Development/Plasticity/Repair

Estrogen Induces Caspase-Dependent Cell Death during Hypothalamic Development

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The sexually dimorphic population of dopamine neurons in the anteroventral periventricular nucleus of the preoptic region of the hypothalamus (AVPV) develops postnatally under the influence of testosterone, which is aromatized to estrogen. There are fewer dopaminergic neurons labeled with tyrosine hydroxylase (TH) in the male AVPV than the female, and sex steroids determine this sex difference, yet the role of cell death in specifying numbers of dopaminergic neurons in the AVPV is unknown. Estradiol treatment of the AVPV, *in vivo* and *in vitro*, was used to manipulate TH-ir cell number. *In vitro*, concurrent treatment with the estrogen receptor antagonist ICI 182,780 rescued TH-ir cells. Cyclosporin A, an inhibitor of cell death dependent on the opening of a mitochondrial permeability transition pore also blocked TH-ir cell loss. *In vivo*, estradiol increased the number of apoptotic profiles, both TUNEL and Hoechst labeled nuclei, in the AVPV. This increased apoptosis was also dependent on the presence of the α form of the estrogen receptor. To test for caspase dependent TH-ir cell loss, the pancaspase inhibitor ZVAD (*N*-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone) was used to rescue TH-ir cells from estradiol-mediated reduction in number. Together, these data suggest that an intrinsic cell death pathway is activated by estrogen to regulate TH-ir cell number. Thus, in contrast to the more widespread neuroprotective actions of sex steroids in the mammalian nervous system, in the AVPV estrogen regulates dopaminergic neuron number through a caspase-dependent mechanism of apoptotic cell death.

Introduction

The number of neurons that reside in brain nuclei are determined by a variety of factors that regulate production and death of neurons during development. Developmental control of cell death through apoptotic mechanisms is a common mechanism for influencing cell number in a variety of brain regions that is actively regulated by both genetic and environmental factors. It is well established that sex steroid hormones act on key components of forebrain pathways during perinatal development to sculpt the architecture of neural systems that are both morphologically and physiologically different in males and females. The increased testosterone secretion that occurs shortly after birth in male rodents acts on the brain to produce greater numbers of neurons in such regions as the central part of the medial preoptic nucleus (MPNc) and principal nucleus of the bed nuclei of the stria terminalis (BSTp), both of which remain larger in males relative to homologous structures in females (for review, see Simerly, 2002). Treatment of female neonates with exogenous testosterone increases the number of neurons in these nuclei and masculinizes patterns of sexual behavior and neuroendocrine physiology (Simerly et al., 1985). In most sexually dimorphic nuclei, testosterone is converted to estradiol via aromatization and acts primarily through the α form of the estrogen receptor (ERα) to alter multiple aspects of brain structure and function (for review, see McCarthy 2008). The anteroventral periventricular nucleus (AVPV), which plays a critical role in promoting the preovulatory surge in gonadotropin secretion, is unusual among sexually dimorphic nuclei in that it is smaller in males than in females (Simerly, 2002). Dopaminergic neurons are 3–4 times more abundant in the AVPV of females, and this sex difference, as well as the ability to generate a preovulatory gonadotropin surge, is dependent on postnatal exposure to sex steroids and ERα (Simerly et al., 1997).

Despite the prevalence of sex differences in the CNS we know surprisingly little about the molecular mechanisms that produce them (for review, see Forger 2006). It is generally believed that sex steroids act via steroid receptors to promote neuronal survival by reducing the incidence of cell death. For example, testosterone exposure reduces loss of neurons in the rat spinal cord and BSTp by reducing the incidence of cell death mediated by apoptotic proteins of the Bcl-2 family (Forger 2009). Less is known about how estradiol impacts apoptosis, but gonadal steroids appear to increase markers of cell death during sexual differentiation of the AVPV (Murakami and Arai, 1989; Sumida et al., 1993). In males, the testosterone surge after birth has been linked to increased levels of the activated form of caspase 3 and altered expression of antiapoptotic and proapoptotic proteins, including Bcl-2 and Bax in the AVPV (Tsukahara et al., 2006). However, none of these previous studies has conclusively demonstrated the mechanism underlying gonadal steroid regulation of AVPV sexual differen-

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tiation. Using tyrosine hydroxylase (TH) as a marker of dopaminergic neurons in the AVPV, we used inhibitors of caspase activity and an *in vitro* model of AVPV sexual differentiation to demonstrate that estradiol acts directly on the AVPV to induce the caspase-dependent loss of dopaminergic neurons during hypothalamic development.

Materials and Methods

Tissue culture. All animals were maintained in accordance with the National Institutes of Health animal care guidelines. AVPV explants were prepared from 2-d-old female Sprague Dawley rat or C57BL/6 wild type and estrogen receptor α knock-out (ERKO) mouse pups using a modified version of Gähwiler's slice culture technique (Gähwiler et al., 1997). Sprague Dawley rats were used for all experiments, except for those that involve comparison between wild-type and ERKO mice. Pups were cryoanesthetized and the brain was removed and submerged in ice-cold Gey's plus glucose (2.5 g/250 ml). Sections (300 μm) were collected using a Vibratome (Electron Microscopy Sciences) and stored in ice-cold serum-free defined media EOL-1 (containing thyroid hormone but lacking testosterone, estradiol, and progesterone) (Annis et al., 1990). The AVPV was microdissected using the ventricles and anterior commissure as guides, mounted onto collagen-coated (1 mg/ml 60% EtOH, Upstate) Millicell-CM membranes (0.4 μm pore size, 30 mm diameter, Millipore) over culture media and incubated at 37°C (in 95% O_2 and 5% CO_2). After 24 h in culture, the medium was replaced with EOL-1 medium containing estradiol and inhibitors. Forty-eight hours after estradiol treatment, cultures were collected by fixation in 4% paraformaldehyde, cryoprotected in 20% sucrose, and embedded in OCT (Sakura).

Steroid and inhibitor treatments. For explants, stock solutions of 17-β-estradiol (E_2) (Sigma), *N*-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone (ZVAD) (Calbiochem), ICI 182,780 (ICI) (Sigma), and cyclosporin A (CsA) (Biomol Research Laboratories) were suspended in 100% EtOH. All were diluted to their final concentration in EOL-1 media. Vehicle consisted of 1% EtOH in culture media. For rat or mice pups, 25 μg of estradiol benzoate (EB) (Sigma) was suspended in 10 μl of sesame oil and injected subcutaneously at the nape of the neck.

Whole brain collection. Animals were perfused transcardially with 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB), postfixed for 2 h, and cryoprotected overnight in 20% sucrose in PB.

Tyrosine hydroxylase immunohistochemistry. Tissue was cryosectioned at 20 μm and thaw-mounted onto gelatin-coated slides. Tissue sections were incubated in (1) LKPBS [KPBS containing 2% normal goat serum (Upstate) and 0.4% Triton X-100 (Bio-Rad)] overnight at 4°C; (2) mouse anti-TH (1:1000; Immunostar) for 72 h at 4°C; (3) Alexa Fluor 568 goat anti-rabbit IgG (1:200; Invitrogen) for 2 h at room temperature; and (4) Hoescht 33258 (1:4000; Invitrogen). All incubations were separated by washes of KPBS.

TUNEL. The In Situ Cell Death Detection Kit (Roche) was used according to the manufacturer's instructions. Tissue was pretreated with 0.5% TTX in PBS at 60°C for 15 min. The labeling reaction mixture was diluted 1:1 with diluent and incubated with tissue for 1 h at 37°C.

Analysis. All analysis was performed with the investigator blind to the experimental conditions. The rostral, caudal, and lateral edges of the AVPV were identified by using ERα-ir to delineate the nucleus in fluorescence-labeled tissue; morphological landmarks were used to define the nucleus in peroxidase-labeled tissue. For all experiments, numbers of labeled cells were counted unilaterally in the AVPV in all tissue sections through the rostrocaudal extent of the nucleus. Total numbers of TH-ir neurons were counted for each experimental group and compared statistically by using a one-way ANOVA and Fischer's PLSD *post hoc* analysis using Statview software (SAS Institute). $p < 0.05$ was considered statistically significant. All data are shown as mean + SEM.

Images of immunolabeling in the AVPV were captured using a charge-coupled device camera mounted on a Zeiss Axioplan 2 microscope by using a 20× objective and saved using OpenLab software (Improvision). Images were processed in Adobe Photoshop to adjust gray levels, enhance brightness and contrast, and differentiate the tissue from other background regions.

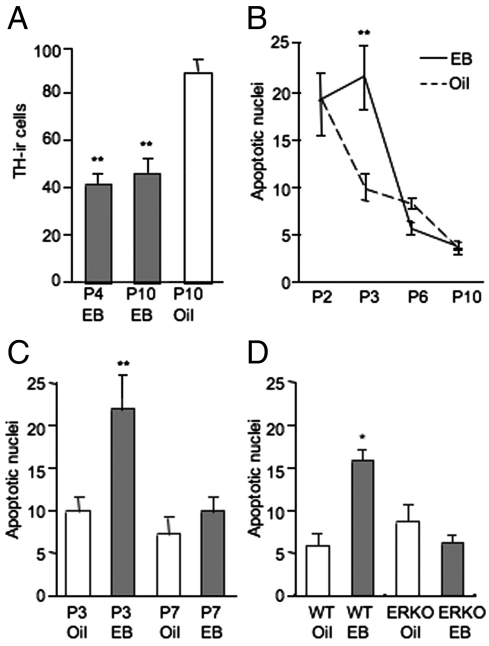

Figure 1. Estrogen treatment increases apoptotic-like changes in nuclear morphology of AVPV neurons *in vivo*. Treatment of neonatal rats with EB for 24 h increased nuclear blebbing on P3 but not P7 compared with oil. ***A***, The number of TH-ir neurons in the AVPV of rats treated on P2 with EB was significantly reduced within 24 h (P3) and remained low at P10. ***B***, Hoescht labeling was used to visualize condensed, fragmented apoptotic nuclei. On P2, the number of apoptotic nuclei was high and began to decrease over time in oil-treated control animals; however, 24 h after EB treatment there was an increase in numbers of apoptotic nuclei, which then decreased to control levels by P6. ***C***, EB treatment of rats on P2 increased numbers of apoptotic nuclei in the AVPV 24 h later, but not if the treatments were administered on P7. ***D***, Apoptotic nuclei increased in the AVPV of wild-type mice but did not change in ERKO mice after 24 h of EB treatment. $n = 3$–5 animals per group. *$p < 0.05$, **$p < 0.01$ compared with oil.

Results

Estradiol increases markers of apoptotic programmed cell death

Apoptotic cells undergo stereotypical changes in cellular morphology including nuclear condensation and fragmentation. To begin to determine whether estrogen-induced cell loss in the AVPV is caused by apoptotic cell death, we used Hoescht labeling to visualize apoptotic blebbed nuclei (Clarke, 1990). Subcutaneous injections of EB on postnatal day 2 (P2) reduced TH-ir cell number within as little as 24 h after treatment, and within 48 h of hormone exposure numbers of TH-ir cells were reduced to levels similar to those typically observed in adult males (Fig. 1*A*) ($n = 3$–5, $F_{(2,10)} = 16.972$, $p < 0.01$). In contrast, no decrease in TH-ir cells was observed with oil treatment. In the oil-treated group, the incidence of Hoescht-labeled apoptotic nuclei, cells with condensed, fragmented nuclei, decreased with increasing age. However, EB treatment increased the number of condensed, fragmented nuclei on P3, 24 h after injecting rats on P2 with EB, compared with oil-treated control pups (Fig. 1*B*) ($n = 3$–4, $F_{(7,19)} = 6.769$, $p < 0.01$). The ability of EB treatment to increase the incidence of apoptotic nuclei appears to be dependent on the timing of the treatment: EB administered to P6 rats did not increase numbers of fragmented nuclei 24 h later (Fig. 1*C*) ($n = 3$, $F_{(3,9)} = 6.69$, $p < 0.01$). Moreover, the hormone-dependent induction of apoptotic morphological changes appears to be dependent on an intact ERα. Comparison of wild-type mice versus ERKO mice treated with estradiol on P2 revealed that fragmented nuclei were increased in wild-type mice, but not in ERKO mice (Fig. 1*D*) ($n = 3$, $F_{(3,8)} = 14.588$, $p < 0.05$). In addition to the

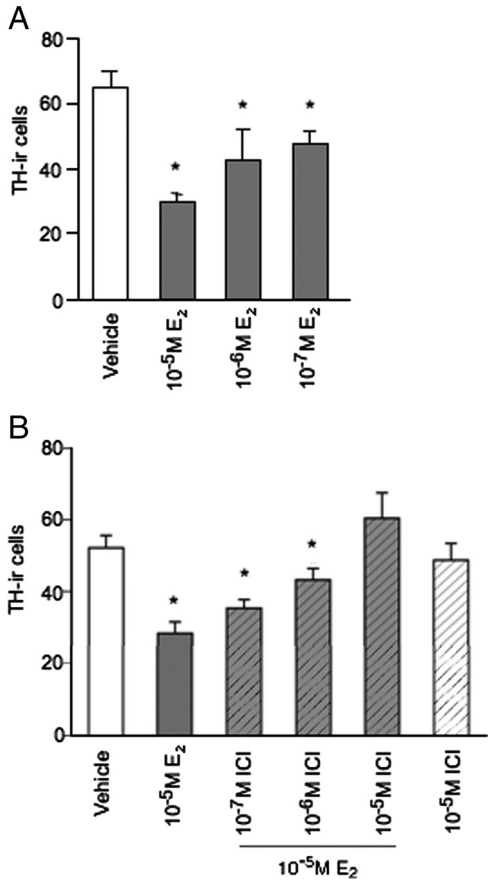

Figure 2. Rapid differentiation of AVPV explants is ER dependent. Exposure of AVPV explants derived from postnatal female rats to 10^{-5} M E_2 reduced TH-ir cell numbers to that typical of adult males. The effect of E_2 exposure on the number of TH-ir cells was blocked by concurrent application of the ER antagonist ICI 182,780 (ICI). ***A***, Addition of three doses of E_2 to the culture medium reduced the number of TH-ir neurons in AVPV explants, but only the 10^{-5} M E_2 dose caused a complete masculinization. ***B***, Concurrent incubation of ICI, 182,780 with 10^{-5} M E_2 caused a dose-dependent blockade of loss of TH-ir cells in AVPV explants. Exposure of AVPV explants to 10^{-5} M ICI 182,780 alone had no effect on TH-ir cell number. $n = 3$–5 explants per group. *$p < 0.05$ compared with vehicle.

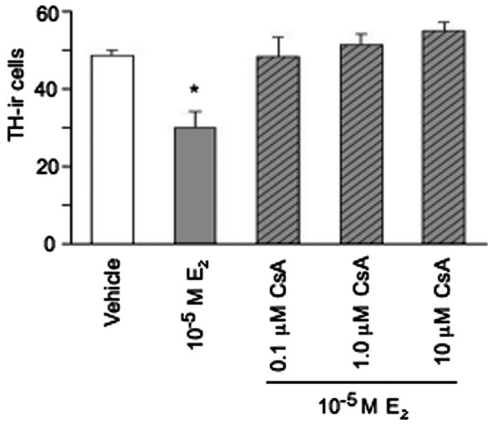

Figure 3. CsA blocks E_2-induced reduction of TH-ir cells in AVPV explants. Concurrent treatment with E_2 and 0.1, 1.0, or 10.0 μM CsA prevented E_2-induced loss of TH-ir cells. *$p < 0.01$ compared with vehicle.

marked increases in nuclear morphology observed in the AVPV of EB-treated mice and rats, DNA fragmentation as visualized with TUNEL revealed a significant increase in the number of TUNEL-labeled nuclei in the AVPV within 24 h after EB treatment, relative to that of control rats on P3 (data not shown) ($n = 3$, $F_{(1,4)} = 9.031$, $p < 0.05$).

Rapid differentiation of TH-ir cell number is dependent on estrogen receptors

To study molecular events governing estrogen induced TH-ir cell loss, we sought to develop an *in vitro* experimental model that would induce a rapid reduction in TH-ir cell number in the AVPV to numbers normally observed in adult male animals *in vivo*. In the experiments described below, females were used exclusively to avoid potentially confounding influences of prenatal exposure to sex steroids that occurs in prenatal male rodents. When AVPV explants were treated with 10^{-5} M estradiol for 48 h, TH-ir cell number was dramatically reduced compared with vehicle treated explants (see Fig. 4*A*, left and center panels). Although addition of 10^{-7} M and 10^{-9} M estradiol to the culture medium also decreased the number of AVPV TH-ir cells after 48 h, only 10^{-5} M estradiol treatment achieved reductions in TH-ir cell number that were comparable to the numbers reported in adult male rats (Fig. 2*A*) ($n = 3$–4, $F_{(3,9)} = 7.543$, $p < 0.05$) (Simerly and Swanson, 1987; Simerly, 1989). This rapid reduction in numbers of TH cells appears to be dependent on activation of ERs, since simultaneous application of estradiol and the ER antagonist ICI 182,780 blocked alterations in TH cell number in AVPV explants. AVPV of cultures treated with 10^{-5} M ICI 182,780, and 10^{-5} M estradiol displayed TH-ir cell numbers equivalent to the numbers present in vehicle-treated cultures (Fig. 2*B*), and significantly more TH-ir cells than cultures treated with 10^{-5} M estradiol alone ($n = 3$–5, $F_{(5,18)} = 6.795$, $p < 0.05$), confirming involvement of ERs in mediating the estradiol-induced reduction of TH-ir cell number in AVPV explants. Other doses of ICI 182,780 tested, 10^{-7} M and 10^{-6} M, also attenuated TH-ir cell loss, but only 10^{-5} M ICI 182,780 was fully effective in rescuing TH-ir cells from estradiol-induced reduction in cell number. Treatment with 10^{-5} M ICI 182,780 alone had no effect on TH-ir cell number in the AVPV. In a parallel set of experiments, AVPV explants derived from postnatal wild type and ERKO mice were treated with 10^{-5} M estradiol for 48 h. Numbers of TH-ir cells were markedly reduced in wild-type female, but not in either male or female ERα knock-out mice (ERKO; data not shown), confirming dependence of the rapid effects of estradiol on TH cell number in agreement with previous *in vivo* findings (Simerly et al., 1997).

CsA, an inhibitor of the mitochondrial permeability transition pore, blocks estrogen-induced TH-ir cell death

Mitochondria play a central role in the initiation of apoptotic cell death. Actions of proapoptic molecules at the mitochondrial membrane lead to formation of mitochondrial permeability transition pores (mPTPs) and result in the activation of caspases. The role of mPTPs in TH-ir cell loss was examined through concurrent treatment of AVPV explant cultures with 10^{-5} M estradiol and CsA, which inhibits mPTP opening (Broekemeier et al., 1989). Exposure of AVPV explants to estradiol in the presence of either 0.1 or 1 μM CsA completely blocked estradiol-induced TH-ir cell loss, compared with cultures treated with estradiol alone (Fig. 3) ($n = 3$–5, $F_{(4,11)} = 6.496$, $p < 0.01$). Increasing the CsA concentration to 10 μM also blocked estradiol-induced loss of TH-ir cells in AVPV explants, yet this higher dose had no apparent adverse effects on TH immunostaining or neuronal morphology in the AVPV.

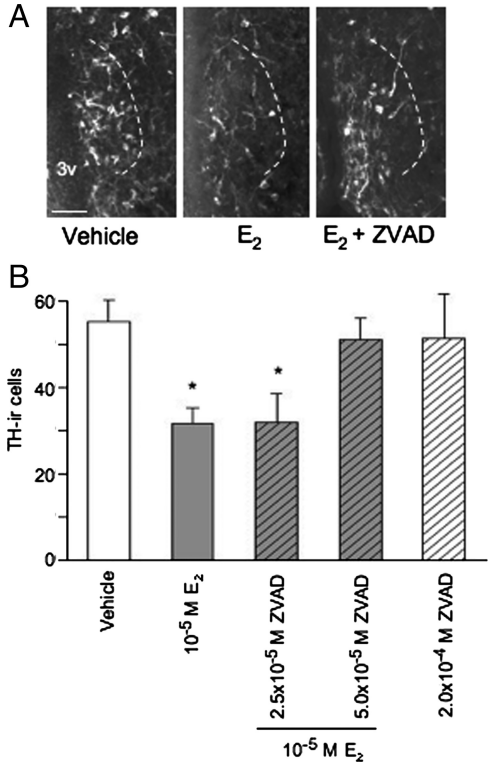

Figure 4. Caspase inhibition blocks E_2-induced loss of TH-ir cells. ***A***, Immunofluorescence images of TH-ir cells in AVPV explants treated with 10^{-5} M E_2, 10^{-5} M E_2 plus 5×10^{-5} M ZVAD, or vehicle for 48 h. 3v, Third ventricle. ***B***, Concurrent treatment with E_2 (10^{-5} M) and 2.5×10^{-5} M ZVAD did not prevent loss of TH-ir cells in AVPV explants, but 5×10^{-5} M ZVAD fully blocked E_2-induced loss of TH-ir neurons. Treatment with 2.0×10^{-4} M ZVAD alone had no effect on the number of TH-ir cells. $^*p < 0.05$ compared with vehicle. Scale bar, 25 μm.

The pan-caspase inhibitor ZVAD prevents estrogen-induced TH-ir cell loss

One of the hallmarks of apoptotic cell death is activation of caspases, thus we tested whether the ability of estradiol to induce loss of TH-ir neurons in the AVPV was dependent on caspase activity. AVPV explant cultures were treated with 10^{-5} M estradiol and the pan-caspase inhibitor ZVAD and stained for TH-ir 48 h later. Concurrent exposure of AVPV explants to estradiol and 5.0×10^{-5} M ZVAD completely blocked the estradiol-induced TH-ir cell loss observed in cultures treated with estradiol alone (Fig. 4) ($n = 3$–5, $F_{(4,15)} = 3.724$, $p < 0.05$). Moreover, the number of TH-ir cells in the estradiol and ZVAD-treated cultures were indistinguishable from those observed in control cultures that had never experienced estradiol treatment. A lower dose of ZVAD (2.5×10^{-5} M) had no effect on estradiol-induced TH-ir cell loss. The effective dose of ZVAD did not appear to be toxic since a fourfold higher dose had no effect on TH-ir cell number in the absence of estradiol. Most TH-ir cells are located in the periventricular zone of the AVPV and this distribution was not altered by ZVAD treatment.

Discussion

Despite the widespread incidence of sexually dimorphic regions in the mammalian forebrain, the underlying mechanisms that regulate cell number have not been identified. Although alterations in neurogenesis, migration, and survival may contribute to sexual differentiation (Forger, 2006), in the AVPV estrogen-mediated differentiation of TH-ir cells number depends on caspase-dependent programmed cell death. Using AVPV organotypic explants, we were able to replicate the estradiol-induced, ERα-dependent reduction in the number of TH-containing neurons that occurs *in vivo*, and demonstrate a cellular mechanism of programmed cell death involved in determining TH-ir cell number. Our findings demonstrate that both mitochondrial permeability and caspase activation are required for estradiol-induced loss of TH-ir cells. In addition, it appears that the ability of estradiol to increase the incidence of apoptotic cells in the AVPV is restricted to a postnatal critical period during the first week of life.

Previous studies of AVPV sexual differentiation provided evidence for steroid-induced cell death, but were not able to identify the molecular mechanism. The results presented here, together with previous reports, demonstrate that gonadal steroids increase the incidence of apoptotic cells in the AVPV and add further support for the importance of the ERα in this process (Murakami and Arai, 1989; Sumida et al., 1993; Arai et al., 1996). More importantly, the role of estrogen-induced caspases was substantiated by the complete rescue of TH-ir cells after blockade of caspase activation. This suggests that during sexual differentiation of the AVPV estradiol induces dopaminergic neuron cell death through a caspase dependent mechanism of apoptotic cell death. Upstream of caspase activation, apoptotic pathways also use mitochondria to regulate initiation of the cell death program. Proapoptotic signals, which trigger mitochondrial permeability transition pore opening and subsequent caspase activation, can be blocked by CsA, thereby preventing caspase activation (Capano et al., 2002). Estrogen-induced loss of TH-ir was blocked by CsA and confirms mitochondrial involvement in the death of AVPV dopaminergic neurons.

TH-ir cell death may be initiated by estrogen-mediated transcription of proapoptotic Bcl-2 family proteins that are upstream of the mitochondria. Expression of proapoptotic and antiapoptotic proteins is sexually dimorphic in the postnatal AVPV. Expression of the antiapoptotic protein Bcl-2 is lower and the proapoptotic protein Bax is higher in the AVPV of 1-d-old males compared with females (Tsukahara et al., 2006). Interestingly, overexpression of Bcl-2 in male mice did not rescue TH-ir cells; however, it did increase overall neuronal density suggesting that Bcl-2 may play a role in cells where estradiol is neuroprotective (Zup et al., 2003). Similar results were found when Bax, a proapoptotic protein, was removed. In Bax knock-out mice TH-ir cells were not rescued, but overall neuronal density increased, suggesting that, although the loss of TH-ir cells in the AVPV does not depend on Bax, there are AVPV neurons whose numbers are regulated by the expression of proapoptotic and antiapoptotic proteins (Forger et al., 2004). The presence of other proapoptotic proteins, such as Bak, or compensatory changes in the expression of other proteins associated with regulation of apoptosis could act to drive TH-ir cell loss in the Bcl-2 overexpressing and Bax knock-out mice (Scorrano and Korsmeyer, 2003).

Steroid-mediated cell death has been reported in other model systems. In *Manduca sexta* and *Drosophila melanogaster*, ecdysone-induced cell death selectively reduces the number of motoneurons during metamorphosis (Weeks, 2003). More commonly, steroid exposure tends to prevent cell loss in a number of species (Madeira and Lieberman, 1995). Recently, caspase inhibitors were used to rescue neurons after hormone-withdrawal in the vocal learning system of white-crowned sparrows. *In vivo* treatment reduced neuronal loss in adults during the transition from the breeding state (high steroid levels) to the nonbreeding state (low steroid levels) (Thompson and Brenowitz, 2008). In general, sex steroids, particularly estrogen, are viewed as neuro-

protective (Wise et al., 2000). However, the findings presented here contribute to the emerging body of evidence that steroids can also be neurodegenerative, as well as neuroprotective. This dual mode of action for estrogen is in keeping with previous reports of cell type specific developmental actions for sex steroids (Simerly, 1998). Future studies aimed at unraveling the regulatory mechanisms that determine the survival of the persistent TH-ir cells, as well as clarification of molecular events that define closure of the developmental critical period for sexual differentiation, may provide insight that can be used to manipulate cell survival during times of cellular dysregulation, including neurodegenerative injuries and diseases.

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