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 dif-

ference 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).

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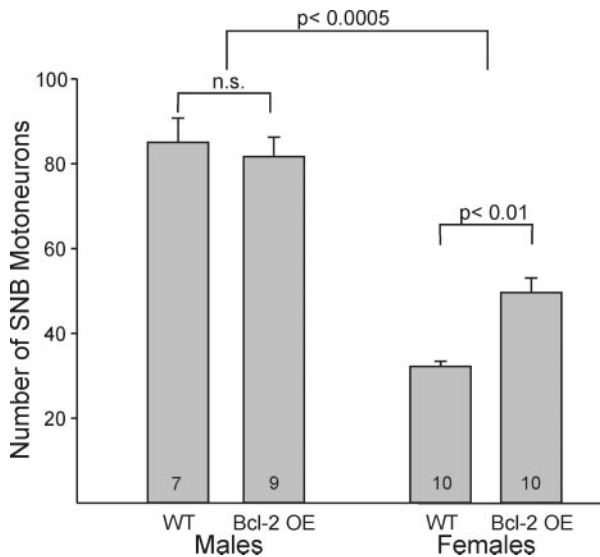
Figure 1. The mean ($\pm$ SEM) number of SNB motoneurons in wild-type (*WT*) and Bcl-2-overexpressing (*Bcl-2 OE*) mice. Bcl-2 overexpression increased motoneuron number in females but not in males. $n =$ *numbers* at base of bars. *n.s.*, Not significant.

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

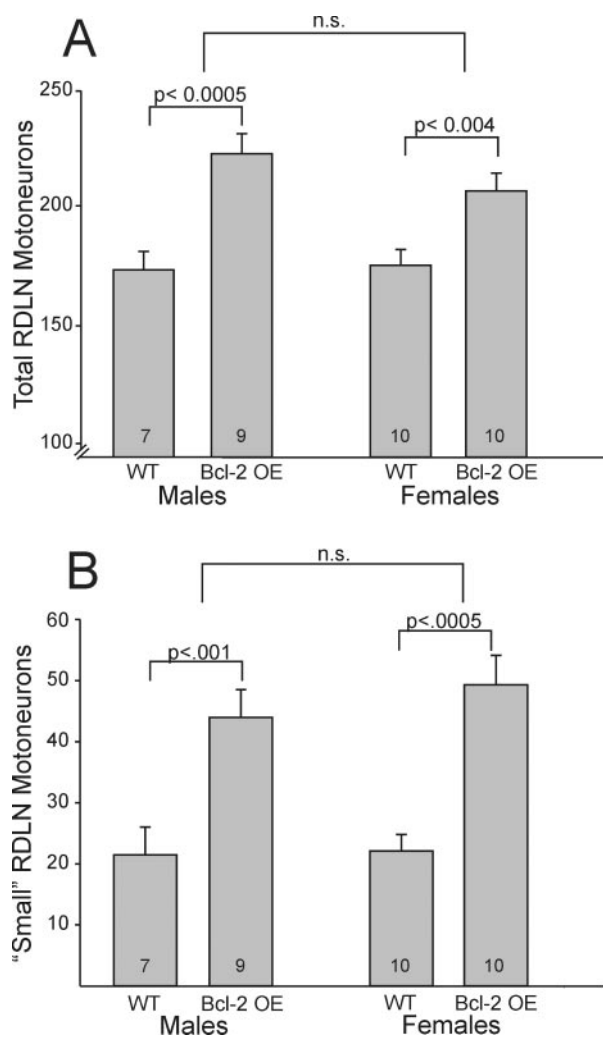|	SNB		RDLN		AVPV	
	Soma	Nucleus	Soma	Nucleus	Soma	Nucleus
Males						
Wild type	346 ± 22	120 ± 8	302 ± 21	121 ± 7	50 ± 2	33 ± 1
Bcl-2 OE	358 ± 13	121 ± 4	278 ± 13	101 ± 5	47 ± 1	34 ± 1
Females						
Wild type	226 ± 11	84 ± 4	301 ± 12	117 ± 5	48 ± 3	33 ± 1
Bcl-2 OE	235 ± 10	84 ± 3	255 ± 6	98 ± 4	47 ± 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).
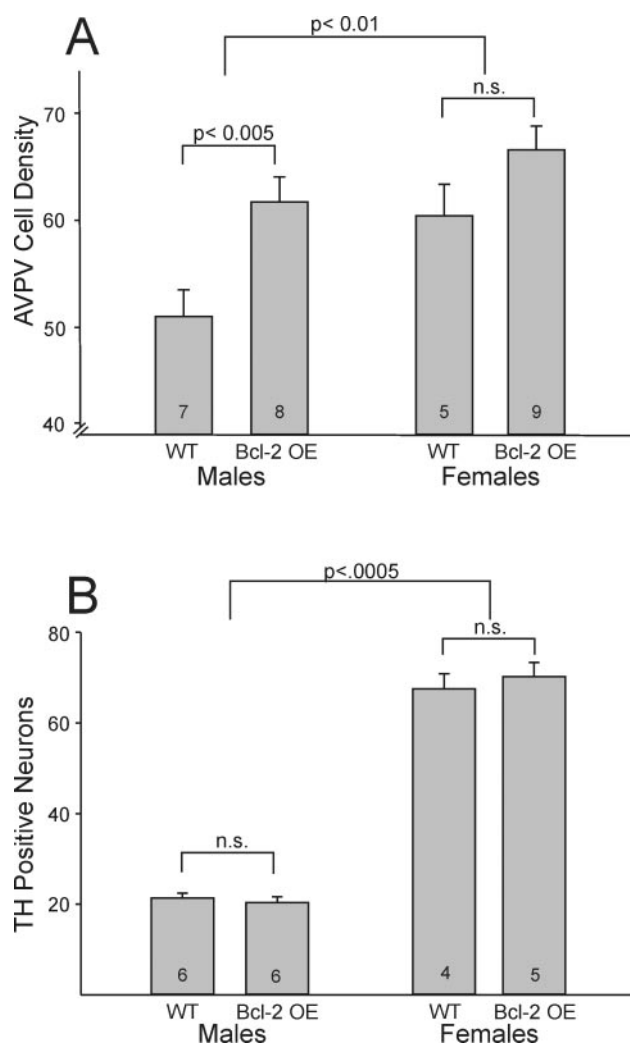
The final common pathway for many types of cell death is the activation of cysteine proteases, known as "caspases," which degrade cellular proteins (for review, see Hengartner, 2000). Preliminary findings of Waters and Simerly (2002) indicate that estradiol markedly decreases TH cell number in organotypic 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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