. DPN had no effect in the uterus and was comparable to the ovariectomized animals. For body weight gain, ANOVA indicated a significant effect of treatment [$F(4,63) = 52.8$, $p<.0001$] (*Fig. 3-7B*). Post hoc analysis revealed that the E2 replacement and sham controls were comparable and different from the DPN replaced and ovariectomized controls. The PPT replaced animals gained less weight than all other treatment groups. Cell counts in the combined experimental groups revealed protection of TH-IR cell bodies in the SNpc and VTA by both the ERα and ERβ-selective agonists (*Fig. 3-8A and B*). ANOVA indicated a significant effect of treatment in the SNpc [$F(4,43) = 6.6$, $p<.001$] and VTA [$F(4,42) = 4.0$,

p<.01]. In the SNpc, post hoc analysis showed ovariectomy decreased cell number relative to the sham controls. Cell counts in the E2, PPT (ERα) and DPN (ERβ) replaced animals were comparable to the sham controls and different from the ovariectomized animals. In the VTA, no difference was observed between control groups. However, post hoc tests showed that E2, PPT (ERα) and DPN (ERβ) were different from the ovariectomized animals.

A parallel study in mice revealed similar effects of ER agonists. Uterine weights were collected to determine the effectiveness of hormone replacements over the one month period. In mice, ovariectomy resulted in a decrease in uterine weight relative to the sham controls (*Fig. 3-9*). ANOVA revealed a significant effect of treatment [F(4,32) = 14.3, p<.0001]. Post hoc analysis revealed that E2 and PPT (ERα-selective agonist) replacement increased uterine weight relative to the ovariectomized controls. However, E2 replaced uteri were greater than the sham controls. DPN replaced uteri were not different from either control group. After confirmation of successful replacement, cell counts in the SNpc and VTA of hormone replaced mice revealed that TH-IR cell number is reduced after ovariectomy (*Fig. 3-10A and B*). ANOVA revealed a significant effect of treatment in both the SNpc [F(4,32) = 3.7, p<.05] and VTA [F(4,32) = 5.2, p<.01]. Post hoc tests revealed that ovariectomy resulted in a decrease in TH-IR cell number in the SNpc and VTA. In the SNpc, more cells were counted in E2, PPT and DPN replaced groups than the ovariectomized animals. However, these counts were lower than the sham

controls. In the VTA, ovariectomy decreased TH-IR cell number. All replacement groups had cell counts comparable to the sham controls and were greater than the ovariectomized animals.

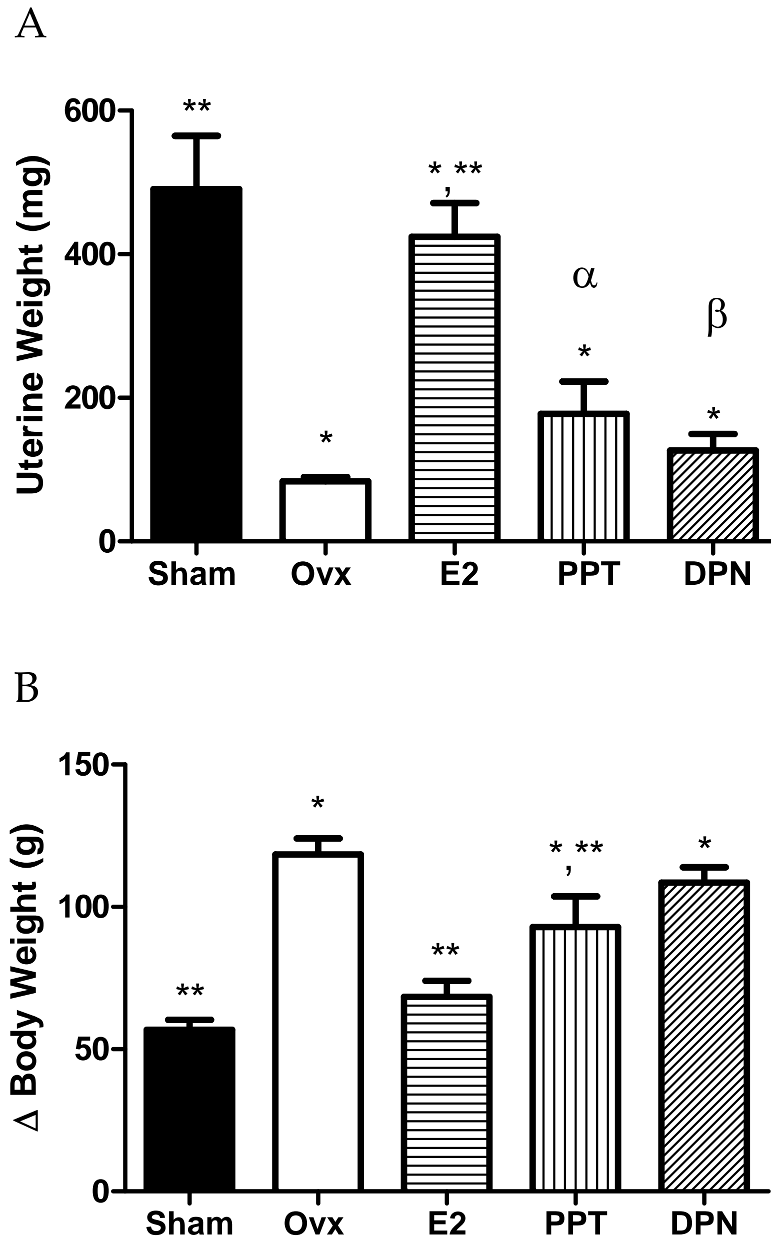


Fig. 3-4. Effect of E2, PPT or DPN on uterine weight (A) and body weight gain (B) in female rats compared to vehicle-treated sham ovx and ovx controls. Animals were ovx or sham ovx at PN60 and treated with vehicle or estrogenic compounds for one month post-surgery. Data are expressed as mean ± SEM. * indicates different from sham ovx ($p<.0001$) and ** indicates different from ovx. ($n = 4-11$)

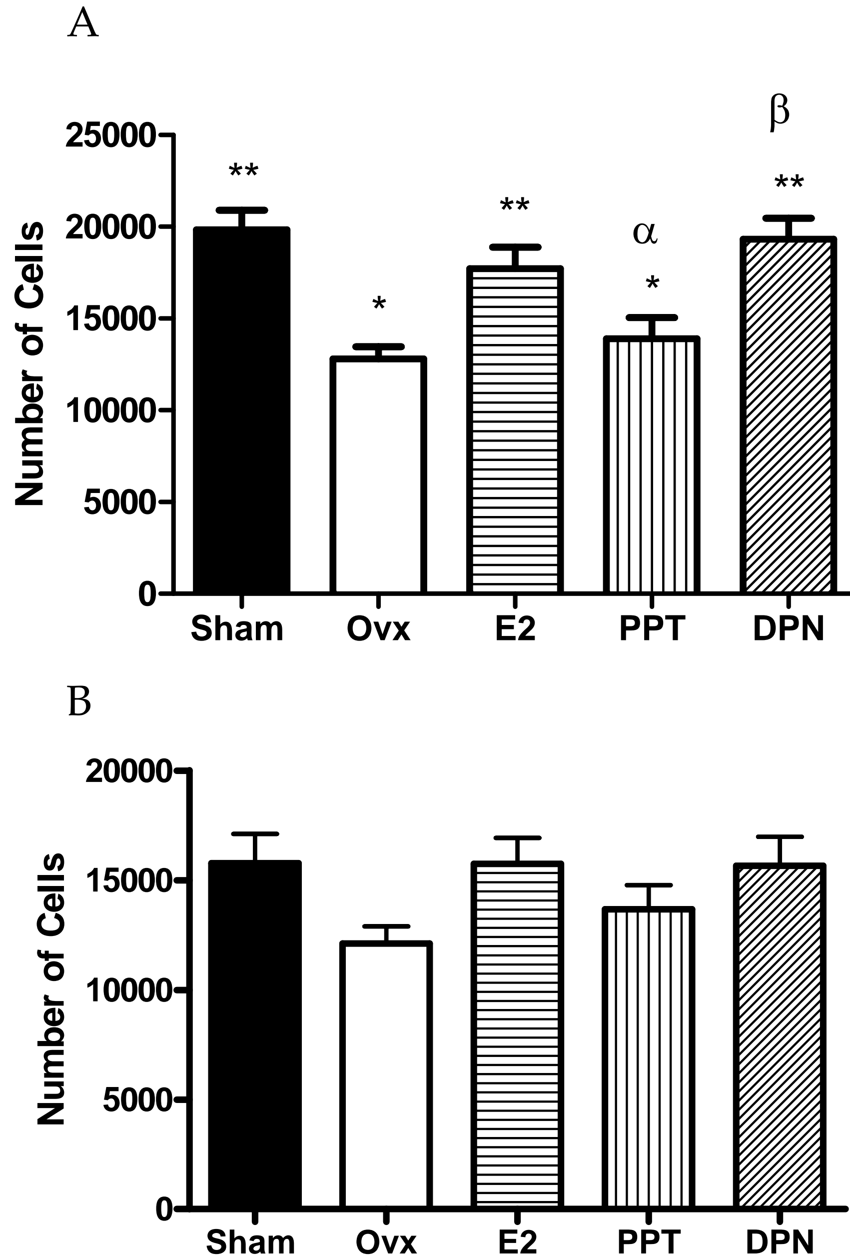


Fig.3-5. Effect of E2, PPT or DPN on TH-IR cell number in the SNpc (A) and VTA (B) of female rats compared to vehicle-treated sham ovx and ovx controls. Animals were ovx or sham ovx at PN60 and treated with vehicle or estrogenic compounds for one month post-surgery. All compounds, including PPT, were administered via minipump. Data are expressed as mean ± SEM (n = 6-8). * indicates different from sham ovx and ** indicates different from ovx, for A (p<.0001).

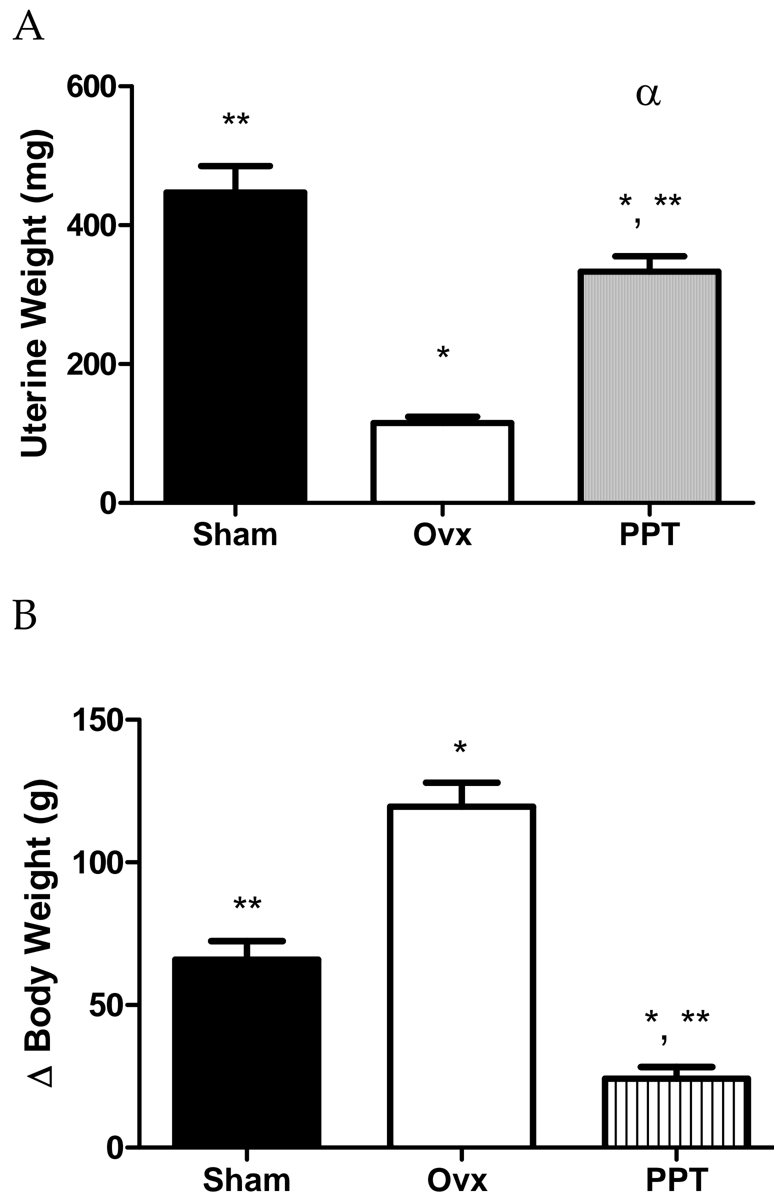


Fig. 3-6. Effect of PPT administered via subcutaneous injection on uterine weight (A) and body weight gain (B) in female rats compared to vehicle-treated sham ovx and ovx controls. Animals were ovx or sham ovx at PN60 and treated with vehicle or PPT for one month post-surgery. Data are expressed as mean ± SEM. For A: * indicates different from sham ovx and ** indicates different from ovx (p<.001). For B: * indicates different from sham ovx and ** indicates different from ovx (p<.0001). (n = 4-7)

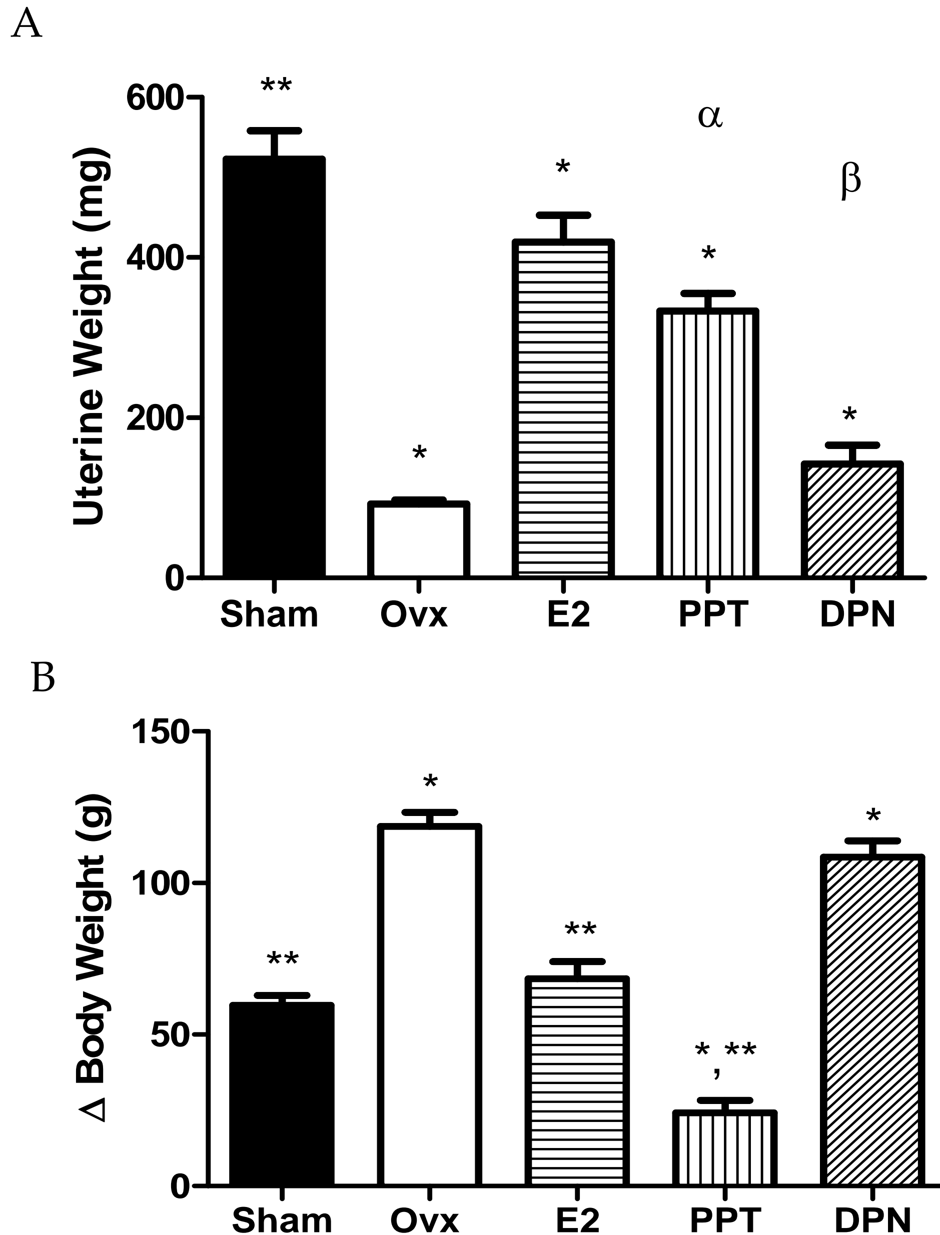


Fig. 3-7. Effect of E2, PPT and DPN uterine weight (A) and body weight gain (B) in female rats compared to vehicle-treated sham ovx and ovx controls for the combined study. Animals were ovx or sham ovx at PN60 and treated with vehicle or PPT for one month post-surgery. Data are expressed as mean ± SEM. For A: * indicates different from sham ovx and ** indicates different from ovx (p<.0001). For B: * indicates different from sham ovx and ** indicates different from ovx (p<.0001). (n = 6-20)

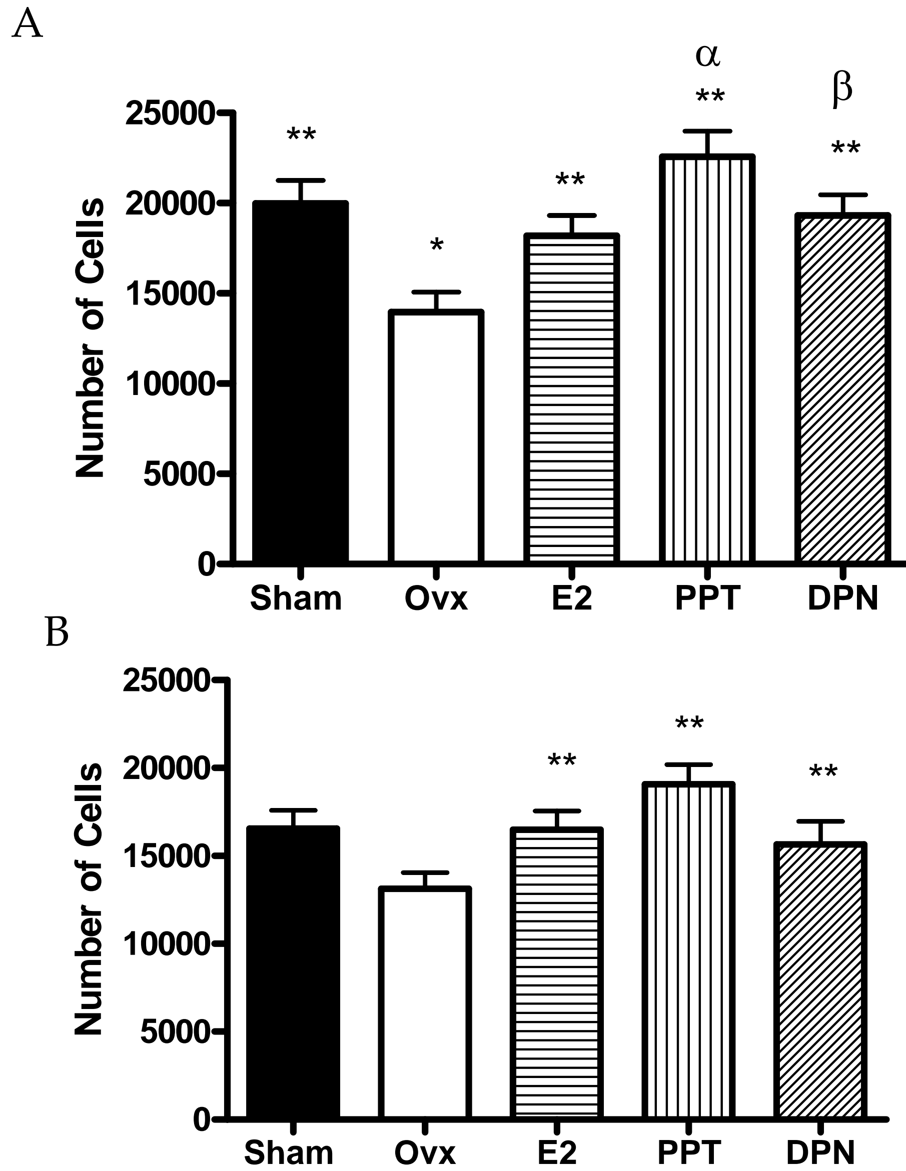


Fig. 3-8. Effect of E2, PPT or DPN on TH-IR cell number in the SNpc (A) and VTA (B) of female rats compared to vehicle-treated sham ovx and ovx controls for the combined study. Animals were ovx or sham ovx at PN60 and treated with vehicle or estrogenic compounds for one month post-surgery. Data are expressed as mean ± SEM (n = 7-12). * indicates different from sham ovx and ** indicates different from ovx. For A: ($p<.01$) and for B: ($p<.05$).

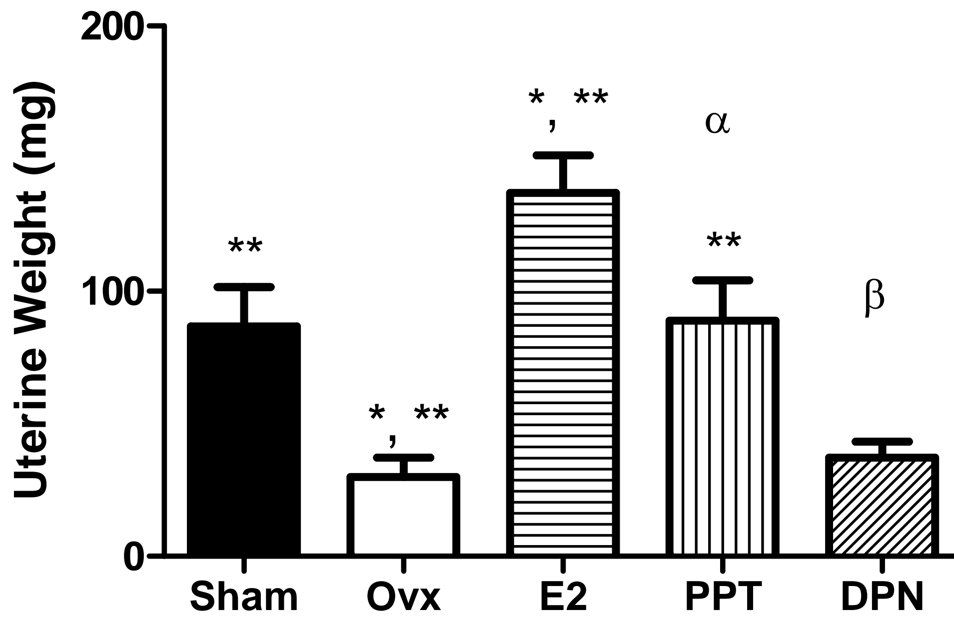


Fig. 3-9. Effect of E2, PPT and DPN uterine weight in female mice compared to vehicle-treated sham ovx and ovx controls. Animals were ovx or sham ovx at PN60 and treated with vehicle or PPT for one month post-surgery. Data are expressed as mean ± SEM. * indicates different from sham ovx and ** indicates different from ovx ($p<.0001$). (n = 5-8)

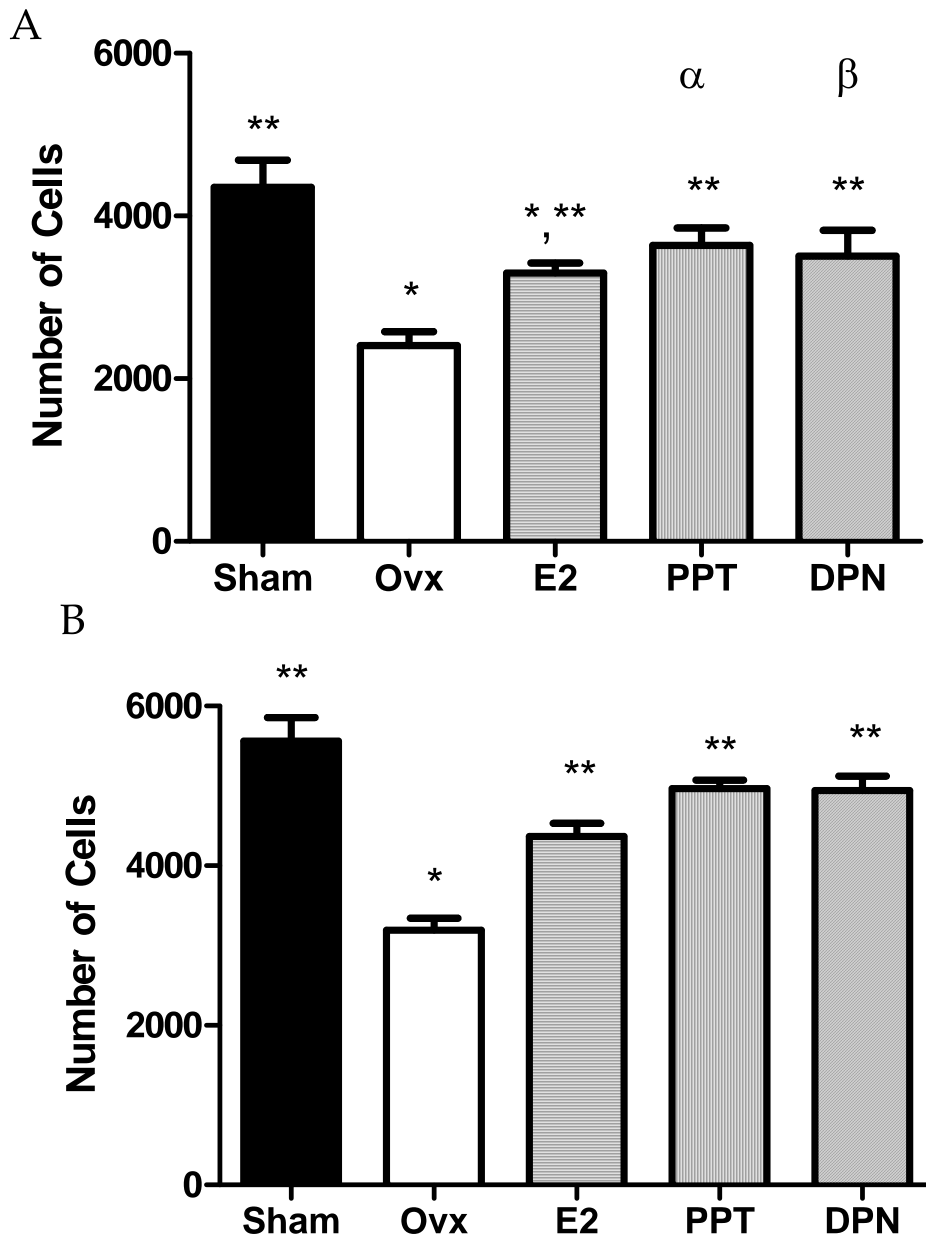


Fig. 3-10. Effect of E2, PPT or DPN on TH-IR cell number in the SNpc (A) and VTA (B) of female mice compared to vehicle-treated sham ovx and ovx controls. Animals were ovx or sham ovx at PN60 and treated with vehicle or estrogenic compounds for one month post-surgery. Data are expressed as mean ± SEM (n = 5-8). * indicates different from sham ovx and ** indicates different from ovx. For A: ($p<.01$) and for B: ($p<.05$).

TH-IR Cell Number in Transgenic Mice

TH-IR cell number in the SNpc and VTA was quantitated in adult ER knockout animals to further elucidate the role of each ER subtype (*Fig. 3-11A and B*). ANOVA revealed a significant effect of genotype in the SNpc [$F(2,15) = 16.9$, $p<.001$] and VTA [$F(2,15) = 11.3$, $p<.01$]. Post hoc tests showed that TH-IR cell number was reduced only in the αERKO mice.

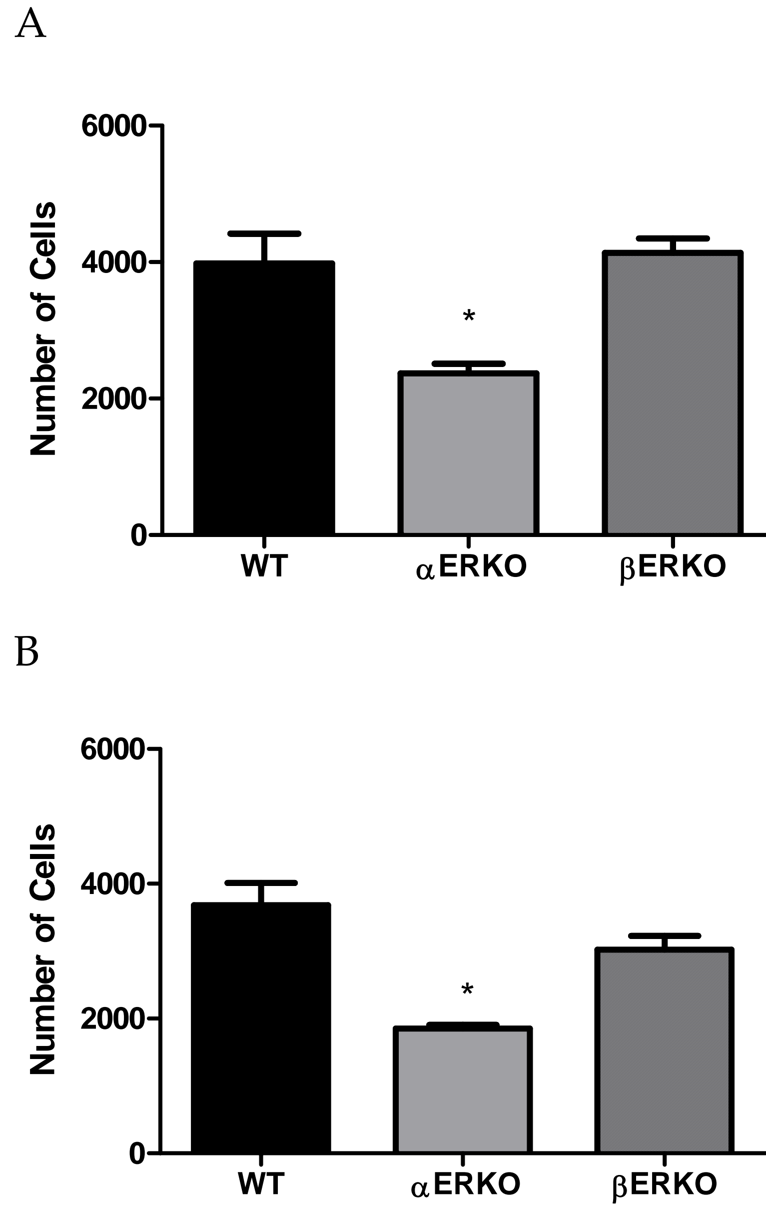
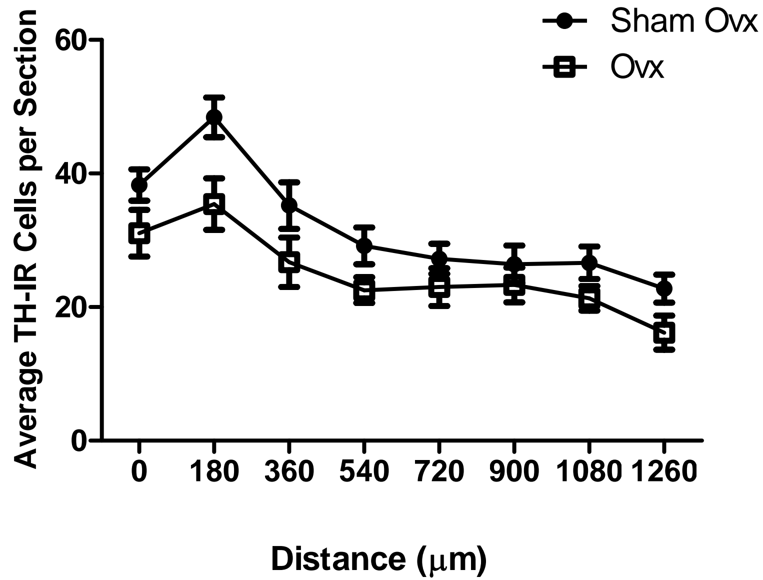


Fig. 3-11. Total TH-IR cell number in the SNpc (A) and VTA (B) of αERKO and βERKO mice at PN85. Data are expressed as mean ± SEM (n = 5). * indicates different from WT (p<.01).

Topographical Analysis of Sham Ovariectomized and Ovariectomized Animals

To investigate whether the decrease of TH-IR cell number observed with ovariectomy was due to region-specific changes in cell number, cell counts were evaluated by section. In this study, the data from sham ovariectomized and ovariectomized animals from both Experiments 1 and 2 were combined to increase statistical power. In the rat SNpc, a global cell loss was observed in the ovariectomized animals relative to the sham controls. In rat, ANOVA indicated a main effect of section [$F(7, 203) =, 14.1, p<.0001$] and a significant effect of surgery [$F(1,203) = 8.1, p<.01$] (*Fig. 3-12A*). In the VTA, ANOVA indicated a significant effect of section [$F(7, 127) = 10.0, p<.0001$] (*Fig. 3-12B*). Similar observations were made in the mouse SNpc and VTA. In the SNpc, there was a significant effect of surgery [$F(1, 165) = 21.9, p<.001$]. In the VTA, ANOVA indicated a significant effect of surgery [$F(1, 165) = 27.5, p<.0001$] and a significant effect of section [$F(6, 165) = 10.7, p<.0001$], suggesting that a global loss occurs in both regions.

A



B

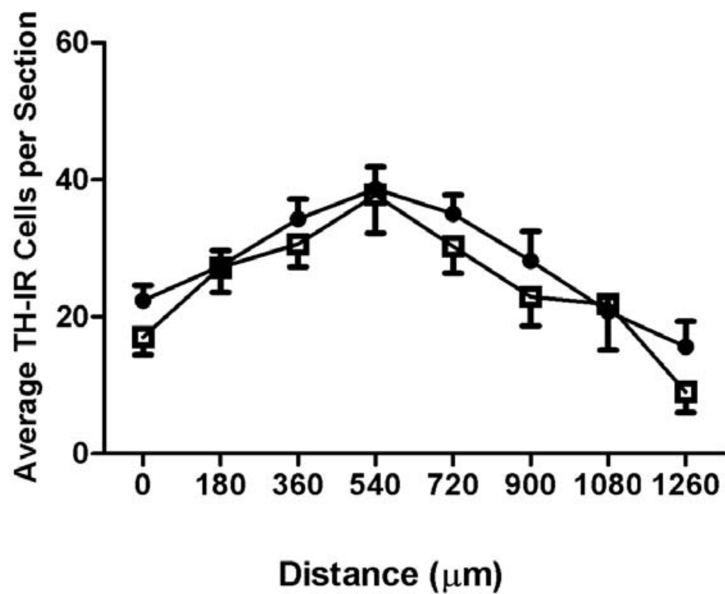


Fig.3-12. Topographical analysis of TH-IR cell number throughout the extent of the SNpc (A) and (B) VTA in sham ovx and ovx rats. These graphs show the average of the left and right SNpc and VTA at each distance through the extent (rostral to caudal) of each region with 0 μm being the most rostral section. Sham animals are represented by the closed shapes and ovx are represented by open shapes. Data are expressed as mean ± SEM (n = 12 for SNpc and n = 14-18 for VTA).

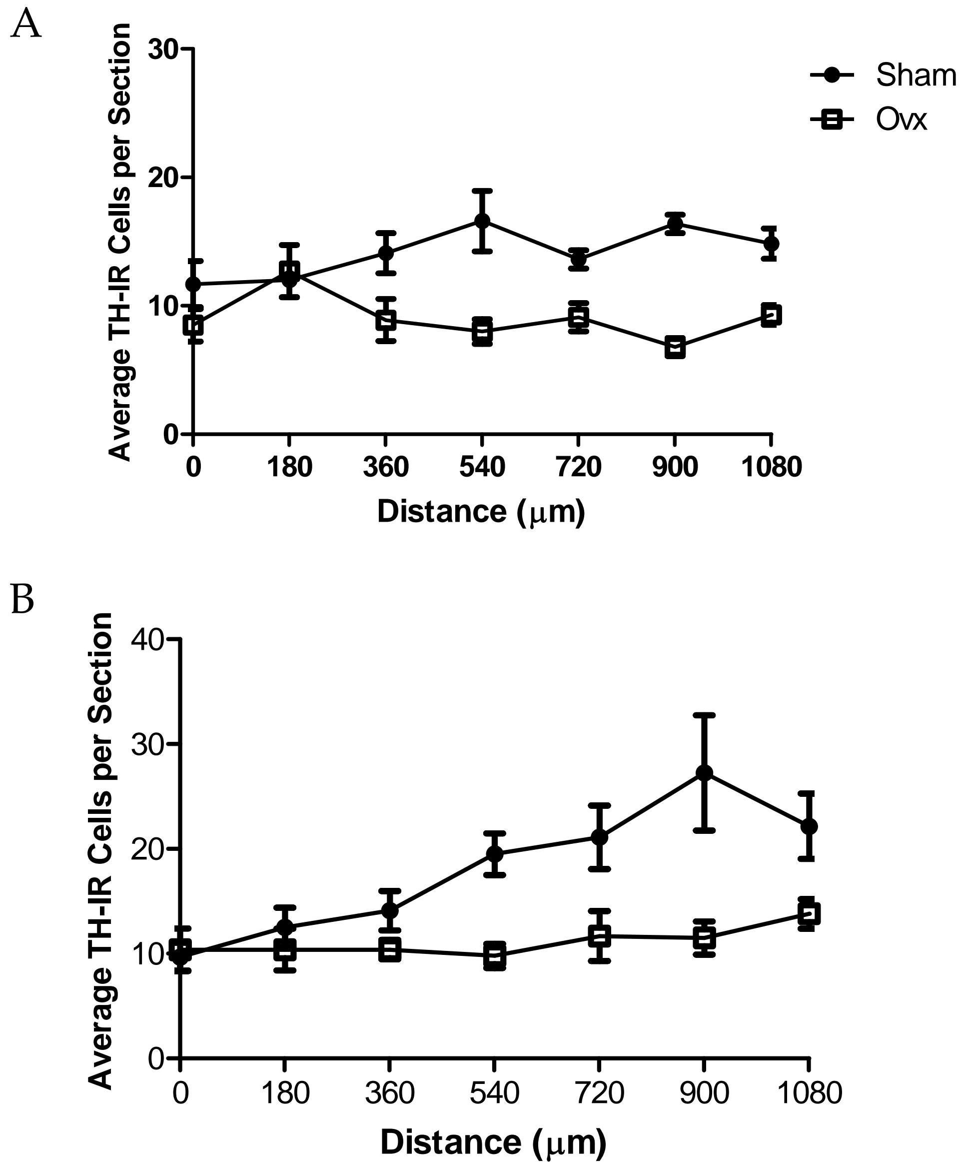


Fig. 3-13. Topographical analysis of TH-IR cell number throughout the extent of the SNpc (a) and (b) VTA in sham ovx and ovx mice. These graphs show the average of the left and right SNpc and VTA at each distance through the extent (rostral to caudal) of each region with 0 μm being the most rostral section. Sham animals are represented by the closed shapes and ovx are represented by open shapes. Data are expressed as mean ± SEM (n = 8 for SNpc and n = 8 for VTA).

Estrogen Does Not Regulate TH Expression in Cells

Studies have shown that TH expression is regulated by estrogen in the SNpc of neonatal mice and in the rat LC. Therefore, one possibility for the loss of TH-IR cells observed with ovariectomy could be that a loss of estrogen results in an inability to detect some TH-IR cells in the SNpc and VTA. To investigate the possibility that the reduction in cell number observed with ovariectomy was not simply due to a loss of TH expression in midbrain cells, we quantitated cell number in the SNpc and VTA of sham and ovariectomized rats in sections counterstained with cresyl violet to determine the number of TH immunonegative (IN) cell bodies in each region. For TH-IR cell number, ANOVA indicated a significant effect of surgery in the SNpc [$F(1,24) = 11.4$, $p<.01$] and VTA [$F(1,24) = 11.3$, $p<.01$] (*Tables 1 and 2*). There were no significant differences in the SNpc and VTA in TH-IN cell number. For the total number of cells (TH-IR + TH-IN), ANOVA indicated a main effect of surgery in the SNpc for TH-IR cell number, ANOVA indicated a significant effect of surgery in the SNpc [$F(1,24) = 17.5$, $p<.001$] and VTA [$F(1,24) = 11.9$, $p<.01$]. Post hoc analysis revealed that TH-IR cell number and mean total cell number the in both the SNpc and VTA decreased after ovariectomy. The percentage of TH-IR cells is similar in both groups suggesting that despite cell loss with ovariectomy, the percentage of TH-IR cells remains the same in rat.

Cresyl violet cell counts were also performed in the mouse hormone replacement study presented in Fig. 3-10 (*Tables 3 and 4*). ANOVA revealed a significant effect of

treatment in the SNpc [F(4,32) = 9.0, p<.0001] and VTA [F(4,32) = 21.8, p<.0001] for TH-IR cell number. No significant differences in TH-IN cell number in the SNpc and VTA between treatment groups were observed, which was consistent with the data presented for the sham and ovariectomized rats (*Tables 1and 2*). For total cell number, ANOVA indicated that there was a main effect of treatment in the SNpc [F(4,32) = 8.3, p<.001] and VTA [F(4,32) = 16.3, p,.0001]. Post hoc test showed that TH-IR and total cell numbers were reduced in the ovariectomized and E2 animals relative to sham. However, E2 replaced animals have both greater TH-IR and total cell numbers than the ovariectomized animals. The PPT and DPN replaced animals were comparable to the sham controls and different from the ovariectomized controls.

In Tables 5 and 6, cresyl violet cell counts for the αERKO mice are presented. No significant difference in TH-IN cell number in the SNpc and VTA of each genotype was observed. ANOVA indicated a significant effect of genotype as in the SNpc [F(2, 15) = 16.9, p<.001] and VTA [F(2,15) =11.3, p<.01] as presented in Fig. 3-11. Total cell number was significantly reduced in the αERKO mice in both the SNpc [F(2, 14) = 25.0, p<.0001)] and VTA [F(2,15) = 12.7, p<0.01].

Table 1. Estimated total number of TH-IR and TH-IN cells in the SNpc of sham ovariectomized (sham ovx) and ovariectomized (ovx) rats

Surgical Condition	*TH-IR*	*TH-IR*	*Total Cells*	*% TH-IR*
Sham Ovx	26169± 759	3437 ± 296	30241 ± 768	86.6 ± 1.6
Ovx	*21187± 1337	2467 ± 296	*23936 ±1367	88.3 ± 1.6

TH-IN,TH-IR and total cell number were determined in sham ovx and ovx animals. No significant differences in TH-IN number were observed in TH-IN cell number, $p = .17$. Significant differences were found in TH-IR cell number and total cells. No significant differences were observed for % TH-IR cells, $p = .47$. Data are expressed as mean ± SEM. * indicates different from sham ovx, $p < .01$ for TH-IR cell number and $p < .001$ total cell number. ($n = 10$-13/group)

Table 2. Estimated total number of TH-IN and TH-IR cells in the VTA of sham ovariectomized (sham ovx) and ovariectomized (ovx) rats

Surgical Condition	*TH-IR*	*TH-IN*	*Total Cells*	*% TH-IR*
Sham Ovx	23528± 1013	2302 ± 296	26317 ±1217	89.7 ± 1.6
Ovx	*18736± 989	1834 ± 218	*20570 ±1098	91.2 ± 0.8

TH-IN,TH-IR and total cell number were determined in sham ovx and ovx animals. No significant differences in TH-IN number were observed in TH-IN cell number, $p = .15$. Significant differences were found in TH-IR cell number and total cells. No significant differences were observed for % TH-IR cells, $p = .47$. Data are expressed as mean ± SEM. * indicates different from sham ovx, $p < .01$ for TH-IR cell number and $p < .01$ total cell number. ($n = 10$-13/group)

Table 3. Estimated total number of TH-IR and TH-IN cells in the SNpc of hormone replaced mice.

Surgical Condition	*TH-IR*	*TH-IR*	*Total Cells*	*% TH-IR*
Sham Ovx	**4353 ± 337	654 ± 96	**4807 ± 403	86.1 ± 2.6
Ovx	*2409± 168	683 ± 54	*3293 ±307	79.4 ± 5.1
Ovx + E2	*,**3297± 126	665 ± 24	*,**3962 ±119	83.2 ± 0.8
Ovx + PPT	**3635 ± 216	691 ± 66	**4326 ± 200	83.8 ± 1.9
Ovx + DPN	**3506 ± 319	695 ± 100	**4202 ± 317	83.0 ± 1.6

TH-IN,TH-IR and total cell number were determined in hormone replaced mice. No significant differences in TH-IN number were observed in TH-IN cell number, p =.99. Significant differences were found in TH-IR cell number and total cells. No significant differences were observed for % TH-IR cells, p =.86. Data are expressed as mean ± SEM. * indicates different from sham ovx, p<.01 for TH-IR cell number and p<.001 total cell number. (n =5-8/group)

Table 4. Estimated total number of TH-IR and TH-IN cells in the VTA of hormone replaced mice.

Surgical Condition	*TH-IR*	*TH-IR*	*Total Cells*	*% TH-IR*
Sham Ovx	**5564 ± 291	657 ± 98	**5930 ± 507	89.2 ± 1.8
Ovx	*3193± 152	631 ± 40	*4116 ±301	84.3 ± 1.9
Ovx + E2	*,**4368± 163	658± 75	*,**5027 ±124	86.8 ± 1.6
Ovx + PPT	**4966± 108	655 ± 63	**5621 ± 109	88.4 ± 1.1
Ovx + DPN	**4942 ±178	690 ± 84	**5633 ± 131	87.7 ± 1.6

TH-IN,TH-IR and total cell number were determined in sham ovx and ovx animals. No significant differences in TH-IN number were observed in TH-IN cell number, $p = .99$. Significant differences were found in TH-IR cell number and total cells. No significant differences were observed for % TH-IR cells, $p = .28$. Data are expressed as mean ± SEM. * indicates different from sham ovx, $p < .01$ for TH-IR cell number and $p < .001$ total cell number. (n =5-8/group)

Table 5. Estimated total number of TH-IR and TH-IN cells in the SNpc of estrogen receptor knockout mice.

Surgical Condition	*TH-IR*	*TH-IR*	*Total Cells*	*% TH-IR*
WT	**3685 ± 328	535 ± 63	**4220 ± 507	86.6 ± 2.3
αERKO	*1851 ± 55	326 ± 81	*2177 ± 301	85.2 ± 3.4
βERKO	**3023 ± 207	407 ± 47	**3430 ± 124	83.7 ± 4.8

TH-IN,TH-IR and total cell number were determined in sham ovx and ovx animals. No significant differences in TH-IN number were observed in TH-IN cell number, $p = .17$. Significant differences were found in TH-IR cell number and total cells. No significant differences were observed for % TH-IR cells, $p = .83$. Data are expressed as mean ± SEM. * indicates different from sham ovx, $p < .001$ for TH-IR cell number and $p < .0001$ total cell number. ($n = 5$/group)

Table 6. Estimated total number of TH-IR and TH-IN cells in the SNpc of estrogen receptor knockout mice.

Surgical Condition	*TH-IR*	*TH-IR*	*Total Cells*	*% TH-IR*
WT	**3981 ± 437	491 ± 80	**3981 ± 437	88.1 ± 3.3
αERKO	*2372 ± 142	561 ± 175	*2372 ± 142	81.3 ± 5.4
βERKO	**4138 ± 208	437 ± 21	**4138 ± 208	90.4 ± 0.8

TH-IN,TH-IR and total cell number were determined in sham ovx and ovx animals. No significant differences in TH-IN number were observed in TH-IN cell number, $p = .17$. Significant differences were found in TH-IR cell number and total cells. No significant differences were observed for % TH-IR cells, $p = .23$. Data are expressed as mean ± SEM. * indicates different from sham ovx, $p < .01$ for TH-IR cell number and $p < .01$ total cell number. ($n = 5$/group)

3.4 Discussion

In the present study we investigated the effect of estrogen and the role each of ER subtype in the maintenance of TH-IR cell number in the rodent SNpc and VTA. The findings of this study suggest that ovariectomy reduces TH-IR cell number in the SNpc and VTA of adult female rodents. Replacement with E2, PPT (ERα-selective agonist) and DPN (ERβ-selective agonist) in ovariectomized rodents prevented cell loss, suggesting that estrogen is required for the maintenance of TH-IR cells and that both ER subtypes contribute to the trophic effects of estrogen in the midbrain. However, studies in transgenic mice suggest that the mechanism for cell maintenance is more dependent upon ERα activation. This marks the first study demonstrate the role of each ER subtype in the maintenance of midbrain TH-IR cell number.

There is an extensive literature suggesting that estrogen is required for cell survival and neuroprotection on various cell types in the brain although the effects of monoamine neurons are little studied. Studies in primates have shown that both short- and long-term ovariectomy reduces the number of TH-IR cell bodies in the SNpc and that estrogen replacement prevents cell loss [23]. In rodents, little is known about how ovariectomy influences cell number. For the first time, we show that ovariectomy at PN60 in both rats and mice results in a decrease in TH-IR cell number in the SNpc and VTA. We selected this time point for gonadectomy based on previous studies in our laboratory suggesting that sex differences in cocaine-stimulated behavior and dopamine

uptake in release in adult rats emerge during early adulthood [12, 15]. This is also the first quantitative study to report prevention of TH-IR cell loss in the midbrain regions with estrogen replacement in rodents. We also demonstrate that the effect of ovarian hormone loss on TH-IR cell number in midbrain regions extends to the VTA.

One possible explanation for the present result is that the decrease observed in cell number may due to a decrease in TH expression in midbrain regions. One technical reason for this apparent loss of dopamine neuron number could be that a loss of TH expression in individual cells makes them undetectable by the antibody. There are studies that suggest that estrogen regulates TH expression, but only if ERα is present . TH has been shown to be regulated by estrogen in cultures of PC12 cells transfected with ERα and in the LC, in which ERα mRNA and protein is expressed [68, 135]. Low to moderate expression of ERα has been detected in the LC [45, 62, 68]. However, immunohistochemical and in situ hybridization studies reveal that there is little to ERα in the rodent SNpc [45, 62, 68]. Studies in the midbrain suggest that estrogen regulates TH expression in neonatal mouse SNpc and in rat primary midbrain cultures [118, 136, 137]. If TH is regulated by estrogen, we would expect to see fewer TH-IR cells but more TH-IN cells in the midbrain of ovariectomized rats. However, cell counts in cresyl violet counterstained sections reveal no differences in TH-IN cell number, only in TH-IR cell number. This is consistent with findings in the primate study. We also found that approximately 85-90% of cells in the SNpc and VTA are TH-IR, which is consistent with

studies in employing similar cell quantification methods in the SNpc [31]. Immunohistochemical and in situ hybridization studies have shown that there is very little ERα expression in the midbrain regions of rats and mice. A recent study suggests that TH mRNA is decreased in the SNpc of αERKO mice [138]. Based on the findings of this study, it is possible that diminished TH mRNA expression in this study reflects a loss of cells in the SNpc.

Both the ERα agonist PPT and ERβ agonist DPN were shown to maintain neuron number in the SNpc and VTA. These findings are supported by studies performed in both *in vitro* and *in vivo* models of dopamine neuron degeneration [139]. In primary cultures derived from mouse and rat, E2 protects cells from MPP+-induced degeneration. In the mouse-derived cultures, ERα-selective agonists prevents MPP+-induced cell loss [140, 141]. In a mouse MPTP model of PD, PPT mimicked the effects of E2 in the protection of striatal dopamine content. In αERKO mice, low doses of MPTP reduce striatal dopamine content more than intact mice [21]. βERKO mice are more protected from striatal MPTP depletion at lower doses than αERKO mice [21]. Studies suggest that ERβ may not have a clear role in neuroprotection. DPN failed to prevent depletion of striatal dopamine in a mouse PD model [20]. In addition to this, treatment of primary midbrain cultures with DPN failed to rescue TH-IR cells from degeneration [34]. However, other measures of dopaminergic function have been shown to be ERβ-mediated. Dopamine D2 receptor and DAT specific binding are both reduced with

ovariectomy [36, 37]. Treatment with E2 and DPN resulted in restoration of specific binding observed in the intact controls, suggesting that ERβ may play a role in the control in the maintenance of dopaminergic function. ERβ plays a role in normal brain development as the cerebral cortices of βERKO mice are underdeveloped and the SNpc contains smaller and fewer neurons compared to WT mice.

The use of transgenic models has helped us to further understand the roles of these receptors. TH-IR neuron number was only reduced in midbrain regions of αERKO mice relative to WT. This finding is discordant with the hormone replacement findings that both ER-selective agonists contribute to the maintenance of cell number. In the hormone replacement study we are addressing the activational effects of estrogenic drugs. It is possible that in animals that lacking hormone receptor throughout development, the appropriate number of TH-IR cells in midbrain regions is not established. Another explanation for this may be that a high dose of DPN was used in the hormone replacement study causing some ERα activation. Although having approximately 70-fold greater affinity for ERβ, DPN can also activate ERα, which has been shown to occur at high doses [142]. We administered a dose (8 mg/kg/day) of DPN that is reported to have protective and anti-inflammatory effects in mice [35, 39]. A study involving long-term replacement in mice receiving this dose of DPN showed that DPN was not uterotrophic [132]. In rat, we found that DPN did not have any estrogenic effects in the uterus. In mice, the effect of DPN in the uterus was inconclusive. Despite

this, these results confirm that ERα plays a key role in neuroprotection, whereas ERβ may simply contribute to the activational effects of estrogen.

We performed topographical analysis of cell number through the extent of each midbrain structure in sham ovariectomized and ovariectomized animals to determine if cell loss was confined to one specific region in the SNpc and VTA of both rats and mice. Although studies show that there is low to no ERα expression in the midbrain, *in situ* hybridization studies have shown that there is low to moderate expression of ERβ in the SNpc and VTA of both rats and mice [62, 68, 69]. Immunohistochemical studies reveal that a small subset of cells in midbrain regions expresses ERβ [40, 41]. We observed ~ 25% loss in both regions in rat after ovariectomy and ~20% loss in mice. Studies have shown that a small subset of TH-IR cells express ERβ in midbrain regions. However, a more recent study suggests that as many as 40% of TH-IR neurons in the SNpc express ERβ [42]. With a small amount of loss, it was possible that this subset would be targeted. However, we observed a uniform loss of cells in the SNpc and VTA of rats and mice suggesting that loss is global and occurs throughout the extent of each structure.

In conclusion, the findings of this study provide evidence that estrogen is required for maintenance of TH-IR cells in the female midbrain and that both ERα and ERβ play a role in the protection of these cell populations. This is the first study to quantitate cell number in animals replaced with ER-selective agonists and to show the effects of estrogenic drug replacement on midbrain TH-IR cell number in the VTA. We

also present the first study to quantitate cell number in midbrain regions of ER knockout mice. The results of this study suggest that estrogen mediates its protective effects through ERα and but may have trophic effects through ERβ as well. The modulation of TH-IR cell number by estrogenic drugs in midbrain regions, specifically the SNpc, may provide insight into the sex differences observed in psychostimulant drug-stimulated behavior, dopamine uptake and release and neurotoxin-induced damage in rodents. This study also has implications for PD and other neurological disorders from which females are less susceptible. In the case PD, the finding that DPN could serve as a possible substitute for estrogen therapy in the protection of cells may open doors for hormone replacement treatements as DPN does not have agonist effects in the mammary gland [43] and based on this study and others has a limited effect in the uterus.

4. Ovarian Hormone Modulation of the Relationship between Dopamine Neuron Number, Dopamine Release and Cocaine-stimulated Behavior

4.1 Introduction

Studies have shown that women are more sensitive to the effects of cocaine as they progress faster to addiction and are more likely to relapse than men [83, 84]. Our laboratory and others have shown that adult female rats exhibit greater locomotor responses to cocaine in the open field than males [6, 101]. Female rats also self-administer cocaine more and have enhanced place preference for cocaine than males, suggesting that ovarian hormones may mediate the sex differences in the behavioral responses to cocaine [93, 94, 127]. Ovariectomy results in an attenuation of the locomotor responses to cocaine, which can be restored with estrogen replacement. However, replacement with progesterone has been shown to either suppress or have no effect on cocaine-stimulated behavior, suggesting that estrogen mediates the stimulatory effects in the response to cocaine [79]. Our laboratory has also reported that adult female rats exhibit greater electrically-stimulated striatal dopamine uptake and release than males. Ovariectomy results in an attenuation of dopamine release in rats treated with acute doses of cocaine, suggesting that the neurochemical responses to cocaine parallel the behavioral responses.

In Chapter 2 of this thesis, data were presented that showed adult female rats have a greater number of TH-IR cell bodies in the SNpc and VTA than males. In Chapter 3, we showed that ovariectomy in adulthood results in a decrease in TH-IR cells in midbrain regions and that replacement with estrogenic compounds can restore TH-IR cells number in the SNpc and VTA of both rats and mice. However, little is known about whether differences in TH-IR cell number mediate sex differences in cocaine-stimulated behavior and dopamine release. To answer these questions, we investigated the effect of ovarian hormones on the relationship between TH-IR cell number in the SNpc, electrically-stimulated dopamine release and cocaine-stimulated behavior. To accomplish this experiment, all three measures were assessed in the same sham ovariectomized and ovariectomized rats. Cocaine-stimulated locomotor behavior, dopamine release and cell number were assessed in individual animals and the measures correlated to determine a relationship between these measures.

4.2 Materials and Methods

Animals

Female rats were gonadectomized or sham gonadectomized at PN55 by the supplier (Charles River, Raleigh, NC) and shipped. Animals were housed under a 12:12 hour light-dark cycle in a temperature and humidity controlled environment with *ad libitum* access to food and water until PN90 when they underwent behavioral and neurochemical testing.

Behavioral Testing and Fast Scan Cyclic Voltammetry (FSCV)

Behavioral testing and FSCV were performed by Joe Caster and Q. David Walker, PhD, respectively. Animals sham ovariectomized and ovariectomized at PN55 were habituated on PN90 in the open field for one hour. Upon completion of habitutation, animals were injected with an acute dose of cocaine (10 mg/kg i.p) activity was recorded for 60 minutes in the open field. Behavioral data were collected using the Kinder software. Two hours after testing cocaine-stimulated behavior, animals were anesthetized with urethane (1.5 g/kg i.p.) and a subjected to FSCV as described in previous studies [5, 7]. Briefly, baseline electrically-stimulated release was determined and then release was determined after the animals received a second injection of cocaine (10 mg/kg i.p).

Tissue Preparation and Immunohistochemistry

Upon collection of neurochemical data, animals were lavaged to determine estrous cycle phase and immediately transcardially perfused with 10% formalin. Uteri and blood were collected to assess effectiveness of hormone replacement and serum estradiol levels, respectively. Brains were postfixed overnight at 4°C and cryoprotected with a 30% sucrose solution. Serial coronal sections (30 µm thick, every 3rd) were cut on a cryostat and thaw-mounted to slides. Sections were allowed to dry overnight at 37°C. Heat mediated antigen retrieval was performed to increase immunoreactivity of the tissue for TH. Sections were pressure cooked at high pressure for 1 minute and 30 seconds in citrate buffer (pH=6.0). Sections were rinsed in PBS and incubated in 0.3% hydrogen peroxide-methanol for 30 minutes to quench endogenous peroxidase. Sections were rinsed and blocked in 0.5% BSA + 0.3% Triton X-100 for 15 minutes at room temperature. After blocking, sections were incubated in primary antibody diluted in blocking buffer (1:10000, Immunostar, Inc.) overnight at 4°C. The next day, sections were rinsed and incubated in a biotinylated horse anti-mouse secondary antibody (1:1000, Vector Labs) for one hour at room temperature. The sections were then rinsed and incubated in avidin-biotin complex for one hour at room temperature. The sections were then rinsed and stained with DAB (Vector Labs). Sections were then counterstained with cresyl violet, mounted and coverslipped.

Stereology

TH-IR cell number in the SNpc and VTA was estimated using the optical fractionator method. Every 6th section through the extent of the midbrain on both sides of the brain was analyzed. Starting sections were selected at random, and all brains were coded and analyzed blindly. Midbrain regions were manually traced at low magnification (4x). Individual cells were visualized for counting with a 100x oil immersion objective (numerical aperture = 1.3). Sections were systematically sampled with counting frames measuring 40 x 40 μm (1600 μm^2 area) spaced randomly 80 μm apart along the x and y axes (sampling grid area = 6400 μm^2). Due to extensive staining methods, some tissue shrinkage occurred resulting in a mounted thickness of 14 μm. Therefore, a disector height of 8 μm was used, with top and bottom guard zones of 3 μm. Only cells that came into focus at the fixed height were counted. Using these parameters, we were able to count enough cells to result in a coefficient of error for each estimate that ranged between 0.05 – 0.10.

Statistics

All statistical analyses for behavioral data and cell counts were performed using ANOVA (NCSS) with a significance level of $p<.05$. Post hoc analysis was performed using the Newman-Keuls test. Correlational analyses were performed in GraphPad

Prism 5.0. Statistical analyses of correlation experiments were performed using Analysis of covariance (ANCOVA, JMP7) with a significance level of $p<.05$.

4.3 Results

Effect of Ovariectomy on Cocaine-stimulated Behavior

Fig. 4-1 shows habituation and cocaine-stimulated activity for ambulations and fine movements, respectively. No differences between surgical groups were observed for ambulations and fine movements during habitutation. Following a cocaine injection (10 mg/kg intraperatoneally (i.p.)), cocaine-stimulated behavior was greater in sham controls compared to the ovariectomized animals. For cocaine-stimulated ambulations, ANOVA indicated a main effect of surgery [$F(1,33) = 5.0$, $p<.05$]. Post hoc tests showed that sham controls were significantly different from ovariectomized animals.

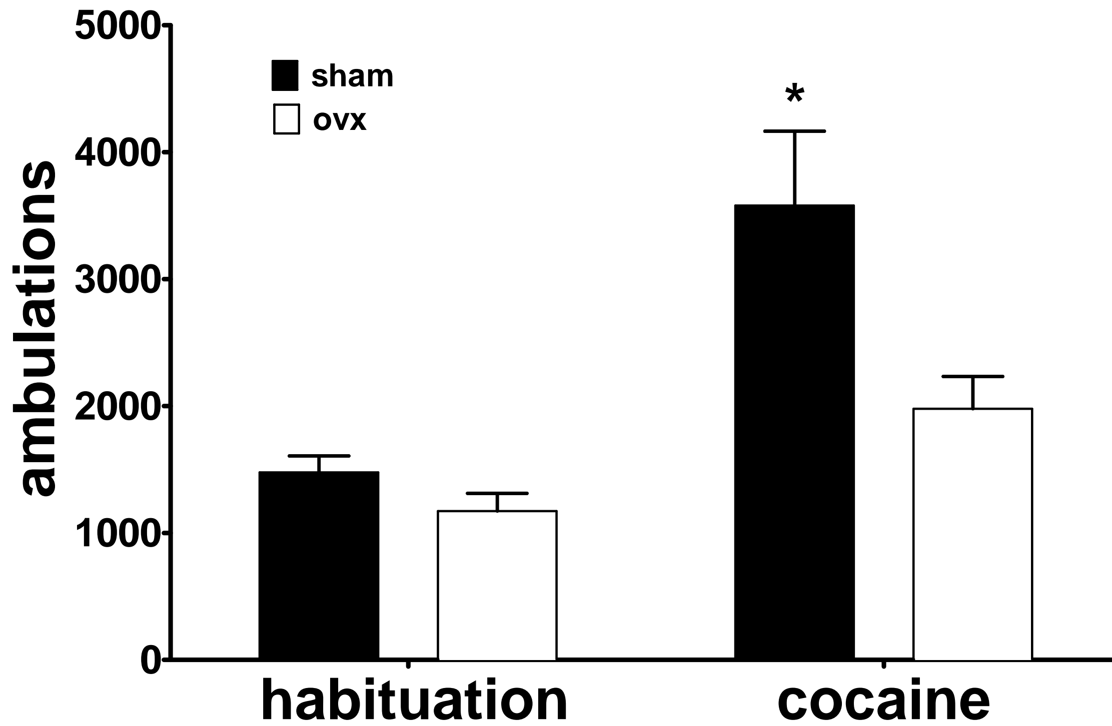


Fig. 4-1. Effect of ovariectomy on behavior during habituation and after cocaine. No differences were observed for ambulations during habituation. After cocaine (10 mg/kg), sham ovariectomized animals exhibited greater ambulations and than ovariectomized animals. Data are expressed as mean ± SEM. * indicates different from ovx, $p<.05$. ($n = 15$-19/group)

Effect of Ovariectomy on Electrically-Stimulated Dopamine Release

Fig. 4-2 shows a comparison of electrically-stimulated dopamine release before and after a 10 mg/kg cocaine injection at various frequencies in sham and ovariectomized rats. No differences were observed between surgical groups without cocaine. After cocaine injection, dopamine release is increased in sham ovariectomized animals at every frequency relative to the ovariectomized. ANOVA indicated a main effect of surgery [$F(1,137) = 21.9$, $p<.001$] and a main effect of frequency [$F(5,137) = 44.5$, $p<.001$]. Post hoc tests showed that sham ovariectomized animals had significantly greater dopamine release at 40, 50 and 60 Hz compared to the ovariectomized animals.

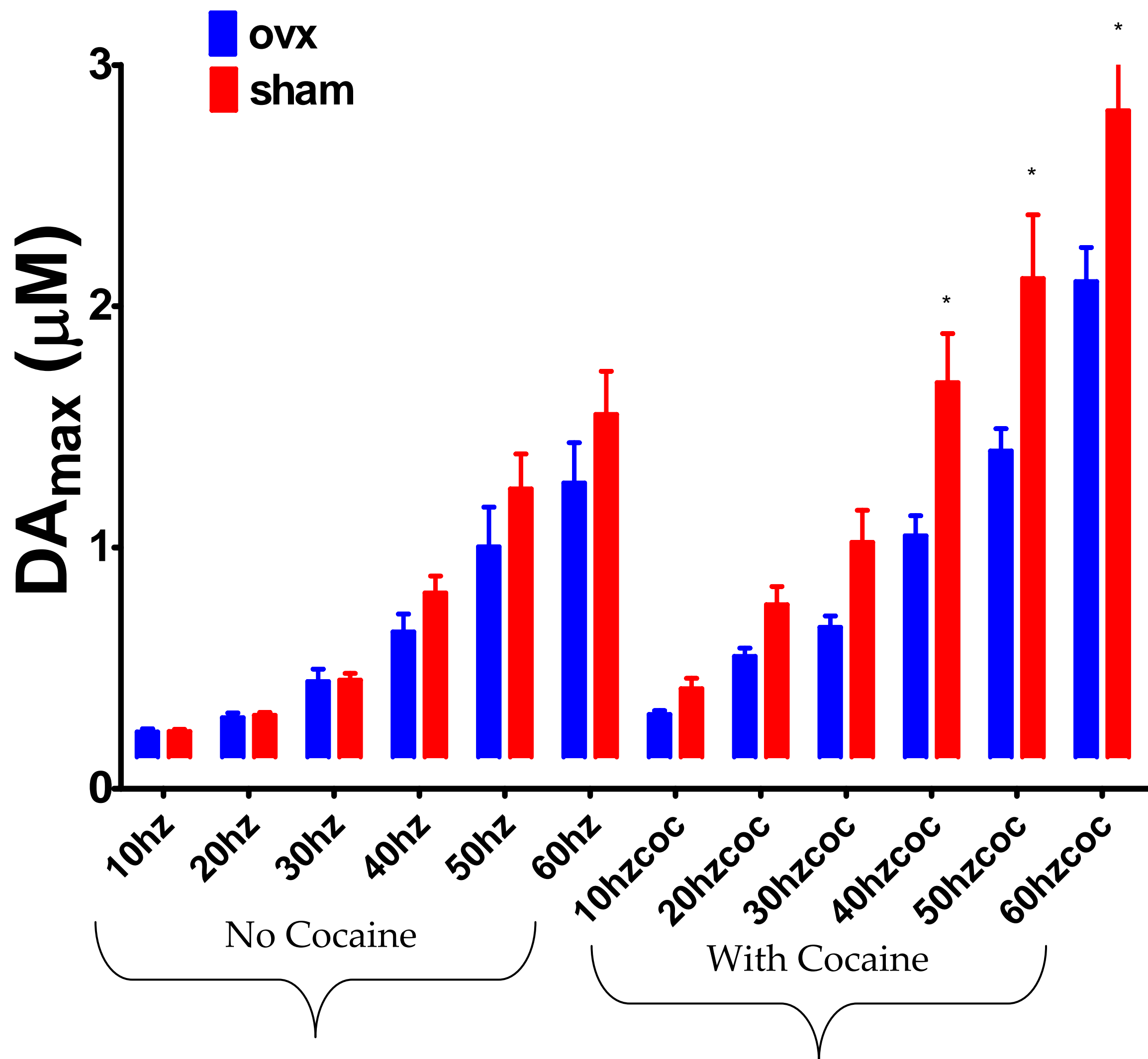
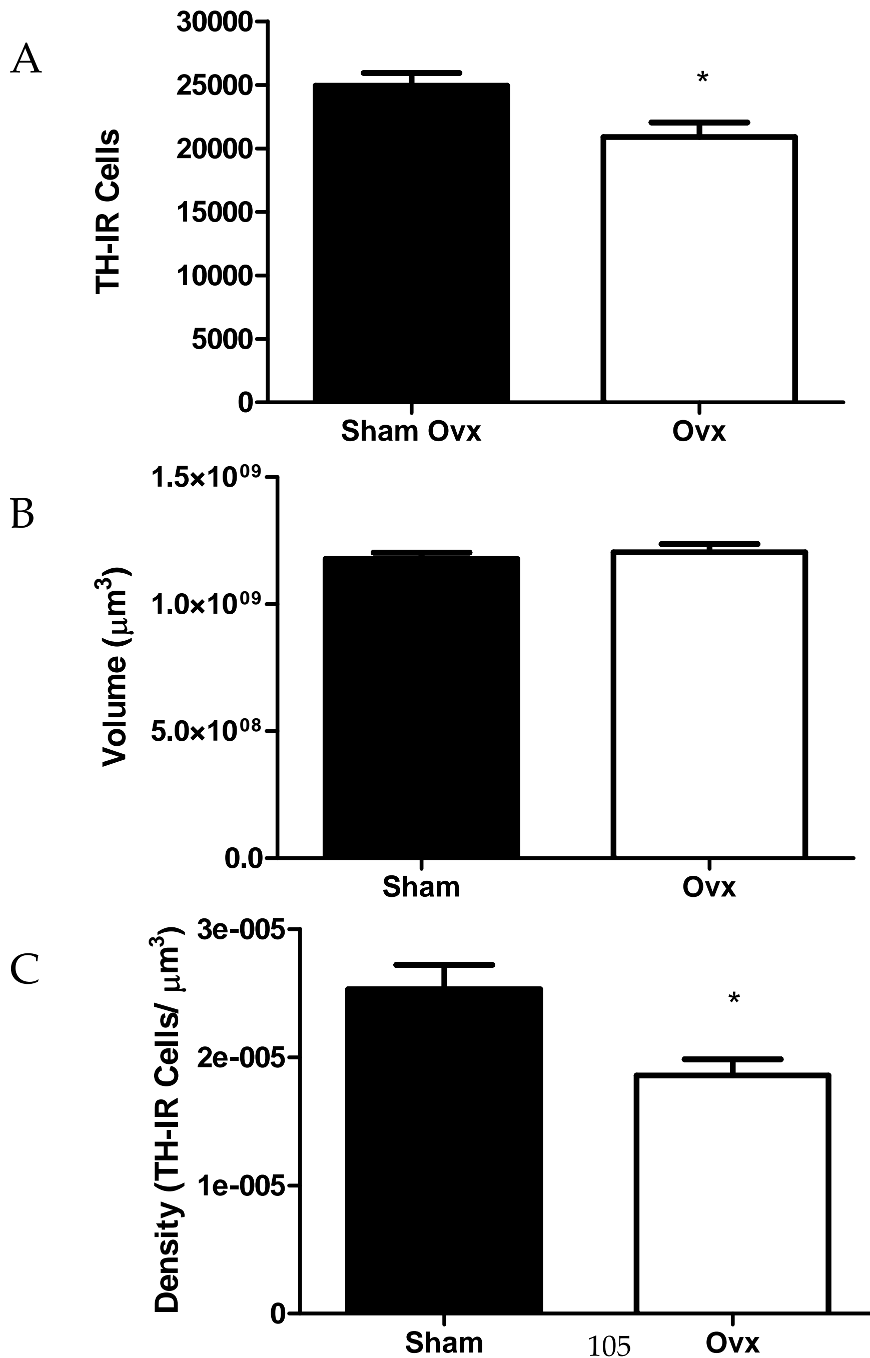


Fig. 4-2. Effect of ovariectomy on maximal dopamine overflow (DA_{max}) in response to a range of electrically-stimulated frequencies before and after cocaine. DA_{max} was increased after cocaine injection (10 mg/kg) in both sham ovx and ovx animals. However, DA_{max} was greater in the sham controls compared to ovariectomized at high frequencies (40-60 Hz). Ovariectomy attenuated this effect. No differences were observed prior to cocaine. Data are expressed as mean ± SEM. * indicates different from ovx, $p<.001$. ($n = 15-19$/group)

Determination of Cell Number and Density in the SNpc

TH-IR cell number was determined in the SNpc of sham ovariectomized and ovariectomized rats. The significant reduction in cell number in ovariectomized animals relative to sham was replicated (*Fig. 4-3A*). ANOVA revealed a significant reduction with ovariectomy [$F(1, 24) = 6.7$, $p<.05$]. There was no difference between surgical groups in SNpc volume (*Fig. 4-3B*). However, there was a significant difference in cell density in the SNpc [$F(1,23) = 8.3$, $p<.01$] (*Fig. 4-3C*). Cell density was used as a normalizing factor for number of neurons in a given SNpc volume (size).

Fig. 4-3. Effect of Ovariectomy on TH-IR cell number (A), region volume (B) and cell density (C) in the SNpc. Ovariectomy results in a loss of TH-IR cells in the SNpc relative to the sham controls. There is no difference in SNpc volume between surgical groups. Ovariectomy also results in a decreased cell density relative to the sham controls.
* indicates different from sham. $p<.01$ for panels A and C. ($n = 11\text{-}12$ per group)



Topographical Analysis of Cell Number

The previous findings of uniform loss throughout the SNpc with ovariectomy were replicated in the animals used for the correlation experiments (*Fig. 4-4)*. Again, analysis revealed a common pattern of cellular distribution in sham and ovariectomized rats. Fig. 4-5 illustrates differences in cell number in brain sections of sham ovariectomized (*Fig. 4-5A*) and ovariectomized (*Fig. 4-5B*) rats. This was consistent with our previous findings shown in Chapter 3. ANOVA revealed a main effect of surgery [(F 1, 181) = 28.5, $p<.0001$)] and a main effect of section [(F 1, 181) = 27.1, $p<.0001$] but no interaction. These data confirm results from the previous studies, suggesting that cell loss occurred in the ovariectomized animals and that loss is not limited to a specific region of the SNpc.

We assessed the effects of gonadectomy on striatal dopamine content to determine the effects of ovariectomy in terminal regions by HPLC (Fig. 4-6). ANOVA indicated no significant effect of surgery.

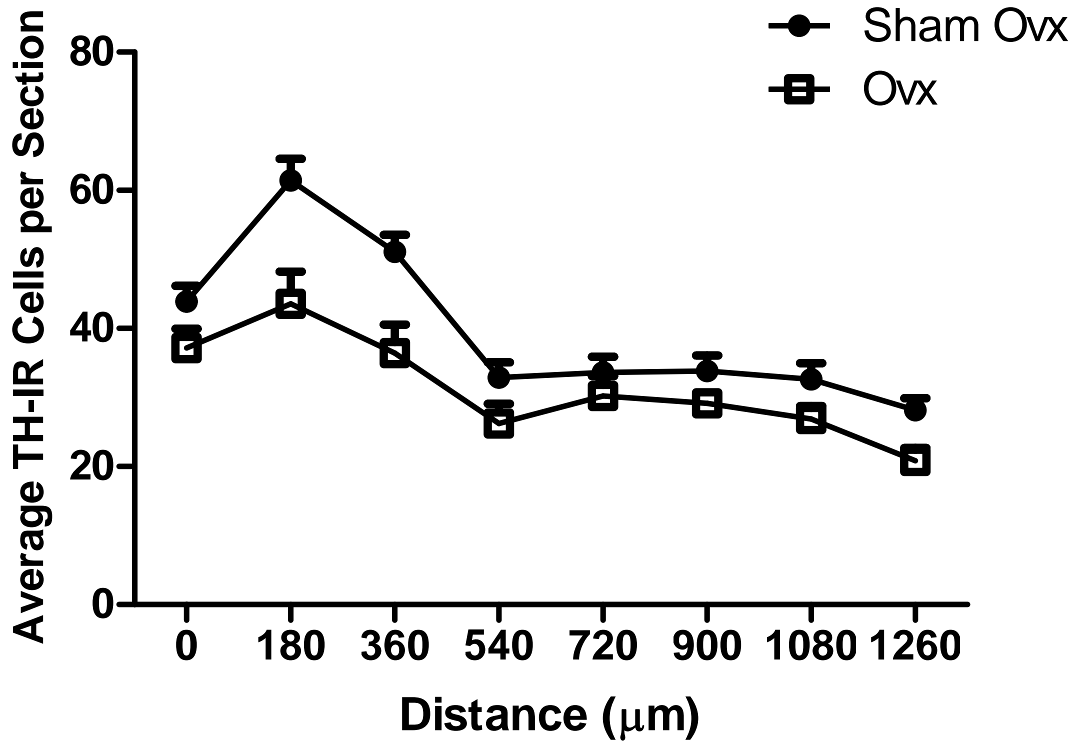


Fig. 4-4. Topographical Analysis of TH-IR cell number in the SNpc. The average TH-IR cell number from the right and left SNpc was quantitated per section through the extent (rostral to caudal) of the region starting with the most rostral section (0 μm). Data are presented as means ± SEM. n = 11-12 animals per group and n = 6-8 sections per animal.

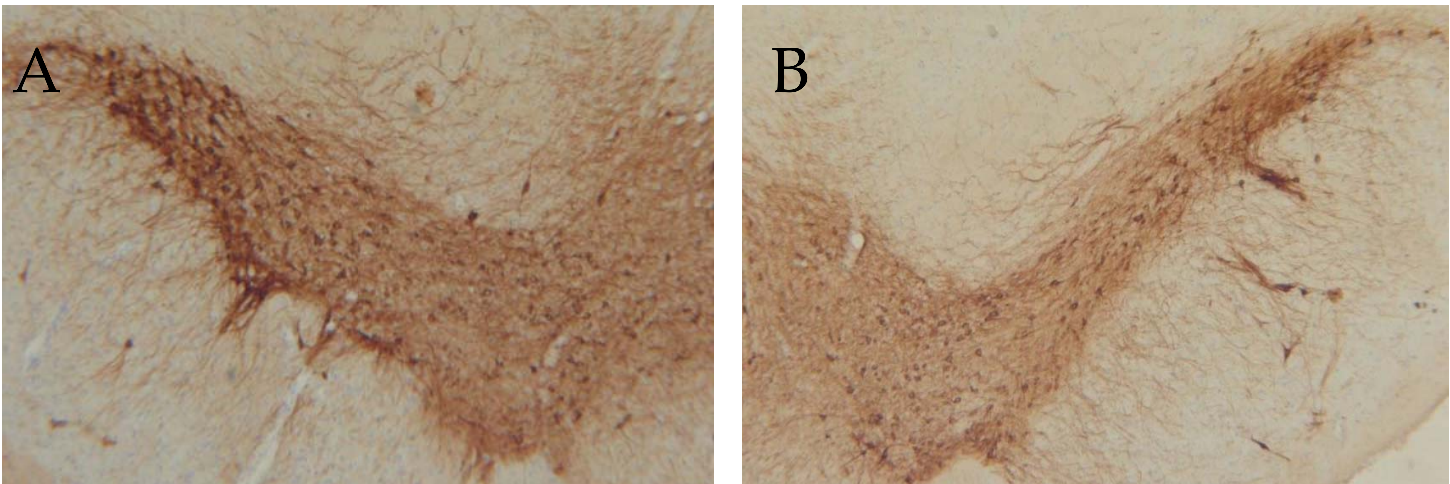


Fig. 4-5. Representative photomicrographs of TH-stained sham ovariectomized (A) and ovariectomized (B) female rats at 180 μm. Taken at 2.5 x magnification.

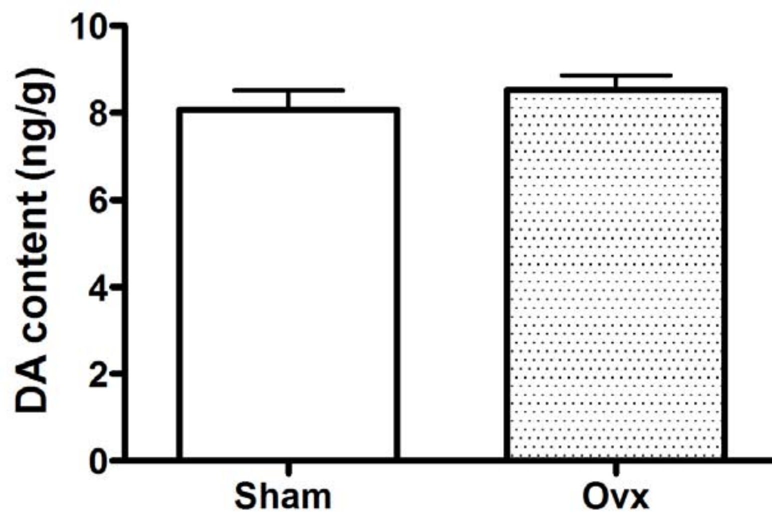


Fig. 4-6. Effect of ovariectomy on striatal dopamine content. Dopamime content was determined by HPLC in PN90 rats sham ovariectomized and ovariectomized at PN60. Data are presented as mean ± SEMs. ANOVA indicated no significant differences between treatment groups, p =.55. (n = 9 per group).

Density of TH-IR Neuron Number Correlates with Behavior and Dopamine Release

Fig. 4-7 shows representative time courses of cocaine-stimulated ambulations and dopamine release. Both ambulations and dopamine release (maximal dopamine overflow) at 20 Hz stimulation are lower in the ovariectomized animals, showing that the attenuation of cocaine-stimulated behavior with ovariectomy extends to dopamine release.

The relationship between nigral TH-IR cell density and cocaine-stimulated behavior was investigated to determine if loss of cells observed with ovariectomy played a role in the behavioral responses to cocaine. Correlational analyses revealed that positive correlations were found between cell density in the SNpc and cocaine-stimulated behavior, as an increase in TH-IR cell density mirrored an increase cocaine-stimulated behavior (*Fig. 4-8*, $r^2 = 0.64$, $p<.001$). ANCOVA [$F(3,25) = 7.1$, $p<.01$] revealed a significant effect of SNpc cell density ($p<.05$) and a surgery x SNpc density interaction ($p<.01$). No correlation was observed in the ovariectomized animals. These data suggest that there is a relationship between SNpc density and behavior in the sham ovariectomized animals only.

The relationship between TH-IR cell density in the SNpc and dopamine release was also determined. A positive correlation between cell density and dopamine release was observed (*Fig. 4-9*, $r^2= 0.44$, $p<.05$) in the sham controls. ANCOVA [$F(3,25) = 3.8$, $p<.05$] revealed a significant effect of density ($p<.01$). No correlation was observed in

the ovariectomized animals. A cell density x dopamine release interaction was not observed.

Correlational analysis of cocaine-stimulated ambulations and dopamine release at 10 Hz stimulation revealed a positive correlation in the sham ovariectomized animals (*Fig. 4-10,* $r^2 = 0.35$, $p<.05$). ANCOVA [$F(3,25) = 5.7$, $p<.01$] indicated a significant effect of dopamine release ($p<.05$). This relationship was also lost after ovariectomy. Slopes for the sham ovariectomized and ovariectomized animals were similar. Therefore, no cell density x dopamine release interaction was observed.

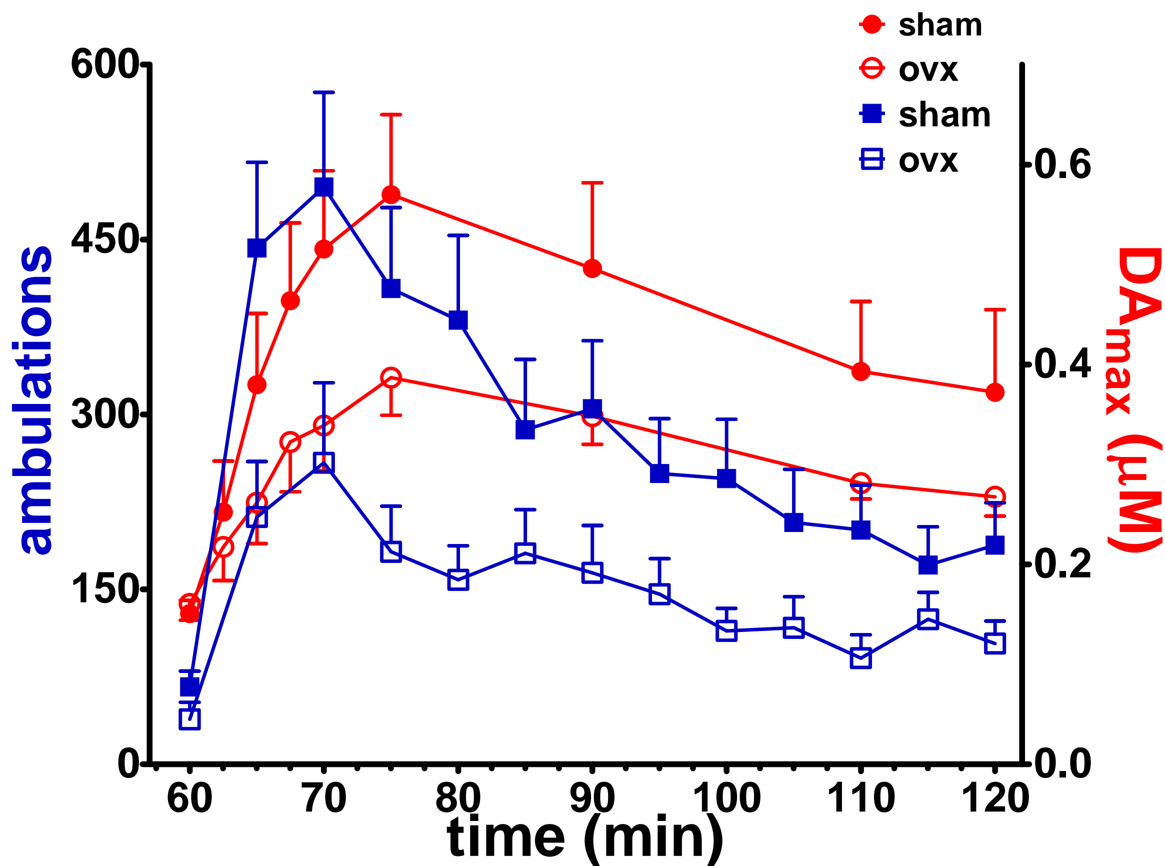


Fig. 4-7. Time course of ambulations and maximal dopamine overflow (DA_{max}) in the same sham and ovariectomized rats. Cocaine-stimulated behavior was first determined andthen rats were anesthetized two hours later. After baseline voltammetry, each rat was again injected with 10 mg/kg cocaine and time course of stimulated-dopamine release was determined. Ovariectomy results in a decrease in ambulations (blue, left y-axis) and DA_{max} (red, right y-axis). Sham animals are represented by closed shapes and ovx are represented as open shapes.

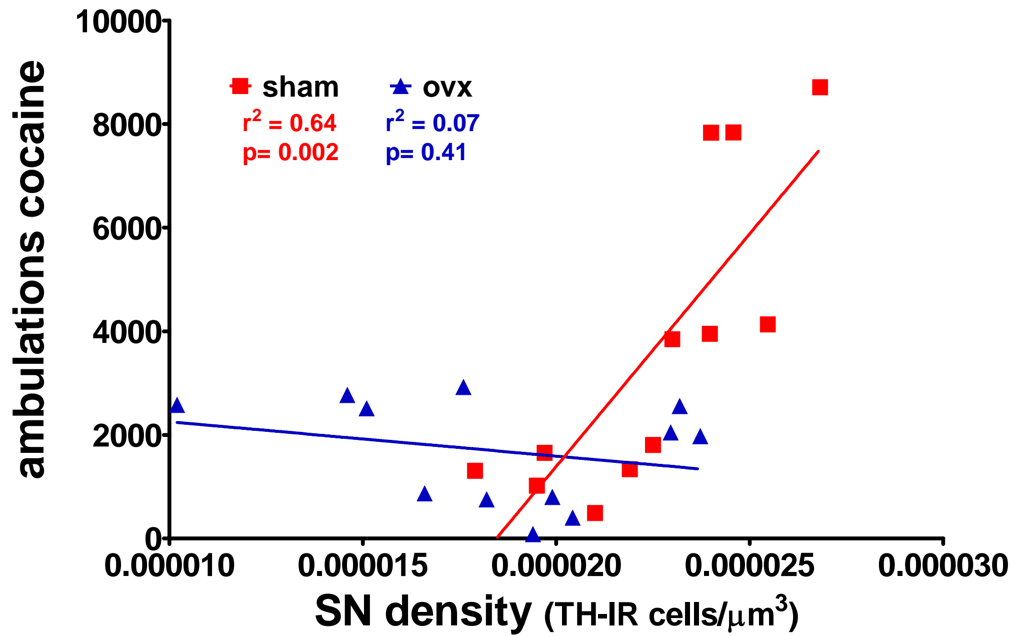


Fig. 4-8. Effect of ovariectomy on the relationship between cell density and cocaine-stimulated behavior in individual animals. Correlational analysis of the cocaine-stimulated ambulations and TH-IR cell density. A positive correlation was observed in the sham ovariectomized animals (red), r^2 =0.64, $p<.01$. ANCOVA indicated a main effect of density, $p<.05$ and a surgery x density interaction, $p<.05$. (n = 12-13 animals per group)

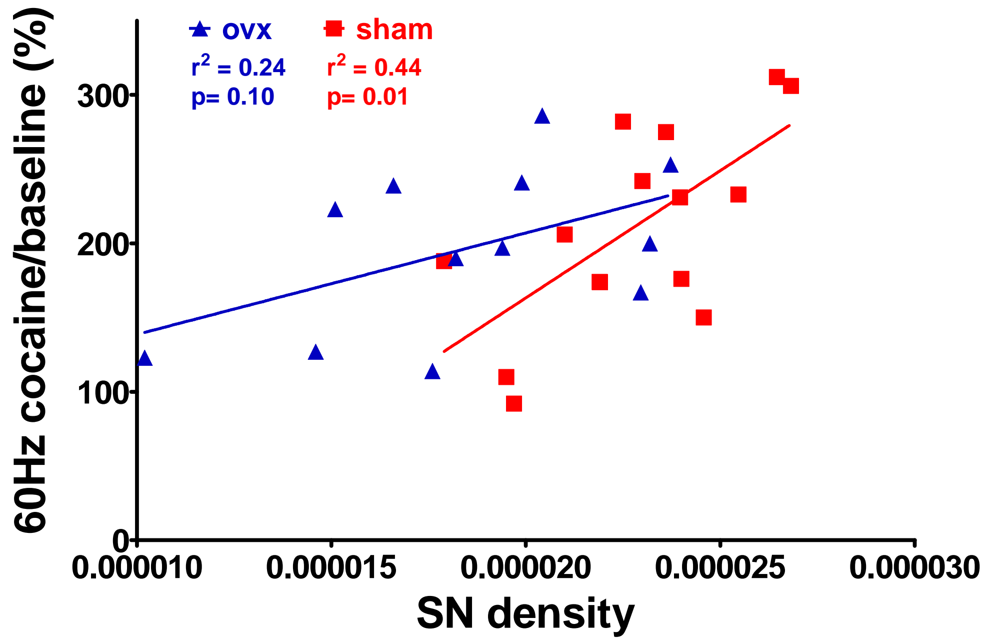


Fig. 4-9. Effect of ovariectomy on the relationship between cell density and dopamine release in individual animals. Correlational analysis of TH-IR cell density and electrically-stimulated dopamine release (presented as percent stimulation with cocaine over baseline stimulation at 60 Hz). $r^2 = 0.44$, p=.01 for a positive correlation in sham controls (red). ANCOVA indicated a main effect of release, p<.01 and a main effect of surgery, p<.01. (n = 12-13 animals per group)

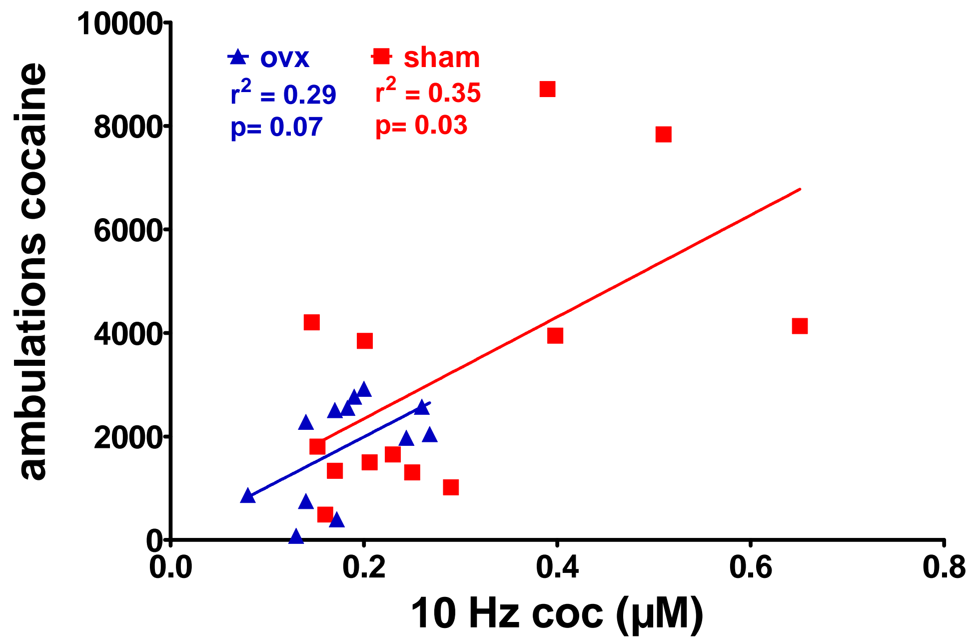


Fig. 4-10. Effect of ovariectomy on the relationship between cocaine-stimulated behavior and dopamine release in individual animals. Correlational analysis of the cocaine-stimulated ambulations and electrically-stimulated dopamine release at 10 Hz stimulation after cocaine injection. $r^2 = 0.35$, $p<.05$ for a positive correlation in sham controls (red). ANCOVA indicated a main effect of release, $p<.05$. (n = 12-13 animals per group)

4.4 Discussion

The prior chapters of this thesis have examined estrogen effects on dopamine neuron maintenance. In the present study, the functional aspects of ovarian hormone effects in the midbrain were investigated. To determine if the loss of cells observed with ovariectomy played a role in the differences in cocaine-stimulated behavior, we sought to link the effects of gonadectomy shown to occur in cocaine-stimulated behavior with cell density in the SNpc of the same individual animals. We replicated previous findings that ovariectomy results in a loss of TH-IR cell number in the SNpc as well as a decrease in cocaine-stimulated behavior [6, 101, 143] and dopamine release relative to the sham controls. Correlational analyses of all three measures in the same individual animals revealed that cocaine-stimulated behavior, dopamine release and nigral cell density are positively correlated in sham ovariectomized animals. Ovariectomy results in a loss of these relationships, suggesting that estrogen is required for the maintenance of dopamine neurons that regulate the behavioral and neurochemical responses to cocaine in adult female rats.

Ovariectomy had no effect on cocaine-stimulated behavior and electrically-stimulated dopamine release during habituation and at baseline, respectively. However, ovariectomy attenuated the behavioral response to cocaine and reduced the increase in electrically-stimulated dopamine release observed after cocaine injection relative to the sham ovariectomized controls. These findings are concordant with previous studies

from this laboratory and others. Ovariectomy has been shown to attenuate cocaine-stimulated behavior [6, 101, 143], and estrogen replacement in ovariectomized animals increases cocaine-stimulated locomotor acivity [102, 127]. Estrogen replacement increases cocaine-induced sensitization relative to ovariectomized rats [100, 127, 144]. Ovariectomy results in a decrease in DAT specific binding in the striatum of adult rats [74]. After three months, ovariectomy has been shown to reduce DAT mRNA expression in the rat striatum and SNpc [145]. In addition to influencing monoamine transporter binding and expression, ovariectomy decreases dopamine D2 receptor specific binding in the rat striatum [75, 146].

The loss of TH-IR cells with ovariectomy was also replicated in this study. Topographical analysis of nigral sections was performed to determine if cell loss was confined to a specific region in the ovariectomized animals. Cell numbers were globally reduced throughout the extent of the SNpc. A study in rats has reported that TH-IR cell number is correlated with the number of axons in the striatum [147]. However, striatal dopamine content did not mirror this loss, as dopamine content was the same in the sham and ovariectomized animals, suggesting that there is some compensation for cell loss at the terminal level. One possible explanation for the lack of difference in dopamine content is the sprouting of processes by the remaining cells in the SNpc of ovariectomized rats as a compensatory mechanism for reduction in neuron number.

In this study, ovariectomy resulted in ~20% loss of TH-IR cell number in the SNpc relative to sham controls, which parallels the attenuation of the behavioral responses to cocaine. Our explanation for these findings suggests that there may be a small population of cells that are dependent upon estrogen for maintenance and that these cells may influence the behavioral responses to cocaine. Immunohistochemical studies show a select population of TH-IR cells in the SNpc express ERβ and may be responsive to estrogen stimulation [70, 77]. This notion is further supported by studies in ovariectomized rats showing that estrogen administration increases firing rate in specific neurons in the SNpc [103].

These findings raise a question about the relationship between cell density and behavioral and neurochemical responses. Does cell density below a certain level result in a loss of these relationships? To answer this question, we investigated the role of ovarian hormones on the relationship between cell density and cocaine-stimulated behavior and electrically stimulated dopamine release. A positive correlation between TH-IR cell density and cocaine-stimulated ambulations in sham ovariectomized rats was observed. Ovariectomy resulted in a loss of this relationship. Studies in female 6-OHDA lesioned rats show that TH-IR cell number is positively correlated with apomorphine-induced rotational behavior [147]. Studies in aging primates also show a correlation between TH-IR cell number and performance on motor tasks. Animals with low TH-IR cell number performed more poorly on tasks than animals with higher cell numbers

[148]. We also observed a positive correlation between cocaine-stimulated behavior and electrically-stimulated dopamine release in the sham ovariectomized rats. Studies in adult male rats show that peak dopamine signal and clearance in the nucleus accumbens and striatum are correlated with locomotor behavior [149]. These studies as well as the present data suggest that behavior and neurochemical responses are correlated and that nigral cell number and density strongly influences these responses.

In conclusion, the data presented in this chapter show that estrogen augments cocaine-stimulated behavior and dopamine release and that these responses are mediated by estrogen-maintained TH-IR cell number in the SNpc of intact animals. This study may also suggest that animals with higher cell densities may be more sensitive to the effects of cocaine and other psychostimulant drugs as the intact animals were the highest responding individuals in the behavioral and neurochemical tests. This is the first study to determine the role of ovarian hormones on the within-animal relationships between cocaine-stimulated behavior, dopamine release and dopamine neuron number.

5. The Effect of Androgens on Tyrosine Hydroxylase Immunoreactive Cell Number in Midbrain Regions

5.1 Introduction

Clinical studies show that many disorders of dopaminergic dysfunction have a higher incidence in men than women [4, 23, 24, 83, 84]. For example, men are more prone to the development of drug addiction [83, 84] and PD than women [2-4]. The mechanisms behind these differences are poorly understood. While a great deal of focus has been placed on the stimulatory and protective effects estrogen in dopaminergic systems, considerably less is known about the role of androgens.

Studies from our laboratory and others have reported that adult male rats are less active in the open field than females under normal conditions [6, 80]. This sex difference becomes more exaggerated after treatment with psychostimulant drugs [6, 101]. Castration increases locomotor responses to amphetamine and cocaine and also increases dopamine release [78, 80]. Testosterone replacement results in a decrease of these dopaminergic responses, suggesting that androgens may have a suppressive effect on dopaminergic function [78, 104, 105].

Animal models of neurotoxin-induced damage support these findings. In rat models of PD, males suffer more severe behavioral deficits and cell loss than females after 6-OHDA lesion [96]. Castration in rat results in a protection of striatal dopamine content from 6-OHDA lesion compared to intact males [97, 98]. Testosterone

replacement in castrated mice does not protect depletion of striatal dopamine from MPTP, suggesting that testosterone may have neurotoxic effects [81, 82].

The data presented in previous chapters support the observations of a suppressive role of androgens in dopaminergic pathways. In Chapter 2, adult male rats were shown to have fewer TH-IR cells in the SNpc and VTA than females, a finding that is concordant with the previously observed sex differences in cocaine-stimulated behavior and dopamine release reported by our laboratory. Therefore, the purpose of this study was to determine how androgens affect TH-IR cell number in the SNpc and VTA through replacement with testosterone, nandrolone and DHT in male rats castrated at PN60.

5.2 Materials and Methods

Animals, Surgery, Hormone Replacement and Housing

Male rats castrated or sham castrated on postnatal day (PN) 60±5 were purchased from Charles River Laboratories (Raleigh, NC). Animals were segregated by surgical condition and housed in plastic cages under a light-dark cycle (12:12 hour) with *ad libitum* access to food and water. In a parallel study, female C57Bl/6 mice ovariectomized at PN60±5 (Charles River Laboratories, Raleigh, NC) were separated by surgical condition and housed in plastic cages under a light-dark cycle (12:12 hour) with *ad libitum* access to food and water. Rats were transcardially perfused with 10% neutral buffered formalin (VWR) on PN90. In a second experiment, castration or sham surgery were performed on adult rats (PN60) while animals were under ketamine (80 mg/kg) and xylazine (10 mg/kg). Starting on the day of surgery, animals received daily subcutaneous injections of vehicle (sesame oil), testosterone (1 mg/kg), DHT (0.5 mg/kg) and nandrolone (0.5 mg/kg). Animals were housed until PN90 for perfusion.

Tissue Preparation and Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused with 10% neutral buffered formalin. After perfusion, the brains were extracted and post-fixed overnight in 10% formalin. Brains were then equilibrated in a 30% sucrose cryoprotectant solution and stored at 4°C. Serial coronal sections (30 μm thick) were cut on a cryostat and thaw-

mounted to slides. For rats, every 3rd section was collected and for mice every 2nd section was collected. Sections were allowed to dry overnight at 37°C. Heat mediated antigen retrieval [24, 25] was performed to increase immunoreactivity of the tissue for TH. Sections were pressure cooked (Deni electric pressure cooker) at high pressure for 1 minute and 30 seconds in citrate buffer (pH = 6.0) [26]. This length of time allowed for optimal staining without compromising cell morphology. Sections were rinsed in PBS and incubated in 0.3% hydrogen peroxide-methanol for 30 minutes to quench endogenous peroxidase. Sections were rinsed and blocked in 0.5% BSA + 0.3% Triton X-100 for 15 minutes at room temperature. After blocking, sections were incubated in primary antibody diluted in blocking buffer (1:10000, Immunostar, Inc., Hudson, WI) overnight at 4°C. The next day, sections were rinsed and incubated in a biotinylated horse anti-mouse secondary antibody (1:1000, Vector Labs, Burlingame, CA) for 1 hour at room temperature. The sections were then rinsed and incubated in avidin-biotin complex for 1 hour at room temperature. The sections were then rinsed and stained with DAB (Vector Labs). Sections were rinsed, dehydrated through graded alcohols, mounted and coverslipped. To quantitate the number of cells that were TH immunonegative (TH-IR) or lacking the DAB stain, sections in hormone replaced animals were counterstained with 0.5% cresyl violet after DAB staining and coverslipped.

Unbiased Stereology

Unbiased stereological estimation of the total number of TH-IR cell bodies in the SNpc and VTA was performed using the optical fractionator method [27]. In rats, every third section was collected through the extent of the midbrain, and every 6th section analyzed for cell counting in rostal-caudal fashion through the extent of the midbrain, resulting in a total of 6-8 sections sampled for both the right and left sides of the brain. A computerized counting system containing a Nikon Optiphot-2 microscope, a camera (Dage) and motorized stage (Ludl) was used to estimate the total number of cells. Each region of interest was projected onto a monitor, traced at low (4x) magnification and a sampling grid was superimposed on the traced region by the StereoInvestigator software (MicroBrightField). After shrinkage, final thickness of the sections used averaged 13 μm. Therefore, a 40 x 40 μm counting frame with a dissector height of 8 μm was used. Each counting frame was randomly spaced 100 μm apart and guard zones of 2.5 μm from the top and bottom of the section were used. Individual cell bodies were visualized at with a 100x oil immersion lens (numerical aperture = 1.3). Enough cells were counted to achieve a coefficient of error that was ≤ 0.10. The stereologer was blinded to all surgical and treatment groups for each experiment.

Topographical Analyses

The actual number of counted TH-IR cells from the left and right side through the extent of the SNpc and VTA were averaged to obtain a number for the cells counted at a given distance in the midbrain. Every 6th section in was analyzed. Therefore, a total of at least six sections (left and right), spaced 180 μm were analyzed. The first (most rostral) section counted at the start of each region was considered 0 μm. Data from rats used in the effects of castration HMAR study (Experiment 1) and the hormone replacement controls were combined to increase statistical power.

Prostate Weights

Prostate weights were collected as a measure of successful replacement in comparison with intact controls. After fixation of tissues by transcardial perfusion, prostates were removed. All connective tissues associated with prostates were removed prior to weighing.

Animals and Bromodeoxyuridine (BrdU) Injections

Rats castrated, sham castrated or sham ovariectomized at PN55 were purchased from Charles Laboratories (Raleigh, NC). Animals were segregated by surgical condition and housed in plastic cages under a light-dark cycle (12:12 hour) with *ad*

libitum access to food and water. Animals received daily intraperitoneal (i.p.) injections of BrdU (100 mg/kg). Injections began two days post-surgery and continued until PN90.

BrdU Immunohistochemistry

Animals were deeply anesthetized by i.p. injection with pentobarbital (Nembutal) and transcardially perfused with 10% formalin. Brains were extracted, postfixed in formalin and cryoprotected with a 30% sucrose solution. After sinking in cryoprotectant, brain sections were cut on a cryostat at 30 μm of thickness in multi-well plates containing PBS and processed using the free-floating method. The BrdU staining method was adapted from Parent et al [150]. Briefly, sections were denatured in 50% formamide/2x SSC for 2 hours at 65°C. Sections were rinsed in 2X SSC for five minutes and incubated in 2N hydrochloric acid (HCl) for 30 minutes at 37°C. Sections were rinsed several times in tris-buffered saline (TBS). Sections were quenched in 1% hydrogen peroxide in 10% methanol and TBS for 30 minutes at room temperature. Sections were rinsed in TBS + 0.1% Triton X-100 (Tris A) and TBS + 0.25% BSA and 0.1% Triton X-100 (Tris B) for 15 minutes each. Sections were blocked in 10% goat serum + Tris B for one hour at room temperature and then incubated in primary antibody overnight at 4°C (mouse anti-BrdU, Roche, 1:1000). The next day, sections were rinsed with TBS and incubated in a biotinylated secondary (mouse IgG, Vector Labs) antibody for one hour at room temperature. Sections were rinsed and incubated in avidin-biotin complex (Vectastain Elite Standard, Vectorlabs) for one hour at room terperature.

Sections were stained with DAB, rinsed in deionzed water, mounted to slides and allowed to dry overnight. The next day, sections were dehydrated through graded alcohols, mounted and coverslipped.

For TH-BrdU double staining, the sections were rinsed with deionized water in wells after DAB treatment. Sections were blocked in 0.5% BSA + 0.3% Triton X-100 in TBS for 30 minutes. Sections were incubated in TH primary (1:3500, Immunostar Inc) overnight at room temperature with gentle agitation. Sections were washed several times the following day in TBS and incubated in biotinylated secondary antibody (1:1000) for one hour at room temperature. Following several washed in TBS sections were incubated in alkaline phosphatase (Vector Labs) for one hour at room temperature. Sections were rinsed in TBS and treated for 30 minutes with Vector Red substrate containing levamesole to block nonspecific phosphatase. Sections were rinsed with water, placed on slides and allowed to dry overnight. Dried sections were dehydrated through graded alcohols, mounted and coverslipped.

Immunofluorescent Staining

Coronal tissue sections 30 μm of thickness were cut on a cryostat collected in multi-well plates containing 0.1 M PBS in each well. Each section was placed in a well and sections were stained using the free-floating method. Sections were blocked at room temperature for 30 minutes in 0.1 M PBS containing 10% normal goat serum and 0.4% Triton X-100. Sections were incubated overnight at 4ºC in a mixture of primary

antibodies (diluted in blocking buffer. The next day, sections were rinsed twice for five minutes in 0.1 M PBS. Sections were then incubated in a mixture of Alexa Fluor-488 and 594 conjugated secondary antibodies diluted in blocking buffer (Molecular Probes, 1:200 each) for two hours at room temperature. Sections were rinsed twice in 0.1 M PBS, mounted and coverslipped with 70% glycerol in 0.1 M PBS. Sections were visualized under a Nikon Optiphot-2 fluorescence microscope. Images were captured using a Sony DXC-S500 color video camera controlled by Adobe Photoshop. An overlay of captured images was performed using MetaMorph (Molecular Devices Corporation). Additional sections were visualized using a Leica TCS SP5 broadband confocal microscope.

BrdU Immunofluorescent Staining

Coronal tissue sections were cut on a cryostat at a thickness of 30 μm and collected in multiwell plates containing PBS to be stained by the free-floating method. For denaturation of DNA, sections were incubated in 2N HCl for 30 minutes at 37°C. Sections were immediately rinsed in for 10 minutes in 0.1 M boric acid (pH = 8.5). After washing sections several times in TBS, sections were blocked in 10% goat serum and 0.4% Triton X-100 in TBS for one hour at room temperature. Sections were incubated in a primary antibody mixture overnight at 4°C with gentle agitation. The following day, sections were thoroughly washed rinsed in blocking solution to reduce nonspecific binding. Sections were incubated in a secondary antibody mixture of Alexa Fluor 488

and either Alexa Fluor 594 or Alexa Fluor 647. Sections were rinsed in blocking buffer, mounted with 70% glycerol in PBS and coverslipped.

Primary Antibodies and Dilutions

Rabbit anti -TH, 1:500 (Chemicon)

Mouse anti-Glial fibrillary acidic protein (GFAP), 1:200 (Chemicon)

Mouse anti-BrdU, 1:100 (Roche)

Rat anti-BrdU 1:100 (Novus Biologicals)

Statistical Analysis

All statistical analyses were performed using ANOVA (NCSS) with a significance level of $p<0.05$. Post hoc analysis was performed using the Newman-Keuls multiple comparison and Fisher's least significant difference tests to determine group differences.

5.3 Results

Effect of Castration on TH-IR Cell Number

In sections counted prior to the incorporation of HMAR into the staining method, ANOVA indicated a significant effect of surgery in the SNpc [F(1,10) =17.1, p<.001]. In the VTA, the trend was similar but ANOVA did not indicate a main effect of surgery (p =.06). After incorporation of HMAR into the staining method, castration at PN60 resulted in an increase in TH-IR cell number the SNpc and VTA of male rats (*Fig. 5-2A and B*) and mice (*Fig. 5-3A and B*) at PN90 relative to the sham surgery controls. In rats, ANOVA revealed a significant effect surgery [F(1,10) = 1, p<.01] in the SNpc and VTA [F(1,9) = 11.6, p<.05]. In mice, ANOVA also revealed a significant effect of surgery in the SNpc [F(1,10) = 23.7, p<.01] and VTA [F(1,10) = 6.0, p<.05].

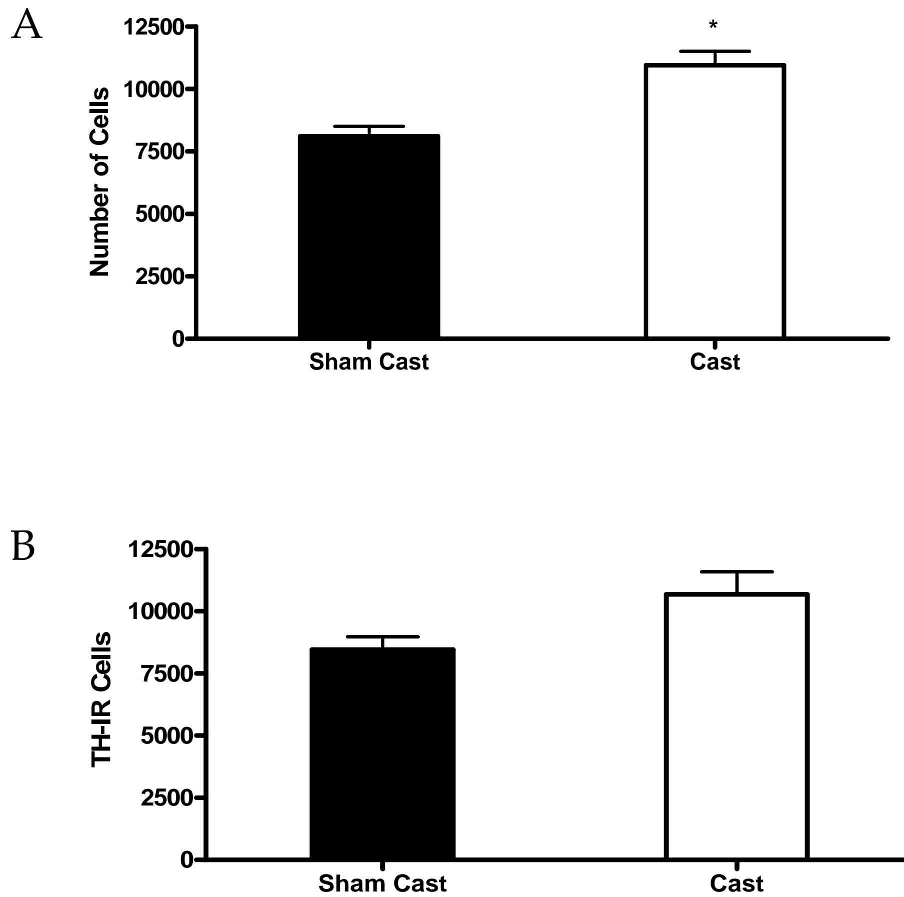
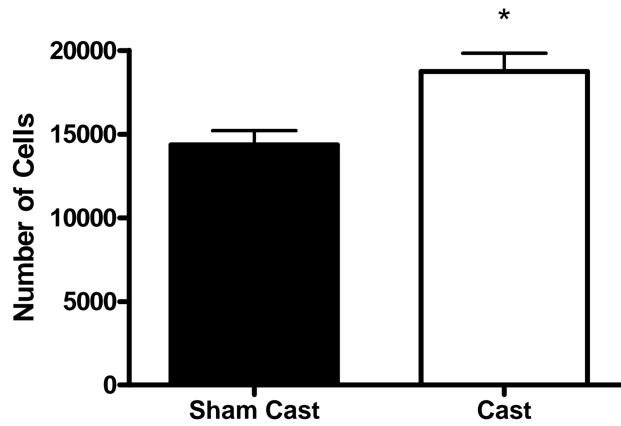


Fig. 5-1. Effect of castration on midbrain TH-IR cell number without HMAR. In the SNpc (A), castration (cast) results in an increase. * indicates different from sham cast, $p<.001$. In the VTA (B), castration did not have an effect ($p = .06$). Data are expressed as means ± SEM. (n = 5-7/group)

A



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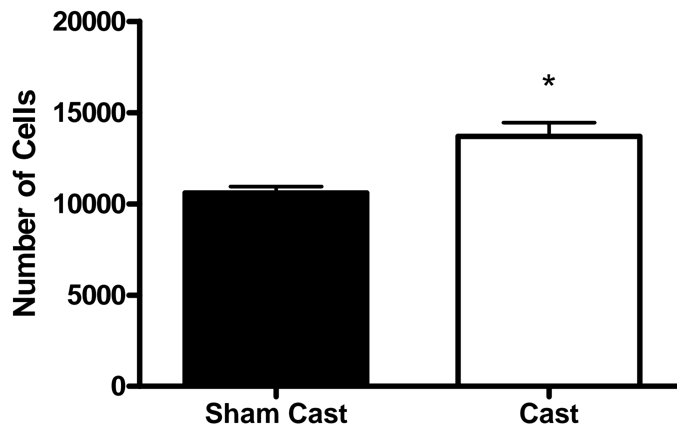
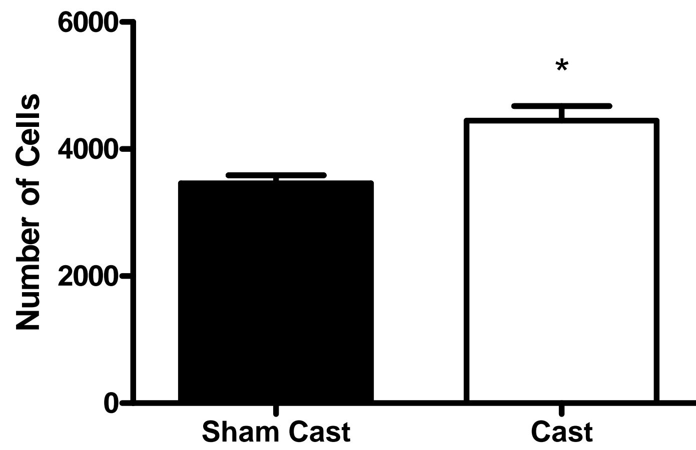


Fig. 5-2. Total TH-IR cell number in the SNpc (A) and VTA (B) of castrated (cast) compared to sham cast male rats after one month of castration during adulthood with HMAR. Data are represented as mean ± SEM (n = 6). * indicates significantly different from sham cast (for A: $p<.01$ and for B: $p<.05$).

A



B

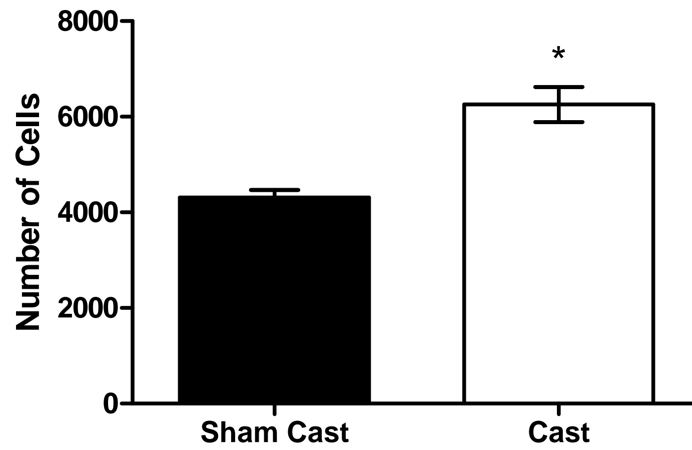


Fig. 5-3. Total TH-IR cell number in the SNpc (A) and VTA (B) of castrated (cast) compared to sham cast male mice after one month of castration during adulthood with HMAR. Data are represented as mean ± SEM (n = 5). * indicates significantly different from sham cast, p<.01.

Testosterone Suppresses TH-IR Cell Number in SNpc

All hormone replacements were confirmed with the collection of ventral prostate weights (*Fig. 5-4*). ANOVA showed a significant effect of treatment [$F(4, 39) = 39.6$, $p<.0001$]. Post hoc analysis revealed that replacement with testosterone and DHT restored prostate weights to that of sham controls. Nandrolone replacement had little effect in the prostate and was found to be ineffective, and therefore, sections were not counted for this treatment group.

TH-IR cell number was increased with castration relative to sham controls. Testosterone replacement resulted in a reduction in cell number that was comparable to sham controls had no effect on cell number in the SNpc (*Fig. 5-5A and B*). ANOVA revealed a significant effect of treatment [$F(3,22) = 3.3$ $p<.05$] in the SNpc and VTA [$F(3,22) = 3.3$ $p<.05$]. No significant differences were observed in TH-IN cell number in the SNpc and VTA (*Table 7*).

Topographical analysis of the SNpc revealed a global increase in average TH-IR cell number with castration (*Fig. 5-6A and B*). In the SNpc, ANOVA indicated a main effect of section [$F(7,107) = 27.9$, $p<.0001$]. In the VTA, ANOVA also indicated a main effect of section [$F(7,120) = 8.3$, $p<.0001$]. No section x surgery interaction was observed for both regions.

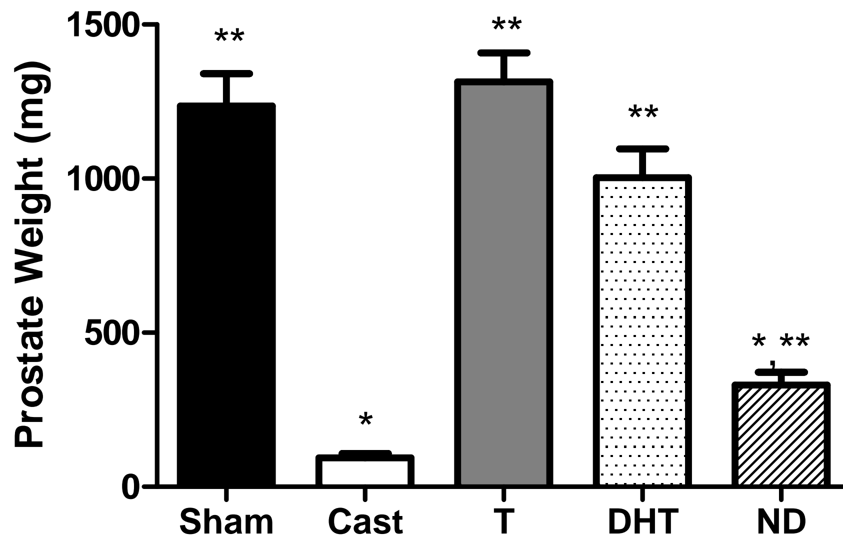
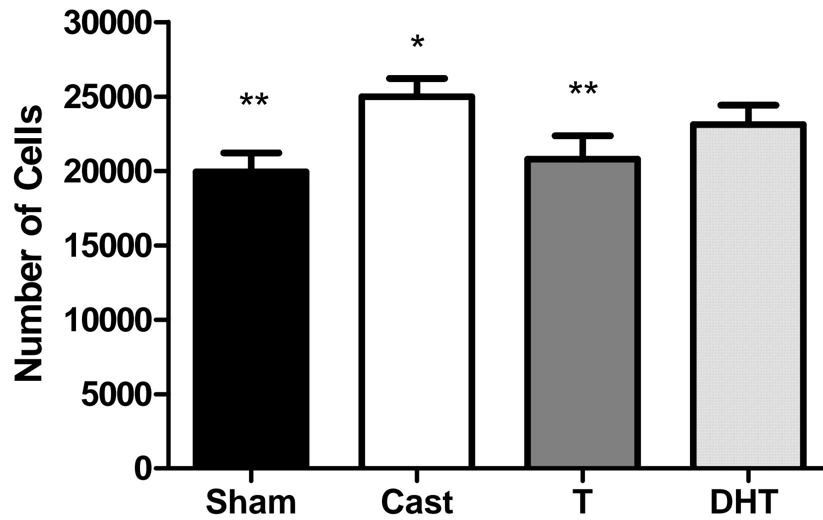


Fig. 5-4. Effect of testosterone (T), DHT and nandrolone (ND) on prostate weight in male rats compared to vehicle-treated sham castrated and castrated controls. Animals were cast or sham cast at PN60 and treated with androgens for one month post-surgery. Data are represented as mean ± SEM. * indicates different from sham castrated and ** indicates different from castrated ($p<.0001$). ($n = 5\text{-}8$)

A



B

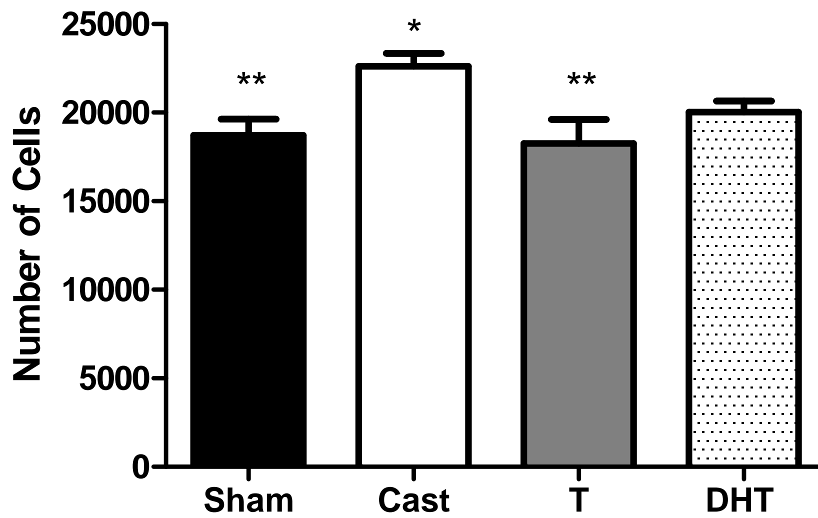


Fig. 5-5. Effect of testosterone (T) and DHT on TH-IR cell number in the SNpc (A) and VTA (B) of male rats compared to vehicle-treated sham castrated and castrated controls. Animals were castrated or sham castrated at PN60 and treated with vehicle or androgens for one month post-surgery. Data are expressed as mean ± SEM (n = 4-7). * indicates different from sham castrated and ** indicates different from castrated, $p<.05$.

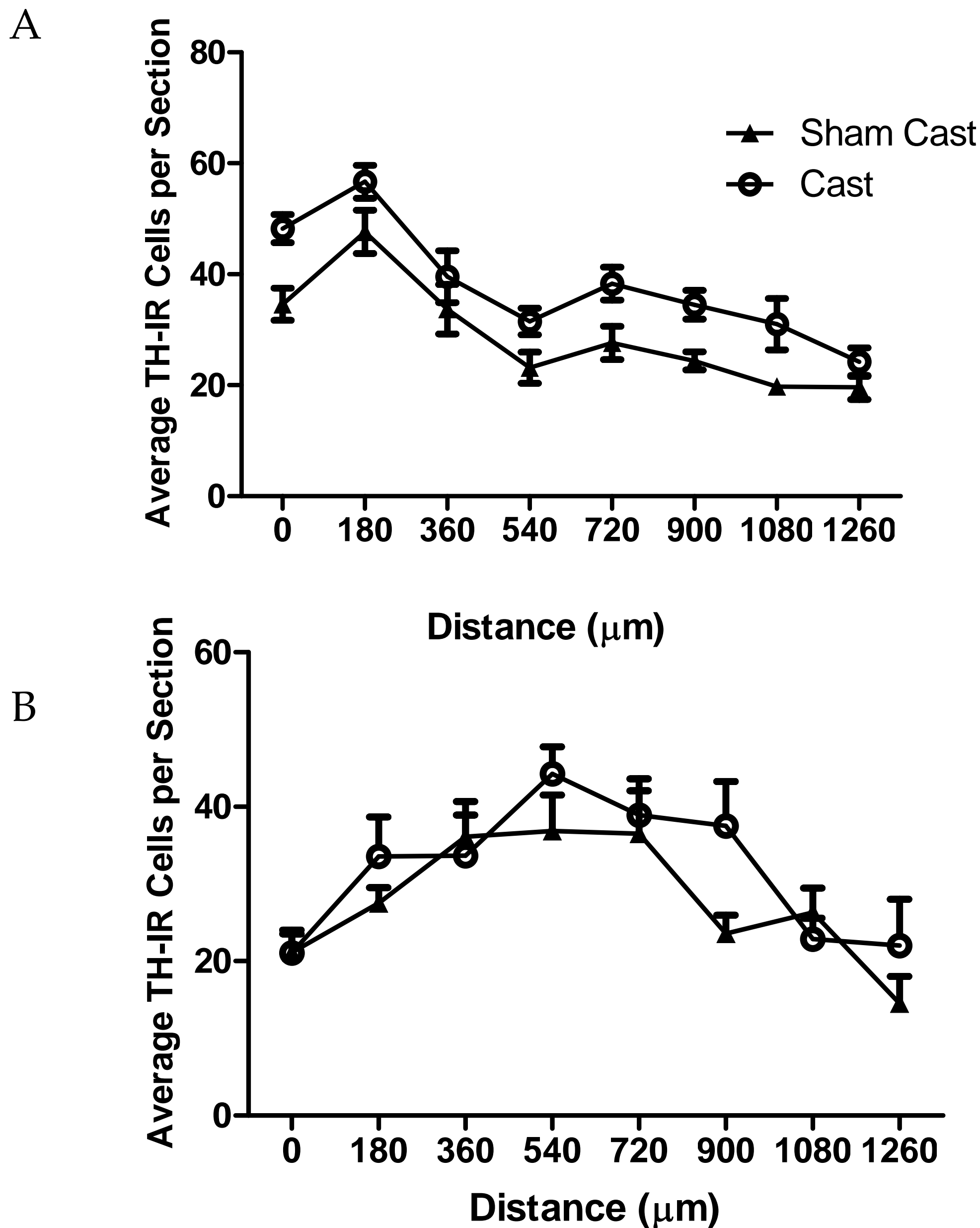


Fig. 5-6. Topographical analysis of TH-IR cell number throughout the extent of the SNpc (A) and (B) VTA in sham castrated and castrated rats. These graphs show the average of the left and right SNpc and VTA at each distance (rostral to caudal) through the extent of each region. The most rostral section is 0 μm. Sham animals are represented by the closed shapes and ovx are represented by open shapes. Data are expressed as mean ± SEM (n = 12 for SNpc and n = 8-10 for VTA).

Table 7. Estimated total number of TH-IN cells in the SNpc and VTA of hormone replaced males

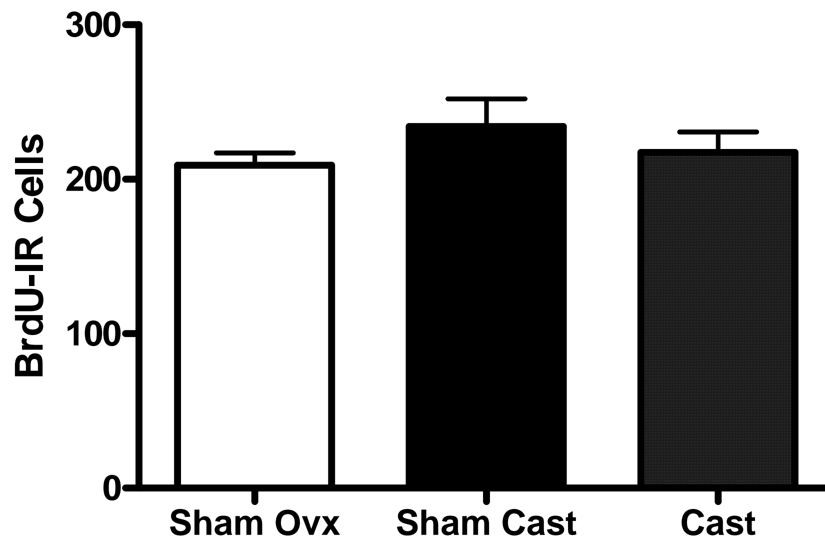
TH-IN Cell Number - SNpc		**TH-IN Cell Number - VTA**	
Sham Castrated	**3775 ± 704**	***Sham Castrated***	**2341± 534**
Castrated	**2588 ± 250**	***Castrated***	**2048 ± 241**
Testosterone	**3930 ± 888**	***Testosterone***	**2380 ± 466**
DHT	**3124 ± 528**	***DHT***	**1749 ± 505**

TH-IN cell number was determined in male hormone replacement animals. No significant differences were observed between surgical groups. In the SNpc, p=.60 and in the VTA, p=.88. (n = 4-7)

Low Rate of Neurogenesis in the Substantia Nigra

To determine whether the increase in cell number during long-term castration was due to the birth of new neurons, we investigated possible neurogenesis in the SNpc of sham ovariectomized, sham castrated and castrated rats. Approximately 200 BrdU-IR cell bodies in the SNpc of each brain were counted (*Fig. 5-7*). No differences were observed in the number of newly formed cells among the surgical groups. Double immunofluorescence revealed that 1-2 of these cells per section were double immunoreactive for BrdU and TH (*Fig. 5-8*). However, confocal imaging revealed no colocalization in cells stained for the astrocytic marker glial fibrillary acidic protein (GFAP) and BrdU, indicating that none of the new cells were astrocytes (*Fig. 5-9).*

A



B

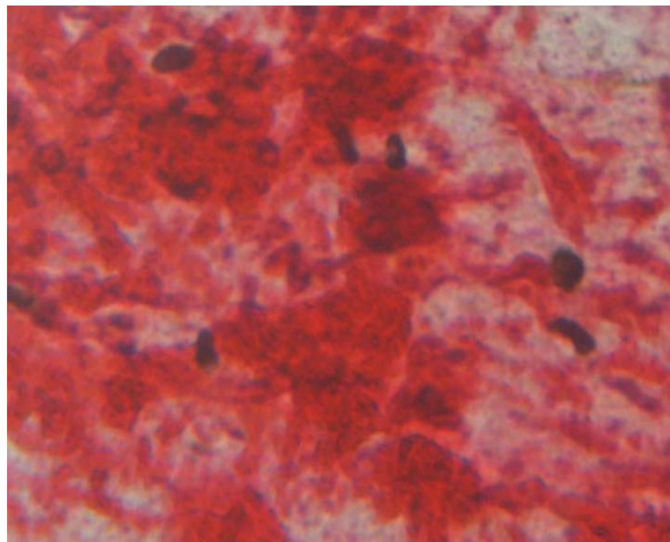


Fig. 5-7. Effects of gonadal hormones on neurogenesis in the SNpc. Total number of BrdU-IR cells in the SNpc of sham gonadectomized and gonadectomized rats (A). Data are presented as means ± SEM. No significant differences were found in BrdU-IR cell number ($p = 0.47$). Representative photomicrograph taken at 40x magnification in a castrated male rat in a BrdU-TH double labeled section (B). BrdU-IR labeled nuclei are the small, black dots and TH-IR cells and fibers are shown in red.

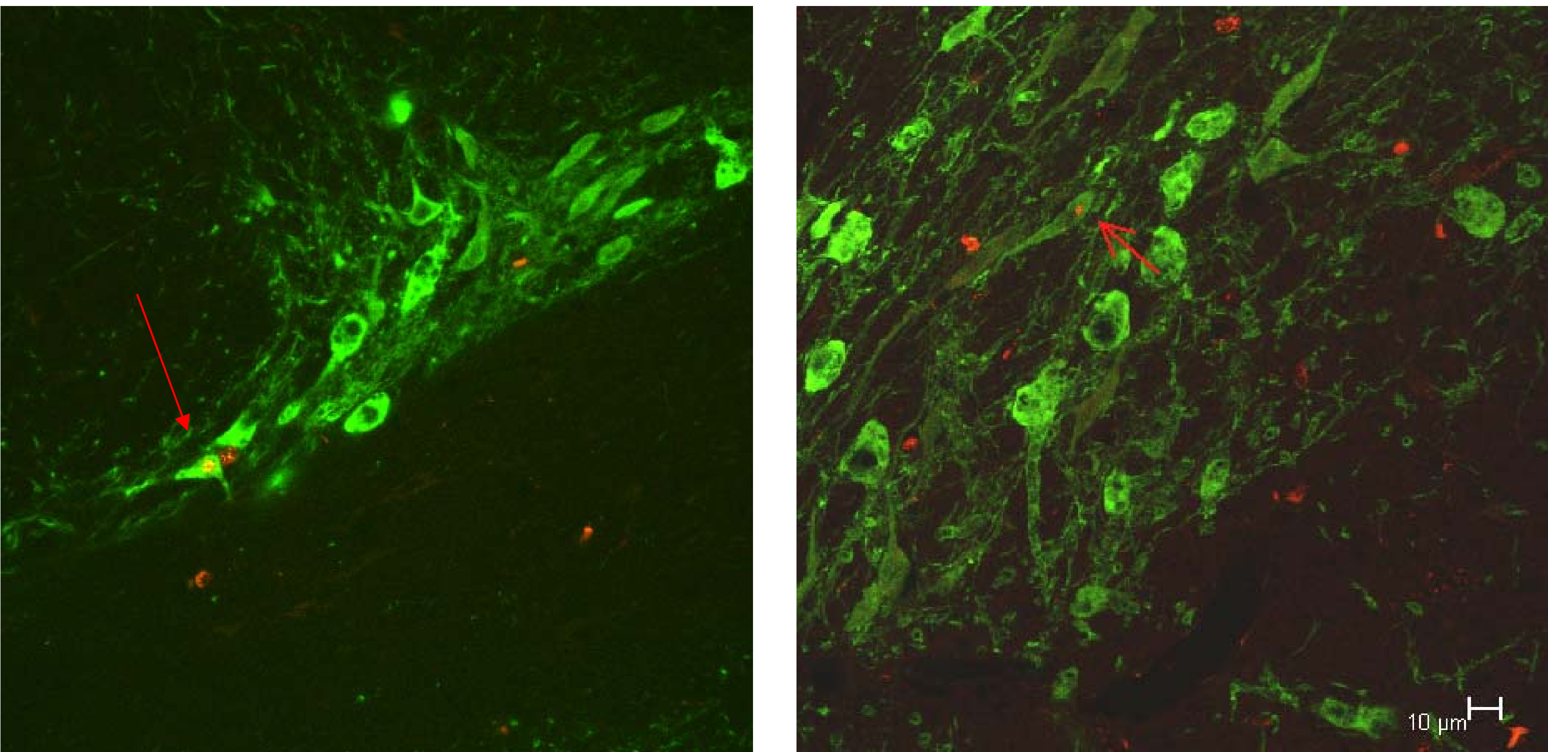


Fig. 5-8. Immunofluorescent staining of TH (green) and BrdU (red) double labelled cells in the SNpc.

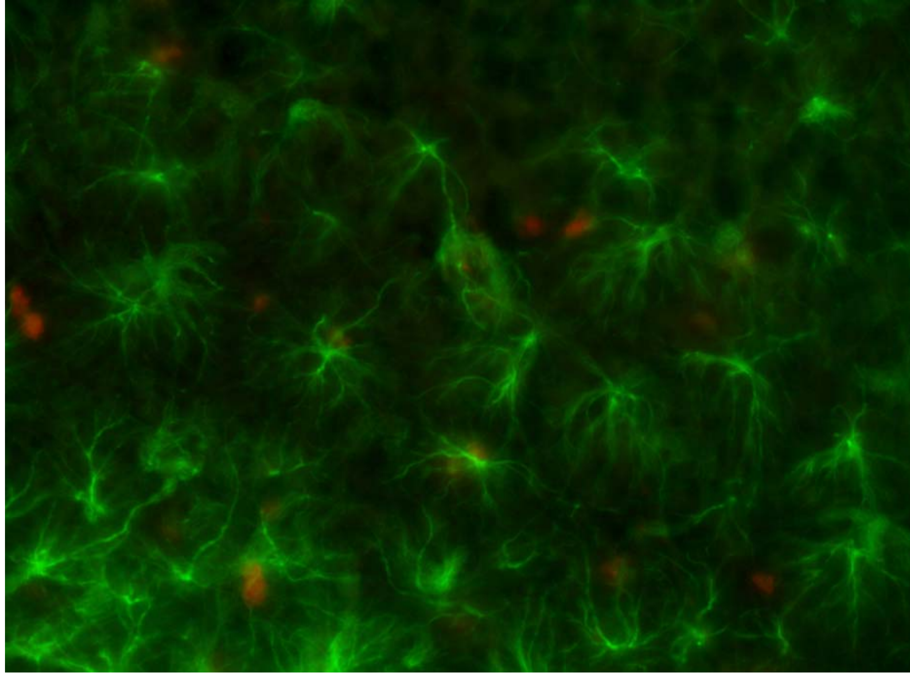


Fig. 5-9. Double immunofluorescent staining of GFAP-IR astrocytes (green) and BrdU –IR nuclei (red). Taken at 20x magnification.

5.4 Discussion

In this study, we found castration in adulthood increases TH-IR cell number in the SNpc and VTA of both rats and mice. In the prostate, nandrolone treatment had minor effects in the prostate, suggesting that a higher dose of nandrolone is needed to evaluate its effects. DHT replacement resulted in a significant increase in prostate weight relative to castrated rats; however, TH-IR cell number was not different from either sham or castrated controls in midbrain regions. In the SNpc and VTA, testosterone reduced TH-IR cell number to that of sham castrated controls, suggesting that testosterone suppresses midbrain TH-IR neuron number. These studies also suggest that mechanisms of cell maintenance in males and females are different. It is possible that while estrogen has protective effects, testosterone naturally suppresses dopamine neuron number in midbrain regions, which may contribute to the sex differences observed in TH-IR neuron number, cocaine-stimualated behavior and electrically-stimulateddopamine release [5-7, 101]. The suppressive effects of testosterone on TH-IR cell number parallel findings in psychostimulant-induced behavior. Testosterone replacement decreases cocaine and amphetamine-stimulated behavior relative to castrated animals [78, 104, 105]. Testosterone in neonatal females suppresses locomotor responses to amphetamine in adulthood. Testosterone has also been shown to suppress dopaminergic function in dopaminergic pathways. In castrated mice, potassium-stimulated striatal dopamine output was greater than in testosterone-replaced mice

[151]. Castration has also been shown to increase innervation density of TH-IR projections in cortical regions, an effect that is suppressed with androgen treatment [152, 153].

Studies in animal models of neurotoxin-induced damage show that testosterone does not have protective effects in the NS pathway. In mice, testosterone does not protect against depletion of striatal dopamine concentration after MPTP lesion [82]. In rats receiving unilateral 6-OHDA lesions, castration was shown prevent the loss striatal dopamine content, while DHT was replacement ineffective [97, 98], suggesting that androgens have no trophic effects in the NS pathway.

One possibility for the increase observed in TH-IR cell number with castration could be that loss of testosterone increases neurogenesis in the midbrain. It is well established that neurogenesis occurs in the adult hippocampus [150, 154]. Studies have shown that hippocampal neurogenesis can be influenced by gonadal hormones [155, 156]. In the dentate gyrus, cell proliferation in female rats is greatly influenced by the estrous cycle as cell proliferation was found to be greater during proestrus, when estradiol levels are highest, compared to cell proliferation in estrus and diestrus. During proestrus, females also produce more new cells than males in the dentate gyrus [157]. Ovariectomy resulted in a decrease in the number of cells BrdU-IR, which was prevented with estradiol replacement [155, 157, 158]. A recent study in adult male mice shows that treatment with estrogen can increase TH-IR cell number in the SNpc.

The role of androgens in neurogenesis is unclear. Studies have shown that testosterone stimulates neurogenesis in the hippocampus, while castration decreases cell proliferation [159]. In contrast, a study in which adult male rats were replaced with the anabolic steroid 19-nortestosterone (nandrolone) revealed that nandrolone significantly reduced neurogenesis in the dentate gyrus [154].

In recent years, the possibility of neurogenesis in the SNpc has been investigated. However, unlike the hippocampus, nigral neurogenesis has been shown to occur at low rates with very few of the newly born cells committing to a dopaminergic phenotype [160-163]. The discovery that castration resulted in the dramatic increase in TH-IR cell number was exciting and suggested that hormone-mediated nigral neurogenesis could be occurring. Our findings, however, were concordant with the literature. We detected BrdU-IR nuclei in the SNpc, but counted approximately 200 BrdU-IR nuclei in each surgical group. No differences were found between surgical groups. Many of the BrdU-IR nuclei that were found were not TH-IR and did not colocalize with GFAP-stained astrocytes. Based on these findings, gonadal hormones do not play a role in the influence of normal adult neurogenesis in the SNpc, and it is unlikely that an increase in TH-IR cell number of the magnitude shown in this study is due to newly generated dopaminergic cells.

6. Conclusions

Previous reports from this laboratory revealed that adult female rats exhibit greater cocaine-stimulated behavior and electrically stimulated dopamine release than males. The purpose of this thesis was to determine an underlying mechanism for these sex differences by quantitating TH-IR cell number in midbrain regions that regulate these behavioral and neurochemical responses. In this study, a sex difference was observed in TH-IR cell number in the SNpc and VTA with females having greater cell numbers than males. This finding was not only consistent with the previously published findings, but suggested that there was an anatomical mechanism underlying these differences. The role of gonadal hormones in the maintenance of neuronal populations in the rat and mouse SNpc and VTA was also investigated. We showed that ovariectomy decreases TH-IR cell number in midbrain regions and that replacement with estrogenic compounds can maintain dopamine neuron number. In addition to this, we found that castration increases TH-IR cell number in the SNpc and VTA relative to the sham control and that replacement with testosterone suppresses this increase. The results of this thesis suggest that both ovarian and testicular hormones mediate the trophic and suppressive effects, respectively, on dopamine neurons in the SNpc and VTA. Finally, we showed a functional link between ovarian hormone-maintained nigral dopamine neuron number and cocaine-stimulated behavior and striatal dopamine release.

In the first specific aim, immuohistochemistry and stereologic techniques were used to determine TH-IR cell number throughout adolescence in male and female rats. Our data showed that a decrease in TH-IR cell number occurs with age in both male and female rats and demonstrate that females stabilize at a younger age and are protected from further loss after puberty. This finding is concordant with other studies. A gradual decrease in dopamine neuron number with age has been observed in the SNpc of primates and humans [21]. The decrease observed throughout ontogeny was surprising because we have found, in agreement with published studies [164], that dopamine content is increasing through adolescence. One explanation for this may be that even though there are more cells in the adolescent SNpc and VTA, these cells are immature and do not innervate target regions. We did not directly answer these questions as the focus of this thesis was to assess cell number in the SNpc and VTA.

We also found that adult female rats have more dopamine neurons in adulthood than males in the SNpc and VTA. The results of Specific Aims 2 and 4 further support this finding. While ovariectomy reduced TH-IR cell number in midbrain regions, replacement with E2 and ER agonists PPT and DPN prevented this loss and maintained cell number comparable to intact females. The incorporation of data from ERKO mice suggest that ERα may play a more significant role as TH-IR number was significantly reduced only in the αERKO relative to WT. These data suggest that ERα may mediate

both the organizational and activational effects of estrogen and that estrogenic effects mediated through ERβ may be purely activational.

The discovery that drugs targeting ERβ are equally as effective as those targeting ERα presents possible therapeutic avenues for the PD field. Studies in models of PD, stroke and Alzheimer's disease suggest that the protective effects of estrogen are mediated through ERα [49, 52–54, 56, 65, 67, 165]. In contrast, other models of brain injury and neurodegeneration, have shown DPN to be an effective therapy [133, 165-167]. E2 and other ERα-selective agonists have problematic side effects. They act as agonists in several tissues and has led to the development of cancers effecting the reproductive tissues in women receiving hormone replacement therapy [168]. Targeting of ERβ may provide an alternative method of treatment that reduces this risk. The data presented in this thesis suggest that an ERβ-selective agonist could be a useful therapy for PD could open the door for future studies examining the role of ER-selective drugs in the rat model of PD to assess recovery and cell protection in response to treatment with these compounds.

Few studies have investigated the role of testosterone in midbrain regions. The data presented in this thesis are the first to describe a suppressive role of testosterone on TH-IR cell number in the SNpc and VTA. Prior to these findings, animal PD models revealed testosterone had no protective effects on striatal dopamine content and that male rats suffered more severe 6-OHDA lesions than females [169]. These findings

suggest that a combination of the maintaining effects of estrogen and testosterone's suppressive effects on midbrain dopamine systems may contribute to a higher incidence of disorders of dopaminergic dysfunction in men than women.

Finally, a functional link between nigral TH-IR cell density and cocaine-stimulated behaviors was made in this thesis. The findings of this study replicated the decrease in cell number, cocaine-stimulated behavior and dopamine release observed with ovariectomy. Ovariectomized rats were not different from the sham controls during habituation in the behavioral experiments, and they were not different from the sham animals at baseline dopamine release. The loss observed with ovariectomy (20-25%) would not be enough to induce severe behavioral deficits [21]. This is further supported by the lack of difference observed in striatal dopamine content of sham ovariectomized and ovariectomized rats. These data suggest that in the ovariectomized rats there is some compensation at the terminal level. We found significant positive correlations in SNpc cell density and cocaine-stimulated behavior and electrically-stimulated dopamine release after cocaine, suggesting that the maintenance of dopaminergic cell populations by estrogen is required to elicit normal dopamine-dependent behavioral and neurochemical responses. Fig. 6-1 shows a three-dimensional representation of the three measures assessed in the correlational study. The animals with the highest cell densities also exhibited the greatest amount of cocaine-stimulated behavior and electrically-stimulated dopamine release. This unifies the effects of

estrogen on several individual measurements. To our knowledge, this is the first demonstration that the highest psychostimulant-induced behavioral responses occur in individuals with higher dopamine neuron density in the SNpc and this relationship is supported by the increase in dopamine release. The high repsonders in this study were all intact females which may provide a mechanism for the increased vulnerability of females to certain aspects of psychostimulant addiction.

In conclusion, this thesis marks the first in-depth study of the effects of gonadal hormones on midbrain dopamine neurons. This is also the first study to demonstrate a functional link between cocaine-stimulated behavior, dopamine release and cell density. These findings should advance understanding of sex differences in both the addiction and neurodegeneration fields.

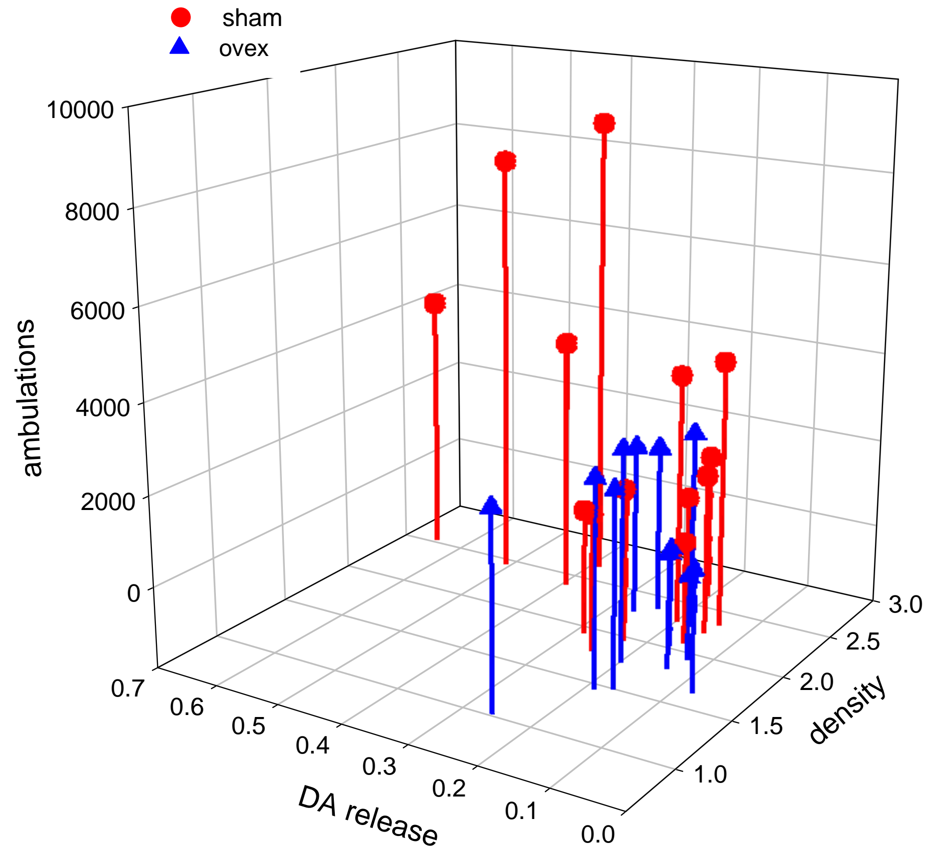


Fig. 6-1. Three-dimensional representation of TH-IR cell density in the SNpc, electrically-stimulated dopamine release and cocaine-stimulated behavior in individual animals presented in Chapter 4. Animals sham ovariectomized (red) and ovareictomized (ovex, blue) at PN55 were subjected to behavioral and neurochemical analysis on PN90. Animals were perfused on PN90 and brain collected for TH immunohistochemistry to determine cell density in the SNpc. Animals with higher cell densities also showed the higher behavioral and neurochemical responses than animals with lower cell densities.

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