

CELL DEATH AND SEXUAL DIFFERENTIATION OF THE NERVOUS SYSTEM

N. G. FORGER*

Department of Psychology and Center for Neuroendocrine Studies,
Tobin Hall, University of Massachusetts, Amherst, MA 01003, USA

Abstract—Sex differences in nuclear volume or neuron number often are attributed to the hormonal control of cell death. In the spinal nucleus of the bulbocavernosus, the central portion of the medial preoptic nucleus, and the principal nucleus of the bed nucleus of the stria terminalis testicular hormones decrease cell death during perinatal life, resulting in a male advantage in neuron number in adulthood. Conversely, males have more dying cells during development and fewer neurons in adulthood than do females in the anteroventral periventricular nucleus of the hypothalamus. This review discusses several limitations and unresolved issues in the literature on sexually dimorphic cell death, and identifies molecular mechanisms by which gonadal steroids may control cell survival. In particular, evidence is presented for the hormonal regulation of neurotrophic factors and involvement of Bcl-2 family proteins in the determination of sex differences in neuron number. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: cell death, sexual differentiation, Bcl-2, Bax, neurotrophic factor.

It has been over 30 years since the discovery of the first neural sex differences, and since then a large number of differences have been found throughout the brains, spinal cords, and peripheral nervous systems of male and female vertebrates. In most cases sex differences in the nervous system are due to the actions of gonadal steroid hormones, usually testosterone or an estrogenic metabolite of testosterone, acting early in development. This single hormonal explanation has been remarkably powerful in explaining much of the variation between male and female brains. What is not known, however, is exactly *how* testosterone and its metabolites engender the myriad differences that have been reported. In fact, it is probably fair to say that for no single neural sex difference can we trace the steps from hormone production to the neural morphology observed in adulthood. In a surprising number of cases, it is not even known with certainty *where* testosterone acts to initiate the cascade culminating in an observed

difference in neuronal size, number, connectivity, or neurochemistry.

Differences in cell number are among the most common types of sex difference reported and may be either a difference in the total number of cells in a given area (e.g. as assessed in a Nissl stain), or in the number of defined neurons of a particular phenotype. In theory, these differences could arise as the result of differential neurogenesis, migration, differentiation of phenotype, or cell death in males and females. Currently, there is no clear evidence linking the hormonal control of neurogenesis to a sex difference seen in the adult brain, and only scant evidence that the hormonal control of migration contributes to sexual differentiation of the brain (Henderson et al., 1999; Oriyasa et al., 2002). In contrast, several well-studied sex differences are attributed to the hormonal control of cell survival (see Forger, 2001 for review).

Given that the hormonal control of cell death is often cited as the best established mechanism for creating neural sex differences, it is interesting to ask: just how well established is it? If the evidence is viewed with a critical eye, it must be admitted that even for this “best established” mechanism, the data are clear in only a few cases. Based on the types of methodologies actually employed in the sexual differentiation literature to date, I propose the following four criteria in order to comfortably conclude that hormonally regulated developmental cell death underlies any given sex difference in the nervous system: 1. There is a sex difference in neuron number in the adult; 2. this sex difference in adult cell number is reversed by hormone treatments during development; 3. there is a sex difference at some point in development in the number of dying cells; and 4. hormone treatments that reverse the sex difference in cell number also reverse the developmental sex difference in cell death. In other words, a hormonally-determined sex difference in cell number in adulthood correlates with a hormonally-determined sex difference in developmental cell death.

If one requires that all four criteria be demonstrated, then cell death is an “established” mechanism in only a handful of neural regions, all in the laboratory rat (Table 1). These are: the spinal nucleus of the bulbocavernosus (SNB), the central portion of the medial preoptic nucleus (MPNc; a subportion of the sexually dimorphic nucleus of the preoptic area), the principal nucleus of the bed nucleus of the stria terminalis (BNSTp), and the anteroventral periventricular nucleus (AVPV). Testosterone decreases cell death in the SNB, MPNc and BNSTp, while increasing cell death in AVPV (Nordeen et al., 1985; Murakami and Arai, 1989; Davis et al., 1996a; Chung et al., 2000) and in

*Tel: +1-413-545-5982; fax: +1-413-545-0996.

E-mail address: nforger@psych.umass.edu (N. G. Forger).

Abbreviations: AVPV, anteroventral periventricular nucleus; BC, bulbocavernosus muscle; BH, Bcl-2 homology; BNSTp, principal nucleus of the bed nucleus of the stria terminalis; CNTF, ciliary neurotrophic factor; CNTFR α , ciliary neurotrophic factor α receptor; ER, estrogen receptor; LA, levator ani muscle; MPNc, central portion of the medial preoptic nucleus; SNB, spinal nucleus of the bulbocavernosus; TH, tyrosine hydroxylase; VP, arginine vasopressin.

Table 1. Neural sex differences attributed to hormonally controlled cell death

| Neural area | Species | Evidence (reference) | | | |
|-----------------------|-------------|-----------------------------------|---|---------------------------------|------------------------------------|
| | | 1. Sex difference in cell number | 2. Cell number reversed by hormones | 3. Sex difference in cell death | 4. Cell death reversed by hormones |
| SNB | Rat | Breedlove & Arnold (1980) | Breedlove & Arnold (1983) | Nordeen et al. (1985) | Nordeen et al. (1985) |
| MPNc | Rat | Dodson & Gorski (1993) | Dodson & Gorski (1993) | Davis et al. (1996a) | Davis et al. (1996a) |
| BNSTp | Rat | Guillamón et al. (1988b) | Guillamón et al. (1988b) | Chung et al. (2000) | Chung et al. (2000) |
| AVPV | Rat | Sumida et al. (1993) ^a | Sumida et al. (1993) ^a | Sumida et al. (1993) | Murakami & Arai (1989) |
| Visual cortex | Rat | Reid & Juraska (1992) | Núñez et al. (2002) ^b | Núñez et al. (2001) | Núñez et al. (2000) ^b |
| RA | Zebra finch | Gurney (1981) | Gurney (1981); Grisham & Arnold (1995) ^c | Kim & De Voogd (1989) | — |
| Laryngeal motoneurons | Frog | Kay et al. (1999) | Kay et al. (1999) | Kay et al. (1999) ^d | — |
| SDApc | Gerbil | Holman et al. (1995) | — | Holman et al. (1996) | — |

^a Total cell number not reported; sex difference inferred from measures of cell density and overall volume of the nucleus.

^b Androgens administered perinatally decrease cell death in females; however, ovarian steroids after postnatal day 20 may normally be responsible for the sex difference in cell number.

^c Early work suggested that androgens increase cell number in RA; later work implicates estrogens.

^d Sex difference in cell death inferred, based on greater disappearance of healthy motoneurons in females than in males during development.

each case, the dying cells exhibit the morphological and/or biochemical hallmarks of apoptosis (i.e. pyknosis and DNA fragmentation). In several other cases, two or three of the four criteria have been demonstrated, making it likely that cell death is the mechanism underlying an observed sex difference in cell number. This latter category includes examples in birds, frogs, and rodents other than rats, suggesting that the mechanism of hormonally controlled cell death is likely to be widespread.

Limitations of the cell death literature

Even for those neural regions in which all four criteria have been satisfied, however, there are reasons for caution. For example, in order for counts of dying cells in development to account for an adult sex difference in neuron number in a quantitative way, we would have to know how long a cell appears apoptotic in the material under study. A common estimate is that the entire process of apoptosis takes only two to three hours (Bursch et al., 1990). If so, then the number of apoptotic cells seen in a tissue section at any time represents only a small fraction of the total number dying that day. However, other investigators have estimated that it may take two days or more for apoptotic cells to be eliminated (Hu et al., 1997). Determining the length of time a cell appears apoptotic in the material under study is a tall order, and has been attempted for only one of the regions listed in Table 1 (Nuñez et al., 2000). The unsettling possibility that the hormonal milieu could affect the time-course of apoptosis has received even less attention; if so, then counts of dying cells would lead to erroneous conclusions about the magnitude of cell death in different treatment groups.

Secondly, it is difficult to rule out other mechanisms. For example, in the AVPV testosterone increases cell death perinatally (Murakami and Arai, 1989; Sumida et al., 1993), but sex differences in nuclear volume and length do not emerge until much later (Davis et al., 1996b). This does not invalidate cell death as the mechanism responsible for the volume or length differences, but leaves plenty of room

for contributions by other mechanisms (e.g. cell size changes, developmental changes in apparent nuclear boundaries, and the recruitment or emigration of cells). Indeed, in the rat locus coeruleus, adult females have more neurons than do males (Guillamón et al., 1988a). Although cell death is greater in males on the day of birth, a detailed study of developmental changes in locus coeruleus cell number suggests that the adult sex difference is primarily due to greater postpubertal cell addition in females than in males (Pinos et al., 2001). Finally, one can be led astray by effects of exogenous hormone treatments. For example, treatment with androgens masculinizes the pattern of cell death in primary visual cortex of neonatal female rats (Nuñez et al., 2000), but androgens may not normally account for the sex difference in neuron number of adults (Nuñez et al., 2002).

Investigators have struggled with the best way to quantify cell death and variously report the absolute number of dying cells in the region under study, the number of dying cells per unit volume, or a ratio of apoptotic to healthy neurons. If cell death is the only process influencing the number of neurons in a given region, ratios of dying cells to total cell number make good sense. If multiple processes are involved, however, ratios can be problematic. This can be appreciated if one imagines a cell group in which a sex difference in neuron number (male>female) is due solely to the fact that more neurons migrate into the nucleus in males. If migration overlaps with the period of cell death and exactly the same number of neurons die in both sexes (i.e. there is no sex difference in absolute counts of apoptotic cells), the result would be a sex difference in the cell death ratio, and the incorrect conclusion that differential cell death in males and females explains the sex difference. This is not far-fetched: a similar scenario has been proposed to explain the development of a sex difference in neuron number in the higher vocal center of zebra finches (Burek et al., 1997), and migration into the nucleus overlaps with the period of cell death in the rat SNB and

gerbil sexually dimorphic area pars compacta (Sengelau and Arnold, 1986; Holman et al., 1995).

Thus, even in cases for which the evidence is quite strong, there are limitations inherent in using counts of dying cells in development to explain sex differences in adult neuron number. When any evidence is found suggesting that cell death contributes to a neural sex difference, the temptation is to conclude that the sex difference has been explained and to look no further. We probably should keep in mind that the data are rarely quantitative enough to conclude that cell death can account for the entire difference and that no other mechanisms are involved. Recently, we have used mutant mice to get around some of these problems and to test more directly the contribution of cell death genes to neural sex differences (see below).

Defining the first steps in the cascade: site of hormone action

If the ultimate goal is to explain how testosterone or its metabolites control cell death, then one must know which cell or cells the hormone acts on. For most neural sex differences, however, we do not really have a clear answer to the “site of hormone action” question. Classical intracellular androgen and estrogen receptors (ER) regulate sexual differentiation of cell number in several systems (Breedlove and Arnold, 1981; Simerly et al., 1997), so it is reasonable to start the search for sites of hormone action by identifying cells expressing the relevant hormone receptor(s). For example, estrogens control cell number in the AVPV and MPNc, and ERs are expressed by neurons in these nuclei during the cell death period (DonCarlos and Handa, 1994). Thus, it is possible that aromatized products of testosterone act directly on cells in AVPV and the MPNc to control their survival. However, there are many other possibilities. Hormones may act on afferents or targets of AVPV or MPNc neurons or on glial cells within the region, for example.

In the SNB, investigators were forced early on to consider indirect effects of hormones in the control of neuron number. Testosterone acts via androgen receptors to rescue SNB cells from death (Breedlove and Arnold, 1981, 1983) yet the motoneurons themselves do not express androgen receptors during the perinatal cell death period (Jordan et al., 1991). In fact, an SNB motoneuron that never expresses a functional androgen receptor can nonetheless be rescued by perinatal androgen treatments (Freeman et al., 1996). A variety of observations indicate that androgens act at the target muscles in this system and that SNB cells are secondarily spared following direct hormone action at the muscle (Fishman and Breedlove, 1985; Fishman et al., 1990; Kurz et al., 1992; Freeman et al., 1996).

It can be more difficult to pinpoint the direct site of hormone action in the brain. Even if one is successful in demonstrating that gonadal steroids act within a given nucleus to control cell number the search is not over, because most brain nuclei are composed of many different cell types. Ultimately, the answer to the site of action

question for most neural areas may have to await the development of animals in which estrogen or androgen receptors are conditionally expressed or silenced *specifically in the population of neurons known to undergo sexually dimorphic cell death*. In the absence of such evidence, it is hard to know for sure where the cascade leading to survival or death starts.

Mechanisms of hormone action: neurotrophic factors

As mentioned above, the direct site of hormone action for controlling motoneuron survival in the SNB is thought to be the perineal target muscles, bulbocavernosus and levator ani (BC/LA). These muscles normally form in both sexes prenatally, but degenerate in female rats and mice around the time of birth; testosterone treatments that rescue SNB motoneurons also rescue the BC/LA (Cihak et al., 1970). Striated muscles throughout the body produce trophic factors that retrogradely influence the survival of innervating motoneurons, suggesting a model in which trophic factors from the BC/LA muscles mediate the sparing effects of androgens on SNB motoneurons (Fig. 1A). Indeed, findings from our laboratory indicate that signaling through the ciliary neurotrophic factor α receptor (CNTFR α) is required for the development of the sex difference in the SNB. For example, the application of exogenous CNTF rescues SNB motoneurons in female rats; CNTF receptors are abundantly expressed by SNB motoneurons during perinatal life; and the normal sex difference in the SNB is absent in knockout mice lacking the CNTFR α (Fig. 1B, C; Forger et al., 1993, 1997; Varela et al., 2000). Finally, the androgenic rescue of SNB motoneurons can be blocked by the local application of antagonists to several trophic factor receptors, including CNTFR α (Fig. 1D; Xu et al., 2001).

Taken together these findings provide compelling evidence that endogenous factors, likely produced by the BC/LA muscles, act through trophic factor receptors on SNB motoneuron terminals to mediate sexual differentiation of the SNB. In the brain, the complexity of neural connections and the relative inaccessibility of neurons make it difficult to perform manipulations such as those described above. However, neurotrophic factors are expressed throughout the developing brain and are viewed as the single most important regulators of neuronal cell death (Oppenheim, 1991). It would be surprising if the hormonal control of trophic support does not contribute to some of the sex differences in neuron number observed in the brain.

Intracellular mechanisms of neuronal cell death and the Bcl-2 family

Gonadal hormones and trophic factors are two classes of extracellular molecules influencing neuronal survival. These signals must then be integrated within individual neurons to determine whether a given cell will live or die. Tremendous strides have been made in understanding the molecular bases of apoptosis over the past several years, and proteins of the Bcl-2 family have emerged as crucial regulators of death in many cell types (reviewed in Merry and Korsmeyer, 1997). Bcl-2 family members can be di-

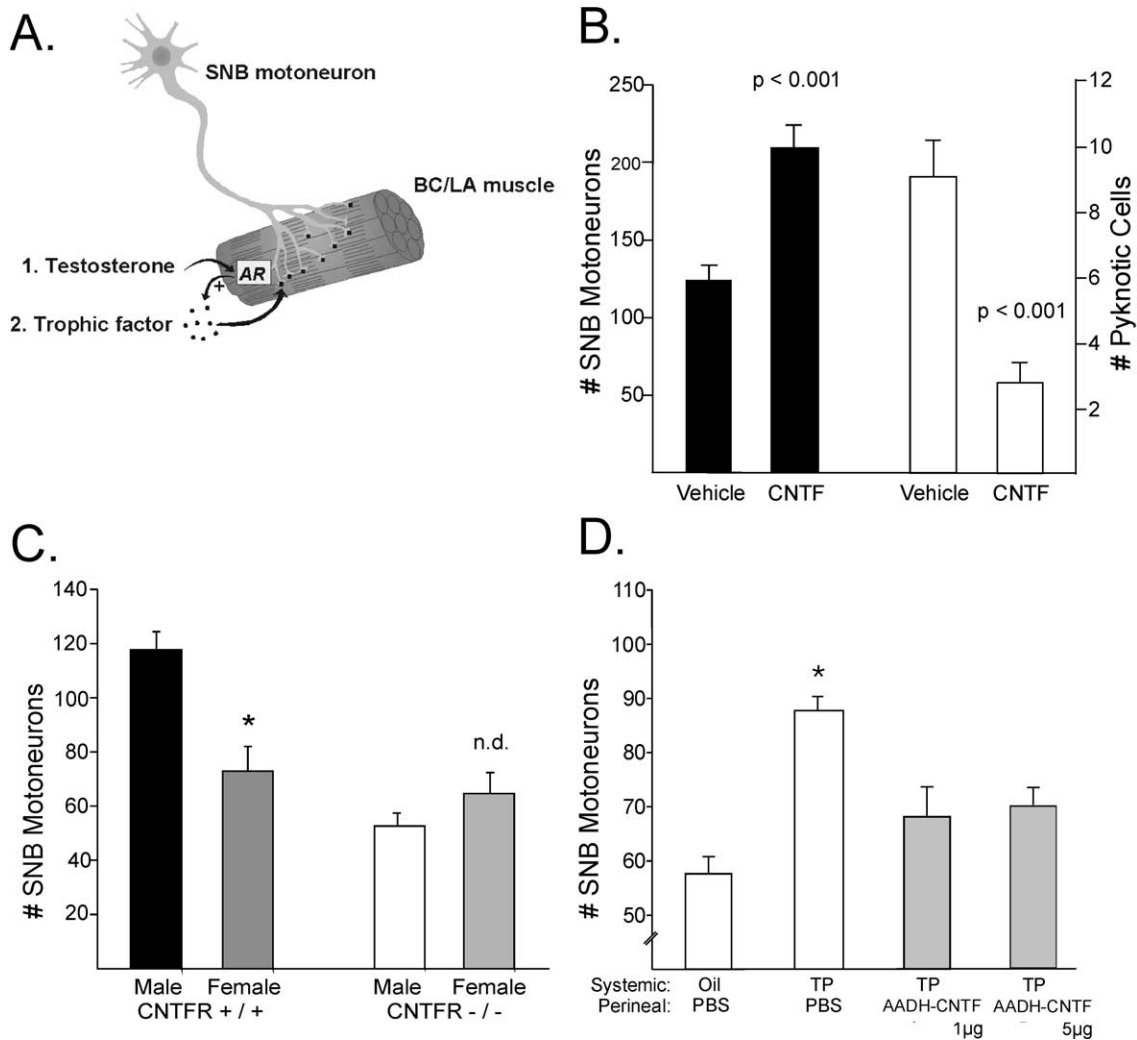


Fig. 1. (A) Model for the androgen-dependent sparing of SNB motoneurons. Testosterone binds to androgen receptors in the BC/LA target muscles, and neurotrophic factors from the muscle then bind to trophic factor receptors on SNB axon terminals (black squares) to regulate SNB motoneuron survival. (B) Daily perineal injections of CNTF from embryonic day 22 to postnatal day 3 increase the number of motoneurons (black bars) and decrease the number of pyknotic cells (white bars) in the SNB of female rats. (C) A sex difference in motoneuron number that is seen in the SNB of newborn, wild-type mice (CNTFR +/+; * $P < 0.001$) is absent in knockout mice lacking the CNTF receptor (CNTFR -/-; n.d., not different). (D) The number of motoneurons in the SNB of female rats receiving systemic injections of sesame oil or testosterone propionate (TP) and perineal injections of 1 or 5 µg of the CNTF receptor antagonist, AADH-CNTF. A blockade of CNTF receptors prevents the androgenic sparing of SNB cells. * Indicates significantly different from all other groups. [B, C, and D adapted with permission from Forger et al., 1993, 1997; and Xu et al., 2001, respectively. Copyrights, the Society for Neuroscience.]

vided into two categories, depending on whether they inhibit (e.g. Bcl-2, Bcl-x_L) or promote cell death (e.g. Bax, Bak). Although the precise mechanisms whereby Bcl-2 family proteins control apoptosis are still not clear, much of the available evidence supports the so-called “rheostat model” in which the balance between death-promoting and death-repressing family members constitutes a critical checkpoint that determines whether a cell will execute an apoptotic program (Gross et al., 1999).

Specifically, the pro-death proteins, Bax and Bak accumulate at the mitochondrial outer membrane when a cell receives a “death signal.” Bax/Bak oligomers disrupt mitochondrial membrane function, allowing for the release of cytochrome c and other signaling molecules, which activate downstream executioners of death such as the

caspases (Fig. 2; see Desagher and Martinou, 2000 for review). Pro-survival members of the Bcl-2 family may act primarily by preventing the translocation or oligomerization of Bax/Bak (Cheng et al., 2001). Although Bax and Bak play redundant roles in most cell types, developing neurons do not express full-length Bak (Sun et al., 2001; Uo et al., 2005), leaving Bax as a singularly important protein for apoptosis in neural development.

We recently asked whether disrupting the balance of Bcl-2 family members would affect the development of those neural sex differences thought to be due to differential cell death in males and females. We first examined mice over-expressing the survival-promoting protein, Bcl-2, and found reductions in the sex differences in neuron number in the SNB and AVPV (Zup et al., 2003). These findings are

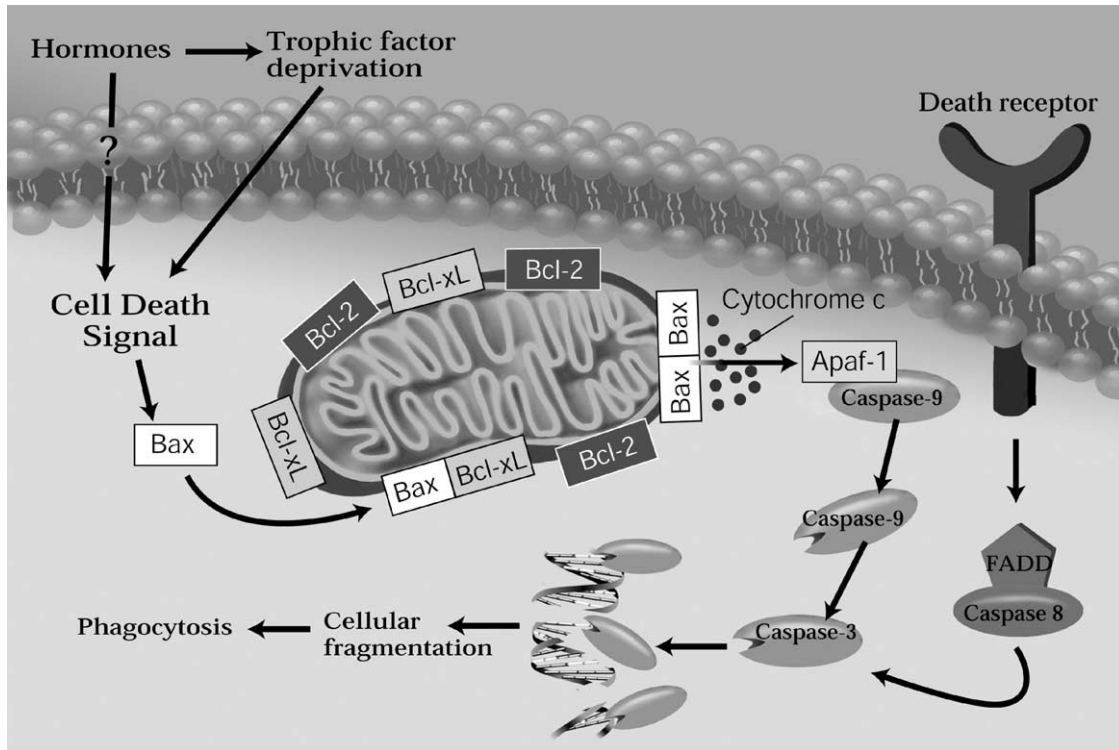


Fig. 2. A model for the control of cell death by Bcl-2, Bcl-xL and Bax in neural cells. Bax normally resides in the cytoplasm, but translocates to mitochondria in response to a death signal. A high ratio of Bax to Bcl-2 (or Bcl-xL) leads to the formation of Bax/Bax oligomers and the release of cytochrome c from the mitochondria, which then activates caspases leading to cellular degradation.

consistent with the idea that testosterone normally controls cell death in the SNB and AVPV via a Bcl-2 dependent pathway. However, the effects of Bcl-2 over-expression were relatively modest, and an over-expression study cannot address the physiological relevance of a given protein.

More recently we addressed these concerns by examining the SNB, AVPV, and BNSTp of *Bax*^{+/+} and *Bax*^{-/-} mice. We find that sex differences in overall cell number are eliminated in each case (Fig. 3; Forger et al., 2004; Jacob et al., 2005), demonstrating that Bax is required for sexually dimorphic cell death in the mouse forebrain and spinal cord. Moreover, because all animals in this study were gonadally intact, *Bax* status overrides endogenous hormonal signals in determining cell number of sexually dimorphic nuclei.

One advantage of cell death mutants is that they allow us to get away from some of the limitations, mentioned above, in relying on correlations between counts of dying cells in development and cell number in adulthood to address the contribution of cell death to neural sex differences. Not all developing neurons require Bax for cell death (Fan et al., 2001; Middleton and Davies, 2001). However, in those regions in which Bax is involved, the requirement is profound (White et al., 1998). If this is also true for sexually dimorphic regions, then cell number in adult *Bax*^{-/-} animals provides a window onto the number originally generated during development in both sexes, and the difference in neuron number between *Bax*^{-/-} and

Bax^{+/+} mice represents the total number of neurons lost, integrated over the entire developmental cell death period.

Thus, one can ask whether cell death completely accounts for a given sex difference, or whether other mechanisms should be considered. In the AVPV, BNSTp, and SNB cell number is equivalent in male and female *Bax*^{-/-} mice, suggesting that cell death alone can explain the sex differences in overall cell number found in these regions in wild-type animals. Of course, there are also limitations to the *Bax* knockout model for the study of neural sex differences. One must assume, for example, that *Bax* deletion eliminated cell death in development but did not affect any other processes. As in any scientific endeavor, the most solid conclusions will rely on converging evidence from independent types of experiments.

Heterogeneity of mechanisms controlling cell number within a single area

Although we talk about brain nuclei such as the AVPV and BNSTp as single cell groups, in fact they are an aggregation of phenotypically heterogeneous cells. In addition to the relatively modest sex difference in overall cell number in the AVPV, for example, there is a marked sex difference in the small subset of dopaminergic neurons, as measured by tyrosine hydroxylase (TH) immunoreactivity. Female rats and mice have three to four times more TH-immunoreactive neurons in AVPV than do males, and this difference requires an intact ER α (Simerly et al., 1985, 1997).

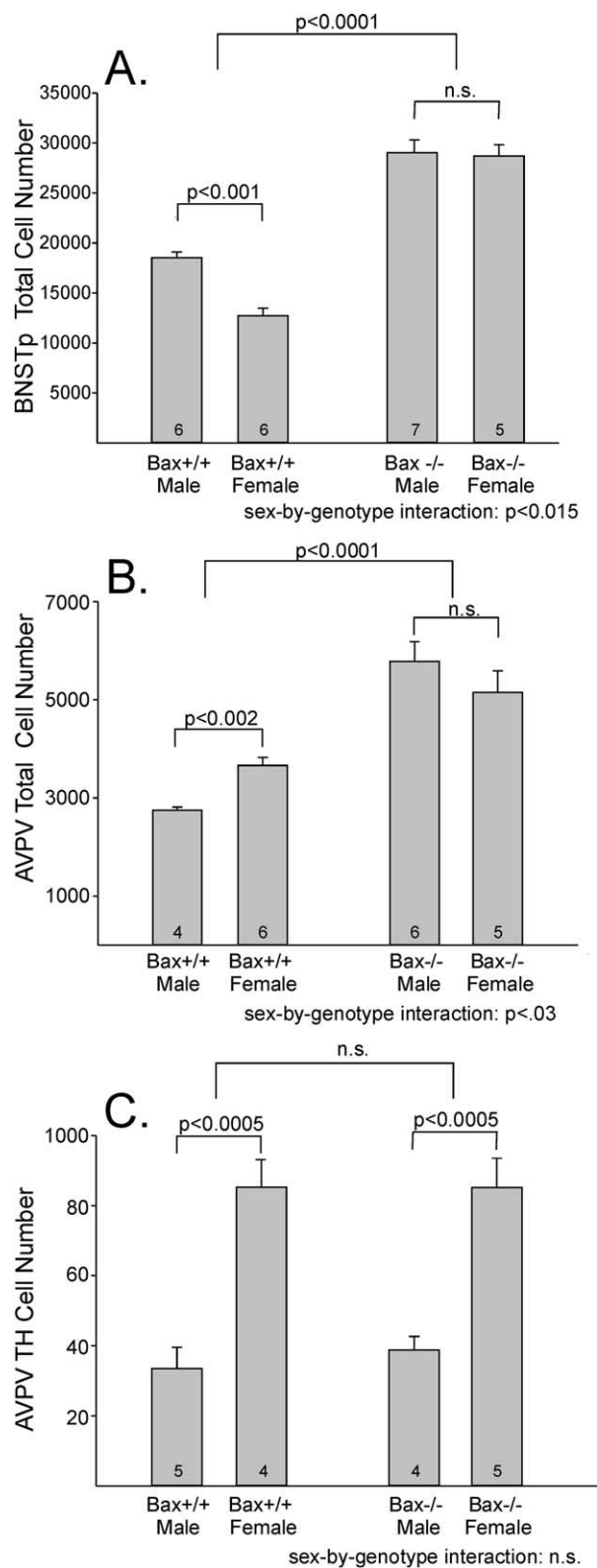


Fig. 3. (A) Wild-type (*Bax*^{+/+}) male mice have more neurons than do females in the BNSTp and this sex difference is eliminated in knockout mice lacking the *Bax* gene. (B) Wild-type females have more cells in AVPV than do males, and this sex difference is also eliminated by *Bax* gene deletion. (C) Female wild-type mice have many more TH-positive neurons in the AVPV than do wild-type males. A deletion of the *Bax* gene has no effect on this sex difference. [Reprinted with permission from Forger et al., 2004; Copyright, National Academy of Sciences (USA).]

Interestingly, although *Bax* gene deletion increased AVPV cell number in both sexes and eliminated the sex difference in overall cell number, there was no effect of *Bax* gene status on the number of TH cells, and the normal sex difference in TH cell number persisted in *Bax* knockouts (Fig. 3B, C). This same pattern was seen in mice over-expressing Bcl-2 (Zup et al., 2003). Thus, different mechanisms control the sexual differentiation of overall cell number and dopaminergic cell number in AVPV. Our findings do not exclude a role for cell death in the differentiation of TH neurons in AVPV, however. In fact, estradiol reduces the number of TH immunoreactive neurons in explants of neonatal rat AVPV and this effect can be blocked by caspase inhibitors (Waters and Simerly, 2002). Thus, the sex difference in the number of dopaminergic neurons in AVPV may be caused by a cell death program independent of Bcl-2 family proteins.

Similarly, in the BNSTp, male rats have two to three times as many neurons that express the neuropeptide vasopressin (VP) than do females (De Vries et al., 1994), and this sex difference depends on neonatal exposure to testosterone (Wang et al., 1993). Whereas the sex difference in total neuron number in BNSTp can be attributed to differential cell death (Chung et al., 2000; Forger et al., 2004), the hormonal control of neuronal phenotype (i.e. testosterone directs more neurons to express VP in males) is proposed to underlie the sex difference in VP cell number (Han and De Vries, 1999). However, direct evidence confirming this explanation or refuting other explanations, such as differential cell death of VP neurons in males and females, has been lacking.

In collaboration with Geert de Vries, we are using *Bax* knockout mice to test the role of cell death in the development of a sex difference in VP cell number in the BNSTp of mice. We reason that if hormones control VP cell phenotype, then preventing cell death should not prevent the sex difference in VP cell number from emerging. Indeed, preliminary evidence indicates that the absolute number of cells expressing VP mRNA is increased in *Bax*^{-/-} mice of both sexes, but the relative sex difference is unchanged (De Vries, Reza and Forger, unpublished observations). Thus, the cells that (eventually) express VP are subject to *Bax*-dependent cell death, but a sex difference in the magnitude of this death does not explain why males have more VP neurons than do females.

These data remind us that not all sex differences are due to cell death, and that the control of cell number varies not only from region to region, but also among subtypes of neurons within a single region. We have to be somewhat sophisticated about the questions we ask regarding whether a given sex difference is due to differential cell death in males and females, and cautious when extrapolating counts of dying cells to functionally distinct classes of neurons within a nucleus. In the future, it may be possible to use *Bax* knockout mice or other cell death mutants as a kind of "litmus test" to determine whether a given neural sex difference is due—in whole or in part—to the hormonal control of cell survival.

Future directions

As of yet, the direct gene targets regulated by estrogens and androgens to control neuronal cell death have not been identified. The data presented above suggest that gonadal steroids might modulate cell death by controlling the expression of Bcl-2 family proteins. There is a fairly large literature linking steroid hormone-regulated death of peripheral tissues and cancer cells to an alteration in the ratio of pro-survival to pro-death Bcl-2 family members (e.g. Wang and Phang, 1995; Kandouz et al., 1996; Huang et al., 1997). In at least some cases this regulation is likely to be direct, since putative estrogen response elements have been described for the Bcl-2 and Bcl-xL genes (Pike, 1999; Perillo et al., 2000).

Steroid hormones have also been shown to regulate the expression of Bcl-2 family members in neural tissue (Garcia-Segura et al., 1998; Dubal et al., 1999; Pike, 1999;

Harms et al., 2001). This work has primarily been motivated by a desire to understand the neuroprotective effects of estrogens in injury models, however, and the hormonal control of Bcl-2 proteins during development is virtually unexplored (but see Hsu et al., 2000). We have shown that testosterone up-regulates Bcl-2 (but not Bax) protein in SNB motoneurons of adult rats (Zup and Forger, 2002), and may have the same effect during perinatal life (Fig. 4; Zup, 2002). Thus, in some cases, gonadal steroid hormones may regulate the expression of Bcl-2 and/or Bax to control neuronal cell death.

In other cases, however, posttranslational modifications and/or the sub-cellular redistribution of Bcl-2 family members are likely to be important. For example, the translocation of Bax from the cytoplasm to the mitochondrial membrane is an important step in Bax-mediated apoptosis (Fig. 2) and can occur quickly, without any change

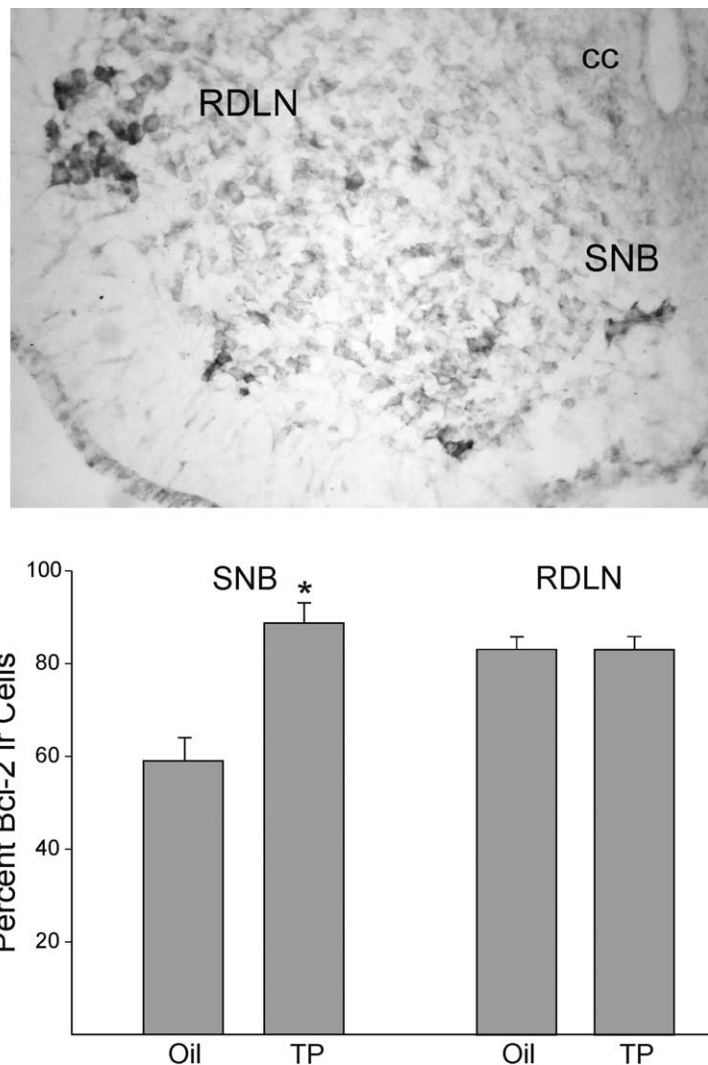


Fig. 4. Top: Bcl-2 immunoreactivity in the SNB and retrodorsolateral nucleus (RDLN) of a female rat on E22. cc, central canal. Bottom: Percent of Bcl-2 positive motoneurons on E22 in females treated with testosterone propionate (TP) or oil from E19-E21. Bcl-2 immunoreactivity was present in a higher percentage of cells in the SNB of TP-treated females ($P < 0.02$). Hormone treatment did not alter immunoreactivity in the RDLN, a cell group in which motoneuron number is not regulated by gonadal steroids [(Zup, 2002)]. Figure used with permission from the author.

in Bax expression. The location and activity of Bax and other full-length Bcl-2 family proteins are often controlled by family members known as “BH3-only” proteins (Cheng et al., 2001). These molecules contain only one Bcl-2 homology (BH) domain (the third), in contrast to full length members of the family which contain three or four BH domains. BH3-only proteins (e.g. Bid, Bim, Bik) are emerging as essential components of the “death signal,” which has until recently been the most mysterious part of the cell death cascade (see Fig. 2). They are almost always pro-apoptotic and transduce extracellular signals, culminating in Bax-dependent cell death (Putcha et al., 2001). Importantly, BH3-only proteins can be dynamically regulated. For example, the withdrawal of nerve growth factor markedly upregulates Bim expression in sympathetic neurons, and estrogen deprivation upregulates Bik mRNA >40-fold in breast cancer cells (Putcha et al., 2001; Hur et al., 2004). These BH3-only members of the Bcl-2 family are therefore important potential targets of steroid hormone regulation in sexually dimorphic brain regions.

With the explosion of apoptosis research in the last decade, there is a dizzying array of other proteins and signaling pathways that could also play important roles in hormone-regulated cell death. It may be possible to narrow the field a bit by taking advantage of the opposite patterns of cell death in regions such as the AVPV and BNSTp. Testosterone (or a metabolite) decreases cell death in the BNSTp while increasing death in the AVPV, yet in both cases the cell death is dependent on Bax. This suggests the existence of a molecular switch someplace between the production of testosterone and the “Bax checkpoint.” Possibilities include differences in the active hormone metabolite, hormone receptor subtypes, expression of steroid receptor co-activators, or differences in expression of any proteins influencing Bax-dependent cell death. Keeping in mind the caveat that the direct site of hormone action for controlling overall cell number has not been demonstrated in either nucleus, a comparison of gene expression in AVPV and BNSTp in response to estradiol, perhaps casting the net widely with techniques such as microarrays, seems like a promising place to start. In the process of searching for the molecular explanation for the opposing patterns of cell death in these two nuclei, one may identify key genes targeted by steroid hormones to control sexually dimorphic cell death.

REFERENCES

- Breedlove SM, Arnold AP (1980) Hormone accumulation in a sexually dimorphic motor nucleus in the rat spinal cord. *Science* 210: 564–566.
- Breedlove SM, Arnold AP (1981) Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen-insensitive rats. *Brain Res* 225: 297–307.
- Breedlove SM, Arnold AP (1983) Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. *J Neurosci* 3:424–432.
- Burek MJ, Nordeen KW, Nordeen EJ (1997) Sexually dimorphic neuron addition to an avian song-control region is not accounted for by sex differences in cell death. *J Neurobiol* 33:61–71.
- Bursch W, Kleine L, Tenniswood M (1990) The biochemistry of cell death by apoptosis. *Biochem Cell Biol* 68:1071–1074.
- Cheng EH-YA, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ (2001) BCL-2, BCL-X_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 8:705–711.
- Chung WC, Swaab DF, De Vries GJ (2000) Apoptosis during sexual differentiation of the bed nucleus of the stria terminalis in the rat brain. *J Neurobiol* 43:234–243.
- Cihak R, Gutmann E, Hanzlikova V (1970) Involution and hormone-induced persistence of the *M. sphincter (levator) ani* in female rats. *J Anat* 106:93–110.
- Davis EC, Popper P, Gorski RA (1996a) The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. *Brain Res* 734:10–18.
- Davis EC, Shryne JE, Gorski RA (1996b) Structural sexual dimorphisms in the anteroventral periventricular nucleus of the rat hypothalamus are sensitive to gonadal steroid perinatally, but develop peripubertally. *Neuroendocrinology* 63:142–148.
- Desagher S, Martinou J-C (2000) Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 10:369–377.
- De Vries GJ, Wang Z, Bullock NA, Numan S (1994) Sex differences in the effects of testosterone and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis of rats. *J Neurosci* 14:1789–1794.
- Dodson RE, Gorski RA (1993) Testosterone propionate administration prevents the loss of neurons within the central part of the medial preoptic nucleus. *J Neurobiol* 24:80–88.
- DonCarlos LL, Handa RJ (1994) Developmental profile of estrogen receptor mRNA in the preoptic area of male and female neonatal rats. *Dev Brain Res* 79:283–289.
- Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM (1999) Estradiol modulates bcl-2 in cerebral ischemia: a potential role for estrogen receptors. *J Neurosci* 19:6385–6393.
- Fan H, Favero M, Vogel MW (2001) Elimination of Bax expression in mice increases cerebellar Purkinje cell numbers but not the number of granule cells. *J Comp Neurol* 436:82–91.
- Fishman RB, Breedlove SM (1985) The androgenic induction of spinal sexual dimorphism is independent of supraspinal afferents. *Dev Brain Res* 23:255–258.
- Fishman RB, Chism L, Firestone GL, Breedlove SM (1990) Evidence for androgen receptors in sexually dimorphic perineal muscles of neonatal male rats. Absence of androgen accumulation by the perineal motoneurons. *J Neurobiol* 21:694–704.
- Forger NG (2001) The development of sex differences in the nervous system. In: *The handbook of behavioral neurobiology*, Vol. 13: Developmental psychobiology (Blass E, ed), pp 153–208. New York: Plenum.
- Forger NG, Roberts SL, Wong V, Breedlove SM (1993) Ciliary neurotrophic factor maintains motoneurons and their target muscles in developing rats. *J Neurosci* 13:4720–4726.
- Forger NG, Howell ML, Bengston L, MacKenzie L, DeChiara TM, Yancopoulos GD (1997) Sexual dimorphism in the spinal cord is absent in mice lacking the ciliary neurotrophic factor receptor. *J Neurosci* 17:9605–9612.
- Forger NG, Rosen GJ, Waters EM, Jacob D, Simerly RB, de Vries GJ (2004) Deletion of Bax eliminates sex differences in the mouse forebrain. *Proc Natl Acad Sci U S A* 101:13666–13671.
- Freeman LM, Watson NV, Breedlove SM (1996) Androgen spares androgen-insensitive motoneurons from apoptosis in the spinal nucleus of the bulbocavernosus in rats. *Horm Behav* 30:424–433.
- Garcia-Segura LM, Cardona-Gomez P, Naftolin F, Chowen JA (1998) Estradiol upregulates Bcl-2 expression in adult brain neurons. *Neuroreport* 9:593–597.

- Grisham W, Arnold A (1995) A direct comparison of the masculinizing effects of testosterone, androstenedione, estrogen, and progesterone on the development of the zebra finch song system. *J Neurobiol* 26:163–170.
- Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 13:1899–1911.
- Guillamón A, de Blas MR, Segovia S (1988a) Effects of sex steroids on the development of the locus coeruleus in the rat. *Dev Brain Res* 40:306–310.
- Guillamón A, Segovia S, del Abril A (1988b) Early effects of gonadal steroids on the neuron number in the medial posterior region and the lateral division of the bed nucleus of the stria terminalis in the rat. *Dev Brain Res* 44:281–290.
- Gurney ME (1981) Hormonal control of cell form and number in the zebra finch song system. *J Neurosci* 1:658–673.
- Han TM, De Vries GJ (1999) Neurogenesis of galanin cells in the bed nucleus of the stria terminalis and centromedial amygdala in rats: a model for sexual differentiation of neuronal phenotype. *J Neurobiol* 38:491–498.
- Harms C, Lautenschlager M, Bergk A, Katchanov J, Freyer D, Kapinya K, Herwig U, Megow D, Dirnagl U, Weber JR, Hortnagl H (2001) Differential mechanisms of neuroprotection by 17 beta-estradiol in apoptotic versus necrotic neurodegeneration. *J Neurosci* 21:2600–2609.
- Henderson RG, Brown AE, Tobet SA (1999) Sex differences in cell migration in the preoptic area/anterior hypothalamus of mice. *J Neurobiol* 41:252–266.
- Holman SD, Collado P, Rice A, Hutchison JB (1995) Stereological estimates of postnatal structural differentiation in a sexually dimorphic hypothalamic nucleus involved in vocal control. *Brain Res* 694:167–176.
- Holman S, Collado P, Skepper JN, Rice A (1996) Postnatal development of a sexually dimorphic, hypothalamic nucleus in gerbils: a stereological study of neuronal number and apoptosis. *J Comp Neurol* 376:315–325.
- Hsu C, Hsieh YL, Yang RC, Hsu HK (2000) Blockage of N-methyl-D-aspartate receptors decreases testosterone levels and enhances postnatal neuronal apoptosis in the preoptic area of male rats. *Neuroendocrinology* 71:301–307.
- Hu Z, Kazunari Y, Ozawa H, Haiping L, Kawata M (1997) The *in vivo* time course for elimination of adrenalectomy-induced apoptotic profiles from the granule cell layer of the rat hippocampus. *J Neurosci* 17:3981–3989.
- Huang Y, Ray S, Reed JC, Ibrado AM, Tang C, Nawabi A, Bhalla K (1997) Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. *Breast Cancer Res Treat* 42:73–81.
- Hur J, Chesnes J, Coser KR, Lee RS, Geck P, Isselbacher KJ, Shioda T (2004) The Bik BH3-only protein is induced in estrogen-starved and antiestrogen-exposed breast cancer cells and provokes apoptosis. *Proc Natl Acad Sci U S A* 101:2351–2356.
- Jacob DA, Bengston CL, Forger NG (2005) Effects of *Bax* gene deletion on muscle and motoneuron degeneration in a sexually dimorphic neuromuscular system. *J Neurosci* 25:5638–5644.
- Jordan CL, Breedlove SM, Arnold AP (1991) Ontogeny of steroid accumulation in spinal lumbar motoneurons of the rat: implications for androgen's site of action during synapse elimination. *J Comp Neurol* 313:441–448.
- Kandouz M, Siromachkova M, Jacob D, Chretien MB, Therwath A, Gompel A (1996) Antagonism between estradiol and progesterin on Bcl-2 expression in breast-cancer cells. *Int J Cancer* 68:120–125.
- Kay JN, Hannigan P, Kelley DB (1999) Trophic effects of androgen: development and hormonal regulation of neuronal number in a sexually dimorphic vocal motor nucleus. *J Neurobiol* 40:375–385.
- Kirn JR, DeVoogd TJ (1989) Genesis and death of vocal control neurons during sexual differentiation in the zebra finch. *J Neurosci* 9:3176–3187.
- Kurz EM, Cover AR, Sengelaub DR (1992) Testosterone fails to save androgen-sensitive rat motoneurons following early target removal. *Dev Brain Res* 70:181–189.
- Merry DE, Korsmeyer SJ (1997) Bcl-2 gene family in the nervous system. *Annu Rev Neurosci* 20:245–267.
- Middleton G, Davies AM (2001) Populations of NGF-dependent neurons differ in their requirement for BAX to undergo apoptosis in the absence of NGF/TrkA signalling *in vivo*. *Development* 128:4715–4728.
- Murakami S, Arai Y (1989) Neuronal death in the developing sexually dimorphic periventricular nucleus of the preoptic area in the female rat: effect of neonatal androgen treatment. *Neurosci Lett* 102:185–190.
- Nordeen EJ, Nordeen KW, Sengelaub DR, Arnold AP (1985) Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. *Science* 229:671–673.
- Nuñez JL, Jurgens HA, Juraska JM (2000) Androgens reduce cell death in the developing rat visual cortex. *Dev Brain Res* 125:83–88.
- Nuñez JL, Lauschke DM, Juraska JM (2001) Cell death in the development of the posterior cortex in male and female rats. *J Comp Neurol* 436:32–41.
- Nuñez JL, Sodhi J, Juraska JM (2002) Ovarian hormones after postnatal day 20 reduce neuron number in the rat primary visual cortex. *J Neurobiol* 52:312–321.
- Oppenheim RW (1991) Cell death during development of the nervous system. *Annu Rev Neurosci* 14:453–501.
- Orikasa C, Kondo Y, Hayashi S, McEwen B, Sakuma Y (2002) Sexually dimorphic expression of estrogen receptor β in the anteroventral periventricular nucleus of the rat preoptic area: Implication in luteinizing hormone surge. *Proc Natl Acad Sci U S A* 99:3306–3311.
- Perillo B, Sasso A, Abbondanza C, Palumbo G (2000) 17beta-Estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol* 20:2890–2901.
- Pike CJ (1999) Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. *J Neurochem* 72:1552–1563.
- Pinos H, Collado P, Rodríguez-Zafra M, Rodríguez C, Segovia S, Guillamón A (2001) The development of sex differences in the locus coeruleus of the rat. *Brain Res Bull* 56:73–78.
- Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 29:615–628.
- Reid SNM, Juraska JM (1992) Sex differences in neuron number in the binocular area of the rat visual cortex. *J Comp Neurol* 321:448–455.
- Sengelaub DR, Arnold AP (1986) Development and loss of early projections in a sexually dimorphic rat spinal nucleus. *J Neurosci* 6:1613–1620.
- Simerly RB, Swanson LW, Handa RJ, Gorski RA (1985) Influence of perinatal androgen on the sexually dimorphic distribution of tyrosine hydroxylase-immunoreactive cells and fibers in the anteroventral periventricular nucleus of the rat. *Neuroendocrinology* 40:501–510.
- Simerly RB, Zee MC, Pendleton JW, Lubahn DB, Korach KS (1997) Estrogen receptor-dependent sexual differentiation of dopaminergic neurons in the preoptic region of the mouse. *Proc Natl Acad Sci U S A* 94:14077–14082.
- Sumida H, Nishizuka M, Kano Y, Arai Y (1993) Sex differences in the anteroventral periventricular nucleus of the preoptic area and in the related effects of androgen in prenatal rats. *Neurosci Lett* 151:41–44.
- Sun YF, Yu LY, Saarma M, Timmusk T, Arumae U (2001) Neuron-specific Bcl-2 homology 3 domain-only splice variant of Bak is anti-apoptotic in neurons, but pro-apoptotic in non-neuronal cells. *J Biol Chem* 276:16240–16247.

- Uo T, Kinoshita Y, Morrison RS (2005) Neurons exclusively express N-Bak, a BH3 domain-only Bak isoform that promotes neuronal apoptosis. *J Biol Chem* 280:9065–9073.
- Varela CR, Bengston L, Xu J, MacLennan AJ, Forger NG (2000) Additive effects of ciliary neurotrophic factor and testosterone on motoneuron survival; differential effects on motoneuron size and muscle morphology. *Exp Neurol* 165:384–393.
- Wang TT, Phang JM (1995) Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Res* 55:2487–2489.
- Wang Z, Bullock NA, De Vries GJ (1993) Sexual differentiation of vasopressin projections of the bed nucleus of the stria terminalis and medial amygdaloid nucleus in rats. *Endocrinology* 132:2299–2306.
- Waters EM, Simerly RB (2002) Estrogen-mediated programmed cell death during development of a sexually dimorphic hypothalamic nucleus. *Soc Neurosci Abstr* 28:33.5.
- White FA, Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD (1998) Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. *J Neurosci* 18:1428–1439.
- Xu J, Gingras KM, Bengston L, Di Marco A, Forger NG (2001) Blockade of endogenous neurotrophic factors prevents the androgenic rescue of rat spinal motoneurons. *J Neurosci* 21:4366–4372.
- Zup SL (2002) The role of Bcl-2 family members in sexual differentiation and adult neural plasticity. Dissertation, University of Massachusetts.
- Zup SL, Forger NG (2002) Testosterone regulates BCL-2 immunoreactivity in a sexually dimorphic motor pool of adult rats. *Brain Res* 950:312–316.
- Zup SL, Carrier H, Waters E, Tabor A, Bengston L, Rosen G, Simerly R, Forger NG (2003) Overexpression of Bcl-2 reduces sex differences in neuron number in the brain and spinal cord. *J Neurosci* 23:2357–2362.

(Accepted 10 July 2005)
(Available online 28 November 2005)