Neuronal cell death: when, why and how

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Apoptosis is recognised increasingly as a prominent event in nervous system development and disease. This form of death appears to obey the same rules in neurones as in other cells, in that it is initiated by similar extracellular perturbations and distinguished by similar morphological and biochemical changes. When neurones die after survival factor withdrawal, gene transcription is important, with the transcription factor c-jun and the cytoplasmic signalling cascade that regulates it being particularly significant in at least some types of cells. However, death can be induced in a transcription-independent manner by agents such as staurosporine. Both types of death involve activation of members of the ICE family of proteases but, surprisingly, the particular protease involved seems to depend very much on the manner in which death is initiated.

Cell death in the nervous system occurs under three sets of circumstances, with perhaps three sets of underlying mechanisms. During embryonic and early postnatal development, a large percentage (perhaps 50% or so) of neurones in each region of the nervous system die by programmed cell death. The timing varies from region to region, but, in each case, this death is thought to be by apoptosis and to be similar to that occurring in other tissues, in that it is the result of competition for a limited amount of one or more extracellular survival factors, generally polypeptide in nature. One difference for neurones may be that these factors are often target-derived and, hence, produced at a distance and transported back to the neuronal cell body. A second phase of cell death accompanies a variety of neurodegenerative disorders, such as Alzheimer's disease. In these cases, the cell death may be quite significant in amount, but it may occur over a period of years, so that at any point in time, there are only a small number of dying cells. The causes of death associated with degenerative diseases are often not known and, until recently, there was little data concerning the types of death that occurred. A final instance of neuronal cell death occurs after the hypoxia that accompanies stroke. In this case, a large amount of neuronal cell death may take place over a period of days, with the type of death being a current topic of debate.

This article will focus on recent studies of neuronal cell death, with an emphasis on apoptosis. Death of cultured neurones in different situations will be discussed first. Cell death modulators and pathways...
will be described, with particular regard to intracellular events that initiate and that retard death. These events will be seen to be similar to those taking place in other types of cells. Finally, there will be an extensive account of how the different types of neuronal cell death should be classified, and of whether it is meaningful, from a mechanistic point of view, to make an absolute distinction between necrosis and apoptosis.

### Neuronal cell death in culture

The fact that developmental or programmed cell death of neurones had been studied in some detail led to the establishment of useful cell culture models of these types of death. The types of cells utilised most often include: rat superior cervical ganglion (SCG) neurones, which are nerve growth factor (NGF)-dependent sympathetic neurones, PC12 cells, an NGF-dependent neurone-like cell line, and cerebellar granule neurones (CGNs), normally grown in the presence of high extracellular K\(^+\), to support survival\(^1\text{--}^3\). When deprived of NGF or, in the case of CGNs, when K\(^+\) is lowered, these cells die by classical apoptosis, with membrane blebbing, neurite fragmentation, chromatin condensation, formation of apoptotic bodies, a decrease in dehydrogenase activity (measured by the MTT reaction) and DNA laddering, all taking place at a time at which the plasma membrane remains relatively intact (non-leaky to dyes such as trypan blue and propidium iodide). The death is relatively slow (the commitment time is 15–20 h, and 50% cell death takes place in 24–48 h, although CGN death is somewhat faster) and asynchronous; this presents problems for certain types of biochemical and molecular biological experiments. One clear observation, pertaining at least to SCG and CGN cell death, is that the process is blocked by inhibitors of mRNA or protein synthesis, as is again typical of apoptosis. Thus, death of neurones in response to survival factor withdrawal is dependent on gene transcription and subsequent protein synthesis.

Following the observation that high concentrations of the non-specific protein kinase inhibitor staurosporine initiate apoptosis in many cell types\(^4\), this drug was also applied to different kinds of cultured neurones. Staurosporine-induced death was typically apoptotic, at least using morphological criteria\(^5\), and the sequence of events seemed roughly the same as with survival factor withdrawal. Mechanistically, however, there was one major difference from survival factor withdrawal death in that the staurosporine type was not blocked by RNA or protein synthesis inhibitors. Thus, neurones, like other cells, appear to have a set of death effectors even when present, in cell culture, in a seemingly healthy state.
While the death initiated by these two procedures was thought to reproduce some aspects of programmed cell death and, perhaps of the death that accompanies neurodegenerative disease, investigators sought to derive a model that might reveal more about the type of cell death that occurs after stroke. Numerous groups have examined cultured rat or mouse cortical neurones maintained for a brief period in an hypoxic and hypoglycaemic state. Under these conditions, cells swell rapidly and die by a process normally agreed to represent necrotic death. The mechanism is presumed to be release of glutamate from depolarised neurones, followed by excess Ca entry into the cells. Another way of studying this type of death is simply to apply high concentrations of glutamate directly to the neurones. Again, many cells die rapidly, mostly by necrosis. However, Choi et al. found that blocking NMDA and AMPA/kainate receptors together slowed much of the rapid necrotic death, leaving the cells to die by apoptosis at later times. Thus, there is an underlying apoptotic component to this glutamate-induced death. Recent work also suggests the existence of a similar underlying component in stroke brain itself (see below).

Intracellular changes during neuronal cell death

The type of neuronal cell death brought on by survival factor withdrawal can be thought of as potentially consisting of three phases. In the first phase, the cell ‘senses’ the absence of the factor. This is accomplished by activation or inactivation of cytoplasmic signalling pathways. Following these cytoplasmic changes, there is a phase of required gene expression. Finally, there is the appearance or activation of the death effectors themselves (meant here as the molecules that produce the changes that define death). It is also necessary to understand that staurosporine, and related initiators of apoptosis, bypass the first two phases and produce direct activation of extant cytoplasmic effectors.

Cytoplasmic signalling, transcription factors and neuronal cell death

NGF activates multiple signalling pathways, including the MAP kinase cascade, that are important for survival, neurite elongation, and other processes associated with differentiation in PC12 cells and sympathetic neurones (see, for example, Nobes and Tolkovsky and Xia et al). When NGF is removed from these cells, and they begin to lose their
differentiated phenotype and die, these signalling pathways turn off, and at the same time, other signalling pathways turn on. Since gene transcription plays an important role in the initiation of apoptosis following NGF removal, it should be the case that the activity of particular transcription factors is altered early in the death process. It is logical to assume that this is achieved via one or more of these signalling events.

This possibility has been examined by several groups. Cultured SCG neurones dying following NGF withdrawal have again been most carefully studied. One of the earliest events in these cells—observable within 4 h or so of NGF withdrawal—was the appearance of the phosphorylated, active, form of the transcription factor c-jun and an increase in its mRNA and protein levels. Both in situ hybridisation and immunocytochemistry revealed an increase in c-jun in most neurones, even before they adopted an apoptotic morphology. Levels of other members of the AP-1 family of transcription factors did not change; in particular, c-fos, once thought to be essential in the death process, only appeared in relatively high concentration in a small number of frankly apoptotic neurones. Nonetheless, because c-jun had been implicated in many types of cellular changes, it was necessary to show that it was functionally important in the onset of death. In one series of experiments, neuronal c-jun was blocked by microinjection of an anti-c-jun antibody. In another, a similar result was obtained by microinjection of an expression plasmid for a transcriptionally inactive, dominant negative, c-jun variant. In both experiments, the rate of cell death was noticeably slowed. Thus, blocking c-jun regulated gene transcription blocks death. It was also important to see what would happen if c-jun levels were increased in the presence of NGF. When this was achieved, again by microinjecting neurones with an expression vector, the rate of death was accelerated. Therefore, high levels of c-jun are sufficient to induce death even in the presence of NGF.

Recent work has suggested that levels of c-jun might increase in other apoptotic situations as well. For example, treatment of cortical neurones with amyloid-β-peptide produced an increase in c-jun mRNA and an accumulation of nuclear c-jun protein, early in the death process. Our laboratory has also shown recently that c-jun increases rapidly when CGNs undergo apoptosis (A. Watson et al, manuscript submitted for publication).

Since apoptosis is associated with an increase in the phosphorylated, transcriptionally active, form of c-jun, it might be expected that the activity of JNK, the kinase that phosphorylates c-jun, would increase after NGF withdrawal. In fact, Greenberg et al have shown this to be true in PC12 cells, and our laboratory has similar data for sympathetic neurones. A further prediction is that over-expression of catalytically
active variants of upstream kinases should lead to death, even in the presence of NGF. This was also tested by Greenberg et al, who found that transient transfection of PC12 cells with constitutively active MEKK1 (which phosphorylates and activates SEK1, which phosphorylates and activates JNK) kills them. This death was blocked by simultaneous over-expression of dominant-negative c-jun. Again, our laboratory has obtained similar results with sympathetic neurones. Finally, Greenberg et al showed that activation of another parallel signalling pathway—that for p38 MAP kinase—can also lead to cell death. Thus, it can be concluded that, under at least certain circumstances, apoptosis is due to the induction of signal transduction pathways that regulate particular transcription factors. Remaining to be established is the generality of these observations. That is, are the p38 MAP kinase/JNK pathways the initiators of death in all kinds of neurones under all circumstances, at least when apoptosis is involved?

Gene activity and neuronal apoptosis

The experimental observation that survival factor withdrawal-induced neuronal apoptosis can be blocked by actinomycin D and cycloheximide was widely interpreted to mean that cell death initiated following factor withdrawal was based on the appearance of new proteins that cause death. These were meant to be proteins that previously were either not present at all or were present at very low levels when survival factors were still available. While this is not the only interpretation of these experimental data, a wide variety of experiments have been designed to address this possibility. Certainly, it has been the case that mRNA levels of a number of potentially interesting proteins, such as cyclin D1 in sympathetic neurones, have been found to increase early in the death process, but still no particular transcriptional event has been implicated in apoptosis. This work will not be described in detail here. However, the identification of particular functionally important transcription factors may assist in the search for neuronal death genes.

Cytoplasmic effectors of neuronal cell death

In this discussion, it is important to distinguish between cytoplasmic changes that accompany or are upstream initiators of the death process and those that actually produce the changes that we associate with death. Sometimes, this can be difficult, though. For example, the role of reactive oxygen species in neuronal cell death is controversial. Although some types of cells clearly are able to die in the total absence of reactive
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oxygen$^{19,20}$, some investigators still feel that reactive oxygen is an essential part of the death process in neurones. We favour the view that, in certain instances, oxygen radicals may be upstream initiators of death, possibly acting via a pathway such as the JNK cascade.

Discussions of death effectors invariably centre around the role of ICE-like proteases (now termed caspases), which undoubtedly are featured prominently in many articles in this issue. In that regard, neurones behave like all other cells in that a cascade of caspases seems to be activated during death, as judged by various criteria, including: (i) initiation of neuronal cell death by over-expression of ICE; (ii) use of fluorescent enzyme substrates; (iii) cleavage of known caspase substrates; and (iv) inhibition of neuronal cell death by caspase inhibitors.

Much of the work concerning caspases and neurones has been carried out on NGF-dependent neurones dying following NGF withdrawal. Gagliardini et al$^{21}$ microinjected cultured sensory neurones with an expression vector for either wild-type murine ICE or enzymatically inactive ICE. Wild-type ICE killed cells maintained in NGF, while inactive ICE had no effect. This suggests that unregulated ICE activity is sufficient to kill cells even when they are maintained under normal survival conditions.

There is also evidence that induction of a caspase cascade occurs during apoptosis and is functionally significant. Direct proof for increased caspase activity in dying neurones has been provided by Schulz et al$^{22}$, who showed cleavage of a fluorescent caspase substrate during the death of CGNs. Further evidence was provided by Gagliardini et al$^{21}$, who microinjected sensory neurones with an expression vector for the viral ICE inhibitor crmA. These cells were consequently less likely to die following NGF withdrawal. Similar studies were done by Martinou et al$^{23}$ who over-expressed, again by microinjection, the baculovirus caspase inhibitor p35 in SCG neurones. They found that this inhibitor also offered a significant degree of protection against death due to NGF withdrawal.

A variety of peptide-type caspase inhibitors of varying specificities towards individual members of the caspase family have also been applied to dying neurones. The general finding has been that several of these inhibitors block growth factor withdrawal death, although they vary in effectiveness to some degree. zVAD-fmk, a somewhat general inhibitor of caspases, has blocked cell death in several different systems, including PC12 cells$^{24}$ and sympathetic neurones (McCarthy, Rubin and Philpott, submitted for publication). However, it was ineffective in blocking low K+ death of CGNs$^{58}$. YVAD-based inhibitors, which block at least ICE itself, were found to block motor neuron apoptosis in culture and during normal avian embryo development$^{25}$ and to block low K+ death of CGNs$^{22}$. DEVD-type inhibitors, derived from the
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cleavage sequence of PARP, a CPP-32 substrate, blocked death of SCG neurones (McCarthy, Rubin and Philpott, submitted for publication). However, for this inhibitor to be effective, it was necessary to microinject it into the cells because it has very limited membrane permeability.

Since staurosporine induces neuronal apoptosis by direct activation of death effectors, it was important to determine if it acted via caspases. Evidence that this is true again comes from several different studies. Philpott et al found that lamin, a known caspase substrate, is cleaved from 69 kDa to 46 kDa during death of PC12 and SCG cells. Taylor et al found that the nuclear enzyme PARP, a caspase substrate, was degraded from a 116 kDa intact form to an 85 kDa proteolytic fragment during CGN death. Thus, caspases are activated during staurosporine death. That this is required for death was suggested by the work of McCarthy, Rubin and Philpott (submitted for publication) who found that over-expression of baculovirus p35 in SCG neurones prevented staurosporine-induced death, much as it did NGF-withdrawal death.

Interesting differences were revealed, however, when the effects of peptide ILP inhibitors were compared. DEVD-fmk was effective on both staurosporine and growth factor-withdrawal types of death in SCG neurones. On the other hand, zVAD-fmk was more effective in blocking NGF death than staurosporine death of these cells (Taylor et al, submitted for publication). Unexpectedly, in CGNs, zVAD-fmk was very effective in blocking staurosporine death, but not very good at blocking low K⁺-death. Differences in the effectiveness of these inhibitors was also found by Troy et al, who compared death of PC12 cells due to either down-regulation of superoxide dismutase or withdrawal of serum or NGF.

These results suggest that there are several ways of inducing neuronal apoptosis that involve the same types of morphological and biochemical pathways. However, it seems that there is variability, both among different types of neurones and among different initiators of death in an individual neuronal type, in the particular caspases that cause death. It will be very important to determine the source of this variability. For instance, are the very upstream activators of the caspase cascade in the different types of cell death identical, with differences arising in at least some of the downstream proteases? Alternatively, do the different stimulators of death generate different intracellular cascades at their onset, with perhaps some overlap of downstream caspases?

Bcl-2, bax, and neuronal cell death

Entry into the death cascade in most cell types involves the participation of bcl-2 and bax-like proteins. Bcl-2 over-expression appears to block
Apoptosis death, probably by inhibiting entry into the caspase cascade, whereas bax seems to stimulate the onset of the cascade. It has been clear for some time that bcl-2 over-expression is anti-apoptotic in neurones. Martinou et al28 injected sympathetic neurones with an expression plasmid for bcl-2 and found that survival of these cells in the absence of NGF was improved. Allsopp et al29 obtained similar results for other types of neurotrophin-dependent neurones, but found, surprisingly, that bcl-2 over-expression did not block death of neurones dependent on ciliary neurotrophic factor (CNTF). This group also found that antisense bcl-2 constructs decreased the ability of neurotrophins to support survival30. However, these constructs failed to affect the activity of CNTF. This might mean that CNTF promotes survival by a mechanism dependent on a bcl-2-related protein. It is clearly the case that other members of the bcl-2 family, such as bcl-x, support survival31. This is reasonable since many types of adult neurones have undetectably low levels of bcl-2 with levels of bcl-x being more substantial. An important role for such related proteins is confirmed by the observation that there is some neuronal cell death in bcl-2 knockout mice, but not massive malformation of the nervous system.

Bax and related pro-death proteins are important regulators of neuronal apoptosis. NGF addition to PC12 cells causes a substantial decrease in their bax levels, suggesting that this is a normal part of differentiation. This is consistent with the finding that bax is high in neurones at developmental times when there is significant programmed cell death, but decreases substantially afterwards32. Interestingly, bax levels remain high in certain neuronal populations, possibly those that are particularly susceptible to dying. For instance, bax levels are high in adult cerebellar Purkinje neurones, which die most readily when the cerebellum is made ischaemic32. The important role of bax can be evaluated directly in several ways. One is to engineer its over-expression. When this is done in sympathetic neurones, they die, even in the presence of NGF. This death can be blocked by concomitant over-expression of bcl-x and is also blocked by p35 over-expression, which indicates that Bax initiates death by activating the caspase cascade59.

Necrosis versus apoptosis in neuronal cell death

One of the most controversial topics amongst investigators focusing on neuronal cell death occurring under pathological conditions relates to classification: is it necrosis or is it apoptosis? Everyone agrees that cell death in development resembles apoptosis. Most investigators now believe that neurodegenerative disorders, such as Alzheimer's disease, involve apoptosis to a substantial degree and, until recently, most
probably accepted the theory that neuronal death in stroke is predominantly by necrosis.

An important question is whether or not it is valuable to categorise neuronal death as being of one type or the other. We will conclude that there is likely to be some sharing of intracellular pathways in the two processes. However, although in extreme cases it is not difficult to distinguish between the two types, one very important issue must centre on the criteria which can be used in a given situation to distinguish between the two types of death.

The classical view is that necrotic cells are swollen, due to early changes in the permeability of their plasma membranes, and are associated with inflammation in response to leaked cytoplasmic constituents. Apoptotic cells have intact membranes, distorted organelles, condensed chromatin and are not associated with inflammation since apoptotic cells are generally engulfed early in the death process. Ultrastructural analysis should be able to determine the death type, but this is a time-consuming technique and not useful as a routine experimental procedure. It is particularly difficult in studies that require the use of biopsied or post mortem human nervous tissue. As already mentioned, the rapid disappearance of apoptotic cells presents another problem in trying to ascertain the type of cell death.

Thus, investigators have sought more convenient procedures, two of which are now routinely used. The first is to look for labelling with the TUNEL technique, which is generally considered to be diagnostic for apoptosis, but can occur during necrosis as well. The second is to isolate tissue from damaged regions and look for evidence of DNA fragmentation. DNA from apoptotic cells is cleaved at 180 bp intervals and runs as a ladder, while that from necrotic cells runs as a smear. However, this technique is also problematic from two points of view. It is not always easy to see DNA laddering even in cultured neurones undergoing apoptosis, and any tissue sample might contain only a small percentage of apoptotic cells, visible by TUNEL staining or, perhaps, by ultrastructural examination, but not able to produce enough DNA to make laddering obvious. So, in the end, many studies simply describe whether or not TUNEL-positive cells or DNA laddering is seen. The presumption is that if either occurs, cell death by apoptosis is involved. However, it is clear that this type of information is often not conclusive in deciding on the type of cell death.

**Death in neurodegenerative diseases**

Despite all of these problems, there has been a recent flurry of papers trying to determine the extent of, especially apoptotic, cell death in various degenerative diseases. These experiments are difficult, for the
reasons just outlined, and compounded by having to use human biopsy samples, which themselves are often processed slowly, perhaps leading to more cell death. Nonetheless, reasonable progress has been made. Using Alzheimer's disease human brain samples, several investigators have found evidence for TUNEL-positive cells, including cortical and hippocampal neurones. However, there is some disagreement as to whether or not these cells have a typically apoptotic morphology. Further, there is some confusion as to whether there is a direct correlation between the location of dying cells and the presence of amyloid plaques and neurofibrillary tangles, the distinguishing pathological features of the disease. In cell culture, the situation is somewhat clearer. It appears that addition of high concentrations of amyloid-β-peptide kills cortical neurones by apoptosis, with chromatin condensation, DNA fragmentation and surface blebbing.

Other neurodegenerative diseases have also been studied. In Huntington's disease samples, TUNEL-positive cells were seen in the striatum, and there was some indication that these cells were dying by apoptosis, although it was difficult to find DNA fragmentation consistently. In status epilepticus in rodents and scrapie, there have been descriptions of apoptosis. Finally, a very interesting case is that of spinal-muscular atrophy, which is associated with extensive apoptosis of motor neurones. The gene affected in this disease is termed NAIP, neuronal apoptosis inhibitor protein, and is homologous to a baculovirus inhibitor of apoptosis. When over-expressed in different cell types, not yet including neurones, NAIP inhibits apoptosis, as expected.

Cell death in stroke

Of all disorders of the nervous system, stroke has been examined most carefully for its cell death phenotype. One reason for this (other than the obvious prevalence and importance of the condition) is the relative availability of animal models (although the exact correspondence between the different models and the human disease is frequently debated). However, another reason is that the neuronal cell death is fairly extensive and relatively rapid, occurring in hours to days. This is a tremendous experimental advantage when compared to the slow degenerative disorders. The common view until recently was that cell death in stroke was entirely by necrosis, with excess glutamate release causing Ca\(^{2+}\)-overload, cell swelling and death. However, when the frequency of apoptosis as a death type became clear, investigators were interested in discovering if it accompanied stroke as well. Reviewing the
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now extensive literature would itself require a review of this size, but it is possible to summarise the essential information fairly succinctly. The most important point is that there is an apoptotic component to neuronal death that follows stroke. This has been established by: (i) the appearance of TUNEL-positive cells; (ii) DNA laddering in tissue samples; (iii) ultrastructure of dying cells; and (iv) partial inhibition of death by cycloheximide. There is variability in the degree of apoptosis, depending on species, time and type of occlusion, time of reperfusion, and so on. The clearest example of apoptosis is in the CA1 region of the hippocampus, in which death is significantly delayed with respect to the onset of ischaemia. Other significant indications that apoptosis occurs in stroke include inhibition by agents normally thought of as being anti-apoptotic — bcl-2 and caspase inhibitors.

Necrosis and apoptosis: how different are they?

The concluding topic in this review will centre around the question of how important it is to divide types of death into discrete categories. A pragmatic position is that the important issue is not categorisation, but prevention. Of course, it seems logical to expect that knowing the type of cell death underlying a particular disorder will be very important in that regard, but this will be difficult for the following reasons. First, some disorders are associated with dying cells that have some characteristics normally thought to be associated with apoptosis and some with necrosis. In some situations, it is very difficult to decide on the dominant phenotype. Second, the same type of stimulus — hypoxia, glutamate, the calcium ionophore A23187 — can lead to either necrosis or apoptosis or both, depending on length of treatment, concentration of drug, etc. Third, certain agents — neurotrophins, bcl-2, caspase inhibitors — normally categorised as anti-apoptotic seem to block types of death often thought of as necrotic.

The situation with A23187 is, in a way, particularly instructive in that low concentrations kill by apoptosis, high by necrosis. This presumably means that events underlying apoptosis must begin to occur when A23187-treated cells are dying by necrosis and would become obvious if they survived for a long enough time. The same is true of hypoxia-induced death of cortical cells that, as already mentioned, undergo delayed apoptosis made apparent if the ‘necrotic-type’ death is blocked pharmacologically.

A recent set of experiments carried out by Tsujimoto et al. is also extremely important. They placed three different types of cells under hypoxic conditions. The cell types all underwent a mixture of necrosis
and apoptosis, with the ratio varying from one cell type to the next. Yet, bcl-2 blocked all types of death in these cells. These experiments suggest immediately that at least some of the events underlying necrosis and apoptosis must be similar or even identical.

**Conclusion**

Apoptosis in neurones, resulting from growth factor withdrawal or occurring in different neurodegenerative disorders, is fundamentally similar to that in other cell types. It is normally activated by cytoplasmic signalling pathways, transmitted via transcription factors and alterations in gene transcription, and carried out by the appearance or activation of cytoplasmic effectors. There are cell-specific events, but ICE-like proteases are key effectors of the death programme, and bcl-2 and bax-like proteins regulate entry into the pathway. While neuronal cell death has two extreme phenotypes, apoptosis and necrosis, there may be many forms of death that are not so simple to distinguish. Nonetheless, substantial progress has been made in understanding these processes and in blocking them, at least in experimental systems.

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