ESTROGEN-DEPENDENT PROGRAMMED CELL DEATH DURING DEVELOPMENT OF THE HYPOTHALAMUS.

by

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A DISSERTATION

Presented to the Neuroscience Graduate Program

and the Oregon Health and Sciences University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

April 2004

School of Medicine
Oregon Health and Sciences University

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Acknowledgements

I would like to thank my advisor, Dr. Richard Simerly, and my committee members, Dr. Philip Copenhaver, Dr. Janis Weeks, Dr. Larry Sherman, and Dr. Mihail Iordanov for their support, suggestions, and criticisms. I also want to thank Melissa Kirigiti, Shin Draper, Dr. Sebastien Bouret, Dr. Eva Polston, and Stephen Chen for technical and editorial assistance. Finally, I want to thank Chris and Aurelia Littlepage for their patience.

ABSTRACT

Brain development requires precision in order to complete the neural circuitry necessary for an animal to perform the wide range of functions linked to survival. Development of the hypothalamus is critically important to successful reproduction. During hypothalamic development, sex steroids direct the refinement of neuroarchitecture to differentiate the brains of females and males and determine the reproductive capability of the animal. The sexually dimorphic hypothalamic areas that develop as a result of sex steroid exposure include the anteroventral periventricular nucleus of the preoptic area (AVPV). Postnatal development of the AVPV depends on the interactions of estrogen with the alpha form of the estrogen receptor to direct both the protection and the destruction of various neuronal populations. Estrogen, similarly to other neurotrophic molecules, affects cell fate through initiation of gene transcription and signal transduction pathways to regulate cell survival and programmed cell death mechanisms. Estrogen-regulated AVPV cell populations include dopaminergic and prodynorphin neurons that are greater in number in the female and proenkephalan neurons that are greater in number in male. I describe an *in vitro* model that recapitulates estrogen-regulated sexual differentiation of AVPV neurons. I also use both an *in vivo* and *in vitro* model of AVPV sexual differentiation to demonstrate that the estrogen-induced loss of dopaminergic neurons is mediated by caspase-dependent programmed cell death.

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INTRODUCTION

During development, a variety of epigenetic factors act on the brain to determine cell number and direct the formation of neuronal architecture. Epigenetic factors integrate the genetic code with environmental conditions to direct the development of an organism into a healthy, fecund adult. Among these factors are the sex steroid hormones that influence the development of sexually dimorphic brain nuclei. These areas, which develop differently in females and males, are altered by steroid-mediated developmental programs. Importantly, the development of sexually dimorphic neural circuitry is required to achieve both reproductive status and sex specific behaviors in mammals. The processes of sexual differentiation, which can be mediated by sex steroid hormones, include differential neurogenesis, cell survival, death, or migration. However, only cell death has garnered sufficient evidence to support its role in the development of sexual dimorphisms.

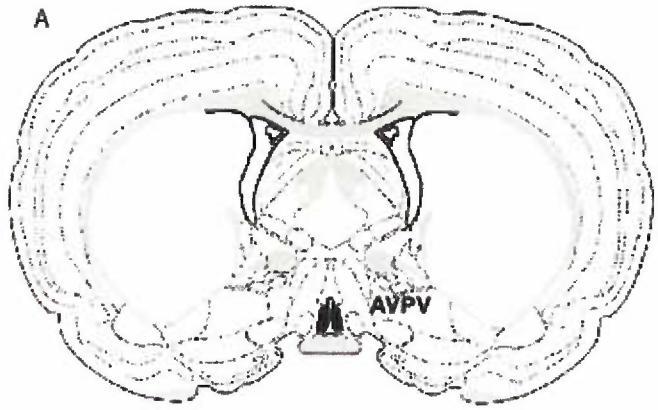
Sexual dimorphisms in the brain are generally established early in postnatal development when levels of circulating gonadal steroid hormones differ markedly between males and females. It is well known that sexual dimorphisms are established early in development under the influence of gonadal steroid hormones and depend on the presence of the

cognate steroid receptors. However, the mechanisms of sex steroid action downstream of steroid receptors are poorly understood. There is a need to understand more completely the effects of sex steroids on brain development by identifying sex steroid-mediated cellular processes, particularly neuroprotective and neurodestructive mechanisms, that alter brain organization.

Development of the preoptic hypothalamus

The hypothalamus is a vastly heterogeneous brain region with complex chemo- and neuroarchitecture that is responsible for coordinating a wide range of information received from other areas. It functions as a neuroendocrine motor system maintaining homeostasis and generating signals that alter hormone secretion and behavior. In rodents, development of the preoptic hypothalamus begins in the neuroepithelial lining of the third ventricle on embryonic day 12. At this time, the neural tube has closed and the forebrain can be distinguished from other parts of the brain (for review see: Markakis 2002). As neurons migrate into the hypothalamus, the earliest born neurons take positions in the lateral portion of the hypothalamus, and the latest born neurons reside in the periventricular zone. The mature hypothalamus is represented by four rostrocaudal levels: 1) preoptic; 2) anterior; 3) tuberal; and 4) mammillary, and three mediolateral

zones: 1) lateral; 2) medial; and 3) periventricular (for review see: Simerly 1998). A particular region of interest in the hypothalamus because it is smaller in size in the male than in the female is the anteroventral periventricular nucleus (AVPV), located in the preoptic periventricular zone (Fig. 1 A). The neurons in the preoptic hypothalamus are generated between embryonic days 12 and 17 (Altman and Bayer 1978a, 1978b); however, AVPV neurons are born between embryonic days 14 and 17 (Nishizuka et al. 1993). Surprisingly, the AVPV consists of mostly neurons, since colocalization with the DNA marker Hoechst and the nuclear neuronal marker NeuN was nearly complete, suggesting that most cells in the AVPV are neurons (Fig. 1 B). The brain's first exposure to the differentiating effects of gonadal steroid hormones is a surge of testosterone that occurs on embryonic day 18 (Baum et al. 1991). AVPV neurons, therefore, are not only post-mitotic at birth but are also born prior to testosterone exposure. Indeed, Arai et al. demonstrated with BrdU labeling that neurogenesis is not steroid-dependent (Nishizuka et al. 1993) and cannot explain the differences in volume that distinguishes the female AVPV from the male AVPV. Instead, sexual differentiation of the AVPV is regulated by steroid-mediated mechanisms that control cell fate: the choice between survival or death.



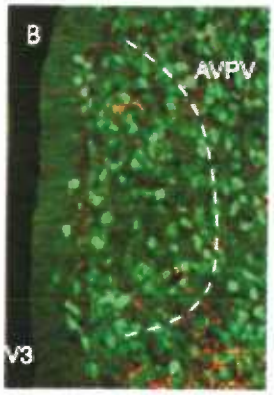


Figure 1. **The AVPV is located in the preoptic periventricular zone of the hypothalamus.** The AVPV is highlighted in black on plane 18 of the Swanson atlas (A). Staining of the AVPV with the nuclear neuronal marker NeuN (green) and glial marker GFAP (red). The majority of AVPV cells appear to be neuronal.

Sexual differentiation

Gonadal hormones act on the brain throughout life, regulating reproductive behaviors and physiological functions in female and male rodents. During development, estrogen and testosterone alter brain morphology by determining cell number and creating specific sexually dimorphic areas that are implicated in the ultimate manifestation of female and male behaviors and reproductive functions. These effects of estrogen and testosterone are mediated by their respective steroid receptors. These receptors are widely expressed in the brain, particularly in sexually dimorphic areas, and confer hormone sensitivity upon these tissues (DonCarlos 1996, McAbee and DonCarlos 1998). In adults, activation of steroid receptors by gonadal hormones alters gene expression events that are required for normal reproductive behavior and physiology (Pleim et al. 1989). In neonates, gene expression of survival and death factors regulated by sex steroids may be linked to differentation of sexually dimorphic neural circuitry.

A variety of anatomical, biochemical, physiological and behavioral sex differences are dependent upon the developmental effects of gonadal steroid hormones. Not surprisingly, the development of sexually dimorphic areas of the hypothalamus have been associated with reproductive function and behavior in both males and females. Sexual

differentiation is widespread in the nervous system and includes hormone-dependent alterations in the volume and numbers of neurons in brain nuclei, in the size and phenotype of individual neurons, and in dendritic arborization and synaptic density (for review see: Cooke et al. 1998, Chung et al. 2002). In addition, sexual dimorphisms in the density of axonal projections between areas containing steroid receptors have been described (for review see: Simerly 2002). Sex differences in dendritic arborization and synapse density have been detected in the ventromedial nucleus of the hypothalamus (VMH) (Matsumoto and Arai 1986, Miller and Aoki 1991), as well as in the arcuate nucleus (Matsumoto and Arai 1980, Perez et al. 1990). Common to all these sexual dimorphisms is the presence of receptors for steroid hormones in the neurons.

Sexually dimorphic brain areas demonstrate a high degree of heterogeneity. Nuclei within each brain area may differ with respect to the type of sexual dimorphism present. For example, the bed nuclei of the stria terminalis (BST) have many components, but only the principal nucleus of this region has been shown to be sexually dimorphic (Simerly 2002). The principal nucleus is larger in volume and contains more neurons in the male nucleus than in the corresponding female nucleus. In general, most sexually dimorphic brain areas have more neurons in males relative to the corresponding nuclei in females. For example, the sexually dimorphic nucleus of the preoptic area (SDN-

POA)(Gorski et al. 1978), medial amygdala (Mizukami et al. 1983, Hines et al. 1992), and accessory olfactory bulb (Segovia et al. 1984, Roos et al. 1988) are all larger in males. Also in the central nervous system, the spinal nucles of the bulbocavernosus is larger in males than in females (Breedlove and Arnold 1983). In contrast, the female AVPV has more cells than the male nucleus. The post-natal reduction in cell number that occurs in males is dependent on gonadal steroids. Sex differences in the numbers of neurons with specific phenotypes have also been described. In the female rat AVPV, there are more dopamine, galanin, calcintonin gene-related peptide, and neurotensin-containing neurons than in the male nucleus (Simerly et al. 1985, Alexander et al. 1991, Herbison 1992, Bloch et al. 1993). In contrast, the male AVPV of the rat contains more enkephalin neurons than the corresponding female nucleus (Simerly et al. 1988).

The actions of gonadal hormones are mediated by steroid receptors, which are nuclear transcriptional activators (Tsai and O'Malley 1994). Binding of the steroid to the receptor results in successive conformational changes that free the receptor from an inactivating complex, transforming it into an active state. In the activated state, receptors dimerize and bind to steroid response promoter elements of steroid-regulated genes to produce changes in gene expression. While this action has largely been described in the periphery, the presence of steroid receptors and their accessory transcriptional

coregulators in the central nervous system suggests that similar mechanisms mediate the central actions of steroids. In the brain, ligand-activated steroid receptors may interact with nuclear receptor coactivators and corepressors that augment or reduce gene expression respectively (McKenna and O'Malley 2002). Regulation of steroid receptors and coregulators in a tissue- or cell- specific manner can contribute to the variations in steroid responses detected during development of sexually dimorphic neuronal populations.

There are two isoforms of the estrogen receptor (ER), alpha and beta, that may have opposite effects on gene expression (Paech et al. 1997, Liu et al. 2002). In particular, ERbeta (ERβ) may play a role in regulating the expression of signaling molecules, perhaps terminating cellular responses instituted by the ERalpha (ERα)(Temple et al. 2001). Two additional mechanisms, ligand-independent transcriptional activation and ligand-dependent non-genomic actions, may also mediate the cellular effects of estrogen receptor activation (for review see: Wise et al. 2001). Estrogen receptors have been demonstrated to mediate kinase activation (Singh et al. 1999, Wade et al. 2001) and to interact directly with phosphatidylinositol 3-kinase (PI3-K),(Simoncini et al. 2000). Interestingly, ER interactions with PI3-K signaling may link ERα signaling to growth factor signaling pathways, including that of insulin-like growth factor (Kahlert et al.

2000). One signaling molecule that may play a role in nongenomic ER effects is cAMP-responsive element binding protein (CREB). Estrogen-dependent induction of phosphorylated CREB (pCREB) has been reported in several areas that contain both ERs and sexually dimorphic features, including the amygdala (Beyer and Karolczak 2000), AVPV (Gu et al. 1996), and BST (Zhou et al. 1996). In addition, estrogen was reported to mediate a sexually dimorphic induction of pCREB in the VMH of adult rats (Auger et al. 2001). Estrogen's effects on genes that lack estrogen response elements could also occur through cAMP response elements (Zhou et al. 1996). Thus, through many different mechanisms, ERs may have opposite effects on gene expression, and also impact diverse signaling cascades.

In the male, testosterone is secreted and freely transported to steroid-sensitive tissues. Testosterone may also be converted locally to estradiol by aromatization (Simpson, et al. 1994) and therefore be able to affect brain areas that lack the androgen receptor but contain ER. In contrast, in the female, the presence of alpha-fetoprotein, which only binds freely circulating estrogen and not testosterone, prevents estrogen exposure in the female brain. Steroid actions that affect brain structure, including cell number, size, and arborization, are generally thought of as *organizational.* In contrast, steroid actions during adulthood, which can affect neurochemical secretion or rapidly affect neuronal

ultrastructure, are designated *activational* (Arnold and Breedlove 1985). However, it is important to emphasize that both of these actions may not be limited to a single time period in an animal's life, but rather they may reflect differences in plasticity among various brain sites. Although hypothalamic sexual dimorphisms in nucleus size and cell number are generally established during the perinatal period in rodents, hippocampal synaptic density in adults increases in an estrogen-dependent fashion during the course of the estrous cycle, a decidely female-specific event (Woolley and McEwen 1992). Moreover, steroid-sensitive plasticity in song birds has also been reported to cause sex differences in cellular architecture and phenotype into adulthood (for review see: Arnold et al. 1996).

The earliest evidence for a sex difference in cell number was observed in the preoptic region by Gorski and colleagues in 1978. Subsequently, a great deal of progress has been made in the characterization of sexually dimorphic features and developmental requirements of the preoptic region, which illustrates many of the properties that are shared amongst sexually dimorphic nuclei. The volume of sexually dimorphic nucleus of the preoptic area (SDN-POA) is approximately 3 fold greater in males due to a greater number of neurons than in the corresponding female nucleus (Gorski et al. 1980). The SDN-POA consists primarily of the central part of the medial preoptic nucleus (MPNc)

(Simerly et al. 1984, Bloch and Gorski 1988). In addition to differences in size and cell number, the SDN-POA also exhibits sex differences in its neurochemical composition. For example, males have more cholecystokinin-containing cells than females (Micevych et al. 1987), while females have more calcitonin gene-related peptide-containing cells than males (Herbison 1992). These differences, as well as those in volume, size, and cell number, can be abolished or reversed by perinatal gonadectomy of males or perinatal testosterone or estrogen treatment of females (Gorski et al. 1978, Jacobson and Gorski 1981, Dohler et al. 1984). After the seventh postnatal day, the "critical period" for differentiation has ended, and cell number can no longer be altered by manipulations of the sex steroid environment (Dohler et al. 1982, Rhees et al. 1990, Herbison and Dye 1993, Dodson and Gorski 1993). Since the initial description of the SDN-POA, many more gonadal steroid hormone-regulated sexual dimorphisms have been reported in the brain, illustrating the widespread occurrence of steroid-directed development (for review see: Madeira and Lieberman 1995).

Sex differences in cell number could result from sex-specific rates of cell birth, differentiation, migration or death. Among these hypotheses, only cell death has been implicated in the development of brain dimorphisms. In the SDN-POA and other sexually dimorphic nuclei whose developmental profiles parallel the SDN-POA, there is

accumulating evidence that cell death occurs in the absence of sex steroids and neuroprotection occurs in the presence of the sex steroids. Programmed cell death (PCD) utilizes a genetic program of cell suicide to eliminate cells through several stereotypic process, the most common process in the mammalian nervous system is apoptosis. During sexual differentiation of nuclei that are larger in females than in males, including the MPNc and in the principal nucleus of the bed nucleus of the stria terminalis (BSTp), more apoptotic cells were detected in females than in males. This sex difference in apoptotic markers was sex-reversed by gonadectomy or steroid treatment (Davis et al. 1996, Chung et al. 2000). In brain nuclei that are larger in males than in females, testosterone treatment also increased the incidence of pyknotic cellular nuclei in the AVPV (Murakami and Arai 1989). These results indicate that actions of sex steroids regulate the degree of cell survival and death as the primary mechanism for sexual differentiation of cell number. Unfortunately, despite the growing evidence implicating PCD in sexual differentiation, possible mechanisms for steroid regulation of cell death and survival remain poorly characterized. Studies, which examine multiple PCD markers within the same system, are required for decisive analysis of the role of PCD in sexual differentiation.

Neuroprotection and estrogen

A role for estrogen in determining cell survivability has been recognized in the field of neuroprotection. Identification of genes regulated by estrogen has been particularly important in elucidating the mechanisms of neuroprotection. The expression patterns of these genes can shed insight into the mechanisms by which testosterone and estrogen may alter cell number via regulation of cell survival and death. In models of neuroprotection, estrogen has been demonstrated to induce the expression of genes for neurotrophins and anti-apoptotic proteins. Estrogen effects also include the reduction of both necrotic and programmed cell death. Both *in vitro* and *in vivo* neuronal insult models have sought to elucidate the estrogen-mediated signaling mechanism that links gene expression to neuroprotection. Estrogen prevented cell loss in hippocampal and cortical cultures after an apoptotic insult, an effect that was inhibited by estrogen antagonists or PI3-K inhibitors (Wilson et al. 2000, Harms et al. 2001). These results suggest a role for estrogen receptors in neuroprotection. Regulation of cell death and survival factors by estrogen has also been described. Estrogen increases Bcl-2 gene expression in cortical neurons and in the arcuate nucleus of the hypothalamus (Garcia-Segura et al. 1998, Dubal et al. 1999). Estrogen regulation of the neurotrophin brain-derived neurotrophic factor (BDNF) mRNA and protein was reported in the rat

hippocampus during development (Solum and Handa 2002). Thus, through interactions with both protective and destructive cellular and molecular mechanisms, estrogen regulates and maintains normal brain function. A description of estrogen receptor coupling to the cell death pathway will be necessary to understand how the neuroprotective and neurodestructive actions of estrogen are balanced and utilized in the nervous system.

Programmed cell death during development

Programmed cell death (PCD) is fundamentally important during the development of most organisms. The removal of excess cells to increase the specificity of a system may be required for proper function. For example, tadpole tails are resorbed before becoming adult frogs (for review see: Tata 1998) and proleg motoneurons are eliminated during metamorphosis in *Manduca sexta* (for review see: Weeks 1999). In the nervous system, an initial profusion of cells born early in development results in excess neurons that hinder formation of functional behavioral and physiological systems. During development, extra cells are eliminated by PCD. Later in development, epigenetic factors may further regulate PCD to refine neural circuitry. In addition, although cell death may be inappropriately initiated in some neurodegenerative diseases such as Alzheimer's or

Parkinson's disease, PCD can also be utilized to eliminate injured or diseased cells related to ischemic and cancerous states.

Apoptosis is a form of programmed cell death, which has been proposed to play an important role in development and plasticity of the nervous system. Apoptosis consists of distinct morphological and biochemical events, characterized by a particular sequence of cellular events. These include activation of caspase proenzymes, externalization of phosphatidylserine to the cell surface, cytoplasmic shrinkage, loss of mitochondrial membrane potential, and membrane blebbing, in which large vesicles extruded from the cell and are subsequently phagocytosed without causing an inflammatory response. In addition to the destruction of structural proteins, PCD impairs DNA repair machinery and enhances protease activation, resulting in chromatin condensation and DNA fragmentation. Throughout the mammalian nervous system, these events are used as specific markers of PCD and blebbing of the nuclear and cytoplasmic compartments as a definitive morphological marker for apoptotic PCD (Fig. 2) (for review see: Clarke 1990, Wyllie et al. 1980).

Other forms of cell death, including autophagy and necrosis, also participate in development of the nervous system (Yuan et al. 2003). Autophagy has characteristics

similar to apoptosis but differs significantly in that autophagic vacuoles appear in the cytoplasm (many more than normal), and chromatin condensation is late or absent. Autophagy is most frequently reported in peripheral tissues of insects and chicks (Butterworth and LaTendresse 1973, Hurle and Hinchliffe 1978) although cases of neuronal autophagy have been observed in larval frogs and chicks (Fox 1973, Decker 1974, Lamborghini 1987). Necrosis, the other form of cell death, has been described in developmental motoneuron death (Chu-Wang and Oppenheim 1978). Necrosis results in the dilation of organelles and loss of ATP synthesis without condensation of chromatin. Although little in known about the mechanism of developmental necrosis, it does not result in the bursting and releasing of cellular contents that is associated with necrosis in pathological cell death.

Thirty years passed between the first description of neuronal cell death by Levi-Montalcini and Hamburger and the discovery of a group of cell death effectors, cysteine proteases, that display a preference for cleaving aspartate residues in *Caenorhabditis elegans*. The proteases, later designated as caspases, and the caspase regulatory proteins, designated as BCLs (from the B-cell lymphoma 2 protein, which was found to inhibit death), were initially discovered in *C. elegans* (Horvitz et al. 1982, Ellis and Horvitz 1986, Cory and Adams 2002). This discovery was rapidly followed by the identification

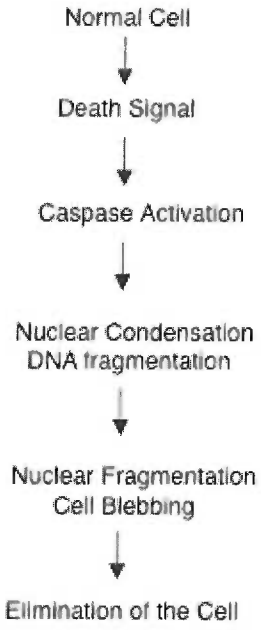


Figure 2. **The apoptotic programmed cell death pathway consists of a stereotyped series of cellular and molecular events.** These events, particularly morphological changes, can be used as markers of PCD.

of the mammalian BCL and caspase cell death molecules (Yuan et al. 1986, Hengartner and Horvitz 1994). Caspases are present constitutively as inactive pro-enzyme zymogens that are activated through two general mechanisms: 1) pro-apoptotic signals cause mitochondrial leakage of proteins capable of activating caspases, which are able to further activate other caspases, and 2) ligand-bound cytokine receptors activate intracellular death domains that assemble cell death proteins into complexes where caspases within close proximity activate each other (Bergeron and Yuan 1998, Meier et al. 2000). Two types of caspases have been identified based on their activity and protein composition. *Initiator* caspases are involved in the assembly of cell machinery that results in the cleavage and activation of executor caspases. *Executor* caspases implement PCD through the activation of proteases and cleavage of a variety of cellular molecules (Villa, Kaufmann et al. 1997). Generation of caspase "knock out" mice confirmed the widespread importance of caspase-mediated PCD: loss of functional caspases results in widely varying development defects, many of which are due to aberrant cell survival in the absence of apoptosis (Zheng and Flavell 2000, Oppenheim, Flavell et al. 2001).

Control of PCD initiation and caspase activation in response to cell death signals has largely been accredited to the BCL family of regulatory proteins (for review see: Earnshaw et al. 1999). Thus far, at least 20 BCL family members have been identified,

and, not surprisingly, they exhibit tissue-specificity and developmentally regulated patterns of expression (Cory and Adams 2002). The BCL family contains both pro- and anti-apoptotic proteins that form heterodimers, and have been implicated in the formation of mitochondrial pores (Martinou and Green 2001). Although the mechanism remains unclear, the BCL-mediated release of mitochondrial cytochrome *c* results in the activation of initiator caspases (Li et al. 1997, Ruiz-Vela, et al. 2002). Expression of BCL molecules may be differentially regulated by apoptotic signals (Martin et al. 1988). Other factors that mediate initiation of PCD continue to be identified, including members of inhibitor-of-apoptosis protein family that bind procaspases and prohibit caspase activity (Deveraux et al. 1998, Silke et al. 2002).

Two BCL family members, the pro-apoptotic Bax and the anti-apoptotic Bcl-2, have been investigated in depth for their roles in transducing apoptotic signals. It has been proposed that the ratio of Bcl-2-to-Bax determines the cellular response to apoptotic insults. Accordingly, the threshold for caspase activation may be reached by decreasing the levels of Bcl-2 or increasing the levels of Bax (Almeida et al. 2000). In the cortex and cerebellum, Bax expression levels are high during the first two weeks of postnatal development, followed by a severe decrease in levels at the same time that PCD is decreasing (Vekrellis et al. 1997). The importance of the Bcl-2:Bax ratio was further

corroborated by evidence suggesting that when the Bcl-2:Bax ratio is decreased due to inverse changes in protein levels, caspase 3 activation was increased, leading to neuronal death (Mooney and Miller 2000).

A variety of experimental manipulations have confirmed the requirement for different components of the PCD cascade (Danial and Korsmeyer 2004). Direct and indirect genetic regulation of PCD has been studied using RNA and protein synthesis inhibitors. These manipulations prevent cell death by blocking the loss of general cellular homeostatic mechanisms or, as recent reports suggest, by increasing or decreasing the ultimate expression of genes that cause PCD (Johnson and Deckwerth 1993, Joo et al. 2002). In addition, caspase inhibitors have demonstrated the requirement for caspases during apoptosis in a variety of cell death models (Milligan et al. 1995, Troy et al. 1996). The use of the pan-caspase inhibitor ZVAD-fmk, which irreversibly binds the caspase proenzymes and blocks caspase activation, has been demonstrated to reliably prevent neuron loss (Bilsland et al. 2002). Although the mechanism for mitochondrial membrane permeabilization during PCD is incompletely described, Bcl-2 and Bax are believed to play important roles in mitochondrial membrane integrity during apoptosis (Gross et al. 1999). Electrophysiological experiments suggest that BCL proteins interact with the mitochondrial membrane and allow unregulated passage of molecules, including

cytochrome c, into the cytoplasm that facilitate the activation of caspases (Zamzami and Kroemer 2001). Prevention of cell loss by caspase inhibitors and induction of morphological markers (i.e., nuclear and cytoplasmic condensation and blebbing) by the potential death signal are generally believed to be sufficient to establish apoptotic PCD as the mechanism for cell loss.

A variety of apoptotic PCD signals have been identified and studied to elucidate the mechanism of PCD initiation through caspase activation (for review see: Oppenheim 1991). For example, in the development of the sympathetic nervous system, the presence of nerve growth factor (NGF) is proposed to inhibit the constitutively present cell death machinery. Absence of NGF signals may result in gene expression that both increases pro-apoptotic protein levels and alters phosphorylation events that activate the PCD cascade (Purves et al. 1988, Martin et al. 1988, Deckwerth and Johnson Jr. 1993, Estus et al. 1994, Deshmukh and Johnson 1997). Other trophic factors implicated in regulating the PCD pathway also affect gene expression to alter cell survival, such as the protective effects of BDNF on hippocampal neurons after ischemia (Kiprianova et al. 1999). In contrast, proapoptotic signals such as the Fas ligand (FasL) can bind to death receptors (Fas) to actively induce PCD (Nagata 1999). Ligand-bound death receptors contain death domains that provide the foundation for the assembly of a death-inducing signal complex,

which in turn activates caspase 8 and the caspase-mediated PCD cascade (Budd 2002). Because steroids have been reported to both prevent and cause PCD, their effects may depend upon tissue-specific expression of steroid receptors and other steroid receptor-associated molecules. For example, in lymphocytes, glucocorticoid receptor dimerization and binding to steroid response elements is required for apoptosis (Kucharova and Farkas 2002). Glucocorticoid- regulated expression of BCL family molecules also plays a role in controlling PCD in the hippocampus, where shifts in the ratio between pro- and antiapoptotic molecules after activation of corticosteroid receptors influence neuronal fate. While glucocorticoid receptor activation increases the Bax:Bcl-2 ratio, resulting in cell death, the opposite effect is seen with mineralcorticoid receptor activation (Almeida et al. 2000). Intracellular signal transduction pathways, particularly protein kinases, may also contribute to the initiation of PCD, suggesting that pro- and anti-apoptotic forces are integrated with other cellular processses and regulated at multiple levels (Xia et al. 1995, Lesuisse and Martin 2002). Developmental regulation of the expression and activation of the molecular components of the PCD process is critical for specific organization of the brain into mature, function neural circuits.

EXPERIMENTAL MODEL

The AVPV will be used as a model of steroid-induced cell loss in the proposed experiments. AVPV sexual differentiation is a temporally and spatially regulated event. As a result of sexual differentiation, the female AVPV contains more dopamine, dynorphin, calcitonon gene-related peptide, neurotensin and galanin-containing neurons, while males demonstrate greater numbers of enkephalin neurons (Simerly, Swanson et al. 1985, Simerly et al. 1988, Alexander et al. 1991, Herbison 1992, Bloch et al., 1993, Eckersell et al. 1993). Changes in AVPV neuron number depend on exposure to testosterone or estrogen that sexually differentiates both AVPV neurons and gonadotrophin releasing hormone (GnRH) release. Although GnRH secretion is pulsatile in both females and males, a large increase in GnRH release in females initiates the preovulatory rise in lutenizing hormone levels (Herbison 1998). Masculinization of the female AVPV decreases AVPV volume, differentiates neuropeptide content, and also disrupts GnRH secretion (Akema et al. 1984, Simerly et al. 1985, Petersen and Barraclough 1989). Additionally, estrogen and progesterone receptors are expressed in the AVPV, suggesting that the AVPV could mediate positive feedback regulation of GnRH neurons (Simerly et al. 1996). Indeed, female estrogen receptor knockout mice (ERKO), which lack ERα, are infertile (Lubahn et al. 1993).

Although both ERα and ERβ are present in the AVPV of adult rats (Shughrue et al. 1997), ERα predominates during perinatal development and the period of AVPV sexual differentiation (Orikasa et al. 2002, Perez et al. 2003). The role of ERα during AVPV sexual differentiation has been clearly established by examination of ERKO mice. In the absence of ERα, the AVPV is not sexually dimorphic. ERKO males contain the same number of dopaminergic neurons, quantified with tyrosine hydroxylase immunoreactivity (TH-ir), as wild-type females (Simerly et al. 1997). In contrast, the AVPV of testicular feminized male mice, which have an androgen receptor deficit, does sexually differentiate (Simerly et al. 1997). This suggests that AVPV sexual differentiation depends on ERα and that the action of sex steroids in the AVPV is mediated by ERα.

Early AVPV development can be studied *in vivo* and *in vitro*, which increases the potential for experimental manipulation. AVPV sexual differentiation depends upon postnatal estrogen exposure that occurs on the day of birth. At this time, male rodents experience a surge of testosterone (Weisz and Ward 1980), which can be aromatized to estrogen in the brain (Simpson et al. 1994). This gonadal steroid surge is required for AVPV masculinization. Castration prior to testosterone exposure prevents AVPV sexual differentiation, which can be restored with testosterone treatment. Likewise, the female AVPV can be masculinized when treated postnatally with testosterone or estrogen

(Simerly et al. 1985, Simerly et al. 1988, Simerly 1989, Waters EM personal observation). These events can also be reconstituted *in vitro* (Ibanez et al. 1998). Treatment of AVPV organotypic explants, prepared on the day of birth, with a single 24 hour testosterone or estrogen pulse during the first postnatal week is sufficient to decrease the number of dopamine neurons, as detected by antibodies for tyrosine hydroxylase (TH), in both male and female AVPV organotypic explants. Furthermore, females and males are masculinized by similar estrogen doses. Preparation of AVPV explants from newborn males, before the testosterone surge, prevents masculinization, which can then be restored with testosterone treatment. Eight days after birth neither testosterone nor estrogen treatment reduces TH-ir cell number and by day P10, TH-ir cell number has stabilized *in vitro,* even though ERα expression persists in AVPV explants (Ibanez et al. 1998). The AVPV can be isolated from the internal environment of the hypothalamus and cultured in defined conditions that allow experimental manipulation of steroids and other trophic factors.

During post-natal development, the potential mechanisms for the steroid-mediated sexual differentiation of AVPV dopaminergic neurons include neurogenesis, migration, phenotype regulation and PCD. Since AVPV neurogenesis is complete prior to gonadal steroid hormone exposure on embryonic day 18 (Nishizuka et al. 1993), neurogenesis

will not be addressed in this study. Migration might also be detectable in AVPV organotypic explants, since lateral migration would result in accumulation of TH-ir neurons around the edges of the explants. Similarly, rostral or caudal migration might result in unusual TH-ir expression patterns in the vascular organ of the lamina terminalis and paraventricular nucleus, which are rostral and caudal, respectively, to the AVPV. However, to date, these scenarios have not been detected, although *in vitro* labeling of cells with a marker for live TH-ir cells could potentially address this issue. In contrast to the lack of evidence for neurogenesis, migration, or phenotype regulation in the AVPV we and other have found evidence for programmed cell death in the control of AVPV differentiation (Waters and Simerly 2000, 2002). Arai and colleagues demonstrated that estrogen treatment increases apoptotic cell numbers in the AVPV compared to vehicle-treated animals (Murakami and Arai 1989, Arai et al. 1994, Arai et al. 1996). As summarized in chapter 3, my data have now confirmed these findings. Using TUNEL labeling and Hoescht 33342 staining as a markers for cell death, I have detected more apoptotic nuclei in estrogen-treated AVPV than in control-treated AVPV, confirming the role of estrogen-mediated PCD during post-natal development of the AVPV

The organotypic explant preparation isolates AVPV tissue from external and internal influences and places it in a defined environment. In the culture environment, explants

can be treated with steroids, enzymes, and imaging reagents and monitored for differentiation. Sexual differentiation of the AVPV is characterized by the number of dopaminergic neurons. This can be quantified by TH-ir, which is a stable marker for dopaminergic neurons that does not require further processing of the tissue, such as colchicine treatment, or the use of *in situ* hybridization. In addition, sex steroids do not acutely regulate tyrosine hydroxylase protein, so levels are not acutely affected by steroid treatments in the experimental protocol (Simerly 1989). Thus, dopaminergic neurons, which are 3-4 times fewer in males than in females, are excellent markers for sexual differentiation. In addition, *in situ* hybridization for proenkephalin or prodynorphin can also be used to monitor the effects of experimental treatment on the health of AVPV explants. Through the use of these markers, I have been able to monitor estrogen activation of ERα, a signal that both increases and decreases cell survival in the same explant (Chapter 2).

The presence of multiple markers for sex steroid effects, the discrete time period over which the events occur and the availability of an *in vitro* model make the AVPv an ideal model for studies of estrogen's action on the developing sexually dimorphic forebrain.

THESIS OBJECTIVE

Estrogen elicits temporally and spatially specific effects by activation of its cognate receptors and subsequent interaction with a plethora of transcriptional proteins and signaling pathways. It is important to understand these interactions, not only in the development of reproductive tissue, but also in the CNS and other steroid receptor-containing tissues. Steroids can regulate the course of diseases such as cancer or Alzheimer's disease, as well as the severity of physical insults such as ischemia. The cellular and molecular mechanisms promoted by estrogen exposure are being pieced together through studies in a variety of *in vivo* and *in vitro* models, naturally occurring and experimentally-induced situations, and neuronally and pathologically derived cell lines.

The effects of estrogen vary because the molecular level of receptors, coregulators and signaling proteins, as well as the location of the receptors may be regulated by development, activity, or other steroids and neuropeptides. In addition to changes in the molecular mileu, estrogen levels change throughout life. Sex steroid levels vary greatly between perinatal, pubertal and aged animals. Together, these steroid events could reinforce or oppose each other, preventing a change in homeostasis or converging to alter other ongoing processes. Although great strides have been made in our understanding,

continued work is needed to characterize the cellular and molecular mechanisms of steroid actions.

How does estrogen affect the outcome of CNS development or protect the brain from stroke? What cellular and molecular mechanisms are employed for the effect? Although estrogen's primary effects are due to classical modulation of gene transcription, non-classical genomic regulation and rapid effects by activation of phosphorylation signalling pathways, Ca^{2+} mobilization, and neuronal excitablity can also conspire to alter cell fate. The future of a cell, then, depends on the balance of cellular events and the summation of molecular events regulated by estrogen actions.

The sexual differentiation of the AVPV represents a novel estrogen action in a naturally-occuring phenomenom: Estrogen induces selective postnatal destruction of neurons in a developmental process critical to the reproductive capability of the female animal. Although demasculinization of the AVPV in the male may not have endocrinological or behavioral outcomes, the estrogen-directed masculinization process cannot be separated *in vivo* from the development of the neuroarchitecture required to produce male specific reproductive behavior. Furthermore, estrogen is also important for the maintenance of select neurons in the AVPV, which parallels the neuroprotective function of estrogen

reported in other dimorphic areas. The parameters that regulate estrogen's cell type specific effects are unknown.

I established an *in vitro* model of AVPv development to study the cellular mechanism regulating TH-ir cell loss. and the molecular regulation of estrogen's action. When I examined cellular events *in vivo* and *in vitro*, I confirmed that estrogen initiates cell death pathways to cause a permanent reduction in TH-ir cell number. This loss depends on the function of ligand-bound ERα. Consequently, the activation of a caspase-dependent cell death pathway suggests that estrogen-induced TH-ir cell loss is most likely due to PCD, probably apoptosis.

CHAPTER 2

Gonadal steroid regulation of neuron number: an organotypic explant model.

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ABSTRACT:

Organotypic cultures maintain the morphology of the brain while exposing it to environmental regulation and experimental manipulation not possible *in vivo*. The anteroventral periventricular nucleus of the preoptic region of the hypothalamus (AVPV) is a well characterized sexually dimorphic brain area that is larger in females than in males and is implicated in the generation of spontaneous lutenizing hormone surges and ovulation. It differentiates postnatally under the influence of steroid hormones resulting in dimorphic cell populations, dopaminergic and prodynorphin-containing neurons that are more abundant in the females nucleus and proenkephalan neurons that are greater in number in the male nucleus. These cell types were examined in the AVPV of postnatal day ten rats and explants prepared on the day of birth and cultured for ten days after steroid or control treatments. Testosterone and estrogen treatment caused differentiation of AVPV. Testosterone treatment was unable to alter dopaminergic neuron number when administered to cultures after eight days in culture. After ten days in culture, the AVPV displayed typical staining patterns of estrogen receptors as well as steroid sensitivity demonstrated by estrogen induction of progesterone receptor immunoreactivity.

INTRODUCTION:

Refinement of the nervous system into a functioning unit requires careful regulation of neuronal number. During development, a wide variety of epigenetic factors act on the nervous system and determine neuronal fate, in part, through the control of cellular and molecular mechanisms that regulate cell survival and consequently cell number. Gonadal steroids, in particular, have a profound effect on cell number during the development of sexually dimorphic nuclei in the hypothalamus. These nuclei, which include the central portion of the medial preoptic nucleus (MPNc), the ventral medial hypothalamic nucleus, and the anteroventral periventricular nucleus of the preoptic area (AVPV) along with other forebrain areas (Madeira and Lieberman, 1995), depend upon early exposure to gonadal steroids not only for the development of their phenotypic changes in neuronal composition but also for the manifestation of their associated sex-specific reproductive functions and behaviors. Receptors for sex steroids, expressed in these and other dimorphic brain areas during development and adulthood, as well as gonadal steroid hormones, play an important role in the regulating of reproduction, in addition to determining of neuronal type and number in these brain nuclei.

There is a great deal that remains to be learned about the interactions between sex steroid and the mechanisms underlying the complex processes of cellular determination.

Elucidation of these mechanisms has been impeded by both the multiplicity of sex steroid actions and the rich environment, including interconnections between hormone-sensitive regions and overlapping neuropeptide and receptor distributions, encompassing sexually dimorphic areas. An experimental model that both simplifies environmental variables and increases tissue access is the organotypic slice culture maintained in a defined serum-free environment. These cultures also maintain cellular relationships that may be important for regulating transmitter phenotype *in vivo*. At the same time, they can be used to partition the highly complex set of regulatory events that exist in the whole animal. In order to increase our understanding of steroid activities during the development of sexually dimorphic brain regions, we focused on the characterization of an *in vitro* model of the AVPV in which we are able to mimic the sexual differentiation of neurotransmitter-specific cell types.

The sexually dimorphic AVPV is an interesting example of cell type-specific steroid effects. As a result of sexual differentiation, the AVPV contains two neuropeptidegic neuronal populations with divergent fates. Those cells whose numbers are greater in females than in males include dopamine and prodynorphin neurons. The male AVPV, however, contains more proenkephalan neurons than the female AVPV. During development, neonatal testosterone alters the neuronal composition of the male AVPV by

reducing the number of dopaminergic and prodynorphin neurons while maintaining proenkephalen neuron number (Simerly et al., 1988). Castration of males prior to a testosterone surge on the day of birth increases dopaminergic neuron number in the adult; however, this effect can be reversed by treatment with either testosterone (T) or estrogen (E), a product of T aromatization (Simerly, 1989). Likewise, DA neuron number in the female AVPV can be masculinized by treatment with either T or E during the first postnatal week, demonstrating a postnatal steroid requirement (Simerly et al., 1985). Examination of dopaminergic neuron number in the AVPV of estrogen receptor alpha (ERα) knock out mice, and in testicular feminized mice, which lack a functional androgen receptor (AR), revealed that ERα but not AR is necessary for differentiation of this cell population (Simerly et al., 1997), even though both receptors are present in the AVPV of males and females during the first postnatal week. In contrast, the beta form of the estrogen receptor is not detectable until the end of the first week (Orikasa et al., 2002, (Perez et al., 2003).

In this study, we confirmed that particular attributes of AVPV sexual differentiation can be recapitulated *in vitro* by employing AVPV organotypic explants prepared from newborn male and female rats and treated with testosterone (T, 10^{-7}M) or estrogen (E,

10^{-7} and 10^{-9} M) for the first 24 hours in culture. Furthermore, females can be used experimentally without the confounds of the T surge on embryonic day 18 in males by treating them with either T or E, essentially providing a clean slate for manipulations of AVPV sexual differentiation. The explants displayed typical expression patterns of ERα immunostaining. Isolation of the AVPV from other hormone-sensitive regions demonstrates that AVPV differentiation relies on the direct local action of steroid hormones. We also demonstrated that sexual differentiation of AVPV dopaminergic neurons is sensitive to the timing of the steroid treatment with exposure to steroids after the sixth day in vitro (DIV 6) being largely ineffective in reducing cell number. However, explants remained steroid-responsive through DIV 8, as shown by the induction of progesterone receptor expression. Immunohistochemistry for tyrosine hydroxylase (TH), which visualized dopaminergic neurons, and *in situ* hybridization for proenkephalen (pENK) and prodynorphin (pDYN) were used to compare the degree of sexual differentiation in the AVPV of explants after ten days *in vitro* (DIV 10) to the AVPV of ten day old animals (P10).

METHODS:

Animals and tissue preparation Untimed pregnant adult female Sprague-Dawley rats were obtained from Animal Technologies Limited. They were housed on a 14:10

light/dark schedule with food and water available ad libitum. Ten day old female and male rat pups were deeply anesthetized with tribromoethanol and perfused trancardially with 4% paraformaldehyde in 0.1 M sodium borate buffer (pH 9.5). Brains were removed, postfixed in the same fixative containing 20% sucrose for 2 hours, and cryoprotected overnight in 20% sucrose in 0.02 M potassium phosphate buffered saline (KPBS).

Tissue culture Explants were prepared using a modified version of Gahwiler's slice culture technique (Gahwiler et al., 1997). Briefly, on the day of birth rat pups were cryoanesthetized and the whole brain removed. Brains were submerged in ice cold Gey's plus glucose (2.5g/250ml) and sectioned at 300 μm on a vibratome (Electron Microscopy Sciences, Hatfield, PA) through the preoptic area. Sections were stored in the serum-free defined media EOL-1 (containing thyroid hormone but lacking T, E, and progesterone) on ice until dissection but no longer than two hours. The AVPV was microdissected using the lateral ventricles and anterior commisure as guides. Three AVPV sections were mounted on each collagen-coated (1 mg/ml 60% EtOH, Upstate, Lake Placid, NY) Millicell-CM membranes (0.4 μm pore size, 30 mm diameter, Millipore, Bedford, MA) prewet with warm EOL-1. The cultures were incubated at 37°C (in 95% O2 and 5% CO2)

and medium was changed every 48 hours after steroid treatment. After ten days cultures were fixed and processed for immunohistochemistry or *in situ* hybridization.

Steroid treatments The stock solutions of 10^{-2}M 17-β-estradiol or testosterone (Sigma, St, Louis, MO) were prepared in 100% EtOH and stored at –20°C. Both were diluted in EOL-1 culture media at the time of use.

Tyrosine Hydroxylase and Estrogen Receptor α and β Immunohistochemistry

AVPV explants were fixed in 4% paraformaldehyde for 15 min, postfixed in 20% sucrose in 4% paraformaldehyde for 1 hour and cryoprotected in 20% sucrose in 0.02 M KPBS for 30 min. Explants were embedded in OCT (Sakura, Torrance, CA), frozen on dry ice, and stored at –80°C until sectioning. All tissues were cryosectioned at 20 um and thaw-mounted onto gelatin-coated slides. Tissue was pretreated with LKPBS [(KPBS containing 2% normal goat serum (Upstate) and 0.4% Triton-X 100 (Biorad, Hercules, CA)] overnight at 4°C. Primary antibodies (mouse anti-tyrosine hydroxylase 1:1000, Immunostar, Hudson,WI, and rabbit anti-estrogen receptor α, 1:40,000, Upstate) were diluted in LKPBS and applied to the tissue for 72 hours at 4°C. Tissue was washed with KPBS, incubated with the respective secondary antibodies, Alexa Flour 488 goat anti-mouse IgG and Alexa Flour 568 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene,

OR), for 2 hours at room temperature, washed again in KPBS and coverslipped with bufferd glycerol, pH 8.6.

Progesterone Receptor Immunohistochemistry

After culture, explants were fixed in 5% acrolein in phosphate buffferec saline (PBS) for 20 min. and cryoprotected in 0.02M KPBS containing 20% sucrose for 90 min. before being embedded in OCT and frozen in dry ice. Explants were sectioned on a cryostat at 20 μm, thaw-mounted onto gelatin-coated slides, allowed to dry at room temperature and stored in 0.02 M KPBS until use on the same day. Antigen retrieval was performed by incubating the sections in 10 μM citric acid in ddH_20 (pH 6.0) at 80°C for 10 min. and allowed to cool in the same solution for 20 min. before being rinsed in KPBS. Sections were pretreated overnight at 4°C in LKPBS, followed by 1% sodium borohydride in 0.02M KPBS for 10 min. at room temperature, then rinsed 3 X 5 min. in KPBS. Sections were then incubated for 72 hours at 4°C in rabbit anti-Progesterone Receptor antibody (DAKO Inc, Glostrup, Denmark) diluted 1:1000 in LKPBS. The rest of the incubations were performed at room temperature and were followed by 5 rinses (5 min. each) in KPBS. Sections were incubated for 60 min. in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a 4.4 μl/ml dilution in KPBS containing 0.3% Triton-X 100 followed by application of the ABC reagent (Vectastain Elite Kit, Vector

Laboratories) for 60 min. Sections were subsequently recycled into each of the previous solutions for 25 min. The antibody labeling was then revealed by incubation in a Nickel/DAB solution for 15 min. (DAB Substrate Kit for Peroxidase, Vector Laboratories). The slides were dehydrated in ascending ethanols, cleared in Xylenes, and coverslipped with DPX (Electron Microscopy Sciences).

pDYN and pENK probe preparation

T7 polymerase (Promega, Madison, WI) was used to transcribe ^{35}S-labeled antisense cRNA probes (^{35}S-UTP from Perkin Elmer Life Sciences, Boston, MA) complementary to a 733 bp *Bam*HI-*Hinc*II fragment of the main exon of the prodynorphin gene. This plasmid was generously supplied by Dr. M. Schaeffer (University of Michigan) who derived this construct from a cDNA provided by Dr. J. Douglass (OHSU). The radiolabeled cRNA probe was purified by passing the transcription reaction solution over a Sephadex G-50 Nick column (Pharmacia, Piscataway, NJ). Four 100 μl fractions were collected and counted by using a scintillation counter (Packard, Meridian, CT). The leading fraction was heated at 65°C for 5 min. with 500 μg/ml yeast tRNA (Sigma) and 50 μM dithiothreitol (DTT) (Stratagene, La Jolla, CA) in DEPC (Sigma) water and then diluted to an activity of $5x10^6$ cpm/ml with hybridization buffer containing 50%

formamide (Boehringer Mannheim, Indianapolis, IN), 0.25 M sodium chloride, 1X Denhardt's solution (Sigma), and 10% dextran sulfate (Pharmacia).

SP6 polymerase (Promega) was used to transcribe cRNA probes complimentary to a 935 bp *Sac*I-*Sma*I fragment of the coding region of rat PENK mRNA as described above, except that 1 μl of a 2 mM solution of digoxigenin-labeled UTP (Boehringer Mannheim) was substituted for the ^{35}S-labeled UTP. This plasmid was generously supplied by Dr. S. Sabol (National Institutes of Health). After transcription, the reaction mixture was treated with DNase (Promega) and RNAsin (Promega) and stabilized with EDTA (Sigma) and salt, and the total volume was adjusted to 100 μl with 20 mM DTT. The cRNA probe was then precipitated with ethanol, dried, and resuspended in 100 μl of DEPC-treated water. The probe was mixed 1:7 with hybridization buffer as described above.

pDYN and pENK in situ hybridization

20 µm frozen sections of fixed explants or perfused P10 rats were cut on the cryostat, thaw-mounted onto gelatin-subbed, poly-L-lysine coated slides, and processed for *in situ hybridization* as described previously (Simmons et al., 1989). After a 15 min. proteinase K digestion (0.6 μg/ml for explants, 0.12 μg/ml for P10 tissue) at 37°C (Boehringer Mannheim) and acetylation (0.0025% acetic anhydride at room temperature), the sections

were dehydrated in ascending alcohols and dried under vacuum overnight. Hybridization solution was pipetted onto the sections (80 μl/slide), which were covered with a glass coverslip, and sealed with DPX (Electron Microscopy Sciences) before incubation for 20 hr at 58°C. After hybridization, the slides were washed four times (5 min. each) in 4X SSC before RNase digestion (10 μg/ml for 25 min. at 37°C; Sigma) and rinsed at room temperature in decreasing concentrations of SSC that contained 1 mM DTT (2X, 1X, 0.5X for 10 min. each) to final stringency of 0.1X SSC at 65°C for 30 min. After dehydration in ascending alcohols, the pDYN-hybridized sections were exposed to Curix Ultra x-ray films (Agfa Corporation, Greenville, SC) for 4 days, together with autoradiographic ^{14}C microscales (Amersham, Arlington Heights, IL), before being dipped in NBT-2 liquid emulsion (Eastman Kodak, Rochester, NY). The dipped autoradiograms were developed 10 days later with Kodak D-19 developer, and the sections were counterstained with thionin through the emulsion. PENK hybridized sections were not dehydrated and dried after the last 0.1X SSC rinse but were processed for localization of digoxigenin-labeled hybrids. Before immunohistochemical detection of digoxigenin-labeled hybrids, the slides were incubated overnight in 2X SSC containing 0.05% Triton X-100 and 2% normal goat serum at room temperature. The next day, the slides were incubated in a 1:1000 dilution of the anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim) for 5 hr at room temperature, rinsed, and then

incubated overnight at room temperature in chromagen solution (BCIP/NBT Liquid Substrate System, Sigma), and the staining reaction was stopped by placing the slides in 10 mM Tris-HCl, 1mM EDTA. The sections were further dehydrated in ethanol, dried under vacuum, and coverslipped with DPX.

Analysis

For all experiments, one side of the AVPV was counted in all tissue sections that contained the area. Explants or whole brains in which an intact AVPV could not be identified were excluded from the analysis. All image analysis was performed blind to the experimental conditions. Brightfield, darkfield, and fluorescent images of the AVPV were captured using a CCD camera mounted on a Zeiss Axioplan 2 microscope at 10X and saved using OpenLab software (Improvision Inc., Boston, MA). The rostral, caudal and lateral edges of the AVPV were identified using ERα-ir in fluorescence-labeled tissue or the third ventricle, optic chiasm, anterior commisure landmarks as well as thionin stain in brightfield in coloremetric- and silver grain-labeled tissue. Total cell or pixel counts for each experimental group were compared statistically with a one-way ANOVA and Fischer's PLSD post hoc analysis using Statview software (SAS Institute Inc., Cary, NC) A p value of 0.05 or less was considered significant.

TH and pENK Analysis: THir or pENK mRNA containing cells were manually counted using a Zeiss Axioplan 2 microscope to visualize the AVPV.

pDYN Analysis: Images collected in Openlab were imported into Metamorph, the AVPV region was selected from the brightfield image of the thionin staining, and this region of interest (ROI) was moved onto the corresponding binarized darkfield image. Pixel density was measured in the AVPV and a background measurement taken from an area adjacent to the AVPV was subtracted.

PR Analysis: Brightfield images of the AVPV were analyzed by Metamorph software. The intensity of background staining was measured in an area lateral to the AVPV and 4-5x the standard deviation was subtracted from the background and used to threshold the AVPV. All objects remaining after thresholding greater in size than 5 pixels were counted as nuclei positive for PR staining.

RESULTS:

Development of dopaminergic neurons

Dopaminergic neuron number was assessed by counting cells immunoreactive for tyrosine hydroxylase (THir). THir cell number and distribution in the AVPV of P10

female and males or DIV10 control and steroid-treated explants cultures appeared similar to that previously reported in adult female and male rats (Fig. 1 A & B, Fig .2 A-D). The AVPV of P10 female rats contained significantly more THir neurons than the corresponding nucleus in male rats (* $p<0.001$) (Fig. 1 C). Similar results were found in AVPV explants prepared on the day of birth and maintained in culture for ten days. Steroid-treated explants were compared to control explants and DIV10 explants treated with either 10^{-7} or 10^{-9} M E2 or 10^{-9} M T. All steroid treatments dramatically reduced the number of dopaminergic neurons compared to non-steroid control treatment of explants, as revealed by the THir cell counts (* $p<0.001$) (Fig. 2 D). THir cell numbers in the AVPV of control explants were similar to the P10 female AVPV. Interestingly, female AVPV explants differentiated as well as male explants. Thus, AVPV cell loss can be reproduced *in vitro* by treatment of isolated AVPV explants with either T or E.

Development of proenkephalen neurons

The number of AVPV cells expressing pENK mRNA *in vivo* are sexually dimorphic ten days after birth (Fig. 3 A & B,). The pENK mRNA expression is significantly higher in the male AVPV than in the female AVPV (*$p<0.05$) (Fig. 3 C); however, the numbers of cells in both males and females were lower than previously reported in adults (Simerly et al. 1988). Similarly, AVPV explants treated with 10^{-7}M T and 10^{-7} or 10^{-9} M E contained

significantly more pENK cells than control explants (*$p<0.05$) (Fig. 4 E). The distribution of pENK cells *in vivo* and *in vitro* was similar to that of adult rats. The differences in cell number between P10 and adult rats suggests two possible explanations: either the expression level in young animals was below our detection limits or all the cells that will ultimately express this phenotype have not yet begun to do so. However, the sexual dimorphism has begun to be apparent by ten days after birth. Exposure to sex steroids, either *in vitro* or *in vivo*, prevented the reduction in pENK-expressing neuron number seen in control explants and females rat pups.

Development of prodynorphin neurons

PDYN mRNA expression levels were constant in all AVPv examined both *in vivo* and and *in vitro*, regardless of sex or steroid treatment. Only low levels of pDYN mRNA were detected in the AVPV of P10 female and male animals (Fig. 5 A & B) or DIV 10 steroid and control treated explants. Individual cells were not identifiable in the perinatal AVPV, while they are easily counted in adult AVPV. There are no differences in pDYN mRNA expression between P10 female or male AVPV (Fig. 5 C). Although the mRNA levels are slightly higher *in vitro* than *in vivo* due to increased non-specific binding, there is also no differenrence in pDYN mRNA expression between control and steroid-treated AVPV explants on DIV 10 (Fig. 5 D). These results correspond with previous work in

our lab demonstrating that pDYN mRNA expression is not sexually dimorphic until after puberty (RB Simerly unpublished data).

Timing of steriod exposure

Steroid treatment of AVPV explant cultures during the first four days *in vitro* differentiates AVPV THir cell number compared to control cultures (Fig. 6 A & B). Steroids do not differentiate THir cell number when steroid treatment is delayed until DIV 8 (Fig. 6 C). Testosterone was added to the culture media beginning on DIV 0, DIV 2, DIV 4 or DIV8 for the duration of the culture period (until DIV10). Explants exposed to estrogen beginning on DIV 0, DIV 2 or DIV 4 showed reductions in TH cell number on DIV 10 ($*p<0.05$)(Fig. 6). However, THir cell numbers were not reduced in AVPV explants exposed to steroids after eight days *in vitro* and were indistinguishable from control cultures.

Progesterone receptor induction

To determine whether the AVPV explants remained steroid-sensitive in culture, explants were treated with 10^{-9} M E on DIV 8 for 48 hours, and then fixed and stained for progesterone receptor. Estrogen treatment induced an increase in progesterone receptor immunoreactivity (PRir) in DIV 10 AVPV explants compared to control treatment (Fig. 7

A & B). The number of PRir nuclei was significantly increased after estrogen exposure (*p< 0.05) (Fig. 7 C). These results confirm that AVPV explants remained steroid-sensitive after ten days in culture.

ERα expression

The staining pattern for ERα immunoreactivity (ER-ir) in the AVPV resembles the outline of the AVPV edges on all sides. The pattern of ER-ir in the AVPV explants is similar to that in the AVPV of P10 females (Fig. 8).

DISCUSSION

As a result of sex steroid exposure *in vitro*, AVPV explants undergo alterations in neuronal number that parallel changes in the content of the AVPV *in vivo*. By ten days after birth, the rat AVPV exhibits sex differences in TH and pENK cell number. These differences can be reproduced *in vitro* by steroid treatment of AVPV cultures made from either female or male rats (male data not shown). Interestingly, THir neurons in female cultures were as responsive to steroids as the male cultures were. Females had limited prior exposure to sex steroids in as a result of E binding to alpha-fetoprotein effectively reducing free circulating estrogen to very low levels. Treatment of female explants with either T or E *in vitro* was successful in differentiating AVPV THir and pENK-containing

cells. These results agree with reports of T-induced differentiation in adults treated as neonates (Simerly, 1989). THir cell number ten days after birth, both *in vivo* and *in vitro*, equals that detected in adult AVPV. A sex difference in pENK cell number is also apparent *in vivo* and *in vitro* by ten days; however, the number of cells containing pENK are considerably fewer than reported in the adult AVPV (Simerly et al., 1988). PDYN expression in the neonatal animals and cultures is not yet dimorphic. Evidence suggests that pDYN expressing cells do not differentiate until after puberty (RB Simerly unpublished results). However, TH, pENK and pDYN cell numbers in the adult rat AVPV are permanently altered by T treatment of neonates. While the effects of T on pDYN number occur outside of the developmental period examined in our culture system, cellular changes may be due to long term effects on the intracellular relationships of pDYN with other neuronal cell types.

The distributions of neuropeptidergic and receptor containing cells *in vitro* were similar to those of the AVPV *in vivo* suggesting that the morphology of the AVPV explants is highly organotypic. In addition to the TH, pENK and pDYN cells discussed above, we examined the distribution of ERα *in vitro*. The expression pattern was similar to that *in vivo*. ERα-ir cells are present throughout the AVPV and were used experimentally to identify the lateral edges of the AVPV as well as the caudal border. We believe that the

beta form of the estrogen receptor (ERβ) does not play a role in AVPV sexual differentiation during the first postnatal week, because the levels of ERβ-expressing cells have been reported to be very low or undetectable in animals younger than P7 (Orikasa et al., 2002). Furthermore, examination of mice deficient for ERα revealed that sexual differentiation of THir cell number did not occur in male AVPV when compared to WT male AVPV. Taken together, these reports suggest that ERα and not ERβ is the mediator of estrogen's effects on cell number during AVPV sexual differentiation.

PR-immunoreactivity was increased in AVPV cultures treated with 10^{-7} M E for 48 hours compared to control cultures. In addition, the PRir expression patterns were consistent with those reported for the induction of PRir by T treatment of neonatal rats (Quadros et al., 2002). This result may suggest a role for progesterone in AVPV sexual differentiation. Although the effect of steroids on TH and pENK cell number detected in this explant culture model occurred in the absence of progesterone in the culture media, estrogen has been reported to induce the synthesis of progesterone in the hypothalamus (Sinchak et al., 2003), which may contribute to AVPV sexual differentiation.

AVPV sexual differentiation resulted from either E or T treatment *in vitro*. Aromatase, the enzyme that synthesizes E from T, is present in the AVPV of adults rat (Roselli et al.,

1998) and is likely present in the neonatal AVPV. Because E treatment was equally effective as T treatment in differentiating AVPV explants, these results confirm that T acts through E to cause sexual differentiation. While steroid exposure early in the culture period decreases the numbers of some cell types, later on it can induce PR protein expression. This induction of PRir confirms that the explants remain responsive to steroids throughout the culture period. Although AVPV neurons remain sensitive to E through DIV 10, TH cell number can no longer be altered by steroid exposure. Steroid treatment on DIV 8 failed to reduce the number of TH cells, suggesting that sex steroid effects on TH cell number can only occur during the first postnatal week. In addition, pENK cell number is also affected by steroid exposure during the first postnatal week; however, the timing of E treatment to determine pDYN cell number remains unknown.

This explant model presents the opportunity to study the activity of sex steroids on opposing cell fates, survival and death, in a naturally occuring developmental context. Steroids have important physiological roles during early development, puberty, and adulthood. Our understanding of steroid actions have become increasingly complex, as both receptor-dependent and -independent actions have been implicated in genomic and non-genomic effects. Steroid responses, both cellular and molecular, may also depend upon the context of each individual cell at a given time. The multiple possible outcomes

of E exposure offers both solutions and further questions in the regulation of AVPV cell number. E has been reported to increase the incidence of cell death markers (pyknotic and TUNEL labeled nuclei) in the AVPV while preventing the increase of these markers in another sexually dimorphic nucleus, the MPNc (Arai et al., 1994, 1996). E as a neuroprotective agent has been also been described in models of acute insults (Wise et al., 2000). Furthermore, E has been demonstrated to increase the expression of neuroprotective agents such as BCL-2 (Garcia-Segura et al., 1998) and other neurotrophic molecules like BDNF (Solum and Handa, 2002).

Models of steroid actions have led to important advances in our understanding of steroid effects and regulation of neuronal plasticity, including changes in cell number, neuropeptidergic phenotype, morphology, and excitability. One of the earliest demonstrations of estrogen as a trophic factor was the increased neurite outgrowth detected in hypothalamic preoptic area organotypic explants treated with estradiol (Toran-Allerand, 1980). More recently, estrogen has been shown to increase synaptic density *in vivo* and *in vitro* in mouse hippocampal primary cultures (Li et al., 2004). The complexity of astrocyte branching is also increased by estrogen treatment, which hints at the indirect steroid regulation of neuronal function (Amateau and McCarthy, 2002). Estrogen also alters neuronal morphology and function through interactions with trophic

factors by regulating their gene expression, such as BDNF, or through interactions with signal transduction pathways (Lee et al., 2004).

We have described a new model system for studying the outcome of estrogen effects on cell fate, as well as the cellular and molecular mechanisms utilized by gonadal steroid hormones. By ten days after birth, AVPV sexual differentiation is underway, TH and pENK cell number is already dimorphic; however, it is not complete, pDYN cell number is not yet dimorphic. These cellular alterations can be linked to physiological outcomes in the whole organism; females treated neonatally with testosterone do not ovulate and experience changes in AVPV content that parallel the male AVPV and are similar to those reported here (Simerly, 1998). The accessiblity of the organotypic explant to various environmental manipulations, while maintaining the intracellular relationships that may be important to determination of cell fate, holds a distinct advantage over primary cell cultures and cell lines.

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The contributions of each author were as follows. E. M. Waters: Prepared AVPV tissues from whole brain and explant cultures for pDYN and pENK in situ hybridizations, PR and ERα immunohistochemistry *in vitro*, and TH and ERα immunohistochemistry *in vivo*. Analyzed PR expression and prepared manuscript. M. Kirigiti: Performed pDYN and pENK *in situ* hybridizations, and PR immunohistochemistry. Analyzed pDYN and pENK expression. M.A. Ibanez: Prepared AVPV explant cultures for TH immunohistochemistry. R.B. Simerly: Analyzed TH stained AVPV explant tissue and is the supporting author.

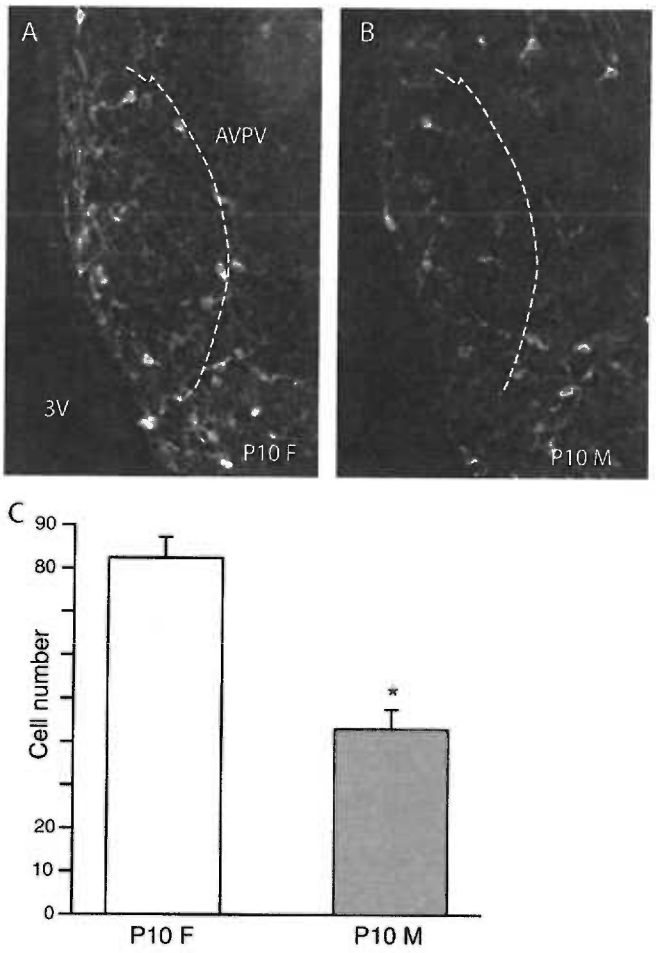

Figure 3. **TH-ir cell number is sexually dimorphic in the rat AVPV at P10.** Low magnification images of TH-ir in the P10 Female (F)(A) and Male (F) (B) AVPV. TH-ir neuron number is 3 fold higher in the Female AVPV than the Male at P10 (C).

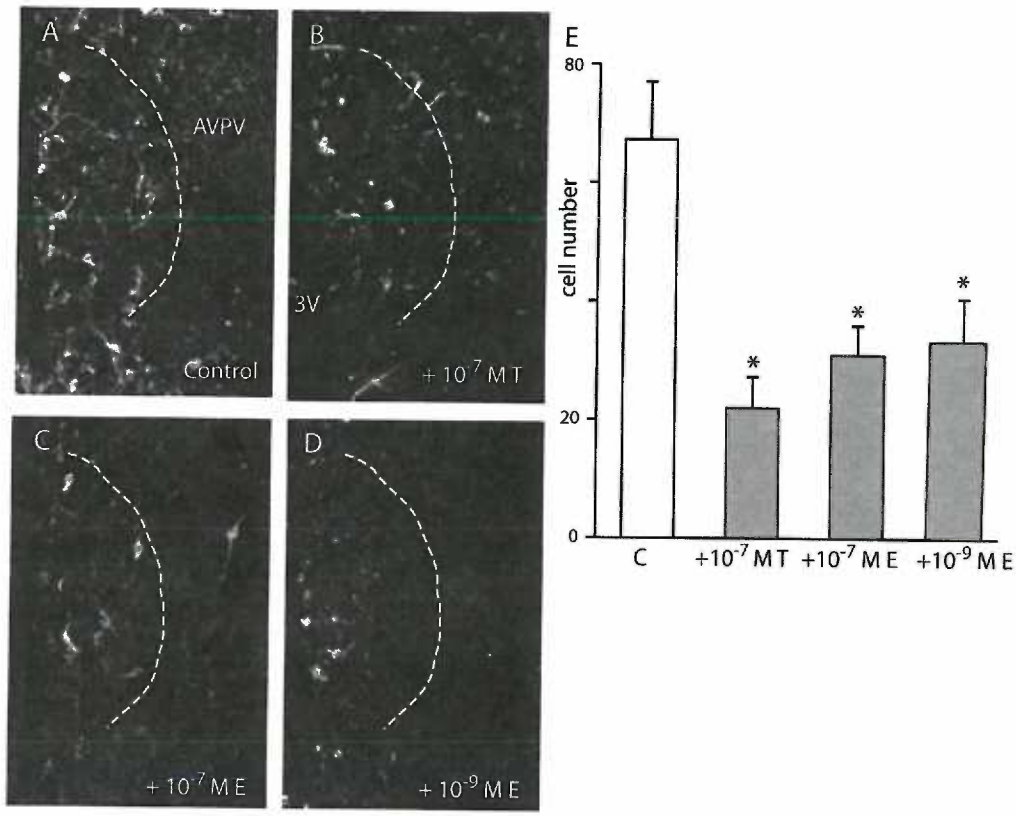

Figure 4. **Loss of AVPV TH-ir cells can be replicated *in vitro*.**
Low magnification images of TH-ir in AVPV explants prepared from female rats on P0, treated for the first 24 hours with either vehicle, Testosterone, or Estradiol, and fixed on DIV 10 (A-D). All steroid treatments resulted in a significant decrease in TH-ir cell number compared to controls (E).

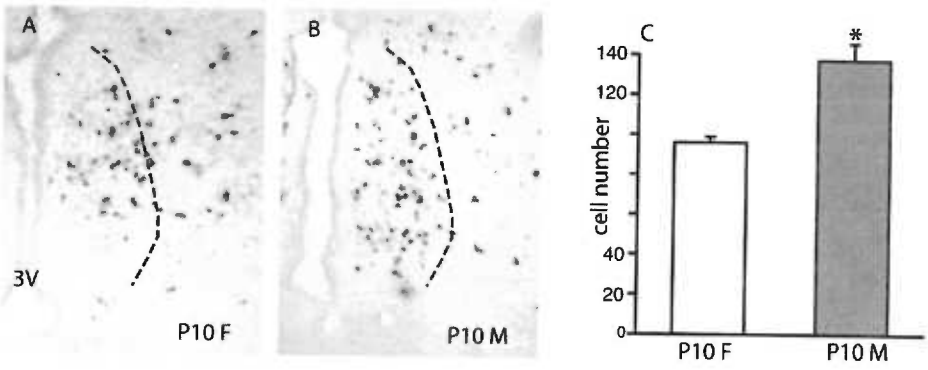

Figure 5. **PENK mRNA levels in the P10 rat AVPV are sexually dimorphic.** Low magnification images of the AVPV of P10 Female (A) and Male (B) rats illustrating pENK mRNA detected by in situ hybridization. The number of pENK mRNA containing cells in the AVPV is significantly higher in male than in female (C).

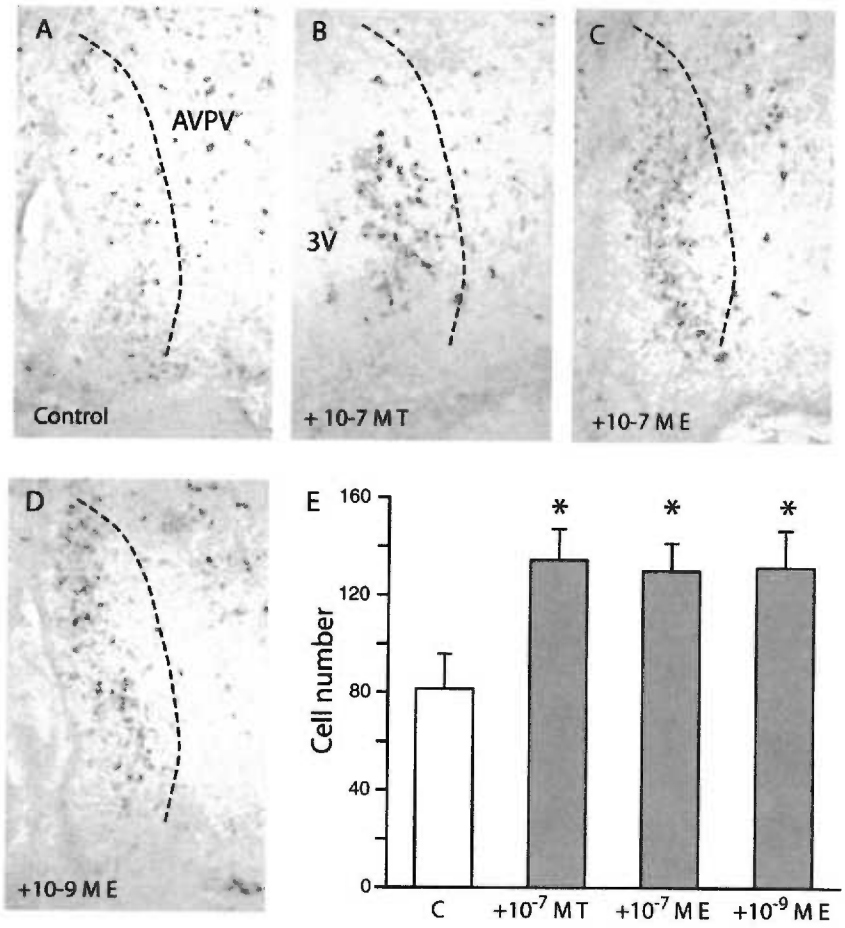

Figure 6. **PENK mRNA levels in the female rat AVPV are sex-reversed by steroid treatment in vitro.** Low magnification images of pENK mRNA in AVPV explants prepared from female rats on P0, treated for the first 24 hours *in vitro* with Testosterone, Estradiol, or vehicle, and fixed on DIV 10 (A-D). Each steroid treatment resulted in a significant increase in pENK mRNA-containing cells detected at DIV 10 compared to the control female explants (E), mimicking the sex difference that exists *in vivo* .

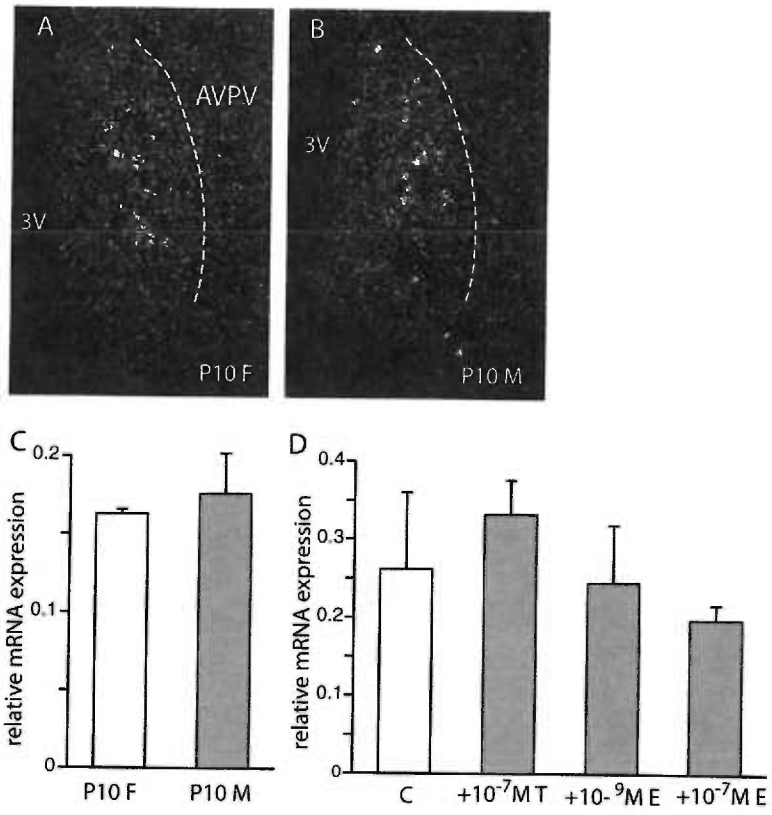

Figure 7. **PDYN mRNA levels in the P10 and DIV10 rat AVPV are not sexually dimorphic, and not altered by steroid treatment in vitro.** Low magnification darkfield images of the AVPV of P10 Female (A) and Male (B) rats illustrating pDYN mRNA detected by *in situ* hybridization. The pDYN mRNA levels the AVPV are not different between males and females at P10 (C). PDYN mRNA in AVPV explants treated for the first 24 hours in vitro with Testosterone, Estradiol, or vehicle, and fixed on DIV 10 all had similar levels of pDYN mRNA (D).

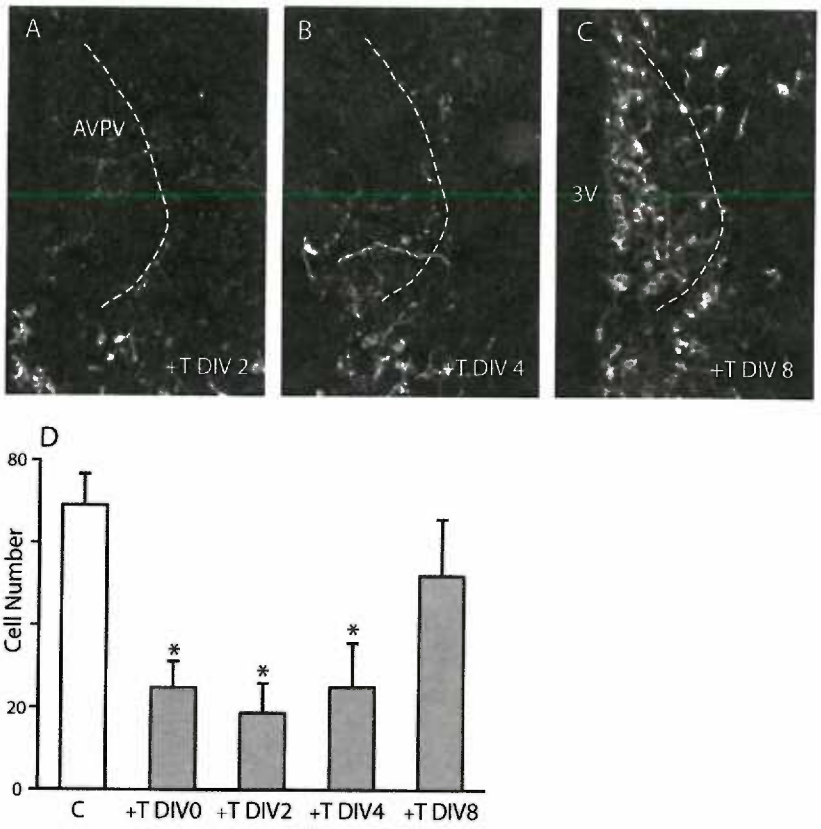

Figure 8. **TH-ir cell number is sensitive to the timing of steroid exposure.** Low magnification images of AVPv explants prepared on P0 and treated with Testosterone after 2, 4, or 8 days in vitro (A-C). Steriod treatment on DIV 0, 2, or 4 resulted in a significant decrease in TH-ir cell number compared to control explants (D). Delaying treatment until DIV 8 failed to cause a loss of TH-ir cells, suggesting a discrete critical period for the sexual differentiation of AVPV TH-ir cells.

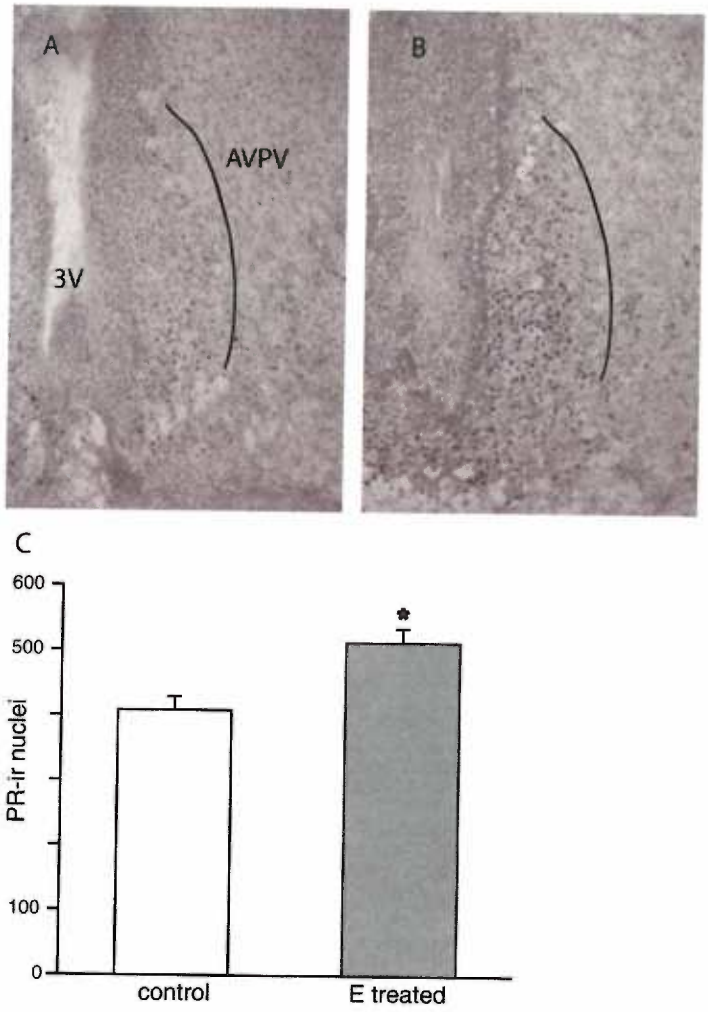

Figure 9. **AVPV explants remain steroid responsive for at least ten days in culture.** Low magnification images of AVPV explants cultured on P0 and treated on DIV 8 for 48 hours with either Estradiol (B) or vehicle (A) then examined for progesterone receptor immunoreactivity. Estrogen treatment resulted in a significant increase in PR-ir nuclei (C).

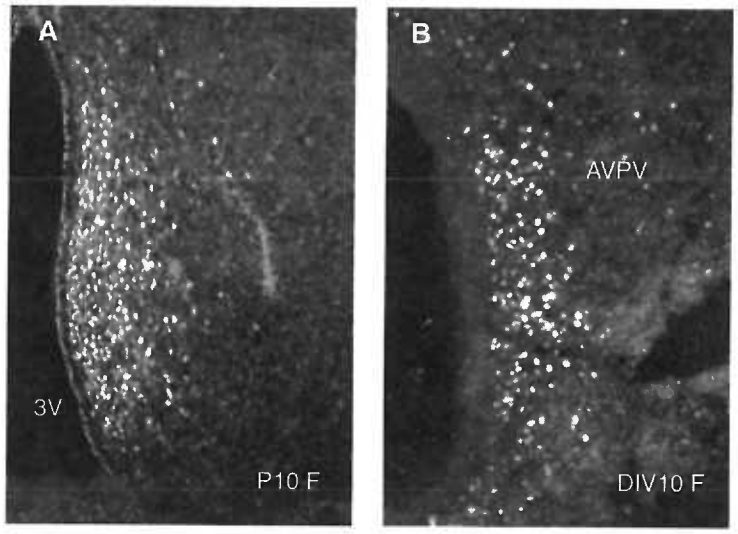

Figure 10. **Estrogen receptor alpha expression in ten day old rat AVPV.** ERalpha-ir cells in the AVPV of P10 Females (A) and DIV 10 Female explants (B).

CHAPTER 3

Estrogen-induced caspase-mediated cell death in the rat hypothalamic area, the anteroventral periventricular nucleus.

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Abstract

Sexual differentiation of the anteroventral periventricular nucleus of the hypothalamus (AVPV) results in an reduction in the volume of the area and in the number of dopaminergic neurons. This represents a novel estrogen action in a naturally occuring phenomena: estrogen induces selective postnatal destruction of neurons in a developmental process that is critical to the reproductive capability of the female animal. Estrogen is also important for the maintenance of select neurons in the AVPV, which parallels the neuroprotective function of estrogen reported in other sexually dimorphic hypothalamic areas. The parameters that regulate each cell type-specific effects of estrogen are unknown. We utilized a model of AVPV development to study, first, the cellular mechanism regulating dopaminergic neuron loss and, second, the molecular regulation of estrogen's action. Examination of cellular events *in vivo* and *in vitro* confirmed that estrogen initiates caspase-dependent cell death pathways to cause the permanent reduction in the number of dopaminergic cells recognized by antibodies for tyrosine hydroxylase. This loss depends on the function of ligand-bound ERα. Estrogen induced increases in the number of cells identified by programmed cell death markers and the activation of a caspase-dependent cell death pathway suggests that estrogen-induced loss of tyrosine hydroxylase immunoreactive cells is likely due to apoptotic programmed cell death.

Introduction

Estrogen is responsible for determining cell number, content, and morphology in brain areas containing steroid receptors. The sexually dimorphic regions of the hypothalamus differentiate early in postnatal development under the influence of estrogen's activities. In the sexually dimorphic AVPV, estrogen's effects sustain some neuropeptidergic populations but eliminate others, resulting in a nucleus that is sexually dimorphic in many aspects (for review see: Simerly 1998). While it is larger in size and contain mores dopaminergic neurons in the female rat than in the male rat; the male AVPV contains more proenkephalan neurons than female AVPV (Simerly et al., 1985, Simerly et al., 1988). This dichotomy suggests that estrogen influences both cell survival and cell death through cell type-specific mechanisms during development of the AVPV. Estrogen's role in neuroprotection has been recently described in variey of models (Wise et al., 2000). In contrast, estrogen-induced reduction in dopaminergic neuron number is a rare example of estrogen promoting the loss of cells over the survival of cells. Arai suggested (Murakami and Arai, 1989, Arai et al., 1996), that estrogen could increase measures of cell death, mainly pyknotic nuclei labeled by TUNEL or Nisel, in postnatal females treated with estradiol benzoate. In addition, the many reports of estrogen-regulated trophic factor expression and kinase activation suggest both genomic and non-genomic mechanisms for interactions of the ligand-bound receptor with programmed cell death

machinery. While sex steroid actions have been implicated in the regulation of the PCD pathway, these actions have been demonstrated to supress rather than stimulate the activation of the PCD pathway (Wise et al., 2001a,b).

Development of the sexually dimorphic AVPV has been coupled to sex steroid exposure during the first week of postnatal life. In males, a surge of testosterone (T), which occurs a few hours after birth (Weisz and Ward, 1980), is freely transported to the brain where it can be synthesized into estrogen (E) by aromatase, resulting in high local E levels (Amateau et al., 2004). In females, because of low ovarian production of E and the presence of alpha-fetoprotein, the level of circulating free estrogen is undetectable and presumed unable to affect the brain. The female brain is capable of generating spontaneous lutenizing hormone (LH) surges and ovulation; however, females treated with T perinatally did not produce LH surges as adults. This T treatment, which mimics the male experience, has also been shown to masculinize the number of AVPV dopaminergic neuron measured by tyrosine hydroxylase immunoeactivity (TH-ir) (Simerly et al., 1985). Moreover, castration of males prior to the T surge rescues dopaminergic neurons; however, cell number in these animals was restored by T treatment during the first postnatal week (Simerly, 1989). The dependence of sexual differentiation of TH-ir cell number on E synthesized from T was further demonstrated in

mice lacking a functional alpha form of the estrogen receptor (ERα, ERKO mice) (Simerly et al., 1997); the AVPV of these animals did not possess dimorphic TH-ir cell numbers. Development of the AVPV can also be studied an *in vitro* explant model that has been used to demonstrate the direct action of E on the AVPV to differentiate TH-ir cell number (Ibanez et al., 1998).

To examine the mechanism of E induced reduction in TH-ir cell number, we developed a model of AVPV sexual differentiation, *in vivo* and *in vitro*, that is rapid and complete by 48 hours after steroid exposure. We used this rapid AVPV differentiation model to demonstrate that the estrogen-induces an increase in PCD markers, which is dependent upon the timing of the steroid treatment and the presence of ERα. We also demonstrated that the E-induced decrease of TH-ir cells can be prevented through the application of ZVAD-fmk, a pan caspase inhibitor or ICI 182, 780, an antagonist of ERα. These results suggest that E-induced PCD during AVPV differentiation is mediated by ERα and caspase activation.

Methods

Animals All animals were housed under long day conditions (14:10) with food and water available *ad libitum* in accordance with institutional animal regulations. Pregnant rats

were purchased from Animal Technologies Limited. ERKO mice on a C57BL/6J background were obtained from Jackson Laboratories (Bar Harbor, MA). Animals processed for immunohistochemistry were anesthetized with tribromoethanol and perfused transcardially with 4% paraformaldehyde in borate buffer (pH 9.5). Animals prepared for TUNEL labeling were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were postfixed in the same fixative containing 20% sucrose for 2 hours, cryoprotected in 20% sucrose in 0.02 M potassium phosphate buffered saline (KPBS) overnight, frozen on dry ice and stored at –80°C until sectioning.

Tissue culture One day after birth, pups were cryoanesthetized and the whole brain removed and submerged in ice cold Gey's plus glucose (2.5g/250ml). The brain was sectioned at 300 μm on a vibratome (EMS, Hatfield, PA) through the preoptic area and the AVPV was microdissected using the lateral ventricles and anterior commisure as guides. Sections were mounted on each collagen coated (1mg/ml in 60% EtOH, Upstate, Lake Placid, NY) Millicell-CM membranes (Millipore, Bedford, MA) prewet with the serum-free defined media, EOL-1, containing thyroid hormone but lacking T, E, and progesterone. The cultures were incubated at 37°C (in 95% O2 and 5% CO2) and medium was changed after 24 hours. Cultures were fixed and processed for immunohistochemistry 48 hours after the application of experimental treatments. AVPV explants were fixed in 4% paraformaldehyde in borate buffer pH 9.5 for 15 minutes,

postfixed in 20% sucrose in fixative for 1 hour and cryoprotected in 20% sucrose in 0.02 M KPBS for 30 minutes. Explants were embedded in OCT (Sakura, Torrance, CA) and frozen until processed for immunohistochemistry.

Experimental treatments The stock solutions of 10^{-2}M 17-β-estradiol (Sigma, St. Louis, MO) and ICI 182,780 (Tocris, Ellisville, PA) were prepared in 100% EtOH and stored at –20°C. ZVAD-fmk (Calbiochem, San Diego, CA) was reconstituted in DMSO. All were diluted in EOL-1 culture media at the time of use. Estradiol benzoate (EB) (Sigma) was resuspended in corn oil at a concentration of 500 μg/ml. Each animal was injected with 50 μl (25 μg) subcutaneously 2 days after birth during the last twelve hours of the day.

Immunohistochemistry

All tissue was cryosectioned at 20 μm and thaw-mounted onto gelatin-coated slides. Tissue was pretreated overnight with LKPBS (0.02 M KPBS containing 2% normal goat serum (Upstate) and 0.4% Triton-X 100 (Biorad, Hercules, CA)). Antigen retrieval by 10 minute incubation in 10 μM citric acid (pH 6.0) at 80°C, was performed on tissue stained for activated caspase 3 and TH or ERα prior to pretreatment with LKPBS. Primary antibodies, mouse anti-tyrosine hydroxylase (1:1000, Diasorin, Hudson, WI), rabbit anti-estrogen receptor alpha (1:40,000, Upstate), rabbit anti-activated caspase 3 (CM-1, 1:40,000, BD Pharmigen, San Diego, CA), rabbit anti-tyrosine hydroxylase (1:500, Protos Biotech, New York, NY) or mouse anti-estrogen receptor alpha ((6F11), 1:1000,

Novocastra, Burlingame, CA)were applied for 48 hours at 4°C. TH and ERa staining was visualized with Alexa Flour 488 goat anti-mouse IgG and Alexa Flour 568 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene,OR), counterstained with Hoechst 33342 (1:10,000, Molecular Probes) in KPBS and coverslipped with buffered glycerol pH 8.6. For detection of activated caspase 3 staining, tyramide applification was used. Briefly, the tissue was incubated in biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) for 1 hour followed by ABC reagent (Vectastain Elite Kit, Vector Laboratories) for 1 hour and Biotynl tyramide (1:200,PerkinElmer, Boston, MA) for 20 min. and washed in KPBS between each step. Finally, tissue was incubated with strepadvin-FITC (1:200, Amersham, Pistacaway, NJ) to recognize the amplified activated caspase 3 signal and Alexa Flour 568 goat anti-mouse IgG to visualize TH or ERa, counterstained with Hoechst 33342, and coverslipped.

TUNEL labeling DNA fragmentation was visualized by TUNEL labeling with the In Situ Cell Death Detection Kit, flourescin used according to the manufacturer's instructions (Roche, Indianapolis, IN). Tissue was pre-treated with 0.5% TTX at 60 °C for 15 minutes then washed in PBS. The labeling reaction mixture was diluted 1:1 with diluent and was applied for 1 hour at 37°C. Finally, the tissue was rinsed in PBS and coverslipped.

Analysis

For all experiments, one side of the AVPV was counted in all tissue sections that contained the area. Explants or whole brains in which an intact AVPV could not be identified were excluded from the analysis. All cells were counted from one side of the AVPV in alternate sections from a one-in-two series through the rostral-caudal extent of the AVPV. All image analysis was performed blind to the experimental conditions. Cells containing TH, TUNEL label or blebbed nuclei were manually counted using a Zeiss Axioplan 2 microscope to visualize the AVPV. Flourescent images of the AVPV were captured at 10X using a CCD camera mounted on the microscope and saved using OpenLab software (Improvision Inc., Boston, MA). Colocalization of immunoreactivity was characterized using a Lecia confocal laser microscope. Uncorrected total cell counts for each experimental group were compared statistically with a one-way anova and Fischer's PLSD post hoc analysis using Statview software (SAS Institute Inc., Cary, NC) A p value of 0.05 or less was considered significant.

Results

Rapid differentiation of TH-ir cell number is induced by increasing E dose.

In order to study the molecular events governing estrogen induced TH-ir cell loss, we modified our AVPV explant system, which has been previously shown to reproduce AVPV sexual differentiation *in vitro* (Ibanez et al., 1998), to induce complete

differentiation of TH-ir cell number within 48 hours of steroid exposure. The AVPV was treated with estrogen on the second day after birth and processed 48 hours later for TH immunohistochemistry to quanitate the degree of sexual differentiation that had occurred. AVPV organotypic explants prepared from one day old female rats were maintained in culture for 24 hours and then exposed to experimental treatments. 48 hours after the addition of steroids or inhibitors the explant cultures were fixed and processed for immunohistochemistry. In the current set of experiments, we sought to reduce TH-ir cell number to adult male levels within 48 hours after steroid treatment. To facilitate this effect of estrogen, the dose given to each explants was increased to 10^{-5} M E. Although E treatments of 10^{-7} M and 10^{-9} M (data not shown) significantly decreased the number of AVPV TH-ir cells after 48 hours, only 10^{-5} M E treatment fully realized the decrease in TH-ir cell number that equals those numbers reported in male adult rats (Fig. 1A, *$p<0.005$),(Simerly et al., 1987). Increasing levels of E to 10^{-5} M caused no further loss of AVPV TH-ir cells when compared to TH-ir cells in AVPV treated with 10^{-7} M E after 48 hours or 10 days in cultures (Fig. 1B *$p<0.001$). Interestingly, in a few 10 day cases of AVPV treated with 10^{-5} M, E seemed to protect TH-ir cells from the reduction in number seen in 10^{-7} M E treated AVPV. In a similar set of experiments we treated mice AVPV explants with 10^{-5} M E for 48 hours and found that TH-ir cell number did differentiate in C57BL\6 wildtype females but did not in ERKO females or males (data

not shown). Parallel studies in vivo demonstrated that 25 μg estradiol (EB) injected subcutaneously on P2 also reduced TH-ir cell number to male adult levels by 48 hours after treatment. Changes in cell number were detected earlier *in vivo* than they were *in vitro*, TH-ir cell number *in vivo* was reduced and equaled to 10 day old animals by 24 hours after treatment (Fig. 2A). Distribution of TH-ir cells *in vitro* (Fig 4A and B) and *in vivo* (Fig 2B) were largely confined to the periventricular zone and were comparable to that previously described in adults (Simerly et al., 1987).

Markers of PCD are increased by estrogen exposure.

Apoptotic cells often undergo stereotypical changes in cellular morphology, including nuclear condensation and fragmentation. DNA fragmentation is also a marker of cells undergoing PCD. We examined the AVPV *in vivo* to determine whether or not E could induce these changes characteristic of dying cells. Similar to previous descriptions (Arai et al., 1996), EB increased the incidence of fragmented nuclei 24 hours after treatment in P2 rat pups compared to oil treated control pups (Fig 3A,*$p<0.01$). This effect is dependent on the timing of the treatment: EB administered to P6 rats did not increase fragmented nuclei 24 hours later (Fig. 3C). A comparison of wildtype mice versus ERKO mice treated with estrogen on P2 revealed that fragmented nuclei were increased in EB-treated wildtype similar to P2 rats but were not increased in EB treated ERKO

mice (Fig. 3D, *p<0.05). DNA fragmentation was also increased by E: 20 hours after steroid treatment, the number of TUNEL-labeled nuclei was increased in the AVPV of EB treated animals versus oil treated animals (Fig. 3B, *p<0.01).

ZVAD-fmk prevented estrogen-induced TH-ir cell loss.

The role of caspase activation in TH-ir cell loss was examined through the treatment of AVPV explants cultures concurrently with 10^{-5} M E and the pan-caspase inhibitor ZVAD-fmk. After 48 hours of treatment, TH-ir cells were counted to quanitate the degree of AVPV sexual differentiation that had occurred. TH-ir cell loss was completely blocked when cultures were treated with $5.0x10^{-5}$ M ZVAD plus 10^{-5} M E (Fig. 5, *p<0.005). TH-ir cell number in these cultures were indistinguishable from TH-ir cells in control cultures that had never experienced E treatment (Fig. 4C). In contrast, $2.5x10^{-5}$ M ZVAD had no effect on E-induced TH-ir cell loss, while a four fold higher dose, used to test ZVAD toxicity, also had no effect on TH-ir cell number in the absence of E (Fig. 5). The distribution of TH-ir cells within AVPV was not altered by ZVAD treatment; most TH-ir cells were detected in the periventricular zone of the AVPV, as they were in control AVPV.

TH-ir cells with apoptotic nuclei stained with Hoechst were documented in an AVPV explant 2 hours after E treatment. A representative cell is shown in a single confocal image plane (Fig. 6A). Also detected were AVPV cells that colocalized staining for activated caspase 3 and apoptotic nuclei (Fig. 6B). Infrequently, AVPV TH-ir cells that was colabeled for activated caspase 3 and contained an apoptotic nucleus were seen.

ICI 182,780 blocked estrogen-induced TH-ir cell loss.

The dependence of estrogen's effect on the activation of ERα was tested by simultaneous application of E and the ERα antagonist ICI 182,780. The AVPV of cultures 10^{-5} M ICI 182, 780 and 10^{-5} M E displayed TH-ir cell numbers equivalent to the AVPV of control cultures (Fig 4D). Both of these groups contained significantly more TH-ir cells than cultures treated with 10^{-5} M E alone (Fig 7, *$p<0.005$). The other doses of ICI 182,780 tested, 10^{-7} M and 10^{-6} M, attenuated TH-ir cell loss, but only 10^{-5} M ICI 182,780 fully rescued TH-ir cells from E induced loss. ICI 182,780 alone had no effect on TH-ir cell number in the AVPV.

Discussion

Estrogen has a permanent effect on cell number during development. In the hypothalamus, E actions differentially regulate cell survival thereby altering the neuronal

content of sexually dimorphic brain areas. In this study, we demonstrated that E increases PCD of TH-ir cells during postnatal development of the AVPV, resulting in sexually dimorphic cell numbers. We have previously shown that AVPV TH-ir cell number is dependent upon E exposure during the first postnatal week (Simerly, 1989, Ibanez et al., 1998), and requires the presence of a functional ERα (Simerly et al., 1997). For the current experiments, we developed rapid differentiation models of the AVPV, *in vitro* and *in vivo*, that mimics and accelerates the naturally occuring phenomena, and used it to show that E increases in apoptotic nuclei. We also demonstrated that cell loss is dependent on the timing of the E exposure and the presence of ERα. Finally, E-dependent decreases in TH-ir cell number requires caspase activation.

TH-ir cell loss during AVPV sexual differentiation occurs via an estrogen-mediated caspase-dependent mechanism. Caspase activation is a hallmark event in PCD and the role of caspase dependent cell death during development in the brain has been established through the development of caspase knockout mice (Oppenheim et al., 2001). Caspase activation occurs in both apoptotic and autophagic forms of PCD (Clarke, 1990) Interestingly, caspase-independent cell death was also revealed by the caspase knockouts. Concurrent treatment of AVPV organotypic explants with E and the pan-caspase inhibitor, ZVAD-fmk, realized a complete rescue of TH-ir cell number. The capacity of

caspase inactivation to neutralize E effects on TH-ir cells together with the increased apoptotic nuclei induced by E treatment illustrates the potential role of apoptosis in AVPV development.

Caspase 3 is the effector caspase most closely tied to developmental PCD. It was detected in its activated form 8 hours after E treatment of AVPV explants (data not shown). Interestingly, although TH-ir cell number is dimorphic at 48 hours after E treatment *in vitro*, it is not dimorphic at 24 hours after E exposure (data not shown). This delay between caspase activation and TH-ir cell loss suggests that TH downregulation is not required for the death of TH-ir cells. Although TH mRNA expression is acutely regulated in adults, we have seen no evidence for acute regulation of TH protein levels in perinatal rat AVPV. *In vivo* TH-ir cell number is completely dimorphic 24 and 48 hours after EB exposure. In addition, TH-ir cell number is reduced significantly 16 hours after treatment but not to the final levels detected at P10 (data not shown). Together the *in vivo* and *in vitro* timelines suggest that an E-induced genomic event, rather than a rapid non-genomic action, is necessary for caspase activation and cell death of TH-ir cells.

Estrogen induction of PCD is mediated by ERα. The first mechanism of ERα described was its function as a transcription factor and many of its actions depend upon ligand-activated gene transcription. Application of ICI 182,780 prevented E-induced TH-ir cell

loss in AVPv explants. The dose of ICI 182,780 that effectively blocked TH-ir cell loss was equimolar to the E treatment, rather than several fold greater. This fact suggests that ICI 182, 780, which blocks genomic effects of ligand-bound ERα, inhibits a PCD pathway that would otherwise activated by E-induced gene transcription. Although E has been implicated in both transcription-dependent and -independent neuroprotection (Wise et al., 2001b), PCD activation remains largely transcription dependent. ERα-ir is present in TH-ir cells at the time that estrogen treatment induces capase activation and TH-ir cell death. However, because ERα-ir was detected in just 30 percent of the TH-ir cells (data not shown), ERα-mediated PCD maybe directly or indirectly activated by E in TH-ir cells.

It is possible that an indirect action of E is responsible for TH-ir cell death and the direct action of E on TH- and ERα-ir cells is neuroprotective. The action of E on nearby ERα-containing neurons could be responsible for an indirect action of E on TH-ir cell death. Also, it's possible that paracrine action of growth factors or death signals induced by E in ERa expressing neurons contributes to the loss of TH-ir cells. For example it maybe that TH-ir containing ERα are neuroprotected while TH-ir that do not contain ERα are suspectible to E-mediated cell death signals because they do not express anti-apoptotic molecules. This hypothesis is supported by recent studies of Bcl-2-overexpressing mice

in which there was effect on AVPV TH-ir cell number detected in adults (Zup et al., 2003). Moreover, E induction of anti-apoptotic BCL family members protects neurons in the hypothalamus (Garcia-Segura et al., 1998), hippocampus (Harms et al., 2001), and cortex (Dubal et al., 1999) after the experimental induction of cell death. Estrogen's neuroprotective effect is modulated by ERα in the hippocampus and cortex and results in the increased expression of Bcl-2 and Bcl-x (Dubal et al., 1999, Pike, 1999, Harms et al., 2001, Stoltzner et al., 2001).

Estrogen regulation of pro-apoptotic proteins may lead to caspase activation in TH-ir cells. This effect could be due to direct actions of E on TH-ir cells, resulting in the expression of molecules that facilitate cell death. Pro-apoptotic molecules implicated in the developmental regulaton of PCD include Bax and Bid, whose actions results in the formation of the apoptosome and activation of effector caspases. However, studies performed on Bax knockout mice revealed no alteration in TH-ir cell number, suggesting that other proteins are involved in E-induced TH-ir cell loss (data not shown). Another death signalling protein that could play a role in the hypothalamus is the Fas ligand (FasL). FasL expression is regulated by estrogen in the endometrium (Selam et al., 2001) and in humans, its promoter contains both an estrogen response element and an AP-1 site (Mor et al., 2003). FasL binding to Fas receptor initiates caspase 8 cleavage, whose

actions then result in the activation of caspase 3 and the death of the cell. E regulation of FasL expression has not been exmained in the hypothalamus during development.

The beta form of the estrogen receptor (ERβ) is likely not involved in differentiation of TH-ir cell number. The expression of ERβ mRNA is very low in P2 rats and rises to levels similar but not equal to ERα in P10 rats (Kirigiti and Simerly, 1999). However, ERβ protein is undetectable until P7 (Orikasa et al., 2002, Perez et al., 2003). In addition, the effectiveness of the ERα antagonist ICI 182,780 in blocking TH-ir cell loss suggests that the E effect is genomic through ERα and not ERβ. The abolition of sex differences in TH-ir cell number in ERKO mice also indicates that ERβ activation does not play a role in the control of TH-ir cell number. The increasing presence of ERβ at the end of the first postnatal week may regulate the end of the critical period for the ERα-mediated induction of TH-ir cell loss and apoptotic markers. ERβ may oppose the transcriptional effects of ERα through interactions that alter ERα-mediated gene transcription (Paech et al., 1997).

Differential expression of steroid receptor coregulators could also be responsible for the cell-type and time-specific actions of E (McKenna et al., 1999). Coactivators can alter interactions of ERs with DNA, selectively promoting gene transcription. Alternatively,

corepressors could inhibit transcription of E-induced proteins, effectively blocking transcription-dependent E effects. Furthermore, coactivators have been implicated in the organization of brain areas important to reproductive behavior. Creb binding protein (CBP) expression is dimorphic in the hypothalamus and important for defeminizing reproductive behavior (Auger et al., 2002). The p160 coactivators, steroid receptor coactivator-1 and 2 (SRC1 and 2) are also implicated in the development of female reproductive behavior. Infusion of SRC1 and 2 antisense into the hypothalamus during development disrupted lordosis (Apostolakis et al., 2002).

The initiation of PCD pathways has been likened to tightrope walking. The balance of pro- and anti-apoptotic forces within the cell determines its response to environmental stimuli aimed at pushing it over the edge towards cell death. The threshold that integrates the pro- and anti-apoptotic forces ensures the specificity of cell death activation during development, resulting the definition of the nervous system. E effects during development of the AVPV may contribute to the induction of pro-apoptotic events as well as determination of the threshold for TH-ir cell death. Thus, reception of the pro-apoptotic E signal does not guarantee that TH-ir cells will die- not all TH cells do.

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The contributions of each author were as follows. E. M. Waters performed all experiments and analysis described in the paper, as well as prepared the manuscript.

K. S. Korach provided the estrogen receptor alpha knock out mice.

R.B. Simerly provided support for the experiments.

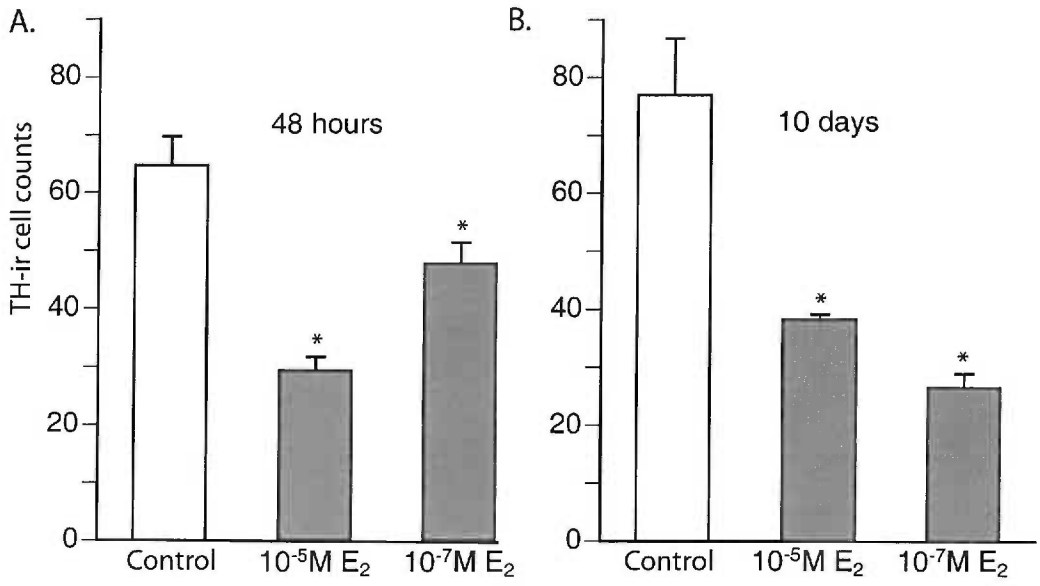

Figure 11. **AVPV TH-ir cell number *in vitro* is dimorphic 48 hours after estrogen treatment.** Both 10-7 and 10-5 M 17-b-estradiol reduced TH-ir cell number within 48 hours (A). The change in cell number induced by 10-5 M represents the complete reduction of TH-ir cell number that is detected at P10 or in adult AVPV. Both estrogen doses result in completely dimorphic TH-ir cell number ten days after treatment of AVPV explants (B).

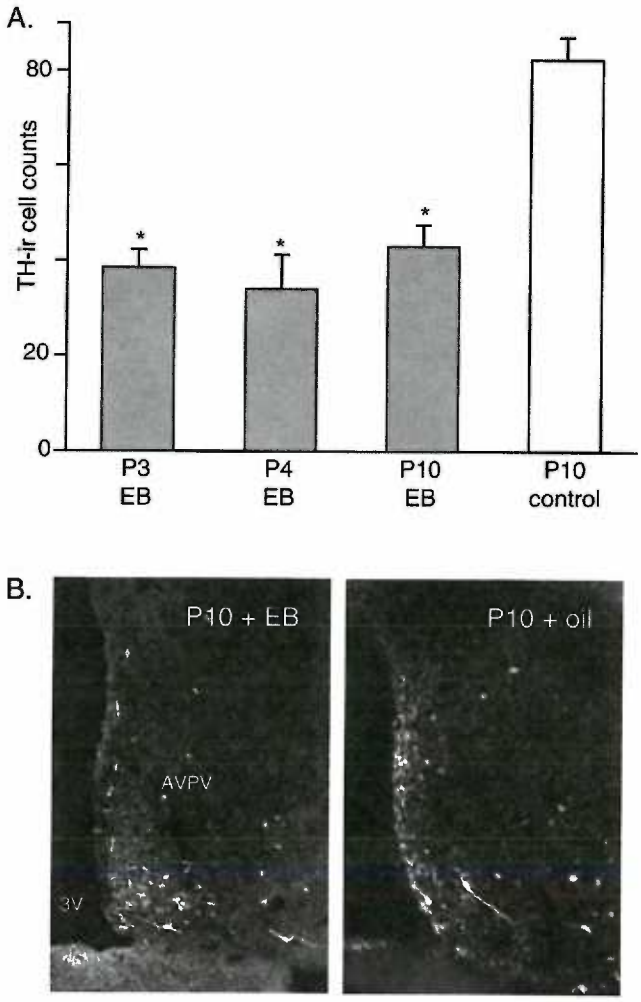

Figure 12. ***In vivo* rapid differentiation of TH-ir cell number is detected 24 hours after treatment with estradiol benzoate (EB).** TH-ir cell number after 24 and 48 hours and ten days in estrogen treated animals is significantly reduced compared to control animals (A). Representative photomicrographs of TH-ir staining in the AVPV of ten day old females treated with estrogen (left) or control (right) (B).

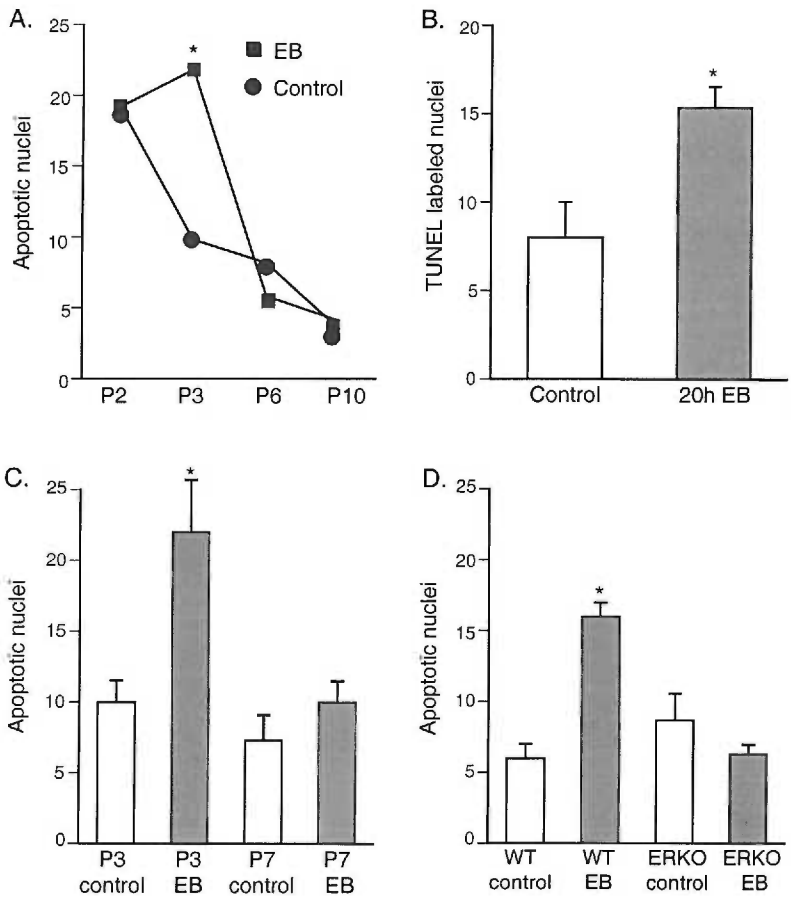

Figure 13. **Apoptotic nuclei labeled by TUNEL and hoechst staining are increased by estrogen treatment *in vivo*.** The number of EB-induced apoptotic nuclei peaks 24 hours after treatment then returns to control levels (A).TUNEL-labeled nuclei are increased by two-fold in EB- versus control-treated AVPV (B). 24 hours of EB treatment increased the number of apoptotic nuclei in the AVPV of P3 rats and mice but not in the AVPV of P7 rats or P3 ERKO mice (C),(D).

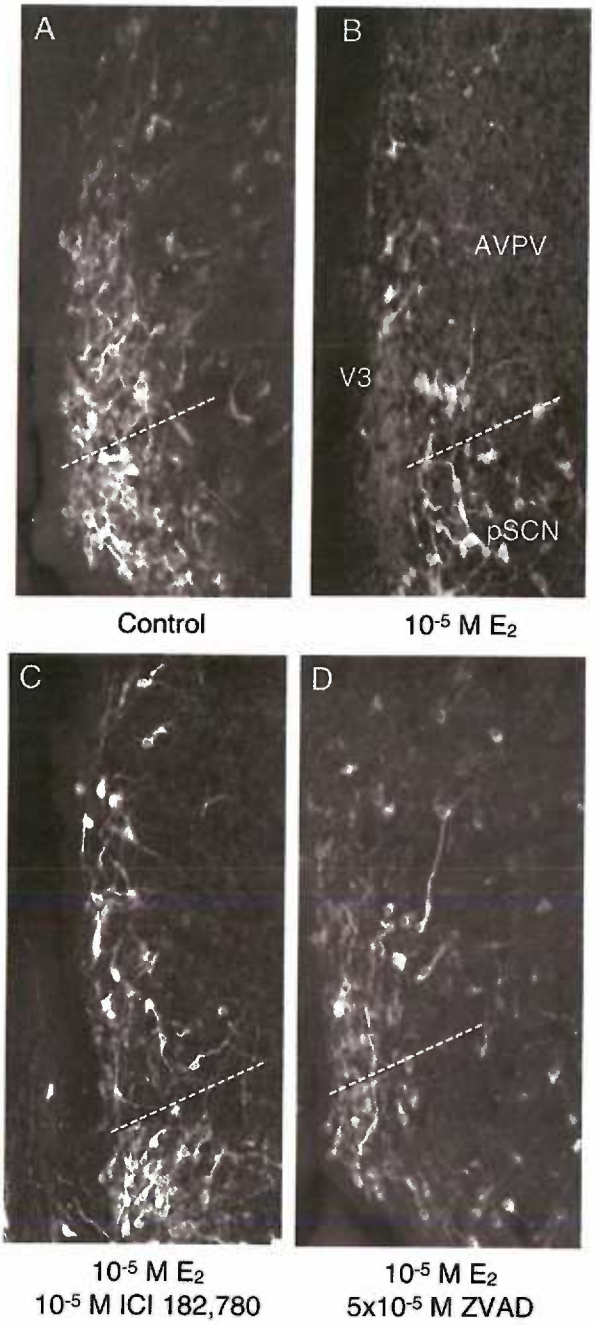

Figure 14. **Representative flourescent photomicrographs of TH-ir cells in AVPV explants** treated with (A) control media, (B) 10-5 M estrogen, (C) estrogen and ICI 182,780, both 10-5 M, or (D) 10-5 M estrogen plus 5x10-5M ZVAD-fmk for 48 hours.

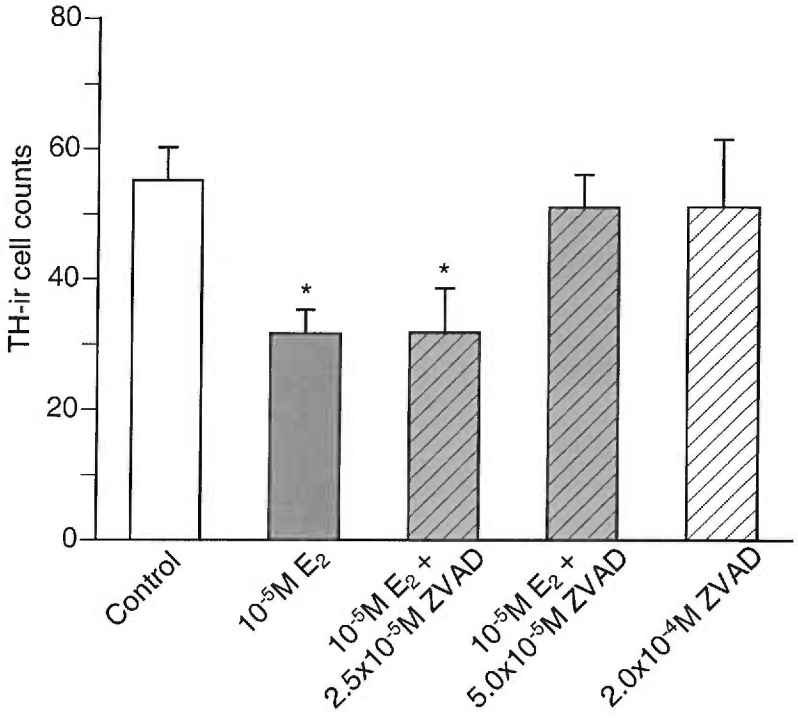

Figure 15. **Inhibition of caspase activation completely rescues TH-ir cell number after estrogen treatment.** Treatment of AVPV explants concurrently with 10-5 M estrogen and 5x10-5M ZVAD-fmk blocked reduction in TH-ir cell number, while 2.5x10-5M ZVAD-fmk did not prevent E-induced TH-ir cell loss. Treatment with a four-fold higher dose of ZVAD-fmk alone has no effect on TH-ir cells.

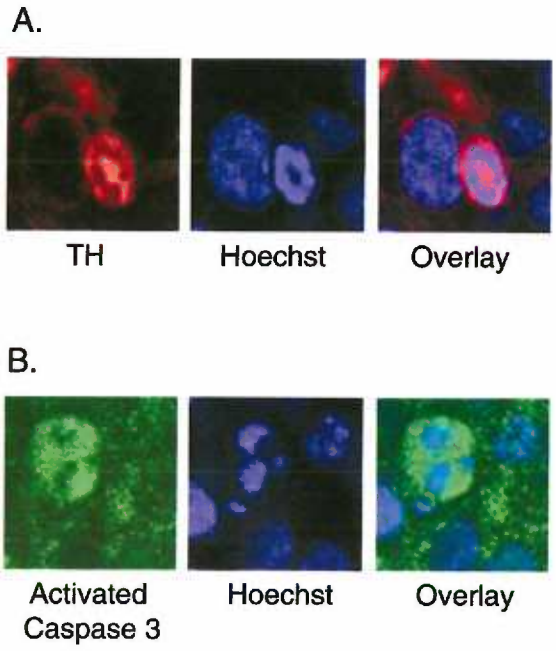

Figure 16. **Representative confocal images of apoptotic nuclei in the AVPV** colocalized with staining for TH (A) or activated caspase 3 (B).

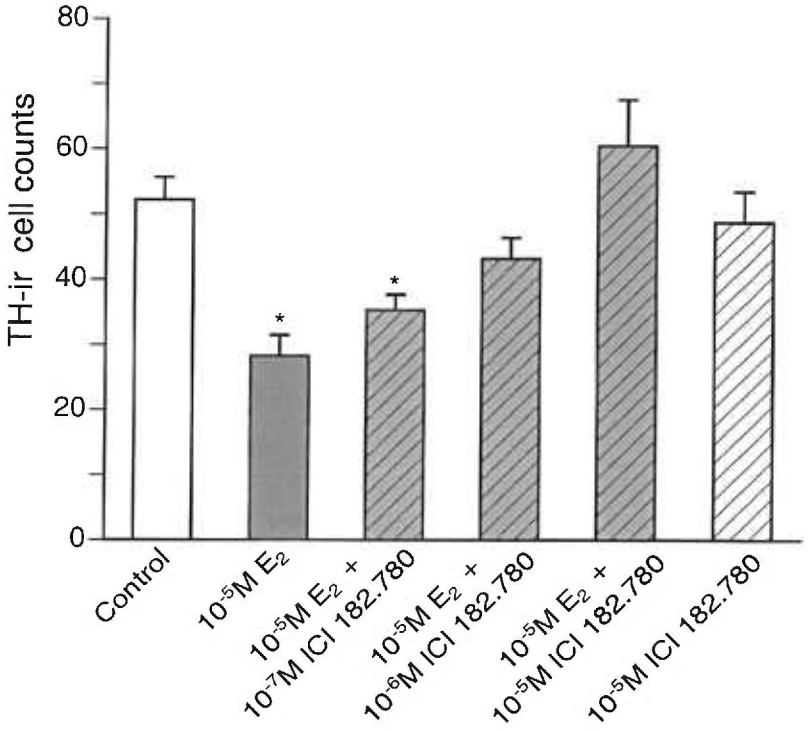

Figure 17. **Estrogen's effect on TH-ir cell number is dependent on ERα.** ICI 182,780 blocks E-induced AVPV TH-ir cell loss in a dose depedent manner but only the 10-5 M treatment fully prevented reduction in TH-ir cell numbers.

CHAPTER 4

Overexpression of Bcl-2 reduces sex differences in neuron number in the brain and spinal cord.

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Journal of Neuroscience. 2003; 23(6):2357-62.

My contribution to this paper consisted of immunohistochemical staining of the AVPV tissue for tyrosinse hydroxylase and the analysis of cell number.

Overexpression of Bcl-2 Reduces Sex Differences in Neuron Number in the Brain and Spinal Cord

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Several sex differences in the nervous system depend on differential cell death during development in males and females. The anti-apoptotic protein, Bcl-2, promotes the survival of many types of neurons during development and in response to injury. To determine whether Bcl-2 might similarly control cell death in sexually dimorphic regions, we compared neuron number in wild-type mice and transgenic mice overexpressing Bcl-2 under the control of a neuron-specific promoter. Three neural areas were examined: the spinal nucleus of the bulbocavernosus (SNB), in which neuron number is greater in males; the retrodorsolateral nucleus (RDLN) of the spinal cord, which exhibits no sex difference in neuron number; and the anteroventral periventricular nucleus (AVPV) of the hypothalamus, in which both overall cell density and the number of tyrosine hydroxylase immunoreactive (TH-ir) neurons are greater in females. Bcl-2 overexpression significantly increased SNB cell number in females, overall cell density of AVPV in males, and RDLN cell number in both sexes. Bcl-2 overexpression did not alter the number of TH-ir neurons in AVPV of males or females. These findings indicate that Bcl-2 can regulate sexually dimorphic cell number in the brain and spinal cord and suggest that Bcl-2 may mediate effects of testosterone on cell survival during neural development. In contrast to the regulation of overall cell density in AVPV, the sex difference in TH cell number apparently is not caused by a Bcl-2-dependent mechanism.

Key words: Bcl-2; sex difference; cell death; nervous system; spinal nucleus of the bulbocavernosus; anteroventral periventricular nucleus

Introduction

Sex differences in neuron number have been described in the mammalian nervous system and often can be attributed to the hormonal control of cell death (Tobet and Hanna, 1997; Forger, 2001). Testosterone, produced by the testes during perinatal life, increases cell death in some neural regions, whereas it decreases death in others. In the spinal nucleus of the bulbocavernosus (SNB), a cluster of motoneurons in the lumbar cord which innervates penile muscles, testosterone acts via androgen receptors to prevent cell death (Breedlove and Arnold, 1983; Nordeen et al., 1985). As a result, adult males have many more SNB cells than do females (Breedlove and Arnold, 1980). Conversely, the anteroventral periventricular nucleus (AVPV), a nucleus at the rostral extreme of the third ventricle implicated in the control of gonadotrophin release, is larger and exhibits greater cell density in females than in males (Bleier et al., 1982; Sumida et al., 1993). These sex differences are attributable, at least in part, to the fact that testosterone or its estrogenic metabolites increase cell death in AVPV during perinatal life (Murakami and Arai, 1989; Nishizuka et al., 1993; Sumida et al., 1993; Arai et al., 1996). Females also have many more dopaminergic neurons in AVPV than do males (Simerly, 1998), but it is not known whether this sex difference arises as the result of differential cell death in males and females or some other mechanism. Although the development of sex differences in neuron number has been best studied in rats, the sex differences in the SNB and AVPV and their developmental dependence on gonadal steroid hormones are similar in mice (Wee and Clemens, 1987; Wagner and Clemens, 1989; Forger et al., 1997; Simerly et al., 1997).

In many neural regions, developmental cell death is critically controlled by the Bcl-2 family of proteins. Some family members, such as Bcl-2 itself, promote cell survival, whereas other family members promote cell death. According to the "rheostat" model of cell death, it is the ratio of pro-life to pro-death molecules that determines whether a cell will live or die (Yang and Korsmeyer, 1996), and elevated expression of a survival-promoting family member should reduce the likelihood of cell death. Indeed, overexpression of Bcl-2 has been shown to prevent neuronal cell death in several experimental paradigms (Allsopp et al., 1993; Dubois-Dauphin et al., 1994; Martinou et al., 1994; Bonfanti et al., 1996). The effect of Bcl-2 overexpression on developmental cell death in sexually dimorphic regions, however, has not been explored.

In the present study, the SNB and AVPV were examined in transgenic mice overexpressing Bcl-2 under the control of a neuron-specific promoter. We reasoned that if Bcl-2 normally promotes cell survival in these regions, then Bcl-2 overexpression might prevent cell death, thereby reducing or eliminating sex differences that depend on differential apoptosis in males and females. For comparison, we also examined the retrodorsolateral nucleus (RDLN), a spinal nucleus in which cell number is neither sexually dimorphic nor affected by perinatal hormone treatments (Jordan et al., 1982; Leslie et al., 1991).

Received Aug. 27, 2002; revised Dec. 30, 2002; accepted Dec. 31, 2002.

This work was supported by National Institutes of Health Grants HD33044 (N.G.F.), HD01188 (N.G.F.), and NS37952 (R.B.S.). We are grateful for the excellent technical assistance provided by Jennifer Pfau and Sabrine Montaldo.

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Materials and Methods

Animals. Transgenic male mice overexpressing human Bcl-2 under the control of the neuron-specific enolase promoter [line NSE73a in Martinou et al. (1994)] were obtained from Dr. J. C. Martinou (University of Geneva, Geneva, Switzerland) through Dr. Flint Beale (Massachusetts General Hospital, Boston, MA). This line was chosen because expression of the transgene is targeted to the nervous system and can be detected in essentially all postmitotic neurons of the brain and spinal cord by embryonic day 13. Moreover, expression of the transgene far exceeds the production of endogenous mouse Bcl-2, i.e., there is true "overexpression" of Bcl-2 in these animals (Martinou et al., 1994). We used antibodies specific for human Bcl-2 to verify that the transgene is abundantly expressed in the SNB, RDLN, and AVPV of the overexpressors (Park et al., 2002; E. M. Waters and R. B. Simerly, unpublished observations).

Wild-type and Bcl-2 overexpressing offspring were generated by mating Bcl-2 overexpressing males with B6D2F1 females (The Jackson Laboratory, Bar Harbor ME) and were genotyped by PCR amplification of tail DNA using published primer sequences (Coulpier et al., 1996). Mice were housed in groups of three to four in a 12 hr light/dark cycle and held at 24°C. Adult mice (60–90 d old) were overdosed with pentobarbital and then perfused intracardially with saline followed by 4% paraformaldehyde.

Motoneuron size and number. Motoneurons in the SNB and RDLN were examined in 36 mice (n = 7–10 animals per group). Lumbosacral spinal cords were dissected out, postfixed in 10% formalin, and immersed in Bouin's solution for 48 hr before paraffin embedding. Serial, 12 μm coronal sections were collected, mounted on slides, and thionin stained. Mean cell size for both SNB and RDLN motoneurons was determined by camera lucida tracings of the somas and nuclei of at least 20 motoneurons per nucleus, per animal. Sections chosen for tracing were spaced equally throughout the rostrocaudal extent of each cell group, and all possible motoneurons from these sections were traced to avoid experimenter bias.

SNB and RDLN motoneurons were counted in alternate sections as in previous studies (Forger et al., 1997; Park et al., 1999). Only motoneurons with a clearly visible nucleus and nucleolus were included in the analysis; the SNB was counted bilaterally, and motoneurons in the RDLN were counted unilaterally. The Konigsmark correction (Konigsmark, 1970) was then used to correct raw motoneuron counts for sampling ratio and for overcounting of split cells on the basis of the size of the object counted (motoneuronal nucleus) in each animal. In preliminary counts, the RDLN population appeared heterogeneous, containing a subpopulation of cells that were small but nonetheless exhibited the classic staining characteristics of motoneurons. Therefore, counts of RDLN motoneurons were divided into "small" or "average" cells on the basis of simple visual inspection by an experimenter blind to treatment groups.

AVPV overall cell density and tyrosine hydroxylase cell number. Brains were removed from the skulls of 29 mice (n = 5–9 per group), blocked, placed in 15% sucrose overnight, and then sectioned at 30 μm in the coronal plane on a freezing microtome. Serial sections were mounted on slides and thionin stained. Cell density counts were made on two sections in AVPV of each animal, at a final magnification of 1000×. For counting, an eye-piece reticule was used to superimpose a grid (192 × 240 μm) on AVPV. The grid was lined up against the third ventricle, and only darkly staining cells with the characteristic morphology of neurons were counted by an observer blind to treatment groups. The mean of the counts was determined and designated as "total cell density" for that animal. This method of counting is similar to that used previously to estimate neuron density in AVPV (Sumida et al., 1993). Cell size was determined by tracing the soma and nucleus of at least 50 neurons in two sections through AVPV, and tracings were imported into SigmaScan to determine cross-sectional areas. Because there was no effect of sex or genotype on cell size in AVPV (see below), no corrections were made for double-counting of split cells. Thus, the cell densities reported may slightly overestimate the density of neurons present in AVPV, but between-group comparisons should remain valid. Nucleus length was determined by multiplying the number of sections containing the AVPV by the section thickness.

A small subset of all neurons in AVPV are dopaminergic, as identified by tyrosine hydroxylase (TH) immunoreactivity, and females have many more TH-immunoreactive (TH-ir) neurons in AVPV than do males (Simerly et al., 1985, 1997). To determine whether Bcl-2 influences the number of dopaminergic neurons in AVPV, brains were harvested from a separate cohort of mice (n = 4–6 per group) perfused with 4% paraformaldehyde in borate buffer, pH 9.5. Coronal sections (20 μm) through AVPV were immunostained for TH and estrogen receptor α, as described previously (Simerly et al., 1997), using mouse anti-tyrosine hydroxylase antiserum (1:1000; DiaSorin, Stillwater, MN) and rabbit anti-estrogen receptor α (1:40,000; Upstate, Waltham, MA). The primary antibodies were localized with goat anti-mouse secondary conjugated to Alexa 488 and goat anti-rabbit secondary conjugated to Alexa 586 (Molecular Probes, Eugene, OR). Estrogen receptor α-immunoreactive cells were used to select sections containing AVPV and to identify the borders of the AVPV from each animal. TH-ir cells with clearly visible nuclei were counted in all sections containing the AVPV.

Data analysis. The effects of sex and Bcl-2 overexpression on SNB and RDLN motoneuron number and size as well as AVPV length, cell density, cell size, and TH-ir cell number were evaluated using separate two-way ANOVAs. Planned comparisons were performed after significant main effects using Fisher's LSD.

Results

Effects of Bcl-2 overexpression on SNB motoneurons

The robust sex difference in SNB cell number seen previously in rats and mice was confirmed, with males having many more motoneurons than females ($F_{(1,32)} = 73.0$; $p < 0.0005$) (Fig. 1). There was no main effect of Bcl-2 overexpression on SNB cell number ($F_{(1,32)} = 3.0$; $p = 0.09$). However, there was a significant sex-by-genotype interaction ($F_{(1,32)} = 6.7$; $p < 0.05$), attributable to the fact that Bcl-2 overexpression increased SNB motoneuron number by 53% in females ($p < 0.005$) and did not affect cell number in males ($p > 0.56$) (Fig. 1). In accord with previous observations, males had larger SNB motoneuron somas and nuclei than did females ($p < 0.0005$ in both cases) (Table 1). There was no effect of Bcl-2 overexpression and no sex-by-genotype interaction on SNB cell size.

Effects of Bcl-2 overexpression on the RDLN

Bcl-2 overexpression significantly increased the total number of RDLN motoneurons, with overexpressing mice having ~22% more RDLN motoneurons than their wild-type siblings ($F_{(1,32)} = 27.0$; $p < 0.0005$) (Fig. 2*A*). There was no main effect of sex and no sex-by-genotype interaction on total number of RDLN cells, and planned comparisons confirm that the increase was significant within both males and females ($p < 0.005$ in both cases). A population of small motoneurons was noted within the RDLN that, across groups, accounted for ~15% of the total. Bcl-2 overexpression significantly increased the number of both "small" and "average" RDLN cells, but the effect was much more pronounced on the small cells: Bcl-2 overexpressors had 114% more small ($p < 0.0005$) (Fig. 2*B*) and 9% more average RDLN cells than did wild-type mice ($p < 0.05$; data not shown). As a result, mean RDLN motoneuron nucleus and soma sizes were decreased in Bcl-2-overexpressing mice ($p < 0.01$) (Table 1). There was no main effect of sex and no sex-by-genotype interaction on RDLN cell size. Thus, Bcl-2 overexpression increased motoneuron number in the RDLN of both males and females, and many of the supernumerary cells were small.

Effect of Bcl-2 overexpression on AVPV cell density, nucleus length, and TH-ir cell number

As reported previously (Bleier et al., 1982; Sumida et al., 1993), there was a modest sex difference in AVPV cell density, with

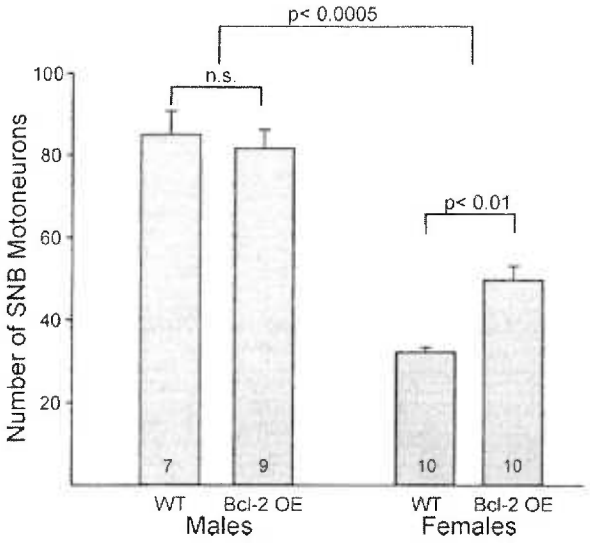

Figure 1. The mean (±SEM) number of SNB motoneurons in wild-type (*WT*) and Bcl-2-overexpressing (*Bcl-2 OE*) mice. Bcl-2 overexpression increased motoneuron number in females but not in males. *n* = *numbers* at base of bars. *n.s.*, Not significant.

higher densities in females ($F_{(1,25)} = 8.1$; $p < 0.01$) (Fig. 3A). Bcl-2 overexpression increased overall cell density in AVPV ($F_{(1,25)} = 11.2$; $p < 0.005$), and planned comparisons indicate that this increase was significant for males ($p < 0.005$) but not for females ($p > 0.1$). As a result, the sex difference in cell density was no longer significant in Bcl-2-overexpressing mice. There was no effect of either sex or Bcl-2 expression on nucleus or soma size in AVPV (Table 1). AVPV volume has also been reported to be greater in female mice than in males, and this sex difference is attributable primarily to the fact that the nucleus is longer in females (Bleier et al., 1982). In the current sample, nucleus length tended to be greater in females than in males, but this difference did not reach statistical significance ($F_{(1,25)} = 3.6$; $p = 0.069$). There was no effect of Bcl-2 overexpression on length of AVPV.

In confirmation of previous reports (Simerly et al., 1985, 1997), the number of TH-ir cells in the present study was 3.3 times greater in females than in males ($p < 0.0005$). However, there was no effect of Bcl-2 overexpression and no sex-by-genotype interaction on TH cell number. Thus, Bcl-2 overexpression increased overall cell density in AVPV of male mice but did not influence the number of TH-positive cells.

Discussion

Bcl-2 rescues many types of neurons from apoptosis caused by injury or disease and can enhance neuronal survival during development (Allsopp et al., 1993; Martinou et al., 1994; Farlie et al., 1995; Bonfanti et al., 1996). Here we examined whether Bcl-2 might also be involved in sexually dimorphic cell death in the CNS. In accord with previous reports, male wild-type mice had many more SNB motoneurons than did females, and, conversely, females had a higher overall cell density in the AVPV than did males. Bcl-2 overexpression significantly increased the number of SNB motoneurons in females and AVPV neuronal density in male mice. In the RDLN, which exhibits no sex difference in neuron number, Bcl-2 overexpression increased motoneuron number similarly in both sexes. Thus, in addition to its well established role in controlling neuronal number in non-dimorphic neural regions, Bcl-2 may control cell number in sexually dimorphic cell groups.

The most likely explanation for the increased number of neurons seen in the overexpressors is that Bcl-2 saved neurons from developmental cell death. The forced overexpression of Bcl-2 prevents the death of developing neurons *in vitro* and *in vivo* (Allsopp et al., 1993; Martinou et al., 1994; Farlie et al., 1995). Moreover, the neuron-specific enolase promoter, which controlled expression of Bcl-2 in the mice used here, is not produced at high levels until neurons become functionally and morphologically mature (Marangos et al., 1980), making it unlikely that Bcl-2 altered neuron number by affecting neurogenesis. Thus, although we cannot definitively rule out other explanations, these considerations support the interpretation that Bcl-2 overexpression increased neuron number by maintaining neurons that would otherwise die in the developing SNB, AVPV, and RDLN.

Although Bcl-2 overexpression significantly increased motoneuron number in the SNB of females, the sparing was not complete: female overexpressors still had significantly fewer SNB cells than did males. The Bcl-2 transgene in the line of mice used here is reported to be expressed in all postmitotic neurons of the spinal cord by embryonic day 13 (Martinou et al., 1994), i.e., well before the perinatal cell death period in the SNB. However, because the level of expression of Bcl-2 specifically within SNB motoneurons during the perinatal period is not known, it remains possible that Bcl-2 protein levels in the SNB were not high enough or produced early enough during the cell death period to rescue all SNB cells of females. It is also possible that Bcl-2 overexpression transiently saved some SNB motoneurons that subsequently died because of lack of trophic support. Target-derived trophic factors are important regulators of motoneuronal cell death during development (Hollyday and Hamburger, 1976; Oppenheim, 1991). SNB motoneurons project to the striated perineal muscles bulbocavernosus (BC), levator ani (LA), and external anal sphincter (Schroder, 1980). Although the BC and LA initially form in both sexes, these muscles remain vestigial (LA) or degenerate (BC) in females during perinatal life, and it is the loss of target muscles that is thought to account for the massive death of SNB motoneurons in females (Cihak et al., 1970; Tobin and Joubert, 1991; Forger et al., 1992). The BC/LA muscles could not be identified by gross dissection of adult females in the present study, regardless of genotype, suggesting that Bcl-2 overexpression does not "masculinize" SNB target muscles. Similarly, reconstructions of BC/LA muscle volumes in newborn females in-

Table 1. Neuronal cell size (μm^2) in wild-type and Bcl-2-overexpressing mice

	SNB		RDLN		AVPV	
	Soma	Nucleus	Soma	Nucleus	Soma	Nucleus
Males						
Wild type	346 ± 22	120 ± 8	302 ± 21	121 ± 7	50 ± 2	33 ± 1
Bcl-2 OE	358 ± 13	121 ± 4	278 ± 13	101 ± 5	47 ± 1	34 ± 1
Females						
Wild type	226 ± 11	84 ± 4	301 ± 12	117 ± 5	48 ± 3	33 ± 1
Bcl-2 OE	235 ± 10	84 ± 3	255 ± 6	98 ± 4	47 ± 2	32 ± 1
ANOVA:						
Sex	$p < 0.0005$	$p < 0.0005$	n.s.	n.s.	n.s.	n.s.
Genotype	n.s.	n.s.	$p < 0.01$	$p < 0.001$	n.s.	n.s.
Interaction	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s., Not significant.

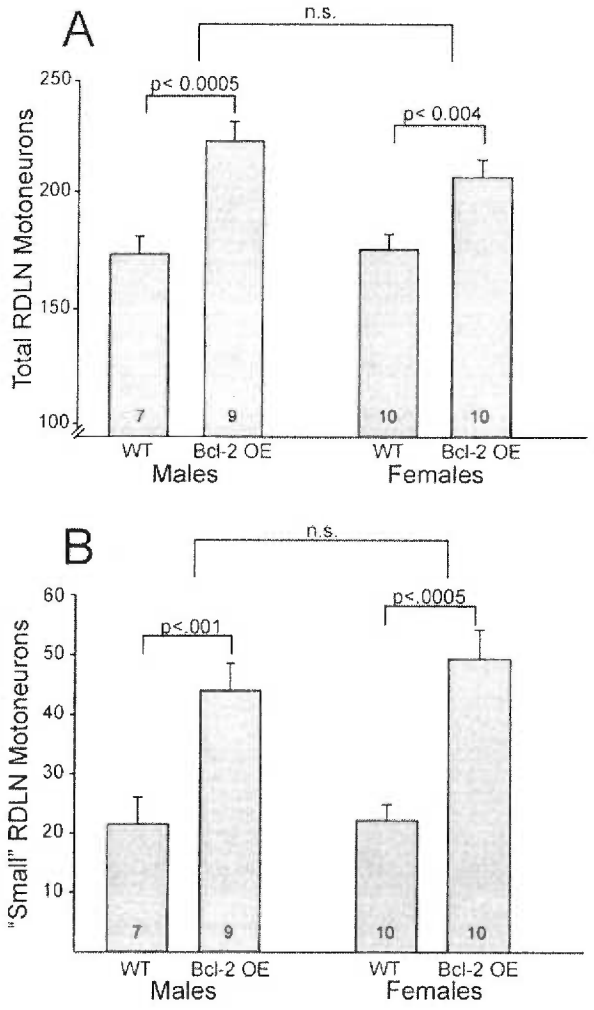

Figure 2. Motoneuron number in the RDLN of wild-type and Bcl-2-overexpressing mice. *A*, There was no sex difference in RDLN cell number. Bcl-2 overexpression increased the total number of RDLN motoneurons by ~22%, and the increase was significant for both sexes. *B*, The number of RDLN motoneurons classified as small was increased by 114% in Bcl-2-overexpressing mice; this increase was significant for both males and females. *n* = *numbers* at base of bars.

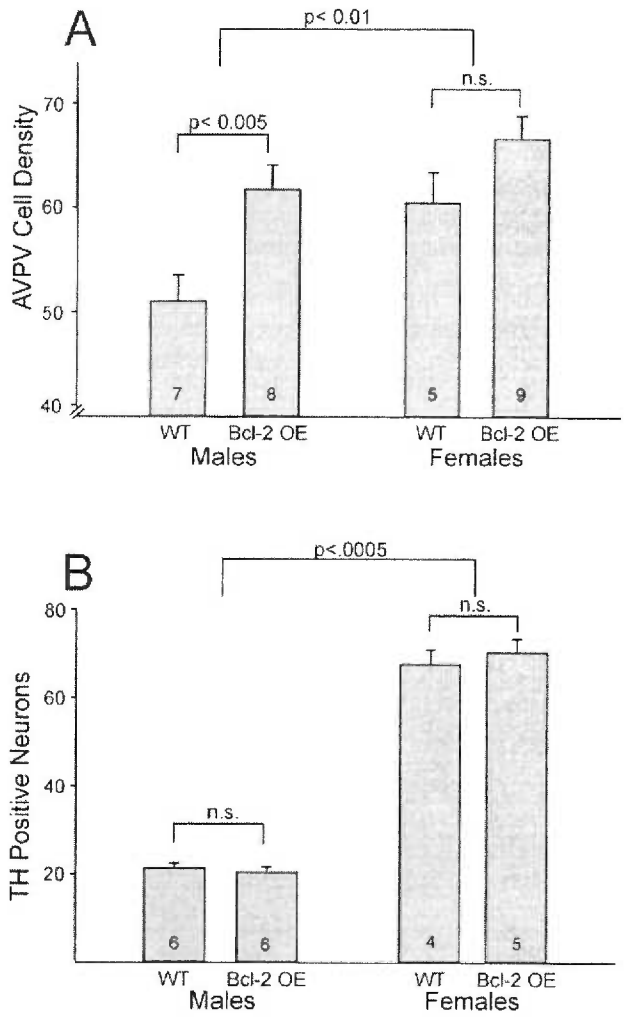

Figure 3. Overall cell density and the number of tyrosine hydroxylase (*TH*)-immunoreactive neurons in AVPV. *A*, Overall cell density was higher in females than in males ($p < 0.01$). Bcl-2 overexpression significantly increased AVPV cell density in males. *B*, Females had 3.3 times as many TH-immunoreactive neurons in AVPV as did males ($p < 0.0005$). Bcl-2 overexpression had no effect on TH cell number in either sex. *n* = *numbers* at base of bars.

dicate no effect of Bcl-2 transgene expression on muscle size during the cell death period (S. Montaldo and N. G. Forger, unpublished observations). The SNB of adult females may therefore provide an unusually stringent test of the rescue ability of Bcl-2, because motoneurons must be maintained indefinitely, despite the loss of major target sites.

Bcl-2 overexpression increased overall cell density in AVPV, but the number of dopaminergic neurons, as measured by TH immunoreactivity, was not affected by transgene expression. There are several possible explanations for this negative finding. First, it is possible that the sex difference in TH cell number arises not as the result of differential cell death of presumptive dompaminergic neurons but by some other mechanism, such as the hormonal specification of neuronal phenotype. Alternatively, the sex difference in TH-ir cell number in AVPV may indeed be caused by hormonally regulated cell death, but by a Bcl-2 independent pathway. For example, other Bcl-2 family members, such as Bcl-xL and Bax, and the cell surface Fas receptor are implicated in controlling the initiation of cell death cascades in developing neurons (Raoul et al., 1999; Martin, 2001).

The final common pathway for many types of cell death is the activation of cysteine proteases, known as "caspases," which degrade cellular proteins (for review, see Hengartner, 2000). Preliminary findings of Waters and Simerly (2002) indicate that estradiol markedly decreases TH cell number in organotypic slice cultures of newborn rat AVPV, and this decrease can be prevented by concomitant treatment with a caspase inhibitor. This suggests that cell death contributes, at least in part, to the hormonally controlled sex difference in TH cell number in AVPV and, taken together with the current findings, underscores the possibility that the molecular control of cell death varies not only from region to region, but also among subtypes of cells within a single neural region. The AVPV exhibits considerable cell type heterogeneity, and in addition to the sex difference in TH neurons, female rats have more neurons that express dynorphin, calcitonin gene-related peptide, neurotensin, and estrogen receptors (Alexander et al., 1991; Simerly, 1991; Bloch et al., 1992; Herbison and Dye, 1993). Although sexual differentiation of these cell types in the AVPV of mice has not yet been examined, overexpression of Bcl-2 may have reduced the sex difference in

overall cell number in AVPV by reducing hormone-dependent cell death in any of these subpopulations of neurons.

Bcl-2 overexpression markedly increased the number of RDLN motoneurons categorized as small. Similarly, many of the facial motoneurons rescued in mice with a targeted deletion of the pro-death gene, Bax, are shrunken in size (Deckwerth et al., 1996). If Bcl-2 overexpression increases motoneuron survival without affecting size of the target muscles, then an increased number of motoneurons may be competing for a constant supply of target-derived trophic factors in the overexpressors. We did not examine RDLN target muscles, which are located in the foot. However, striated muscles are not likely to have been directly affected by Bcl-2 expression in the current study because the Bcl-2 transgene was linked to a neuron-specific promoter. Moreover, as mentioned above, size of SNB target muscles was not affected by transgene expression. Thus, because target-derived trophic factors influence motoneuron size in adulthood (Elliott and Snider, 1996), there may be a subset of motoneurons in Bcl-2-overexpressing mice that do not receive enough trophic support to promote a normal cell size yet are prevented from dying by excess Bcl-2. It is not known whether these cells are functional or, more generally, how neural circuits and the functions that they control may be altered in the brains and spinal cords of the cell death mutants.

Gonadal steroid hormones regulate cell death in the developing SNB and AVPV. Specifically, androgenic metabolites of testosterone reduce cell death in the SNB (Breedlove and Arnold, 1983), whereas estrogenic metabolites increase cell death in AVPV (Arai et al., 1996). It is possible that by increasing Bcl-2 protein expression, some neurons would be maintained that ordinarily die in the SNB and AVPV, even if testosterone does not normally affect the expression or activity of Bcl-2. Alternatively, the present results suggest the possibility that the hormonal regulation of Bcl-2 family proteins may be an important mechanism whereby gonadal steroids sculpt sex differences in neuronal number. Numerous previous studies demonstrated that Bcl-2 mRNA and protein expression are controlled by gonadal steroid hormones in peripheral tissues and in cancer cell lines (Wang and Phang, 1995; Kandouz et al., 1996; Huang et al., 1997, 1999). Within the CNS, estradiol upregulates Bcl-2 in several brain regions of adults (Garcia-Segura et al., 1998; Dubal et al., 1999; Green and Simpkins, 2000; Alkayed et al., 2001), and testosterone increases Bcl-2 immunoreactivity in the SNB of adult male rats (Zup and Forger, 2002). The hormonal control of Bcl-2 expression during neural development has been less well studied, although preliminary findings from our laboratory indicate that testosterone enhances Bcl-2 protein expression in SNB motoneurons of female rats during late embryonic life (Zup, 2002). In addition, an NMDA receptor antagonist that lowers testosterone levels of newborn male rats concomitantly decreases Bcl-2 expression and increases cell death in the preoptic area of the hypothalamus (Hsu et al., 2000). Although in most instances androgens or estrogens increase Bcl-2 expression and promote cell survival, in other cases these hormones decrease Bcl-2 and cell viability (Lapointe et al., 1999). Thus, the same hormonal stimulus can drive Bcl-2-dependent cell death in opposite directions in a cell-type specific manner. Variables such as the steroid receptor subtypes and cofactors expressed by a given cell may determine the response to a given hormone and may explain how testosterone and its hormonal metabolites normally decrease cell death in some neural regions, whereas they increase cell death in others (cf. Nilsen et al., 2000).

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CHAPTER 5

Bax deficiency does not rescue TH-ir cells from estrogen-induced cell death during AVPV sexual differentiation.

Introduction

Estrogen has been demonstrated induce the loss of dopaminergic neurons in the AVPV through a caspase-dependent cell death mechanism. Death of dopaminergic neurons in the AVPV also depends on the activation of the alpha form of the estrogen receptor (ERα). The effects of ERα activation, because it is a nuclear transcription factor, are typically genomic. Among the genes that ERα has been demonstrated to regulate are anti-apoptotic members of the Bcl-2 family of cell death regulatory proteins (Garcia-Segura et al. 1998, Dubal et al. 1999, Pike 1999). Because estrogen-induced dopaminergic neuronal death relies on ERα, the expression of pro-apoptotic Bcl-2 family members could also be regulated by ERα. Estrogen's effect on cell survival or death could be a direct result of ERα-modulated expression of anti- and pro-apoptotic proteins within a cell. Changes in the ratio of anti- and pro-apoptotic proteins prevents or permits the intitiation of the caspase-dependent cell death cascade and ultimately cell death.

The Bcl-2 family of proteins is comprised of members that have either an anti- or pro-apoptotic function. This family includes anti-apoptotic proteins, Bcl-2, Bcl-x_L, and Bcl-w, and the pro-apoptotic proteins, Bax, Bak, Bad, and BH3-only proteins (Cory and Adams 2002), and the ratio of pro-survival-to-pro-death proteins is believed to critically regulate the initiation of the programmmed cell death pathway. The levels of these proteins within a cell reflect the contributions of pro-survival and pro-death forces, both endogenous and exogenous. This threshold may be temporally and spatially regulated. Developmental regulation of Bcl-2 protein expression, in particular, could result in variations of the protein ratio required to push the cell past the threshold for cell death (Vekrellis et al. 1997). Steroids and their cognate receptors also regulate Bcl protein levels, suggesting a means for the integration of environmental signals with the developmental program (Dubal et al. 1999, Almeida et al. 2000). The expression of Bcl proteins, steroid receptors, and other cell death factors varies throughout the central nervous system, suggesting that distinct cell types may respond differently to a similar death-inducing stimulus.

To study the role of the pro-apoptotic molecule Bax in estrogen-induced AVPV sexual differentiation, I utilized a strain of mice deficient for Bax (BaxKO). Sexual

differentiation was assessed with antibodies for tyrosine hydroxylase (TH) to quantify AVPV dopaminergic neurons.

Methods

Animals: BaxKO and wildtype mice on a C57BL/6J background were obtained from Nancy Forger at the University of Massechusetts-Amherst. All animals were housed with food and water available *ad libitum* in accordance with institutional animal regulations. Animals were processed for immunohistochemistry by anesthetizing with tribromoethanol and perfusing transcardially with 4% paraformaldehyde in borate buffer (pH 9.5). Brains were postfixed in the same fixative containing 20% sucrose for 2 hours, cryoprotected in 20% sucrose in 0.02M potassium phosphate buffered saline (KPBS) overnight, frozen on dry ice and stored at –80°C until sectioning. Brains were sectioned on a sliding microtome and collected in three parallel series. All tissue was stored in cryoprotectant until staining.

Immunohistochemistry: One series of brain sections was washed in potassium buffered saline (KPBS) to remove anti-freeze then blocked overnight in LKPBS (0.02M KPBS containing 2% normal goat serum (Upstate) and 0.4% Triton-X 100 (Biorad, Hercules, CA). Primary antibodies, mouse anti-tyrosine hydroxylase (1:1000, Diasorin, Hudson, WI) and rabbit anti-estrogen receptor alpha (1:40,000, Upstate) were diluted in LKPBS

and applied for 48 hours at 4°C. Primary antibody staining was visualized with Alexa Flour 488 goat anti-mouse IgG and Alexa Flour 568 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene,OR) and counterstained with Hoechst 33342 (1:10,000, Molecular Probes). Sections were mounted on gelatin-coated slides and coverslipped with buffered glycerol pH 8.6.

Analysis: All image analysis was performed blind to the experimental conditions. All cells were counted from one side of the AVPV in all tissue sections through the rostral-caudal extent of the AVPV that contained the area. ERα-ir cells were used to visualize the lateral and caudal edges of the AVPV. Cells containing tyrosine hydroxylase immunoreactivity (TH-ir) were manually counted using a Zeiss Axioplan 2 microscope to visualize the AVPV. Uncorrected total cell counts for each experimental group were compared statistically with a one-way anova and Fischer's PLSD post hoc analysis using Statview software (SAS Institute Inc., Cary, NC) A p value of 0.05 or less was considered significant.

Results

Similar to previous reports, TH-ir cell number is three-fold greater in the wild type female AVPV than in the wild type male nucleus (Fig. 1 *$p<0.003$),(Simerly et al. 1997). Deletion of Bax had no effect on the sexual differentiation of TH-ir cell numbers. TH-ir

cells are also three-fold greater in the BaxKO female AVPV compared to the BaxKO male AVPV (Fig. 1 *$p<0.003$). Distribution of the TH and ERα-ir cells in the BaxKO AVPV was also similar to that in the wild type AVPV.

Discussion

Deletion of the pro-apoptotic protein Bax had no effect on estrogen-mediated sexual differentiation of AVPV TH-ir cell number. Bax deficiency alone may not be sufficient to detect the role of Bcl-2 pro-apoptotic proteins in the differentiation of TH-ir cells. Previous reports suggest Bax deletion decreases cell death without causing any gross malformities (White et al. 1998) like those seen by disruption of caspases (Zheng and Flavell 2000). Bax's role in the cell death pathway is upstream of caspase activation. Caspase activation usually commits a cell irrevocably to die, whereas, Bax and other Bcl-2 family proteins have redundant roles that could initiate the caspase pathway in cells lacking Bax. In the absence of Bax, the presence of another pro-apoptotic protein, Bak, is sufficient to induce the activation of cell death pathway and loss of the cell. Animals with the double deletion of both Bax and Bak may yet reveal a role for these proteins in estrogen-induced cell death.

Estrogen regulation of pro-apoptotic molecules has not been described to date. However, ERα-mediated Bcl-2 expression, a pro-survival molecule, has been implicated in neuroprotection. (Garcia-Segura et al. 1998, Dubal et al. 1999). Levels of Bcl-2 may also be regulated by estrogen activation of kinase signalling pathways resulting in CREB binding to cAMP response elements in the promoter region of the bcl-2 gene (Honda et al. 2001). In addition, estrogen receptor binding of hormone response elements increases expression of Bcl-x (Pike 1999). Through these pathways, estrogen can modulate the levels of these anti-apoptotic Bcl-2 family proteins and potentially regulate pro-apoptotic molecules as well.

Over-expression of Bcl-2 had no effect on TH-ir cell number in the AVPV but did increase the density of neurons in the AVPV (Zup et al. 2003). This report together with the current findings, suggests that Bcl-2 and Bax are not alone in regulation of cell survival and death in the AVPV during sexual differentiation. Estrogen regulation of the expression levels of other key modulators in the cell death pathway must translate estrogen signaling into cell survival or death. However, the potential role of Bcl-2 family members in AVPV sexual differentiation remains to be completely clarified.

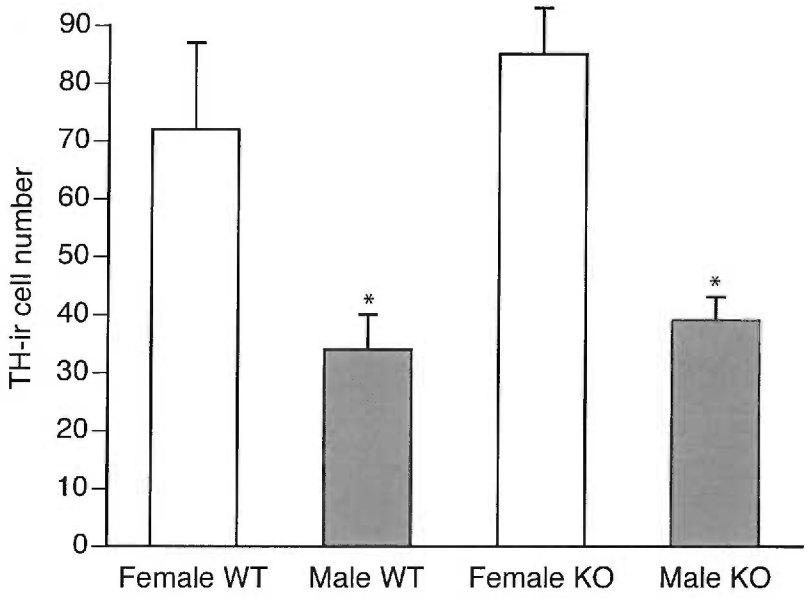

Figure 1. **TH-ir cell number differentiates in wild type and BaxKO mice.** The AVPV of wild type and BaxKO females contain significantly more TH-ir cells than the AVPv of either males.

GENERAL DISCUSSION

This research presents the first characterization of estrogen-induced neuronal cell death. The cellular and molecular mechanisms mediating the estrogen-induced death of dopaminergic neurons were also investigated. Activation of estrogen receptor alpha leads to the initiation of a caspase-mediated cell death pathway during early AVPV sexual differentiation. TH-ir cell number can only be altered during the first postnatal week, as a 'critical period' for steroid exposure limits estrogen regulation of cell death later in development.

The characterization of early AVPV sexual differentiation, both *in vivo* and *in vitro*, was a first step in addressing questions about gonadal steroid hormone regulation of neuronal fate. In the first set of experiments, these parallel experimental systems were used to clarify the developmental timeline of three sexually dimorphic cell types in the AVPV: dopamine and prodynorphin neurons, which are greater in number in female and proenkephalin neurons, which are greater in number in the male. Dopamine and proenkephalin cell number are dimorphic by the end of the first postnatal week, while prodynorphin expression is not.

Proenkephalin neuron number is dimorphic by ten days after birth; however, the total number of proenkephalin neurons at P10 is less than the number detected in the AVPV of adults. While the cellular levels of proenkephalin continue to increase throughout development, the number of cells that are capable of expressing proenkephalin is determine by gonadal steroid hormones. Estrogen exposure, either endogenous or exogenous, prevents the loss of proenkephalin-containing neurons. This neuroprotective action of sex steroids has been reported as the developmental mechanism for other sexually dimorphic hypothalamic areas (Davis et al. 1996, Chung et al. 2000). The molecular characterization of estrogen-induced neuroprotection has been performed for the most in part in models of ischemic or excitotoxic injury (Dubal et al. 1998, Singer et al. 1999, Harms et al. 2001). Estrogen upregulation of Bcl-2, which shifts the ratio of anti- and pro-apoptotic factors to favor survival, is correlated with its neuroprotective actions in the cortex (Garcia-Segura et al. 1998, Dubal et al. 1999). Estrogen regulates the expression of BDNF, which can block activation of caspase 3 (Han et al. 2000). Rapid estrogen effects also protect cells via MAPK signaling pathways (Singer et al. 1999), possibly through MAPK-mediated phosphorylation of NMDA receptors (Bi et al. 2000). The molecular mechanism of estrogen-induced neuroprotection involved in AVPV sexual differentiation remains to be characterized.

Sexual differentiation of dopaminergic neuron number is complete by ten days after birth. The number of dopamine neurons is equal in males and females until exposure to sex steroids differentiates neuron number. After differentiation, the AVPV of females contains more TH-ir cells than it does in males, and the loss of TH-ir cells in the male is estrogen dependent. Estrogen-induced programmed cell death has been reported in the AVPV, but has not been localized to any specific cell type (Arai et al. 1994, Arai et al. 1996). This report suggested that TH-ir cell loss may be due to programmed cell death; however, not all TH-ir cells are lost after estrogen treatment. Thus, within this cell population, there must be further differences in the developmental profiles of individual cells that promote the survival of a subpopulation of TH-ir cells.

Prodynorphin neuron number is not dimorphic after the first postnatal week and does not appear to differentiate until after puberty (Waters, Kirigiti, and Simerly unpublished observation). It is possible that prodynorphin neurons in the AVPV do not contain estrogen receptors perinatally, but then they begin expressing estrogen receptors around the time of puberty. Circulating ovarian hormone levels rise significantly in females as puberty approaches. If the critical period for differentiation of prodynophin neuron number does not occur until puberty, it may be that the number of prodynorphin neurons is greater in the AVPV of females because of neuroprotective effects of estrogen. Even

though the number of both prodynorphin and dopamine neurons are reduced by estrogen exposure, they do not differentiate in the same way. Separate developmental mechanisms are likely required for each neuropeptidergic cell type. Many AVPV neurons express ERα during sexual differentiation (Waters unpublished observation). However, there maybe developmental regulation of steroid receptor expression or, perhaps, a change in the molecular complement of proteins that modify steroid receptor actions, that leads to differences between cell types. These differences may account for the distinct developmental timelines of the two cell types.

Using the *in vitro* model, estrogen-induced reduction of TH-ir cell number was demonstrated to be sensitive to the timing of steroid treatment. This 'critical period' for estrogen exposure begins on the day of birth and ends after the first week of life. Because reductions in TH-ir cell number can only occur during a limited period, ERα may also regulate the period of estrogen sensitivity of these cells. The closing of this critical period is not due to changes in overall steroid sensitivity of the AVPV because estrogen was able to induce progesterone receptor expression in DIV 8 AVPV explants.

Both ERα and ERβ can be detected in the AVPV but exhibit distinct expression patterns. ERα-containing cells are present throughout the AVPV, while ERβ-containing cells are

detected only in the periventricular zone of the AVPV. Additionally, the expression of ERα and ERβ is developmentally regulated in the AVPV and may also be differentially regulated in a cell type-specific manner (Simerly et al. 1996, Kirigiti and Simerly 1999, Perez et al. 2003). In view of the fact that ERβ expression is very low in the AVPV until the end of the first postnatal week (when the levels increase enough to be detectable with antibodies), ERβ is not likely involved in the differentiation of dopamine or proenkephalin neuron number. In contrast, ERα expression is abundant throughout this week and probably transduces estrogen's differentiating effects on cell number. One possible role of ERβ may be to terminate ERα's connection to cell death machinery in TH-ir cells. ERβ may oppose ERα actions by inhibition of ERα-mediated genomic effects through heterodimerization or through transcription of molecules that regulate ERα function. In this way, ERβ may interfere with ERα-mediated gene transcription, such that ERα is no longer able to increase the expression of pro-apoptotic molecules and initiate cell death. ERβ could also induce the expression of coregulators or other molecules that would restrict ERα-induced effects.

Interactions between steroid receptors and coregulators alter the genetic response induced by steroid receptor activation. Developmental and cell type-specific regulation of coregulators may explain the critical period for steroid exposure and the divergent fates

seen among AVPV neuropeptidergic cell populations. There are two major classes of coregulators: coactivators and corepressors either increase or decrease steroid receptor binding at the hormone response elements in gene promoters, thus altering transcriptional activity of steroid responsive genes. Coregulators may also interact with each other to further modulate the range of potential genetic effects available at the time of steroid receptor activation. The molecular profile of these transcriptional regulators within a cell may determine a gene's response to steroid signals and influence the temporal and spatial specificity of steroid hormone activity. Differences in coregulator expression may explain why neonatal masculinization of TH-ir cell number in the AVPV does not occur in females when the level of free estrogen produced by the ovary increases significantly after the first postnatal week. This phenomenom may also determine why the dopaminergic neurons in the preoptic periventricular nucleus, which abuts the caudal edge of the AVPV, are not susceptible to estrogen-induced cell loss. The levels of transcriptional regulators could control the separation of estrogen's effects in the postnatal AVPV from its effects in the adult AVPV or in other brain areas.

Growing evidence suggests that coactivators mediate the effects of estrogen on neurons during sexual differentaion of the hypothalamus. cAMP response element-binding protein (CBP) expression in the brain is estrogen-regulated (Murphy and Segal 1997) and differs

between the sexes during development (Auger et al. 2002). In addition, CBP interacts with another coactivator, steroid receptor coactivator 1 (SRC-1), to regulate the levels of estrogen-induced progesterone receptor expression in the rat brain (Molenda et al. 2002). Reduction in SRC-1 expression disrupts development of the sexually dimorphic MPNc (Auger et al. 2000), and ERα interactions with SRC proteins direct the development of female sexual behavior (Apostolakis et al. 2002). Many other coregulators have also been described in the brain, potentially explaining many of the permutations of steroid receptor actions (for review see: McKenna and O'Malley 2002). Regulation of the specific mixture of coregulators within each neuropeptidergic population could determine the range of estrogen actions and explain why steroids do not behave identically in all cell phenotypes. The difference between TH-ir cells, which die after estrogen exposure, and proenkephalin cells, which survive, could be due to their complements of steroid receptor coregulators.

Estrogen also regulates the expression of progesterone receptor (PR), whose actions could potentially alter AVPV neuronal survival. PR expression was increased by estrogen treatment in DIV8 AVPV explants. In the first postnatal week, gonadal steroid hormones also increase the expression of PR in the preoptic hypothalamus (Quadros et al. 2002). These results suggest that PR-mediated cell survival or death events could alter the

neuronal content of the AVPV. Although progesterone was not required in the cell culture media to induce TH-ir cell loss, local synthesis of progesterone by the AVPV could mediate a potential PR effect on survival of proenkephalin neurons. Progesterone-mediated neuroprotection has been described in hippocampal neurons (Nilsen and Brinton 2002, Nilsen and Brinton 2003). Progesterone also facilitates the production of female sexual behaviors in adults, but this action depends on estrogen pretreatment. The display of female sexual behaviors can be disrupted by exposing females to gonadal steroid early in development or by elimination of ERα. Even though PR expression can be estrogen-induced in ERα knockout mice (Moffat et al. 1998), reproductive behavior remains disrupted (Ogawa et al. 1996). Overall, the development of the AVPv is primarily dependent on effects directly downstream of ERα activation, rather than modulation of progesterone actions. However, given that the greater part of steroid effects occur through genomic mechanisms rather than non-genomic mechanisms, interplay between ERα and ERβ gene transcription may be working together in the AVPV to determine the specificity of estrogen effects.

In the second set of experiments, a rapid AVPV sexual differentiation model was developed to facilitate the characterization of the cellular events regulating the loss of dopaminergic neurons. Pharmacological levels of estrogen induced the complete

sexually dimorphic reduction in TH-ir cell number within 48 hours. This shortened timeline permitted the application of the caspase inhibitor, which would have been toxic to AVPV explants cultured for ten days. The reduction in dopamine neuron number that characterizes AVPV sexual differentiation was confirmed to be due to programmed cell death. Reduction in TH-ir cell number was also a function of ERα activation by estrogen. The rapid AVPV sexual differentiation model synchronized and condensed the events of estrogen-induced cell death into a time period that allowed cell fate to be manipulated *in vitro* and confirmed *in vivo*.

Caspase activation is a hallmark of apoptotic cell death. The concurrent application of estrogen and the pan-caspase inhibitor ZVAD-fmk completely rescued TH-ir cells from estrogen-induced cell loss. These findings confirmed the results of the survey of estrogen-induced apoptotic markers, and provided strong evidence that TH-ir cell loss is due to apoptotic programmed cell death. ZVAD-fmk irreversibly binds caspases, preventing the activation of downstream proteases that destroy the integrity of the chromatin and nuclear and cytoplasmic proteins. The organized destruction of the celllular contents is stereotypical of apoptotic cell death. Chromatin condensation and cytoplasmic blebbing are visual markers of apoptosis that could be colocalized with TH-ir. During development and throughout life, excess or damaged cells are removed through

programmed cell death. This process is critical for refining the function of the nervous system generally and seems to be important for sexual differentiation of the AVPV.

Simultaneous application of the ERα antagonist ICI 182, 780 and estrogen completely blocked estrogen-mediated loss of TH-ir cells. This result confirms the requirement of a functional ERα for the differentiation of AVPV TH-ir cell number, as previously demonstrated by our laboratory (Simerly et al. 1997). That the dose of ICI 182,780 which blocked TH-ir cell loss, was equimolar to the estrogen doses suggests that ERα's actions on the AVPV is a genomic effect rather than a rapid estrogen effect. Interestingly, ERα was detected in only 30 percent of TH-ir cells in the AVPV of four day old females (Waters unpublished observation) when the AVPV is still sensitive to the differentiating actions of gonadal hormones. This percentage corresponds to the number of TH-ir cells surviving estrogen exposure during the first postnatal week and persisting in the AVPV into adulthood. This observation suggests that the presence of ERα in TH-ir cells is neuroprotective. However, the level of ERα protein in TH-ir cells may be below the detectability of the immunostaining procedure. The effect of ERα activation on TH-ir cells that leads to the initiation of cell death pathways could be a direct effect, but this remains to be clarified.

Steroid receptors were the first transcriptional regulators described in detail (Jensen et al. 1968, Yamamoto 1985), and their most prominent activity continues to be genomic regulation. Regulation of differential cell survival during AVPV development depends on the estrogen receptor's ability to increase or decrease gene transcription. This classical action of steroid receptors is mediated by ligand-activated receptor binding to hormone response elements in the promoter regions of steroid-sensitive genes. Phosphorylation of the ERα results in ligand-independent steroid receptor-mediated transcription and also contributes to estrogen's regulatory potential. In these ways, ERα can mediate cellular responses to environmental stimuli through changes in gene expression. Indeed, estrogen-induced expression of trophic factors has been reported, illustrating how estrogen can contribute to the development and maintenance of neural circuitry. BDNF (Sohrabji et al. 1995), and GDNF (Ivanova et al. 2002), as well as NGF receptors (Sohrabji et al. 1994) can alter cell survival. BDNF mRNA expression is greater in the AVPV of P10 male mice than in the AVPV of P10 female mice and is not significantly different in the male and female ERα knockout mice (Kirigiti and Simerly unpublished observation). Sex specific differences in the expression of trophic factors during the first postnatal week have not been characterized but could mediate the death of TH-ir cells or even the survival of proenkephalin cells.

Reduction in TH-ir cell number is not detected until 24 hours after estrogen treatment *in vivo* and 48 hours *in vitro*. The latency of estrogen-induced cell loss also suggests gene transcription is involved in estrogen regulation of dopamine neuron number. This effect could be directly on the TH-ir cells, either by increasing the levels of pro-apoptotic molecules or by decreasing the levels of anti-apoptotic molecules. On the other hand, estrogen's effect on the TH-ir cells could be indirect. Because estrogen sensitive cells surround the AVPV TH-ir cells, their fate may be determined by estrogen-induced gene expression in nearby cells. The induction of a secreted death signal would activate death receptor-mediated PCD in cells that contain the cognate death receptor. The FasL gene contains a hormone response element, and its expression has been demonstrated to be estrogen-regulated (Mor et al. 2003). Estrogen induced apoptosis in immortalized hypothalamic cell lines through interactions with the Fas/FasL death receptor system (Nilsen et al. 2000). Estrogen could also regulate the expression of death receptors within TH-ir cells. Estrogen receptors, together with their coregulators, can alter the transcription of trophic factors and cell death molecules mentioned above in TH-ir cells The fate of individual TH-ir cells may depend on their intracellular molecular complement, including ERs, coregulators, BCL proteins and caspases.

In the fourth and fifth chapters, TH-ir cell number was quantified in mice that either overexpressed Bcl-2 or were deficient for Bax. In the AVPV, TH-ir cell loss was not altered by Bcl-2 overexpression or Bax deficiency. A variety of neurotrophic and neurotoxic signals regulate the activation of PCD machinery either through the regulation of BCL protein expression or the activation of death receptors. Estrogen has been linked to programmed cell death through both of these pathways. Neuroprotection studies demonstrated that estrogen-induction of BCL proteins rescues cells. Although Bcl-2 and Bax expression do not regulate TH-ir cell number, estrogen induction of other BCL family members, such as the pro-apoptotic molecule Bak, may be important for TH-ir cell death. Reports of estrogen-induced FasL expression also implicate death receptor signaling to the caspase pathway. Either BCL or death signal/receptor expression could regulate activation of caspases and commit a neuron to the cell death pathway. Confirmation of the role of either estrogen-induced BCL or death receptor expression as the molecular mechanism downstream of ERα activation remains to be characterized.

Estrogen-mediated programmed cell death plays an important role in AVPV sexual differentiation (Fig. 19). The activation of ERα by estrogen-binding initiates a series of molecular and cellular events that activate the PCD pathway. These events can be blocked by the ER antagonist ICI 182,780. ERα actions also activate the PCD molecule

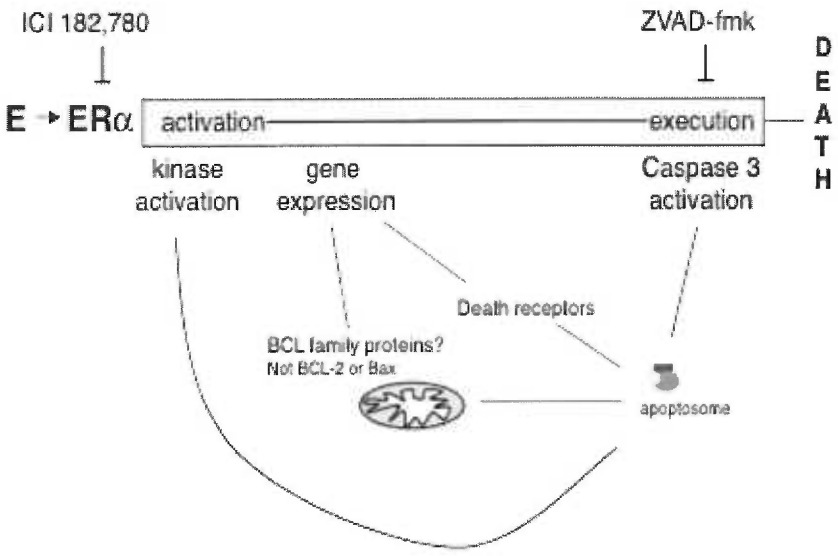

Figure 19. **Estrogen-mediated programmed cell death.**
Estrogen-induced cell loss during AVPV sexual differentiation requires ERalpha andcaspase 3 activation to alter cell number. The molecular events that linkthe actions of estrogen-bound ERalpha to the death machinery are not known.

unknown. Activation of PCD by ERα could occur through several mechanisms, including kinase activation of initiator caspases and formation of an apoptosome that activates caspase 3. Genomic regulation could also mediate estrogen's effect by altering the expression levels of either BCL family members that alters the threshold for death (although not bcl-2 or bax) or death receptors that interact with caspases leading to their activation.

Steroid regulation of behavior, neuroendocrine function and development begins at the level of steroid interaction with individual neurons. The reliance of steroid hormones on steroid receptors for the majority of their effects and the presence of steroid receptors in tissues of interest has made receptor biology a constantly growing field of study. The many roles played by steroid receptors in regulation of cellular mechanisms are derived from their interactions with a wide variety of regulatory proteins and signaling molecules that affect the ultimate outcome of steroid receptor activation. While gene transcription is the foremost effect of steroid receptor activation, it is now known that ligand-dependent and independent non-genomic events are also attributed to these receptors. Genomic effects are augmented by rapid effects mediated through steroid receptor interactions with cellular signal transduction pathways. Characterization of novel steroid-dependent events continues to expand our understanding of the transduction of

steroid signaling into physiological consequences. Targeting the steroid signal to specific tissue types is of particular interest for its potential in the preparation of clinical therapeutics that have fewer side effects.

The AVPV, with its specifically feminine role in ovulation, is sexually dimorphic and highly responsive to sex steroid hormone effects. Early in life, sex steroids permanently masculinize both the structure and function of the male brain, and the absence of estrogen feminizes the female brain. Alterations in the number of various neuropeptidergic cell populations in the AVPV of females have been correlated with the AVPV's role in the control of ovulation and the generation of gonadotropin-releasing hormone (GnRH) surges. In the female, the AVPV sends direct projections to GnRH neurons that lie rostral to the AVPV, while the male does not appear to make comparable connections with GnRH neurons (Gu and Simerly 1997). GABAergic inputs to the AVPV from the principal nucleus of the bed nuclei of the stria terminalis are denser in the male than in the female (Polston et al. 2004). Because the female AVPV receives fewer GABAergic inputs than the male, it may be more responsive to estrogen activation (Simerly 2002). The content of AVPV to GnRH projections have not been demonstrated to be GABAergic; however, the AVPV contains numerous GABAergic neurons, and 30% of these express ERα (Herbison 1997), so its likely that this pathway is GABAergic.

It appears that estrogen regulation of GnRH release is transynaptic. GnRH neurons do not contain ERα (although they may contain ERβ, a role for ERβ in GnRH release has not been established). However, they receive inputs from ERα-positive neurons in the AVPV. Although the role of TH-ir cells in the transduction of the estrogen signal to GnRH neurons is not known, it's likely that these dopaminergic neurons make local connections within the AVPV. Within the AVPV they could function as local interneurons regulating the excitability of the AVPV rather than projecting to other areas, but at present there is no evidence to confirm this. Opioid peptide-containing cell have also been mentioned in the regulation of the AVPV effect on GnRH secretion but, again, there is no direct evidence for this (Simerly et al 1996). In addition, not much is known about glutamatergic input to the AVPV. Glutamate receptors are estrogen-regulated in the AVPV and may play a role in transducing the estrogen signal to the GnRH neurons (Gu et al. 1999).

The mechanisms of steroid actions in development and normal function of the nervous system also play a role in the pathology of neurodegenerative diseases and steroid-sensitive cancers. In addition to the neuroprotective estrogen actions seen in models of ischemic and excitotoxic injury (Wise et al. 2000), estrogen actions regulate the

progression of breast cancers containing ERα (for review see: Shao and Brown 2004). The presence of ERα is considered a good prognostic indicator in breast cancer treatment outcome and has led to the use of the anti-estrogen tamoxifen as a treatment. However, tamoxifen treatment does not eliminate the cancer, and eventually the cancer becomes resistant to this treatment through downregulation of ERα. Present research on breast cancer focuses on ERα. Treatments are sought that regulate the levels of ERα and block ERα activation. The levels of steroid receptor coregulators are being examined as targets for cancer treatments. ERα-mediated cellular signal transduction pathways are being studied to develop therapies targeted specifically to cancer cells containing steroid receptors (for review see: Shao and Brown 2004). Information gathered from the characterization of the estrogen-regulated mechanism that induces dopaminerigc neuron cell death in the AVPV could be also used to develop treatments that target the activation of PCD pathways in estrogen-sensitive cancer cells.

Targeting treatment specifically to particular cell phenotypes is important because of the widespread distribution and diverse actions of estrogen receptors in the body. The danger of general hormone therapies has been illustrated by recent findings in the *Women's Health Initiative* trials. Results from this study demonstrated that for every positive effect of hormone replacement therapy on bone metabolism or cardiovascular function, in other

tissues, there are dangerous negative consequences that lead to the disregulation of cell growth in mammary and endometrial tissues. Not addressed were questions about the relationship of the initiation of hormone therapy to the onset of menopause and the length of treatment. Changes in the cellular response to hormones are due to downregulation of the levels of steroid receptors and proteins essential to steroid receptor actions that may occur during aging or as a result of prolonged steroid exposure. In the brain, the effects of estrogen are profoundly protective; however, there are variations in the responsiveness to estrogen between brain regions. The presence of either ERα or β or both in a brain region may alter the degree of estrogen-mediated neuroprotection that can be detected (for review see: Wise 2003). Again, the cell-type specificity of estrogen's actions are regulated by the ability of estrogen receptors, coregulators, and signal transduction pathways to modulate the expression of survival or trophic factors.

Estrogen-induced neuronal cell death during AVPV sexual differentiation is a naturally occuring developmental phenomena. The activation of cell death in dopaminergic neurons depends on ERα action and initiation of caspase-mediated cell death pathways, and is also limited temporally. The specificity of estrogen's actions on particular cell types during AVPV sexual differentiation suggests that this system may be useful to study the parameters of estrogen regulation of cell survival and death.

Future directions:

The use of alterations in neuronal composition as a functional assay facilitates characterization of the cellular and molecular mechanisms responsible for gonadal hormone effects during sexual differentiation of the brain. I have demonstrated that estrogen can induce ERα-mediated caspase-dependent programmed cell death within the early postnatal AVPV both *in vivo* and *in vitro*. However, with these findings, new questions arise: what signaling mechanisms or molecules facilitate estrogen-induced cell death? What mechanism terminates this estrogen activity to maintain temporal specificity and prevent inappropriate cellular degradation? To understand the molecular mechanisms of estrogen-induced cell death, several aspects of the process must be characterized. It is also important to understand how some TH-ir cells survive estrogen exposure. In these cells, how are the cell death pathways supressed? What survival factors are present and what is the ratio of survival-to-death factors? The ability of estrogen to induce TH-ir cell death is limited to the first postnatal week, while after this 'critical period,' estrogen is unable to alter TH-ir cell number. Changes in gene expression, developmentally regulated or otherwise, presumably limit a cell's ability to die after the critical period. What changes in gene expression prevent estrogen-induced cell death?

These questions require examination of individual neurons. The development of TH-GFP mice and perhaps the future development of pENK-GFP mice would allow for meaningful comparisons of ER activities in individual neurons. Elucidation of tissue- and cell- specific regulatory mechanisms could shed new light on the multiple roles of gonadal steroid hormones. This knowledge could provide information for the development of targeted hormone therapies for the treatment of neurological and pathological disease without deleterious affects on other parts of the body.

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