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Control of Cell Number in the Sexually Dimorphic Brain and Spinal Cord

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Abstract

The hormonal control of cell death is currently the best-established mechanism for creating sex differences in cell number in the brain and spinal cord. For example, males have more cells than do females in the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) and spinal nucleus of the bulbocavernosus (SNB), whereas females have a cell number advantage in the anteroventral periventricular nucleus (AVPV). In each case, the difference in cell number in adulthood correlates with a sex difference in the number of dying cells at some point in development. Mice with over- or under-expression of cell death genes have recently been used to test more directly the contribution of cell death to neural sex differences, to identify molecular mechanisms involved, and to determine the behavioural consequences of suppressing developmental cell death. *Bax* is a pro-death gene of the Bcl-2 family that is singularly important for apoptosis in neural development. In mice lacking *bax*, the number of cells in the BNSTp, SNB, and AVPV are significantly increased and sex differences in total cell number in each of these regions are eliminated. Cells rescued by *bax* gene deletion in the BNSTp express markers of differentiated neurones and the androgen receptor. On the other hand, sex differences in other phenotypically identified populations, such as vasopressin-expressing neurones in the BNSTp or dopaminergic neurones in AVPV, are not affected by either *bax* deletion or *bcl-2* over-expression. Possible mechanisms by which testosterone may regulate cell death in the nervous system are discussed, as are the behavioural effects of eliminating sex differences in neuronal cell number.

Keywords

sex difference; cell death; bax; bcl-2; androgen; oestrogen

Introduction

Many of the best-studied sex differences in vertebrate brains relate to cell number. These may be either sex differences in the total number of cells in a given brain region, as identified in a Nissl stain, or in the number of cells of a particular phenotype. In most cases, sex differences in cell number have been attributed to gonadal steroid hormones, in particular testosterone, or a metabolite of testosterone, acting early in development. In theory, testosterone could cause sex differences in cell number by regulating any of the major neurodevelopmental events: neurogenesis, migration, the differentiation of phenotype, or cell death. Of these, the hormonal control of cell death is the best-established mechanism for generating sex differences in cell number in the brain, and will be the main focus of this review. It is likely that neurogenesis, cell migration, and the differentiation of cellular

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phenotype also contribute to sexual differentiation of the brain, although evidence is more preliminary for these cellular mechanisms (see ref. 1 for a recent review).

For some neural sex differences, the birth of cells is complete before the testes differentiate (2), making it very unlikely that gonadal steroids control neurogenesis to establish sex differences. In other regions, cell birth overlaps with perinatal testicular steroid production (3). At present there is no sex difference in cell number in the adult brain that has been definitively linked to a sex difference in perinatal neurogenesis, although gonadal steroids may cause sex differences in neurogenesis in the developing CA1 region of the hippocampus (4). Neurogenesis continues throughout life in some neural areas, and a very recent report argues that the addition of cells around the time of puberty could serve to maintain or establish sex differences in the brain (5).

Evidence for sex differences in migration comes from the study of organotypic slices of the embryonic mouse brain. Neurones in the medial preoptic area and anterior hypothalamus show different migratory pathways, depending upon whether the brain slice is from a male or female embryo (6). In addition, the movement of neurones in slice cultures is affected by oestradiol and by GABA receptor antagonists (7–9) raising the possibility that sex differences in hormone levels or in neural activity could lead to different numbers of cells within a given region. Estrogen receptor βcells are more laterally located in male rats than in females in the anteroventral periventricular nucleus of the hypothalamus (AVPV) (10); although the mechanism underlying this sex difference is not known, it could be the result of sex differences in migration.

The third possible mechanism for establishing sex differences in cell number, the hormonal control of cellular differentiation, includes both morphological differentiation (e.g., cell size or the complexity of dendritic trees) and chemical differentiation (e.g., expression of receptors, peptides or neurotransmitters). Although sex differences of this type abound in adult brains, it is actually difficult to determine whether a difference in the number of cells of a particular phenotype is due to the control of cell phenotype, per se, or to some other mechanism (for additional discussion see ref. 1). For example males might have larger cells in a given region because testosterone increases cell size (hormonal control of morphological phenotype), or because testosterone increases the birth or survival of large cells. In at least one case, the sex difference in the number of vasopressin neurones in the forebrain, a good case can be made that testosterone controls cell phenotype (11).

Neuronal Cell Death and Sexual Differentiation

A role for cell death in sexual differentiation of the central nervous system was proposed almost 30 years ago, and differential cell death in males and females underlies several of the most prominent neural sex differences (reviewed in ref. 12). For example, adult males have more neurones than do females in the spinal nucleus of the bulbocavernosus and sexually dimorphic nucleus of the preoptic area, and females have more dying cells in these regions than do males during perinatal life (13,14). The opposite pattern a greater number of cells in adulthood in females and more dying cells during development in males is seen for the AVPV (15). These inverse correlations between cell number in adulthood and rate of cell death perinatally provide circumstantial evidence that cell death contributes to the sex difference, but it is difficult to rule out other mechanisms. We have taken advantage of genetically altered mice to determine whether cell death alone can account for given neural sex differences, and to test the involvement of specific cell death genes.

Naturally occurring cell death is a widespread phenomenon of the developing nervous system, and over 50% of neurones initially generated undergo apoptosis during a restricted, developmental time window that varies from region to region (16). Tremendous strides have

been made in understanding the molecular bases of apoptosis over the past 15 years. A breakthrough in understanding neuronal cell death in particular came with the discovery of the Bcl-2 protein (17). Bcl-2 is now known to belong to a family of apoptosis-regulating proteins that fall into three subgroups: 1) anti-apoptotic members (e.g., Bcl-2 and Bcl-xL), which typically contain four conserved motifs termed Bcl-2 homology (BH) domains; 2) full-length pro-death members (e.g., Bax and Bak), which include two of the BH domains; and 3) BH3-only proteins (e.g., Bim, Bad and Bid), which contain only the third BH domain. BH-3 members are almost always pro-death and act by binding to and inactivating the antiapoptotic Bcl-2 proteins that reside in the mitochondrial membrane, or in some cases by directly activating Bax or Bak (18).

Despite substantial redundancy in the Bcl-2 family, the decision of whether or not a cell undergoes apoptosis really comes down to the full-length pro-death proteins, Bax and Bak, which normally reside in the cytoplasm but translocate to the mitochondrial membrane upon receiving a death signal (19). If not opposed by pro-survival members, they are capable of permeabilizing the membrane, leading to the release of factors such as cytochrome c which trigger activation of the caspases cascade and apoptotic cell death (Figure 1). This forms the basis of the “Bax/Bak checkpoint” within each cell. Interestingly, neurones are unusual in that they appear to lack Bak, or produce an alternately spliced Bak with altered function (20,21), leaving Bax as a singularly important protein for neuronal cell death.

In mice with a targeted deletion of the *bax* gene, neuronal cell death is profoundly decreased in many, though not all, regions (22). We reasoned that if a given neural sex difference was due to cell death, it might be reduced or eliminated in *bax* knockout mice. In the sections that follow, I will first describe the effects of *bax* deletion on total cell number and cell number in several populations of phenotypically identified cells in the BNSTp, AVPV and SNB of mice. I then consider possible molecular mechanisms tying testosterone to changes in neuronal cell death and, finally, raise the question of how the elimination of sexually dimorphic cell death, or for that matter neuronal cell death in general, affects behavior.

Principal nucleus of the BNST

The BNST is a limbic forebrain structure involved in the control of sexual behavior, gonadotrophic release, stress and anxiety (23–27). The principal nucleus of the BNST (BNSTp; also known as the encapsulated region of the BNST, or the medial part of the posteromedial subdivision of the BNST) is larger in males than in females of several species, including rats, mice, guinea pigs, and humans (28–31). In rats and mice, the sex difference in volume is due to an increased number of cells in males, and treatment of females with testosterone on the day of birth completely masculinizes cell number (28,32). Female rats have more dying cells in the BNSTp than do males during postnatal life and testosterone treatment of females or castration of males reverses this sex difference (33). The effect of testosterone 0on cell survival in the BNSTp is likely due to oestrogenic metabolites, as we find that oestradiol, but not the non-aromatizable androgen dihydrotestosterone, completely masculinizes BNSTp cell number of mice (32). Moreover, both oestrogen receptors αand βappear to be involved as treatment of females with agonists that specifically target each receptor increases BNSTp cell number relative to controls (unpublished observations).

We tested whether Bax is required for the sex difference in total cell number in the BNSTp by counting thionin-labeled cells in *bax* −/− mice and their wild-type, *bax* +/+, siblings. *Bax* gene deletion increased BNSTp cell number in both sexes and, more important, eliminated the sex difference in cell number in adulthood (29). At birth, wild-type males and females have equal numbers of cells in the BNSTp, with a significant sex different (male > female)

developing between postnatal day 7 and 9 (34). Females have about twice as many dying cells in the BNSTp [as identified by terminal deoxynucleotidyl nick end labeling (TUNEL)] than do males on postnatal days 5 and 6, whereas *bax* −/− mice of both sexes have virtually no TUNEL-positive cells in the BNSTp (34). Taken together, the sex difference in total cell number in the BNSTp can be accounted for by *bax*-dependent cell death during postnatal life.

Like many brain regions, the BNSTp is heterogeneous, and sex differences have been reported for several cell types (e.g., vasopressinergic neurones and androgen receptor positive cells) (35,36). Cell counts based on a thionin stain, as described above, do not reveal what cells are affected by *bax* gene deletion; it is possible that some populations are affected very little and others a great deal. Phenotyping of BNSTp cells in wild-type and *bax* −/− mice indicates that there are significant sex differences in neurones (NeuN-immunoreactive) and androgen-receptor expressing cells, but not in astrocytes (GFAP-immunoreactive cells). *Bax* deletion increases both NeuN+ and AR+ cell counts and eliminates sex differences in these populations (37).

In contrast, Bax-dependent cell death is *not* responsible for the sex difference in vasopressin cell number in the BNSTp. A deletion of the *bax* gene increases the number of vasopressin cells in the BNSTp of both sexes but does *not* reduce the size of the sex difference (11). This same pattern is observed in mice over-expressing Bcl-2 (11). Thus, the pool of potential vasopressinergic neurones is subject to the mitochondrial cell death pathway regulated by Bcl-2 and Bax, but this is not the mechanism underlying the sex difference in cell number. Instead, testosterone likely acts on a pluripotent set of cells in the BNSTp to direct them to become vasopressinergic.

Anteroventral Periventricular Nucleus

The AVPV sits at the rostral extreme of the third ventricle and is critically involved in control of the luteinizing hormone surge at the time of ovulation (38,39). In contrast to most other sexually dimorphic nuclei, AVPV is larger in volume larger and more “cell dense” in females of several rodent species (15,40,41). In addition, females have fewer dying cells than do males in the perinatal AVPV, and treatment with either testosterone or oestradiol increases cell death in females (15,42,43). Sex differences in cell density and total cell number are eliminated in AVPV of mice over-expressing Bcl-2 (44) or with a deletion of *bax* (29), respectively. Thus, for a region where testosterone increases cell death (AVPV), as well as one where the same hormone decreases cell death (BNSTp), *bax* is required for the sex difference in cell number. In both nuclei the actions of testosterone are likely mediated by oestrogen receptors (32,45), suggesting that there is a molecular switch someplace between the binding of hormone receptors and activation of *bax* that determines whether the hormonal signal will favor life or death.

In addition to the sex difference in total cell number, there is also a large sex difference in the subset of AVPV neurones that are dopaminergic, as defined by tyrosine hydroxylase (TH) immunoreactivity. Female rats and mice have 2–3 times as many TH cells as do males (46,47). Interestingly, this sex difference is not reduced at all by over-expressing Bcl-2 in neurones or by deleting the *bax* gene (29,44). In contrast to the vasopressin story described above, there also is no overall increase in TH cell number in AVPV of Bcl-2 over-expressing or *bax* −/− mice. One possible interpretation of this finding is that cell death is not the mechanism determining the sex difference in dopaminergic cell number in AVPV. Alternatively, cell death may be involved, but controlled by a pathway independent of Bcl-2 or Bax. A recent report demonstrates that trophic factor deprivation triggers a novel cell death pathway in cultured dopaminergic neurones from the mouse midbrain (48). Instead of

triggering the mitochondrial pathway that is critically regulated by Bcl-2 family proteins, the death of these cells occurs via the activation of a cell surface death receptor (see Figure 1, right). It would be interesting to know whether this mechanism also controls dopaminergic cell number in AVPV.

Taken together, the TH cells in AVPV and vasopressinergic neurones in the BNSTp remind us that the control of cell number may differ not only from region to region, but also for subtypes of neurones within a region.

Spinal Nucleus of the Bulbocavernosus

The SNB is a cluster of motoneurones that reside in the lumbar spinal cord and innervate striated muscles involved in copulation. Male mice and rats have more SNB motoneurones than do females (49,50), and a similar sex difference is seen in the homologous cell group of other mammalian species. SNB motoneurones innervate striated muscles of the perineum that control copulation, including the bulbocavernosus (BC) and levator ani (LA). These muscles are also markedly dimorphic, with the BC completely absent and the LA severely reduced in females. The SNB motoneurone number and BC/LA muscle morphology can be masculinized by treating females with testosterone around the time of birth (51). In this case, effects of testosterone do not involve conversion to oestrogens, but instead require functional intracellular androgen receptors (52; for review see ref. 53).

We used retrograde labeling to identify SNB motoneurones in wild-type and *bax* −/− mice of both sexes and find that, as for total cell number in the BNSTp and AVPV, deletion of the *bax* gene eliminates the sex difference in SNB motoneurone number (54). Interestingly, however, the BC and LA muscles remained highly sexually dimorphic in *bax* −/− mice (large in males and virtually absent in females), despite the complete masculinization of their innervating motoneurones (54). This might suggest that differentiation of the perineal muscles does not involve cell death, as suggested previously for the developing LA (55). However, most cell types other than neurones express both full-length pro-death Bcl-2 family members, Bax and Bak. In such cases, either protein may be capable of triggering cytochrome c release and other downstream events of apoptosis (Figure 1), and deletion of both the *bax* and *bak* genes may be required to effectively block cell death (56). This possibility was tested for the BC and LA by comparing the perineal muscles in wild-type mice with mice bearing single deletions of either the *bax* or *bak* gene, or a deletion of both genes (*bax/bak* double knockouts). The BC muscle was absent and the LA was rudimentary in all wild-type females and in females with at least one copy of either *bax* or *bak*. In sharp contrast, *bax/bak* double knockout females possessed a BC muscle and the LA was markedly increased: female double knockouts had over 800 LA muscle fibers, in contrast to the ~30 LA fibers in controls (57). In addition, TUNEL-positive cells were virtually eliminated in the perineal muscles of newborn female *bax/bak* double knockouts (57). Thus, sexual differentiation of both SNB motoneurones and the perineal muscles is dependent on cell death, and particularly on the mitochondrial pathway regulated by Bcl-2 family members; however muscle cell death can be triggered by either Bax or Bak.

How Do Gonadal Steroids Control Sexually Dimorphic Cell Death?

The findings discussed above present cases in which cell death converges on *bax* whether testosterone increases or decreases cell survival, and whether androgen or oestrogen receptors mediate effects of the hormone. What are the molecular links between testosterone, androgen or oestrogen receptors, and Bcl-2 family members? One possibility is that testosterone or its metabolites might control the expression of Bcl-2 family members. Androgens and estrogens regulate the expression of Bcl-2 family members in peripheral tissues and cancer cell lines, and estrogen response elements have been described in the

Bcl-2 and Bcl-xL genes (58,59). In addition, newborn male rats have higher levels of Bcl-2 and lower levels of Bax protein in the SDN-POA than do females, and the opposite pattern of protein expression is seen in AVPV (60). Moreover, oestradiol treatment increases Bcl-2 and decreases Bax in the preoptic area (61). Thus, directly or indirectly, testosterone may regulate the expression of Bcl-2 family proteins to control cell number.

Interestingly, however, sexual differentiation often involves a delay between hormone exposure and some cellular response. For example, a single injection of testosterone or oestradiol on the day of birth results in sex differences in cell death in the BNSTp 5 or 6 days later (33). Effects of neonatal testosterone on AVPV volume are not evident for several weeks (41), long after the hormone is cleared from circulation. Lag times such as these suggest a cellular memory for the hormone signal that is consistent with epigenetic changes. That is, testosterone present during a critical developmental period may cause covalent modifications of chromatin that lead to lasting changes in gene expression, without an alteration in underlying DNA sequences.

Chromatin consists of DNA wrapped around a core of histone proteins. Covalent modifications of the DNA (as in methylation of cytosine residues) or of histone protein tails can have lasting effects on gene transcription (62). The best understood of the histone modifications is acetylation, which is often associated with active gene expression (63). Interestingly, steroid hormone receptors recruit cofactors with histone-acetylating activity to target genes (64,65), and blocking these co-factors can prevent effects of testosterone on sexual differentiation (66,67). Thus, we hypothesized that a disruption in the balance of histone acetylation and deacetylation might interfere with effects of testosterone on sexual differentiation. Preliminary findings support this prediction. When the histone deacetylase inhibitor, valproic acid, is administered to newborn mice, masculinization of cell number in the BNSTp is blocked (68). The deacetylase inhibitor does not affect cell number in the BNSTp of females or in nonsexually dimorphic areas, suggesting that it does not cause a generalized suppression of cell survival, but specifically prevents the sparing actions of testosterone. The genes or promoter regions that are affected in mice treated with valproic acid are not known, and there are many chromatin modifications in addition to histone acetylation that may play a role in sexual differentiation. However, it is noteworthy that histone deacetylase inhibitors preferentially target the expression of genes linked to cell cycle or cell death (69), and that valproic acid regulates the expression of Bcl-2 family members in the brain and in cultured neurons (70,71). Thus, one testable hypothesis is that perinatal exposure to testosterone (or a metabolite) may alter histone acetylation in promoter regions of Bcl-2 family genes, making it more or less likely that the gene is expressed several days later, during the cell death period.

What are the Consequences of Eliminating Neuronal Cell Death?

Cell death during neural development is so ubiquitous that it is noteworthy to identify an area in the mammalian nervous system that does *not* undergo a significant pruning of cell numbers during embryonic or early postnatal life. In addition, Bcl-2 related proteins regulate neuronal cell death in species from roundworms to humans (72). This would suggest that the process of naturally occurring cell death during neural development serves an extremely important function. What that function is, however, is not well understood. In contrast to the explosion of information on the molecular mechanisms of apoptosis, the role(s) of cell death in neural development has received comparatively little attention (for a review of this issue see ref. 73). The most common textbook explanation is that developmental neuronal cell death serves to “match” afferent populations to their targets. Most tests of this idea were performed decades ago and involved neurones projecting to targets outside of the nervous system (16). It should give us pause that *bax* knockout mice, which exhibit a profound

reduction of neuronal cell death in many if not most neural areas, are not only viable, but are behaviorally and physically indistinguishable from wild-type littermates based on simple observation (74; and our own observations). In mice lacking the Bax protein, or over-expressing Bcl-2 under a neurone-specific promoter, performance on basic sensory and motor tests is normal (75–78). Bax knockouts and Bcl-2 over-expressors show some impairment on complex tasks, such as maintaining balance on a high speed RotaRod (76,79), but *bax* −/− mice are actually superior to controls in a test of motor strength (78).

Given that *bax* gene deletion eliminates sex differences in cell number in several neuronal populations, we asked whether sexually dimorphic functions might be altered in the knockout mice. In a test of feminine sexual behavior, wildtype and *bax* −/− animals of both sexes were gonadectomized in adulthood and treated with oestradiol and progesterone. As expected, wild-type females showed high levels of lordosis over six weekly tests whereas feminine sexual behavior was low in males (79). Lordosis quotients were low in *bax* −/− mice of both sexes; the knockout males and females did not differ from each other and were equivalent to wild-type males on this measure (79). Preventing neuronal cell death also eliminates a sex difference in olfactory preference. Wild-type males prefer to sniff female-soiled bedding over male-soiled bedding, whereas females show the opposite preference (80). This sex difference is eliminated in *bax* −/− animals (81). In addition, *bax* −/− mice of both sexes are “like males” in time spent sniffing a female stimulus animal in a social recognition test. Not all sexually dimorphic behaviors are absent in *bax* −/− mice, however, as both wild-type and *bax*−/− males show more aggression than do females in a resident-intruder test (81).

Bax gene deletion affects many neural regions, and it is not known whether the elimination of sex differences in cell number in the BNSTp, AVPV, or SNB (29,54) specifically contributes to the elimination of sex differences in feminine sexual behavior or olfactory preference in *bax* −/− animals. More generally, this work highlights the fact that it really is not known how “extra” cells in one sex in any sexually dimorphic area affect neural function or behavior. Based on projection patterns of BNSTp neurones, Segovia and Guillamon (82) proposed that additional cells in the BNSTp of males serve to suppress feminine sexual behavior, and our results are consistent with that idea. In addition, the BNSTp is part of a well-defined neural circuit for processing sexually relevant olfactory cues that shows differential activation of the immediate early gene product, Fos, in response to male soiled bedding (83–85). It will be of interest to determine whether such sex differences in neural activation are eliminated in *bax* −/− mice and, if so, where in the circuit this occurs. The relatively large effects of *bax* deletion on sexual and olfactory behaviors suggests that sex differences in cell number are important for generating sex differences in behavior. By studying animals with alterations in neuronal cell death it may be possible to address long-standing questions about the function of specific sex differences in cell number in the mammalian brain.

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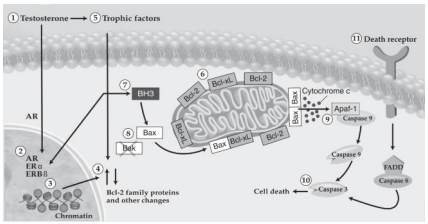

Figure 1. The regulation of neuronal cell death by hormones and Bcl-2 family members

Testosterone, produced by the perinatal testes, regulates cell number in several sexually dimorphic areas. In this simplified model, (1) testosterone binds to (2) androgen receptors (AR), or is aromatized to an estrogen before binding estrogen receptors (ER), such as ERα and ERβ. (3,4) The activation of ARs or ERs leads to changes in the expression of proteins involved in cell death, including those of the Bcl-2 family; this may involve epigenetic changes in chromatin associated with cell death genes. (5) In other instances, testosterone regulates the availability of neurotrophic factors which may also lead, directly or indirectly, to changes in the expression or activity of Bcl-2 family proteins. (6) Pro-survival members of the family, such as Bcl-2 and Bcl-xL, are primarily localized to the outer mitochondrial membrane. (7) Most cell types have two full-length pro-death members, Bax and Bak, but developing neurons lack Bak. Bax normally resides in the cytoplasm, and translocates to the mitochondria in response to a death signal. (8) BH3-only proteins are pro-apoptotic; they bind to and inactivate the pro-survival proteins, or directly activate Bax. (9) If not opposed by pro-survival family members such as Bcl-2 or Bcl-xL, Bax forms oligomers at the mitochondrial membrane capable of releasing of cytochrome c and other factors. Cytochrome c interacts with apoptotic protease activating factor 1 (Apaf-1) to trigger ‘initiation caspases’ such as caspase 9. (10) These in turn activate ‘executioner caspases’ such as caspase 3, which degrade cellular proteins and kill the cell. (11) A second cell death mechanism involves death receptors embedded in the plasma membrane (e.g., fas or the tumor necrosis factor receptor). Although not thought to be the predominant pathway for controlling cell number in the developing nervous system, some sub-populations of neurons may depend on this mechanism. Like the pathway regulated by Bcl-2 family members, the death receptor-mediated apoptosis also converges on executioner caspases.