

MINIREVIEW

Programmed Cell Death in Neurons: Focus on the Pathway of Nerve Growth Factor Deprivation-Induced Death of Sympathetic Neurons

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SUMMARY

Extensive programmed cell death (PCD) occurs in the developing nervous system. Neuronal death occurs, at least in part, because neurons are produced in excess during development and compete with each other for the limited amounts of the survival-promoting trophic factors secreted by target tissues. Neuronal death is apoptotic and utilizes components that are conserved in other PCD pathways. In this review, we discuss the mechanism of trophic factor-dependent neuronal cell death by focusing on the pathway of nerve growth factor (NGF) dep-

riuation-induced sympathetic neuronal death. We describe the biochemical and genetic events that occur in NGF-deprived sympathetic neurons undergoing PCD. Participation of the Bcl-2 family of proteins and the interleukin-1 β -converting enzyme family of proteases (caspases) in this and other models of neuronal death is also examined. The order and importance of these components during NGF deprivation-induced sympathetic neuronal death are discussed.

Death, along with proliferation and differentiation, is of crucial importance during development and in the maintenance of tissue homeostasis (1). PCD is a genetically regulated and evolutionarily conserved process by which cells commit suicide. PCD is essential for the elimination of surplus cells during the sculpting of organs and tissues, cells that are no longer needed as the organism proceeds from one developmental stage to another, or cells that may have become cancerous, infected with a virus, or in any other way harmful to the organism (2). Inappropriate activation of the PCD pathway is thought to be responsible for some neuronal loss during stroke or trauma and perhaps certain neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. PCD contrasts with necrosis in that dying cells do not swell and lyse but instead exhibit characteristic morphological changes of apoptosis. These characteristics include shrinking of the cytoplasm, plasma membrane blebbing, nuclear chromatin condensation, and fragmentation of genomic DNA into oligonucleosomal units; dying cells are eventually engulfed by neighboring cells or phagocytes without causing

inflammation (3). Recently, several factors that influence PCD have been identified in vertebrate and invertebrate organisms, but the precise sequence of events and regulation of this pathway remain unknown. In this review, we discuss the mechanism of neuronal PCD. For a more comprehensive recent review on PCD, the reader is referred to Hale *et al.* (4).

Neuronal Programmed Cell Death during Development

Extensive PCD occurs in the developing nervous system. Depending on the neuronal population, approximately 20–80% of all neurons produced during embryogenesis die before reaching adulthood (5). Most neuronal populations undergo PCD during the developmental period when these neurons innervate their targets; survival of neurons during this period is dependent on trophic factors secreted by the target or other cells. Competition for this trophic factor results in the survival of some and death of other neurons, thus matching the size of the target cell population with the number of innervating neurons (6). The molecular mechanism of target-dependent neuronal death has been studied extensively in

ABBREVIATIONS: PCD, programmed cell death; NGF, nerve growth factor; ROS, reactive oxygen species; SOD, superoxide dismutase; JNK, c-jun/amino-terminal kinase; ICE, interleukin-1 β -converting enzyme; MAP, mitogen-activated protein; PI, phosphatidylinositol; PARP, poly(ADP-ribose) polymerase; SCG, superior cervical ganglion; DRG, dorsal root ganglion.

sympathetic neurons that are dependent on NGF for survival. Sympathetic neurons are acutely dependent on NGF for survival in rats from approximately embryonic day 16 to 1 week postnatally (7); deprivation of NGF during this period causes the sympathetic neurons to undergo PCD both *in vivo* and *in vitro*. Evidence for this comes from experiments in neonatal rats in which administering exogenous NGF increased the survival of sympathetic neurons (8) and reducing the amount of NGF by either neutralization of NGF with blocking antibodies (9) or induction of autoimmunity against NGF (10) decreased the population of sympathetic neurons. In addition, deletion of NGF (11) or its receptor (TrkA) (12) by homologous recombination in mice resulted in the extensive death of sympathetic neurons.

Recently, many other neurotrophic factors and their receptors have been identified. Examination of "knockout" mice in which the genes encoding these neurotrophic factors or their receptors have been deleted reveals significant losses in several peripheral and central nervous system populations, illustrating the widespread dependence of neurons on trophic factors during development (for a review, see Ref. 13). In addition, certain populations of neurons undergo target-independent PCD; death in these situations may be triggered from signals that are cell autonomous or derived from other cells within or outside that population (14). An example of target-independent PCD is the recently described death of cervical motoneurons during early chick embryonic development (15).

Neuronal Models of PCD

The NGF-dependent sympathetic neurons have been used extensively in the study of the mechanism of neuronal PCD. In a frequently used paradigm, sympathetic neurons from the SCG are obtained from embryonic-day-21 rats and are maintained in culture in the presence of NGF for 5–7 days; the removal of NGF initiates PCD and causes the apoptotic death of all neurons 24–48 hr after NGF deprivation in culture (16). The advantage of this model is that its relatively homogeneous population of cells undergoes complete death in a synchronous and reproducible manner. Furthermore, death in this *in vitro* paradigm is representative of the physiologically relevant developmental PCD or the death occurring after axotomy in which neurons become acutely deprived of their trophic support *in vivo*. Difficulties encountered in this experimental model are those that are common to other primary neuronal culture paradigms, including the inability to obtain a large number of cells from each animal and the lack of an efficient transfection method to ectopically express foreign genes. These disadvantages, however, have been partially overcome by the advancement of technologies such as reverse transcription coupled to polymerase chain reaction that are used to examine the expression of genes (17, 18) and microinjection techniques for expressing foreign genes (19). PC12 cells, which are derived from a pheochromocytoma cell line, are also used to study neuronal PCD; these cells attain a neuronal phenotype if differentiated in the presence of NGF and then undergo PCD when deprived of NGF (20, 21).

Other models of neuronal PCD have been described, including those using cultures of sensory and parasympathetic neurons (22), motoneurons (23, 24), retinal ganglion cells (25), and cerebellar granule cells (26). The model of cerebellar granule cell cultures has the advantage, particularly for bio-

chemical studies, in that a large number of cells can be obtained from a single animal.

Mechanism of Neuronal Programmed Cell Death

The fundamental elements of the apoptotic pathway seem to be conserved in organisms as diverse as mammals, *Drosophila melanogaster*, and *Caenorhabditis elegans*. Much of the current research in mammalian cells focuses on the homologs of the cell death genes identified through genetic analysis in *C. elegans* (see below). Although mutations in single cell death genes prevent or augment all cell deaths in *C. elegans*, the homologs of these genes have evolved into multiple, and perhaps redundant, gene families in mammalian cells, and therefore no single gene has been identified that affects all cell death in mammalian cells. Thus, proof of a single death pathway in all mammalian cells remains lacking and probably does not exist. Much of the existing evidence indicates that even though the fundamental elements of the PCD pathway are evolutionarily conserved, variations of this pathway exist that depend on the cell type and the apoptosis-inducing stimulus. For example, death may be fast (4–6 hr, as in Fas-mediated PCD) or slow (24–48 hr, as in trophic factor deprivation-induced neuronal death) and inhibited or augmented by macromolecular synthesis inhibitors. It is beyond the scope of this review to present details on all aspects of the PCD pathway in different cells; instead, we describe the biochemical and genetic features of NGF deprivation-induced death of sympathetic neurons as an example of a neuronal PCD pathway. Important events and molecules that have been identified in other models of cell death and perhaps also conserved in the neuronal PCD pathway are also discussed.

Time course of events in trophic factor deprivation-induced neuronal death. Sympathetic neurons undergoing NGF deprivation-induced death do not exhibit any morphological changes during the first 12 hr after NGF removal *in vitro*. Thereafter, neurites begin to degenerate, the plasma membrane loses its smooth appearance, and the cell outline becomes increasingly irregular. Half of the neurons become atrophied within 24–36 hr, and >95% are dead by 48 hr after NGF deprivation (16, 27, 28). At the ultrastructural level, the nuclei begin to show signs of shrinkage and condensation at 18–24 hr after NGF deprivation and develop significant irregularities at 24–30 hr. Surprisingly, cellular organelles remain intact and show signs of degeneration only during the final stages of death, when the cell loses its structural integrity (16). Similar changes have been described in sympathetic neurons undergoing PCD during development (29), in newborn mice injected with anti-NGF antibodies (30), and in other mammalian cells undergoing PCD *in vivo* (3). Another marker of cells undergoing apoptosis is the fragmentation of genomic DNA. Sympathetic neuronal PCD is accompanied by DNA fragmentation, with the maximum fragmentation detected at 17–20 hr after NGF deprivation; this coincides with the event that irreversibly commits neurons to die (see below) (27, 31).

The PCD pathway initiated by NGF deprivation can be aborted if NGF is added back to the sympathetic neurons. However, NGF can abort PCD only if it is added before the cells become irreversibly committed to die. We and others have defined a "commitment point" that corresponds to the time after NGF deprivation when half the neurons can no

longer be rescued by the readdition of NGF; the commitment point of NGF rescue in sympathetic neurons under the usual experimental conditions is 22 hr (27). The commitment point is dependent on the age of the cells; the longer the neurons are maintained in NGF, the longer it takes for the neurons to become committed to die (28). Because NGF blocks the PCD pathway late and even in the presence of cycloheximide (27, 31), NGF is capable of aborting PCD by post-translationally blocking a late-acting essential event in neuronal PCD. The activation of the ICE family of proteases (see below) is one such late-acting essential event in cell death. However, whether NGF aborts the PCD pathway by directly inhibiting the activation of the ICE family of proteases remains to be determined.

Like NGF, the addition of CPT-cAMP (32) or depolarization with KCl (33) inhibits sympathetic neuronal PCD; CPT-cAMP is a cAMP analog that increases cAMP-dependent protein kinase activity, whereas depolarization with KCl acts by raising the levels of intracellular Ca^{2+} levels (34–36). The survival-promoting activities of CPT-cAMP and KCl have been observed in other neuronal populations, including cultures of cerebellar granule cells (26, 37), retinal ganglion cells (25), and septal cholinergic neurons (38). Interestingly, treatment with CPT-cAMP or KCl aborts sympathetic neuronal PCD with a time course indistinguishable from that of NGF rescue (27, 31, 39). However, whether NGF, CPT-cAMP, or KCl suppresses the death program by inhibiting the same event is not known.

Inhibitors of RNA and protein synthesis block PCD in several neuronal models; these include death induced by trophic factor removal in sympathetic (16), sensory, and parasympathetic (22) neurons *in vitro* and motoneurons both *in vitro* (40) and *in vivo* (41). The simplest interpretation of this result is that synthesis of new gene products is required for neuronal PCD (16), although alternate hypotheses have been considered (42). The cycloheximide inhibitable step occurs ~16 hr after NGF removal; half of the neurons can no longer be rescued by the addition of cycloheximide at that time (27, 28). These results suggest that all the necessary machinery for apoptosis is synthesized several hours before the cells become irreversibly committed to die and that subsequent post-translational processes ultimately trigger the apoptotic events.

Signaling events during neuronal apoptosis. Raff proposed that the basic components of the cell death machinery are present, although inactive, in all cells and that growth factors such as NGF promote the survival of cells, essentially by keeping the cell death pathway inhibited (43). If this is true, two questions become important for determining the signaling mechanism that activates neuronal PCD. First, how does NGF signal to promote neuronal survival (inhibit the cell death pathway)? Second, what signaling events are triggered on NGF removal?

NGF induces both neurite outgrowth and survival by binding to and phosphorylating the TrkA receptor protein tyrosine kinase. Signaling through the TrkA receptor results in the activation of several pathways, including the Ras, MAP kinase, and PI-3-kinase pathways (for a review, see Ref. 44). Inhibition of the MAP kinase pathway inhibits the differentiation and neurite outgrowth in PC12 cells (45) but does not affect the survival (prevention of apoptosis) of PC12 cells (46) or sympathetic neurons (47–49). These results indicate that

the loss of the MAP kinase pathway is not sufficient to cause death. In contrast, neuronal survival seems to be critically dependent on a PI-3-kinase signaling pathway because inhibitors of PI-3-kinase, such as wortmannin or LY294002, inhibit the ability of NGF to prevent apoptosis in PC12 cells (46); LY294002 also inhibits the survival-promoting effects of K^{+} depolarization or insulin-like growth factor-1 treatment in cerebellar granule cells (50). However, exactly how PI-3-kinase signals to maintain neuronal survival is not known.

One of the early events observed in sympathetic neurons on NGF removal is an increase in ROS. Levels of ROS peak at 3 hr and return to base-line within 8 hr after NGF removal (51). The introduction of copper/zinc SOD delays PCD in sympathetic neurons, indicating that the ROS increase may be an important event in neuronal apoptosis (51, 52). Interestingly, the introduction of the SOD protein into sympathetic neurons is ineffective in delaying death if it is injected >8 hr of NGF deprivation (51), when the ROS levels decrease to base-line. ROS have been implicated in several other models of PCD as well (for a review, see Ref. 53). The early and transient increase of ROS in sympathetic neurons deprived of NGF, which coincides with the protective efficacy of SOD, is indicative of ROS functioning as signaling molecules rather than as toxic agents during neuronal PCD. Furthermore, NGF, CPT-cAMP, or KCl prevents sympathetic neuronal death even if added well after the ROS increase, indicating that ROS do not cause irreversible damage in these neurons. ROS are known to function in signaling pathways and regulate gene expression (54). The identification of upstream events and downstream targets of ROS in neurons undergoing PCD will be useful in determining whether the ROS function by regulating gene expression during neuronal death.

The removal of NGF leads to a slow and sustained increase in JNK and p38 kinase activities in PC12 cells (55, 56). JNK activity increases in cells undergoing apoptosis in response to gamma radiation (57), Fas treatment (58, 59), and stress-inducing agents (60), suggesting that JNK activation may be an important signaling event during apoptosis. Consistent with this hypothesis, the expression of dominant-interfering mutants of JNK prevents apoptosis induced by NGF withdrawal in PC12 cells (56). Sympathetic neurons undergoing PCD also show an increase in *c-jun* phosphorylation (as detected with an anti-*c-jun* Ser63 phospho-specific antibody).¹ Moreover, *c-jun*, one of the downstream targets of JNK, is induced in sympathetic neurons deprived of NGF and is important for neuronal PCD (17, 61; see below). Because the JNK pathway is also induced by various non-apoptosis-inducing stimuli (62), activation of JNK alone is apparently not sufficient to induce apoptosis. A combination of an increase in JNK signaling and a decrease in MAP kinase signaling, which would be caused by NGF removal, may be required to activate the apoptotic pathway (56).

Biochemical and molecular events during neuronal apoptosis. Although no visible morphological changes of apoptosis are manifested until 12–18 hr after the removal of NGF, NGF-deprived sympathetic neurons undergo dramatic changes in several biochemical events before that time point. Although the importance of these biochemical changes in the neuronal death pathway have not been established, such

¹ M. Deshmukh and E. M. Johnson, Jr., unpublished observations.

alterations are likely to be an important part of the neuronal death program because they occur well before the cells become committed to die. Furthermore, many of these changes have also been observed in cerebellar granule cells undergoing apoptosis induced by low K^+ in the medium (63). The rate of glucose uptake falls rapidly in neuronally differentiated PC12 cells (20) and in sympathetic neurons, in which glucose uptake is reduced to 35% of control levels within 6 hr after NGF removal (27). The rates of RNA and protein synthesis also decline rapidly in NGF-deprived sympathetic neurons, with both rates reducing to 30% by 12 hr after NGF removal (27). Deprivation of NGF also increases purine efflux, as measured by labeling neurons with [3H]adenine, in sympathetic neurons by 2–3-fold within 10 hr after NGF removal (64).

The dependence of sympathetic neuronal PCD on macromolecular synthesis suggests that the expression of new genes may be required for death. We and others examined the expression pattern of several genes during neuronal death. Although the expression of the overwhelming majority of genes decreases on NGF removal, the expression of a few genes is increased during sympathetic neuronal death; these include *c-jun*, *c-myc*, *mkp-1*, and *cyclin D1*, which are induced within 5–10 hr and show maximum expression 12–18 hr after NGF removal, and *c-fos*, *fos-B*, and *NGFI-A*, which are induced after 10 hr and show maximum expression 15–20 hr after NGF removal (17, 18, 61). Although the absolute requirement of any of these genes for neuronal death has not been demonstrated, numerous experiments point to the importance of *c-jun* in neuronal PCD. First, an increase in *c-jun* mRNA is associated with several other apoptotic cell death models, including those of differentiated PC12 cells (65), cerebellar granule neurons (63), several human tumors of the central nervous system (66), and cultured hippocampal neurons exposed to β -amyloid (67); *c-jun* is also induced during naturally occurring and radiation-induced apoptosis in the developing rat brain (68) and in response to cerebral ischemia (for a review, see Ref. 69). Second, the microinjection of either neutralizing antibodies to *c-jun* or a dominant negative *c-jun* construct, which inhibits *c-jun* function, in sympathetic neurons inhibits neuronal death (17, 61). Third, serum or NGF deprivation of PC12 cells leads to a sustained increase in JNK activity (an event known to cause an increase in *c-jun* expression) (55, 56), and dominant-interfering mutants of JNK inhibit neuronal death in those cells (56).

Although *c-fos* expression also increases during sympathetic neuronal death and in other apoptosis paradigms, its involvement in mediating PCD is less clear. Although the *c-jun* increase is detected in all sympathetic neurons undergoing PCD, <1% of the neurons undergoing PCD are *c-fos* positive, and the increase occurs immediately before chromatin condensation (17, 61, 70). Unlike *c-jun*, microinjection of neutralizing antibodies to *c-fos* does not inhibit sympathetic neuronal death, but neutralizing antibodies to the *c-fos* family (*c-fos*, *fos-B*, *fra-1*, *fra-2*) do inhibit sympathetic neuronal death (17). Although these results argue that the function of the Fos family of proteins is required for neuronal death (the function of *c-fos* specifically may be redundant), one cannot rule out the possibility that inhibition of *c-fos* (or *c-jun*) indirectly affects other activator protein-1-containing complexes that may be more important in mediating death. The specific involvement of *c-jun* or *c-fos* in PCD cannot be satisfactorily

examined in mice deleted for these genes because animals lacking *c-jun* survive only to midgestation (71, 72), which is before the period of the developmental death that we discuss. Consistent with the possibly redundant role of *c-fos* in neuronal death, mice lacking *c-fos* do not have any defects in PCD (73).

The increase in *cyclin D1* during sympathetic neuronal death (18) is of particular interest because it has been suggested that neurons undergo apoptosis due to an abortive attempt to reenter the cell cycle (74). An increase in cyclin D1 protein and cyclin D1-dependent kinase activity occurs in a neuroblastoma cell line undergoing apoptosis; expression of cyclin D1 is sufficient to induce apoptosis in these cells (75). Furthermore, the activation of cyclin D1-dependent kinases seems to be important because expression of the cyclin D-dependent kinase inhibitor p16^{INK4} prevents apoptosis in these neuroblastoma cells. Recent data in support of this model come from experiments in which cell cycle blockers such as mimosine and ciclopirox or inhibitors of cyclin-dependent kinases such as flavopiridol and olomoucine inhibit trophic factor deprivation-induced death of sympathetic neurons and differentiated PC12 cells (55, 76).

The Bcl-2 family. One of the key regulators of mammalian PCD is the Bcl-2 family of genes. Bcl-2 was first identified as a proto-oncogene; its expression is deregulated as a result of a translocation in 85% of follicular lymphomas (for a review, see Ref. 77). Bcl-2 functions as a negative regulator of PCD and therefore contrasts with previously discovered oncogenes in that Bcl-2 promotes oncogenesis by inhibiting PCD rather than promoting proliferation. The evolutionarily conserved importance of Bcl-2 in the regulation of PCD became clear after the discovery that *ced-9*, a *C. elegans* gene that inhibits apoptosis, was homologous to Bcl-2 (78). Bcl-2 is expressed throughout the developing nervous system, with high postnatal expression in the hippocampus, cerebellum, and olfactory bulb. Its expression declines in the central nervous system with aging but remains high in the peripheral nervous system, in which the sympathetic and sensory ganglia retain substantial Bcl-2 protein throughout life (79).

Overexpression of Bcl-2 inhibits apoptosis in a variety of cell types, including rat sympathetic neurons (19, 80) and PC12 cells (81, 82) deprived of NGF. In addition, Bcl-2 inhibits the death of a central neural cell line from treatment with calcium ionophore, glucose withdrawal, and membrane peroxidation (83). However, not all neuronal death pathways are inhibited by Bcl-2; microinjection of a *bcl-2* expression vector rescues NGF-, brain-derived growth factor-, or NT-3-dependent neurons but not the ciliary neurotrophic factor-dependent neurons from the trophic factor withdrawal (84).

Overexpression of *bcl-2* in transgenic mice protects neurons from naturally occurring developmental death and prevents neuronal death induced by ischemic injury or axotomy (85–88). Bcl-2-overexpressing mice have an enlarged brain (12% increase in weight compared with the wild-type littermates) and a 40–50% increase in the number of neurons in the facial nucleus and ganglion cell layer of the retina (88). After optic nerve section, 65% of the ganglion cells survive ≥ 3.5 months in *bcl-2*-overexpressing mice compared with only 10% of the cells that survive in wild-type animals (89). Recently, Alberi *et al.* (90) examined the electrophysiological status of facial motoneurons protected from axotomy-induced death by overexpression of *bcl-2*. Although most electrophys-

ological functions are retained in *bcl-2*-saved neurons, the input resistance of *bcl-2*-saved neurons is higher, presumably because of their smaller diameter; the application of brain-derived neurotrophic factor at the lesion site, however, prevents this axotomy-induced atrophy in *bcl-2*-saved neurons.

The remarkable ability of *bcl-2* overexpression to prevent developmental and injury-induced neuronal death in a variety of neuronal cell types has prompted researchers to examine whether *bcl-2* overexpression prevents the loss of neurons in animal models of neurodegenerative diseases. The results thus far indicate that *bcl-2* overexpression does not prevent pathological neuronal death. *Bcl-2* overexpression increases only the short term survival of photoreceptors in mice with retinal degeneration (*rd* mice); photoreceptors continue to degenerate over time in these transgenic animals (91). The *pmn/pm*n (progressive motor neuropathy) mice, which are a model for motor neuron disease, lose motoneurons and myelinated axons and die at 6 weeks of age. Overexpression of *bcl-2* in *pmn/pm*n mice rescues facial motoneurons and prevents cell body atrophy but does not prevent degeneration of myelinated axons and does not increase the life span of the animal (92). Likewise, overexpression of *bcl-2* did not prevent the degeneration of spinal and brain stem motoneurons in the mutant *wobbler* mice even though the same neurons are protected by *bcl-2* from developmental death (93).

Although these overexpression experiments suggest that *bcl-2* is an important regulator of neuronal death, *bcl-2*-deficient mice do not have an excessive neuronal loss (94, 95); the lack of a dramatic neuronal phenotype in these mice is most likely caused by redundancy in the various *bcl-2* family of proteins (see below). At birth, the number of facial motor, sensory, and sympathetic neurons is not significantly different in the *bcl-2*^{-/-} mice (96). However, postnatally, *bcl-2*^{-/-} mice had fewer DRG (56% of wild-type at postnatal day 44) and SCG (58% of wild-type at postnatal day 44) neurons, indicating that *bcl-2* is required for the maintenance of specific populations of neurons subsequent to the period of developmental neuronal death (96). These results are consistent with the earlier observation that sympathetic neurons from 1-day-postnatal *bcl-2*^{-/-} mice undergo apoptosis more rapidly after NGF deprivation in culture than do SCG from wild-type littermates (80).

Several *bcl-2*-like genes have been identified in mammalian cells. Some of the *bcl-2* family of proteins, like *bcl-2*, are antiapoptotic (e.g., *Bcl-X_L*, *Mcl-1*), whereas others are proapoptotic (e.g., *Bax*, *Bad*, *Bak*, *Bcl-X_S*). Although the exact mechanism by which these *bcl-2* family of proteins regulate cell death is unknown, evidence suggests that protein/protein interactions among the *bcl-2* family members is important for function (77, 97). *Bcl-2* and *Bcl-X* are intracellular membrane proteins localized to the mitochondria, smooth endoplasmic reticulum, and perinuclear membrane. The *bcl-X* gene encodes at least two alternately spliced proteins, *Bcl-X_L*, which inhibits PCD, and *Bcl-X_S*, which accelerates death, presumably by acting as a dominant negative inhibitor of *Bcl-X_L* or *bcl-2* (98). Like *bcl-2*, *Bcl-X_L* expression inhibits sympathetic neuronal death after NGF withdrawal (99, 100). However, unlike *bcl-2*, *Bcl-X_L* is expressed extensively in the adult central nervous system (100–102), and *Bcl-X_L*-deficient mice are embryonic-lethal and have extensive cell loss in the hematopoietic system, developing brain, spinal cord, and DRG (103).

Bax is a proapoptotic *bcl-2* family protein that promotes death when overexpressed in cells (104). However, *Bax* function seems to depend on cellular context because *Bax*-deficient mice display both hyperplasia (e.g., thymocytes and B cells) and hypoplasia (testicular degeneration) (105). Nevertheless, *Bax* seems to be an important regulator of neuronal death. Neonatal sympathetic neurons deprived of NGF and facial motor neurons after axotomy do not undergo apoptosis in *Bax*-deficient mice (106). Furthermore, *Bax*-deficient mice have increased numbers of motor and sympathetic neurons, indicating that *Bax* is required for naturally occurring neuronal death, at least in these cell types.

The ICE proteases family (caspases). The ICE family of proteases, which are also called caspases, are the mammalian homologs of the *C. elegans ced-3* gene and are important mediators of mammalian apoptosis. *ced-3* is required for mediating cell death in *C. elegans*; mutations that inactivate *ced-3* block all naturally occurring cell death in *C. elegans* (2). The caspase family has ≥10 members, all of which are cysteine proteases that have the unique specificity of cleaving after aspartic acid residues. The caspases are translated as inactive precursors and are activated, via cleavage of their prodomains, in cells undergoing apoptosis (107).

Several observations illustrate the importance of caspases in mediating neuronal apoptosis (for a review, see Ref. 108). First, viral proteins such as *crmA* and *p35* are naturally occurring inhibitors of the caspases. The expression of *crmA* in chicken DRG neurons or *p35* in rat sympathetic neurons inhibits apoptosis on NGF deprivation in culture (109, 110). Second, peptide-based compounds that contain sequences corresponding to the caspase-substrate cleavage sites are potent inhibitors of caspases. Milligan *et al.* (111) have shown that one such inhibitor, YVAD-CHO, inhibits motoneuron death *in vitro*. We recently described a small, cell-permeable, aspartate-containing (the amino acid after which all caspases cleave) “pan-caspase” inhibitor [boc-aspartyl(OMe)-fluoromethylketone] that completely inhibits sympathetic neuronal death *in vitro* (70). Using a different strategy, Troy *et al.* (112) synthesized a IQACRG hexapeptide (the IQACRG sequence corresponds to the highly conserved active-site sequence of the caspases) and coupled it to the antennapedia peptide to make the construct cell permeable. The IQACRG peptide prevents sympathetic neuronal and PC12 cell death after NGF withdrawal (112). Third, caspases are activated during neuronal apoptosis. Caspase activity, as assayed by measuring the extent of DEVD-AFC cleaving activity, increases when neuronally differentiated PC12 cells undergo apoptosis after deprivation of NGF (113); increase in caspase activity also occurs during KCl deprivation-induced apoptosis of cerebellar granule neurons (114–116). Although these results point to the importance of the caspases in neuronal apoptosis, the function of individual caspases in apoptosis has not been examined in detail. Of the 10 caspases, reagents (antibodies, specific peptide inhibitors) are currently available to study only a few. Apparent cleavage of pro-ICH-1 (caspase-2) is seen in sympathetic neurons deprived of NGF (70) and on staurosporine treatment of the neuronal cell line GT1-7 (117). Likewise, pro-CPP32 (caspase-3) is processed in KCl deprivation-induced apoptosis of cerebellar granule neurons (114). CPP32 is important in mediating the early morphogenetic neuronal death as the brains of CPP32-deficient mice have supernumerary cells and exhibit disorganized cell

development; these mice die within 1–3 weeks of birth (118). Even though many of the other caspases are expressed in neuronal tissues, the extent of their involvement in neuronal apoptosis is unclear.

What event activates the caspases during neuronal apoptosis? Do the multiple caspases exist to provide redundancy? Or do they cleave different substrates? Thus far, several caspase substrates have been identified. Cleavage of a few, specific substrates rather than global proteolysis seems to be the mechanism by which the caspases execute the death program; these include PARP, DNA-dependent protein kinase, U1 snRNP, D4-GDI dissociation inhibitor for the Rho family, fodrin (nonerythroid spectrin), lamin A, actin, and protein kinase C (for a review, see Ref. 107). Cleavage of these substrates occurs in many models of mammalian apoptosis and is likely to happen during neuronal apoptosis as well (although this has not yet been reported). Little is known of the importance of these cleavage events during apoptosis. Cleavage of PARP is unlikely to be required for death; mice with a targeted disruption of PARP develop normally (119). Although no single cleavage event may be critically important for death, the simultaneous cleavage of all these substrates (and probably others that are currently unknown) ensures that death occurs in a rapid, efficient, and irreversible manner.

Order of Events during Neuronal Apoptosis

Relatively little is known about the exact order of events during neuronal apoptosis. Most of our knowledge is based on the assumption that the temporal sequence in which the events occur after NGF removal from sympathetic neurons is the sequence in which they participate during neuronal apoptosis. In the model that we describe, sympathetic neuronal PCD is initiated on the removal of NGF (Fig. 1). Recent data suggest that the decrease in MAP kinase activity, which is caused by NGF removal, is necessary for PCD; expression of MKK1, a protein kinase that constitutively activates the MAP kinase pathway, prevents apoptosis induced by NGF withdrawal in differentiated PC12 cells (56). However, a decrease in MAP kinase by itself is not sufficient to promote

PCD (47, 48). NGF signals through the PI-3-kinase pathway to maintain neuronal survival (46); therefore, the decrease in PI-3-kinase signaling on NGF removal is also likely to be important in activating the death pathway. Additional knowledge of the PI-3-kinase signaling cascade would help clarify these issues. Consistent with the notion that a fall in MAP kinase signal plays a role in activating PCD is an interesting observation that inhibition of MAP kinase activity (by using the MAP kinase inhibitor, PD 098059), in the presence of NGF, causes an increase in ROS (120). An increase in ROS is an event that occurs relatively early and seems to be important during NGF deprivation-induced sympathetic neuronal death (51, 52). Whether the decrease in MAP kinase activity increases ROS during neuronal apoptosis remains to be determined.

Other events occurring early in sympathetic neuronal apoptosis include a decrease in glucose uptake, which occurs faster than the other metabolic parameters examined and is reduced to 35% within 6 hr after NGF removal; decreases in RNA and protein synthesis, which are reduced to 30% within 12 hr after NGF removal; and reduction in the total amounts of cellular RNA and protein (27). These metabolic events are likely to be part of the cell death program because changes in the metabolic events occur well before the cell becomes committed to die. However, it is not known whether these events are necessary for PCD.

The increase in JNK activity and the resulting increase in *c-jun* mRNA, detected maximally by 15–20 hr after NGF removal, seem to be important for neuronal PCD (as discussed). Although the event that causes the sustained increase in JNK during neuronal PCD is not known, one interesting possibility is that ROS induce JNK activity in NGF-deprived sympathetic neurons; ROS have been reported to activate JNK in other mammalian cells (121). Exactly how the increase in JNK activity promotes neuronal death is unknown. JNK-mediated phosphorylation of *c-jun* causes an increase in activator protein-1 activity and therefore results in increased *c-jun* expression (122). Whether JNK signaling also results in the synthesis of death-promoting genes remains to be determined.

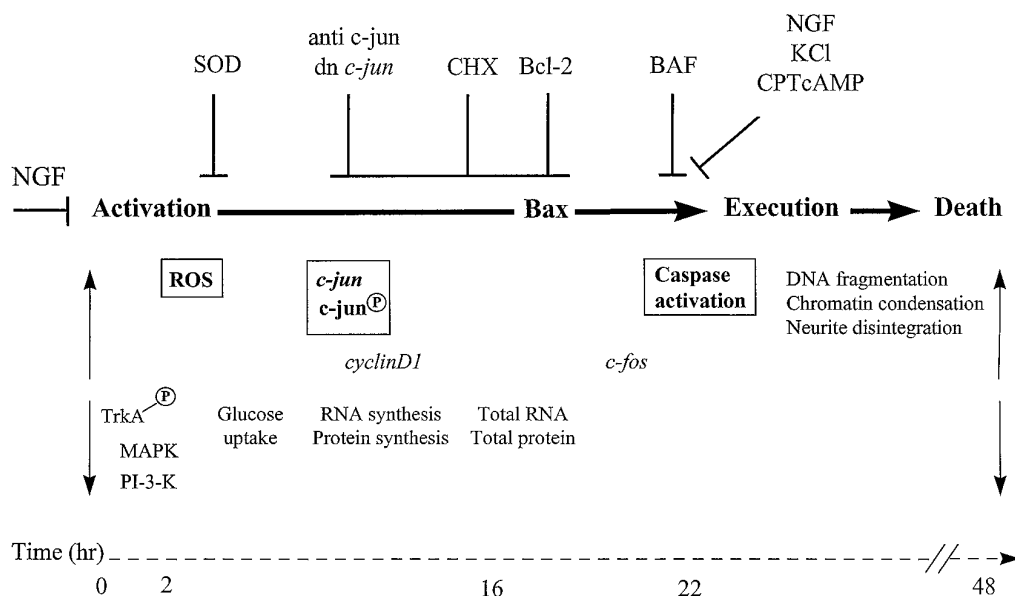


Fig. 1. Temporal sequence of biochemical and genetic events during NGF deprivation-induced sympathetic neuronal death. The removal of NGF activates the death pathway and causes the death of neurons within 24–48 hr. NGF deprivation-induced sympathetic neuronal death is dependent on Bax; other events that seem to be important are boxed. The relative position of the events inhibited by *c-jun* antibodies (and a dominant negative *c-jun* construct), cycloheximide, or Bcl-2 overexpression are not yet known.

Existing data for sympathetic neuronal death suggest that JNK activation and the increase in *c-jun* expression occur before the Bcl-2 family function. Overexpression of Bcl-2 (by microinjection) prevents apoptosis in sympathetic neurons but does not prevent the increase in *c-jun* protein after NGF removal (61). Also, the Bax-dependent step occurs after the increase in *c-jun* mRNA during sympathetic neuronal PCD; Bax-deficient sympathetic neurons show the increase in *c-jun* mRNA during NGF deprivation-induced apoptosis even though the neurons do not complete the apoptosis program.² However, whether the event inhibited by Bcl-2 overexpression and the event that is dependent on Bax during sympathetic neuronal death are identical is not known.

Several reports are consistent with the model that both JNK activation (increase in *c-jun* expression) and the Bcl-2 family of proteins function upstream of the caspases (ICE family of proteases) in neuronal apoptosis. BAF, a cell-permeable inhibitor of caspases, blocks apoptosis in NGF-deprived sympathetic neurons by preventing caspase activation but does not prevent the increase in *c-jun* mRNA that occurs after NGF removal (70). Likewise, z-VAD-FMK, a different inhibitor of caspases, prevents apoptosis but does not prevent JNK activation in PC12 cells (113, 123). Evidence suggesting that the Bcl-2 family of proteins function upstream of caspases in various models of cell death (with the known exception of Fas-mediated death) comes from experiments that show that overexpression of Bcl-2 prevents apoptosis at a step before the activation of caspases (124, 125). Although not yet reported for sympathetic neurons, Bcl-2 overexpression in the GT1-7 neural cells blocks activation of ICH-1 (caspase 2) during apoptosis (117). Likewise, overexpression of Bcl-2 prevents serum deprivation-induced apoptosis of naive PC12 cells before the event that increases caspase activity (DEVD-AFC cleaving activity) (113).

Very little is known about the events between the Bcl-2 family function and the activation of caspases. NGF-deprived sympathetic neurons that are prevented from undergoing apoptosis because of Bax deficiency (blocked at the Bcl-2 family checkpoint) or treatment with BAF (blocked at the caspase family checkpoint) are both atrophic, maintain low levels of protein synthesis, and undergo similar gene expression changes on NGF removal. One important difference, however, is that although Bax-deficient sympathetic neurons plated in the absence of NGF extend an extensive network of neurites (106), BAF-treated neurons plated in the absence of NGF show no capacity to extend neurites.³ This interesting difference between these otherwise similarly saved neurons suggests that NGF-deprived sympathetic neurons lose their ability to extend neurites due to cellular alterations occurring somewhere between the Bax/Bcl-2 checkpoint and the caspase family checkpoint. Consistent with these results, Chen *et al.* (126) recently reported that Bcl-2 overexpression promotes survival and neurite outgrowth of retinal ganglion cells in culture, whereas treatment with zVAD (caspase inhibitor) promotes only their survival and not neurite outgrowth in culture. Because neurons saved as a consequence of either Bcl-2 overexpression or Bax deficiency exhibit neurite extension in culture, we suggest that Bcl-2 overexpres-

sion does not actively promote axonal growth and regeneration, as suggested by Chen *et al.* (126). Rather, we suggest that blocking the PCD pathway at the Bcl-2/Bax checkpoint maintains the neurons in a state in which it is still capable of carrying out its innate ability to extend processes, an ability that is lost due to events distal to Bcl-2/Bax function but before activation of caspases. In effect, Bcl-2 overexpression/Bax deletion is permissive for neurite outgrowth rather than promoting nerve regeneration.

Among the events thus far examined, activation of caspases seems to occur last in NGF deprivation-induced sympathetic neuronal death. Sympathetic neurons that are deprived of NGF in the presence of BAF (caspase inhibitor) are prevented from undergoing apoptosis but nevertheless initiate the cell death program and show decreases in protein synthesis and increases in *c-jun* expression (70). Interestingly, the time courses with which NGF-deprived sympathetic neurons are rescued with the addition of NGF or BAF are identical, suggesting that NGF can abort apoptosis up to a time point indistinguishable from the activation of caspases (70). Thus, the rate-limiting, BAF-inhibitable activation of caspases seems to be the final irreversible event in NGF deprivation-induced neuronal death. Future efforts will undoubtedly focus on how these preceding events result in activation of caspases and how those in turn cause the morphological features of apoptosis.

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³ M. Deshmukh and E. M. Johnson, Jr., unpublished observations.

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