TUTORIAL

Why Neurons Die: Cell Death in the Nervous System

JAMES B. HUTCHINS and STEVEN W. BARGER

It is likely that humans are born with all of the nerve cells (neurons) that will serve them throughout life. For all practical purposes, when our neurons die, they are lost forever. During nervous system development, about one-and-a-half times the adult number of neurons are created. These "extra" neurons are then destroyed or commit suicide. This process of programmed cell death occurs through a series of events termed apoptosis and is an appropriate and essential event during brain development. Later in life, inappropriate neuronal cell death may result from pathological causes such as traumatic injury, environmental toxins, cardiovascular disorders, infectious agents, or genetic diseases. In some cases, the death occurs through apoptosis. In other cases, cell death is random, irreversible, and uncontrollable; to distinguish it from the controlled, planned cell death of apoptosis, we call this necrotic cell death. Understanding the difference between apoptotic and necrotic cell death is essential for designing therapies which will prevent or limit inappropriate cell death in the nervous system. ***Anat. Rec. (New Anat.): 253:79–90, 1998.*** **© 1998 Wiley-Liss, Inc.**

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WHY DO WE STUDY NEURONS?

Brain cells come from two main lineages: nerve cells (or neurons), which have the ability to manipulate charge across their cell membrane and are responsible for processing information in the nervous system, and glial cells (glia), which are thought to mainly support and nutrify neurons.

The human brain is only about 3% of body mass, yet it consumes between one-quarter and one-half of the oxygen and glucose in the bloodstream.

Clearly, neurons are always "riding on the edge"; with their high metabolic requirements and meager provisions, they are extremely vulnerable cells.

This huge energy requirement is a direct result of how neurons work: their proper function depends on the ability to maintain, lose, and then restore electrical potentials in rapid succession. Such a cycle of depolarization occurs 100 or more times per second in an active neuron. Supplying the required energy to accomplish this feat is a formidable task.

Neurons not only consume large quantities of glucose, but they are committed to using glucose as their primary carbon source. The central nervous system (CNS) is protected by the blood-brain barrier (BBB). This barrier serves the same function as the moat and walls of a castle: to restrict the ingress and egress of substances. The BBB results from special properties of the basal lamina and of junctions between the endothelial cells lining the capillaries; these junctions are much tighter in the CNS than in most other regions of the body. The BBB allows glucose, oxygen, vitamins, and some amino acids to pass but not much else. Energy-rich carbon sources other than glucose do not pass the BBB efficiently, making the glucose supply critical. Clearly, neurons are always "riding on the edge"; with their high metabolic requirements and meager provisions, they are extremely vulnerable cells.

Adding to the precious nature of neurons is the fact that they cannot be replaced in any significant numbers in the adult brain. Replacement would

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depend on the existence of neuronal stem cells that can generate small numbers of new neurons. Many vertebrates, including some mammals, can regenerate neurons in the CNS—even as adults. However, evidence of such a regenerative capacity in primates is lacking. Regardless, in any species, most toxic brain disorders involve the death of neurons on a scale that outpaces the ability of stem cells to replace them. Thus, for all practical purposes, humans are born with all the neurons they will ever possess. It is likely that we must trade off the ability to regenerate neurons in order to preserve the staggeringly complex organization of the brain, critical for the normal functions of sensation, learning, memory, motor coordination, creative thought, and consciousness itself.

The unfortunate result of these combined facts is that in the brain we have an organ which is critical for life yet is easily damaged and not readily repaired. There is obvious interest in limiting the damage caused by various insults, triggering endogenous repair mechanisms, or both.

HOW DO WE STUDY NEURONS?

While there are obvious benefits to studying an intact nervous system, cellular and molecular aspects of neuronal function require a more reductionist approach. To facilitate their ability to manipulate neurons in meaningful ways, many neuroscientists remove neurons or neural tissues from the organism for experimentation. One approach is to use a slice preparation, in which the brain tissue is cut into slabs approximately one-tenth of a millimeter in thickness. Brain slices prepared in this way can be kept alive in an oxygenated saline solution for several hours.

Obviously, it is desirable to be able to study the properties of neurons or small groups of neurons for longer periods as well. Investigators have turned to neurons in dissociated cultures, in which neurons are detached from one another at the time of plating and kept alive for up to several weeks, attached and growing on the surface of petri dish–like plates. For this type of culture, neurons usually are obtained from the brain tissue of rodent embryos. These are termed primary cultures.

Another model that has been exploited is the use of immortalized cell lines. These are clonally selected cells that can proliferate indefinitely in culture yet retain at least some neuronal characteristics. Most of these cell lines are derived from tumors of various species and have limited utility to the study of the biological processes unique to neurons. One promising cell line (NT2) is derived from a human teratocarcinoma.[1] Under the influence of the differentiation factor retinoic acid, NT2 cells slowly change to a neuronal phenotype (Fig. 1). Once differentiated, these cells, called hNT neurons, are like primary neurons in that they are not immortal but remain viable long enough for experimentation, up to 2 months.

Another type of neuronal cell line has been generated intentionally by immortalizing embryonic neurons through insertion of a tumor gene (i.e., an oncogene). In the most useful examples, researchers have designed the oncogene so that it can be deactivated on command, thus initiating differentiation of the cells into what appears to be a normal neuron.

APPROPRIATE NEURONAL DEATH*

During development of the nervous system, many more neurons are born than will be needed in the adult.[2] The "die-off" responsible for this difference is not accidental. Indeed, ontogeny seems to have appropriated a form of natural selection to help create the proper organization of the adult nervous system. Major pathways (e.g., spinal sensory tracts to primary sensory cortex) may be dictated by the expression of body plan information stored in the genome and are thus "hard-wired." However, the fine tuning of these connections appears to be orchestrated by environmental cues, including the microenvironments in which individual neurons find themselves. Among the developmental influences impinging on a neuron are its collective efferent and afferent connections. In a process called selective stabilization, viability of the neurons that are properly connected is enhanced, whereas neurons making inappropriate connections (or none at all) are eliminated. Because these cell death events are programmed for the establishment of a mature nervous system, they are considered appropriate. An additional feature is that they occur without tissue inflammation or disruption of surrounding cells (see below for details).

In 1986, the Nobel Prize was awarded to Stanley Cohen and Rita Levi-Montalcini for their discovery of nerve growth factor (NGF) a quarter-century earlier. As the prototype of a set of molecules called target-derived trophic factors, NGF revolutionized our understanding of the mechanisms controlling developmental neuron death. NGF is a secreted peptide produced by neuronal targets in the central and peripheral nervous system. Neurons that both express a specific NGF receptor and grow to innervate a target producing NGF will be stimulated in ways that promote their survival. Those that fail to make this connection, as well as inappropriate neuron cell types that do not express the NGF receptor, will die.

As important as NGF and other target-derived trophic factors are to survival of well-connected neurons, they are not the whole story. As the most obvious evidence of other requirements, neurons can outgrow their dependence on soluble trophic factors yet remain sensitive to loss of their targets. In most instances, a major contribution to neuronal viability is their perpetual electrophysiological activity. The rapid cycle of generating, losing, and restoring an electric potential across a neuron's cell membrane somehow enhances its vitality. During critical developmental periods, electrophysiological activity and soluble trophic factors cooperate through a scenario that has been termed associative target-derived neurotrophism.[3] At later developmental stages, the role of soluble trophic factors may be diminished in some cell populations. But, regardless of the mechanism, individual neurons remain subject to the adage "use it or lose it."

*We thank Steve Estus for introducing us to the terminology of appropriate vs. inappropriate cell death.

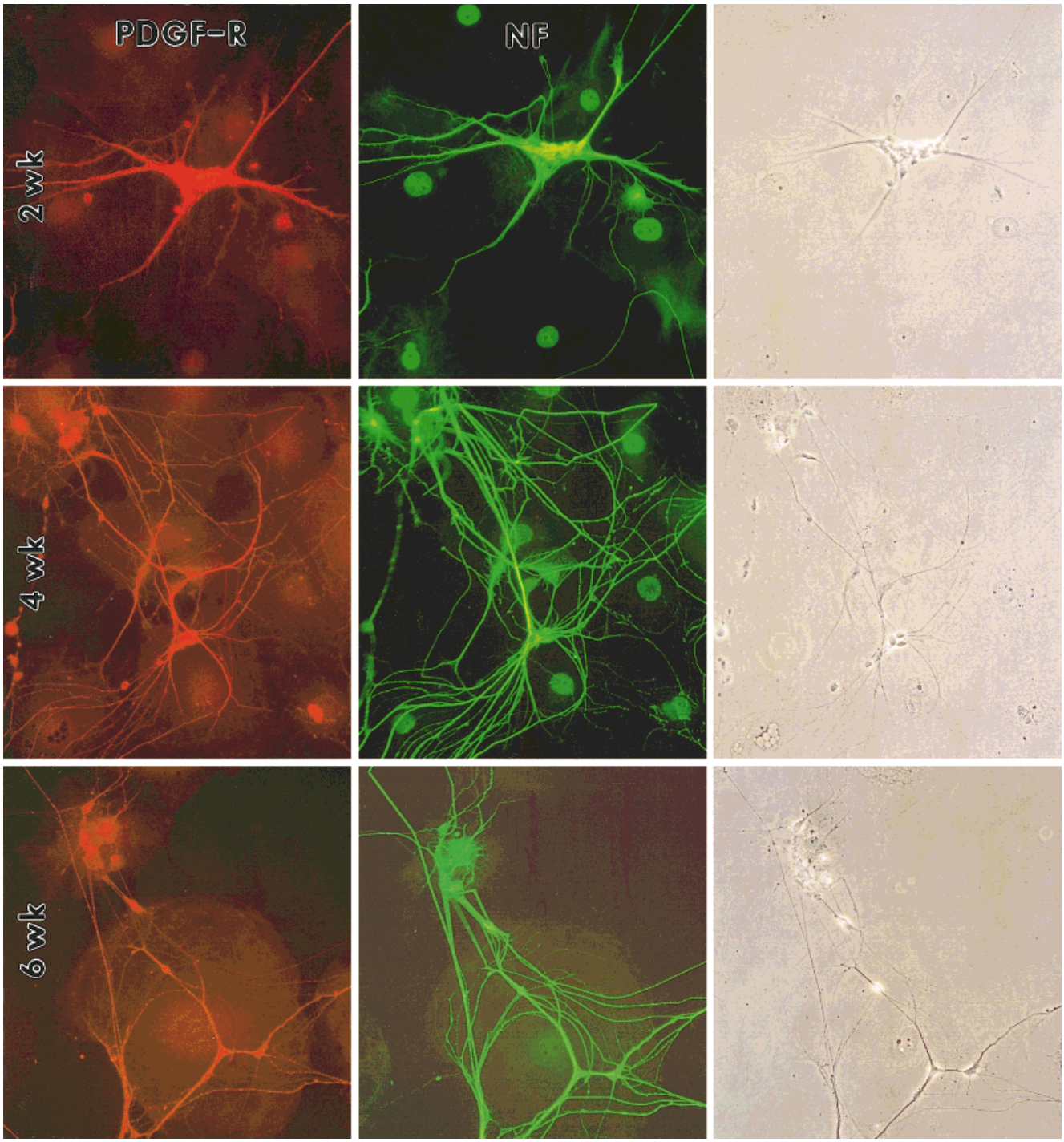

Fig. 1. NT2 cells are human neurons which survive up to 2 months in culture. NT2 cells are maintained in culture for 6 weeks in the presence of retinoic acid; they adopt a neuronal phenotype (hNT neurons). The hNT neurons are shaken loose from the underlying mother cells and are replated into fresh flasks. In these preparations, some of the mother cells have been plated inadvertently as well; they are visible as large, polygonal sheets beneath the neurons. **Left column**: hNT neurons labeled with antibodies to platelet-derived growth factor receptor β (PDGF-Rβ). **Middle column**: hNT neurons from the same field, labeled with antibodies to phosphorylated neurofilament protein. Note that hNT neurons express both PDGF-Rβ and neurofilament, but the NT2 mother cells do not. (The neurofilament antibody cross-reacts with histone proteins, accounting for the ovoid nuclei which are labeled green as well. This serves as a convenient marker for all cell nuclei in the preparation, whether neurons or not). **Right column**: Phase-contrast microscopy of the same area. **Top row**: Two weeks after the end of retinoic acid treatment and replating. **Middle row**: Four weeks. **Bottom row**: Six weeks.

INAPPROPRIATE NEURONAL DEATH

Neuron cell death contributes to some of the most debilitating diseases of the brain, including stroke, brain trauma, and Alzheimer, Parkinson, and Huntington diseases. Even in diseases that are largely considered nonneurological, such as AIDS, there can be neurodegeneration that—due to its irreversibility—may be of lifelong consequence once the pathogenesis is halted.

Stroke may be considered the most acute example of the injury to neurons seen in other conditions. Such "brain attacks" (by analogy with heart attacks) are the third-leading cause of death among adults. A stroke may result from an embolus (e.g., a blood clot, globule of fat, air bubble, or the like) or the growth of an atherosclerotic plaque, either of which can occlude a blood vessel. This is called an ischemic stroke and is the most common form, occurring in six out of seven cases. Less common is the hemorrhagic stroke, which results from bleeding out of a leaky blood vessel in the brain.[4]

Ischemia results in loss of blood glucose, and the residual oxygen within brain tissue is consumed within a few seconds.[5] Initially, phosphocreatinine and what little glycogen is found within the brain are used to maintain ATP concentrations. But, within 2 min, glucose, ATP and phosphocreatinine are all reduced by over 80%.[5] Neurons stop functioning, and consciousness is lost. If oxygen and glucose are not quickly restored, permanent damage to the nervous system will occur within 5–6 min. Although stroke kills more cells more rapidly than chronic conditions such as Alzheimer disease, considerable evidence suggests that some of the same insults lead to neuronal death in both chronic and acute conditions. However, the response of a neuron to these insults can differ dramatically, depending upon its particular neurotransmitter system, metabolic state, and microenvironment of electrophysiological stimulation and trophic support.

NOT ALL DEATH IS CREATED EQUAL

To paraphrase Tolstoy, "Happy neurons are all alike; every unhappy neuron dies in its own way." Neurons do not all die in the same fashion or for the same reasons. The simplest division, and the one that makes the most sense functionally, is to divide neuronal cell death into apoptosis, or cellular suicide, and necrosis, a rapid and messy death.

Apoptosis

Apoptosis is a scientific utilization of the Greek for "a dropping away". It is a fairly recently studied phenomenon.[6] The fact that there is programmed cell death during embryogenesis has been known for some time in a number of well-studied examples (such as the death of cells in the future spaces between digits to form a hand from a paddle-like primordium). Essentially all of the appropriate neuronal death that happens during development occurs through apoptosis. However, there is clear evidence that apoptosis can be inappropriately commandeered by pathological processes to cause cell death in disease.

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Apoptosis seems well-suited to appropriate cell death for two reasons. First, apoptosis is tidy and unobtrusive. Cells that die by apoptosis generally keep their cellular contents walled off in membranous structures and self-digest their structural and genomic elements. The packaged remnants that remain are easily phagocytosed by macrophages to rid the tissue of debris. Second, apoptosis proceeds through a highly ordered program of events and checkpoints that a cell is capable of regulating autonomously. The process of apoptosis is an active one, requiring RNA and protein synthesis.[7] By blocking production of new RNA with actinomycin D and protein synthesis with cycloheximide, the process of growth-factor-deprivation-induced programmed cell death could be delayed. Subsequent studies have shown that the process of apoptosis is not stochastic or accidental but rather part of a developmental plan—hence the tendency to use synonymously the phrase *programmed cell death*. In short (and speaking anthropomorphically), apoptotic cells have chosen to die.

It is important to remember several caveats with respect to apoptosis. First, apoptosis comprises a complex set of physiological events[6] and generally takes several hours to complete, and the early events—and possibly later ones as well—are reversible. For example, loss of mitochondrial membrane potential, one of the earliest events in apoptotic death (see below), may not be tantamount to cell death. Second, some of the techniques for assaying apoptosis are susceptible to artifacts. Third, a cell may commit suicide without exhibiting all the signs of apoptosis. Lastly, instances have been described in which a cell exhibits signs of both apoptosis and necrosis simultaneously. For this reason, the distinction between apoptosis and necrosis is not always as simple as it seems at first.

Necrosis

In contrast to apoptosis, necrosis leads to a more abrupt and uncontrolled damage to cellular structures. There is a swelling and enlargement of the cell. No observable, consistent differences in the morphology of chromatin are seen in the early stages of necrotic cell death, although these changes are seen in apoptosis. The nuclear pores are still present. The plasma membrane loses its integrity and breaks, spilling the cytoplasmic contents and organelles into the extracellular space. Some of the cell contents may induce an inflammatory reaction.

Despite these different mechanisms of death, there is not always a neat, clean distinction between apoptotic and necrotic cell death. At the extremes, they are easily distinguished; but there are frequently situations which resemble both. For example, in a phenomenon known as secondary necrosis, apoptotic cells are shed from their epithelial home into the luminal

space. In this case, phagocytes begin destroying the ectopic cells, and after a time the dying apoptotic cells take on the appearance of necrotic cells. Cytoplasmic volume increases, and therefore cytoplasmic density decreases, a change normally seen in necrosis but not in apoptosis.

Particularly in the early stages of neuronal cell death, before a difference in the morphology of the cell becomes apparent, it is probably impossible to tell with any certainty whether the impending death is apoptotic or necrotic. In addition, some cellular insults appear capable of evoking either form of cell death, depending on the "dosage," time of exposure, and intrinsic characteristics of the affected cell. Nevertheless, mechanistic differences between apoptosis and necrosis create distinct challenges and opportunities for therapeutic intervention. Only by observing events at the molecular level can the processes of apoptosis and necrosis be fully understood and, when desirable, stopped.

HOW NEURONS DIE: ROUTES TO CELL DEATH

Glutamate-Triggered Neuronal Death

The high metabolic activity of neurons means that they are often "pushing the envelope" of their capacity to recover from their demanding job. Obviously, this balance could be upset by decreases in glucose or oxygen supply. However, stress can occur from the other direction as well: modest increases in their activity can tax the ability of neurons to maintain viable homeostasis. The best-characterized example of the latter is excitotoxicity. First studied by Olney[8] and later characterized in detail by Choi[9] and others, excitotoxicity describes neuronal cell death resulting from excessive stimulation of the signal transducing receptors for glutamate. Glutamate is the most widely used excitatory neurotransmitter in the CNS, and its concentration is tightly controlled by several systems of regulated release and uptake. Experimentally, the excitotoxic phenomenon often is studied with the application of various glutamate receptor agonists. In actual neurological disorders, such death can result from either excessive release of endogenous glutamate, failure of glutamate uptake mechanisms, or exposure to drugs and poisons that act as glutamate receptor agonists.

Mechanistically, glutamate-mediated neuronal death involves excessive ion movement across the plasma membrane. Glutamate receptors are divided into categories depending on their effects on the postsynaptic cell; they are further divided into subcategories based on pharmacological properties. Pharmacological receptor classes can be ascribed to different combinations of gene products. Ionotropic glutamate receptors are those that, when stimulated by glutamate or other agonists, cause the entry of ions such as calcium and sodium. Metabotropic glutamate receptors act through a second messenger cascade to change the metabolism of neurons; they will not be considered further here.

The concentration of free calcium ions ($[Ca^{2+}]$) in the neuronal cytoplasm is generally kept very low compared to the extracellular $[Ca^{2+}]$ (less than 10^{-7} M vs. about 10^{-3} M). Recovery from a rise in cytoplasmic $[Ca^{2+}]$ involves a Na^{+}/Ca^{2+} exchanger on the plasma membrane and sequestration of Ca^{2+} into the mitochondria. Mitochondrial calcium accumulation can be harmful because calcium entry depletes the proton gradient which is normally used for ATP production. It may also form calcium phosphate—essentially, bone—in the mitochondrial matrix and thereby reduce the amount of free phosphate available for incorporation into ATP. The endoplasmic reticulum (ER) can also sequester Ca^{2+}. Calcium storage in the ER allows controlled release in response to an intracellular signal, such as a second messenger.

Hyperstimulation of ionotropic glutamate receptors overwhelms the ability of a neuron's homeostatic mechanisms to restore ion balances. Death can come rapidly from the hyperosmotic shock caused by sodium (and its charge-coupled sidekick, chloride) rushing into the cells; this is necrotic death. Alternatively, calcium can initiate a series of events that kills the cell in a more delayed fashion. Calcium does not reach the same ionic concentrations as sodium in excitotoxically challenged cells; thus, its toxicity may result from the overactivation of qualitatively normal cellular signals. Whether the delayed, calcium-triggered component of excitotoxicity is apoptotic has been a question of some controversy, but it clearly involves a relatively complicated program of events and can take from hours to days to occur.

It was thought for some time that those glutamate receptors which respond to N-methyl-D-aspartate (NMDA) are the sole players in ligand-gated calcium conductance. Although it was recognized that AMPA/kainate receptor activation could indirectly activate voltage-gated calcium channels, Choi's original work showed that NMDA receptors were responsible for most of the glutamate-mediated calcium entry and the subsequent neuronal death.[9] Recent evidence has shown that other subcategories of ionotropic glutamate receptors—specifically variants of AMPA/kainate receptors—can act as calcium channels as well.[10] Activation of either type of ionotropic receptor can lead to cell death, but the NMDA receptors appear to make a larger contribution.[11]

Ionotropic glutamate receptors are not distributed evenly across a neuron's surface; they are concentrated in the dendrites, extensions of neurons that receive information. One of the earliest effects of excessive glutamate is "pruning" of the dendrites (Fig. 2). Depending on the severity of the excitotoxic treatment, this dendritic pruning may or may not culminate in the death of the entire cell. This phenomenon has been interpreted as selective death of the dendrites, a concept that may even be extended to dendrite-restricted apoptosis. Loss of dendrites has dire consequences for interneuronal communication. Therefore, any discussion of overt neuropathology should take into account the potential for death of discrete neuronal structures even when counts of cell bodies detect no loss.

The glutamate receptors which allow calcium entry are also concentrated in the dendrites (Fig. 3). Excessive local Ca^{2+} may be responsible for dendritic pruning. The axon, a single, distinct type of cellular extension responsible for sending information, has virtually no glutamate receptors and is preserved in the initial stages of excitotoxic cell death (Fig. 3).

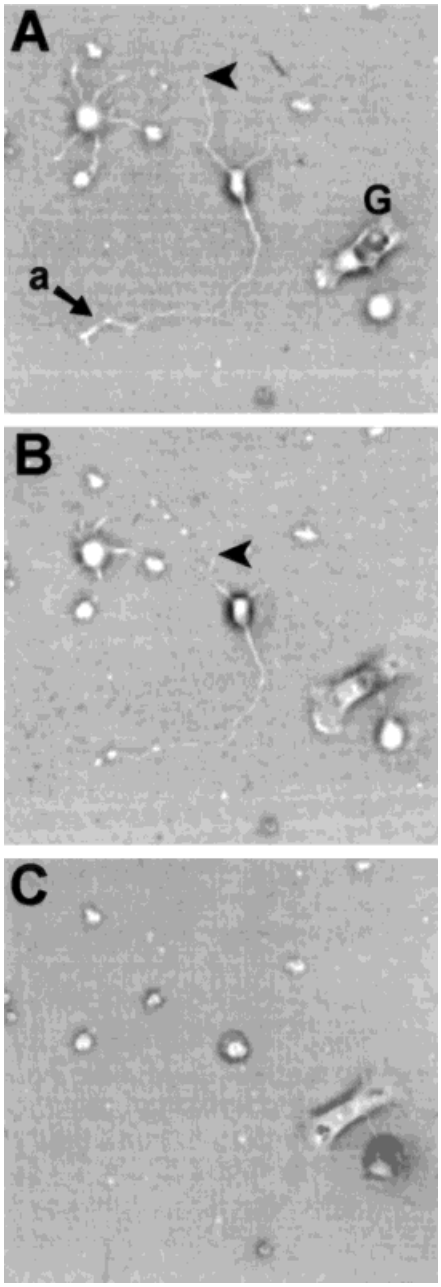

Fig. 2. Time-lapse photography of excitotoxicity. A hippocampal neuron was photographed before (A), 1 h after (B), and 4 h after (C) application of 100 μM glutamate. Note that glutamate evokes a pruning of the dendrites (the regressing end of one of these is marked with an arrowhead) within 1 h. Little change occurs in the cell body or axon within this time period. By 4 h, even the axon (a) is lost, and the cell body has begun to show signs of vacuolization and/or blebbing. A glial cell (G) shows no morphological response to glutamate.

Reactive Oxygen Species and Neuronal Death

Another active area of research and clinical investigation has been the role of reactive oxygen species in the death of neurons. Energy transport and utilization by living organisms is carried out through oxidative phosphorylation, the transfer of electrons in a series of chemical reduction/oxidation (redox) reactions within the mitochondrion. An inevitable by-product of such reactions is the creation of free radicals, molecules with unpaired electrons. Free radicals are extremely reactive, capable of chemically interacting with many biological molecules (including nucleic acids, proteins, and lipids) and thereby functionally destroying them. The oxygen so critical for life is easily capable of reaching a free-radical state. The electron transport chain involved in mitochondrial ATP production is responsible for producing many of the free radicals which must be inactivated by the cell. It has been estimated that 2% of the oxygen consumed by cells is converted to superoxide ($O_2^{\bullet-}$),[12] a particularly damaging radical. As cells with an extremely high metabolic rate, neurons are at a high risk of damage from such reactive oxygen species.

Generally, free radicals are inactivated by enzymes which convert them to relatively harmless forms. For example, superoxide dismutase (SOD), which comes in two forms, converts superoxide to the less-reactive (but still harmful) hydrogen peroxide (H_2O_2). Peroxide in turn is inactivated by (among other enzymes) catalase and glutathione peroxidase. Nonenzymatic free radical scavengers also play an important part in removing reactive oxygen species. Such an antioxidant function is the primary role of some vitamins such as C and E.

In the past decade, it has become clear that a simple gas, nitric oxide (NO) acts as a neuromodulator in many parts of the nervous system. Although NO has discrete and important signal transduction roles, it reacts with superoxide to form peroxynitrite ($ONOO^-$), a free radical that is just as reactive as superoxide and which largely escapes SOD inactivation. It is thought that this peroxynitrite modifies proteins (primarily at tyrosine residues). It may also rearrange to NO_3^-, which in turn joins with a proton to form hydroxyl radical (OH) and NO_2.[12] The hydroxyl radical is a much more reactive and cytotoxic species than superoxide. Peroxynitrite also activates poly[ADP-ribose]polymerase, an enzyme which rapidly consumes cellular electron carrier nicotinamide dinucleotide (NAD) and/or ATP. Both NAD and ATP are crucial for oxidative phosphorylation and glycolytic energy production. The responsiveness of NO synthase to calcium appears to account for much of the calcium-dependent damage manifest in excitotoxicity and discussed above. Superoxide is also generated by excitotoxins.[13] Regardless of the species or mechanism

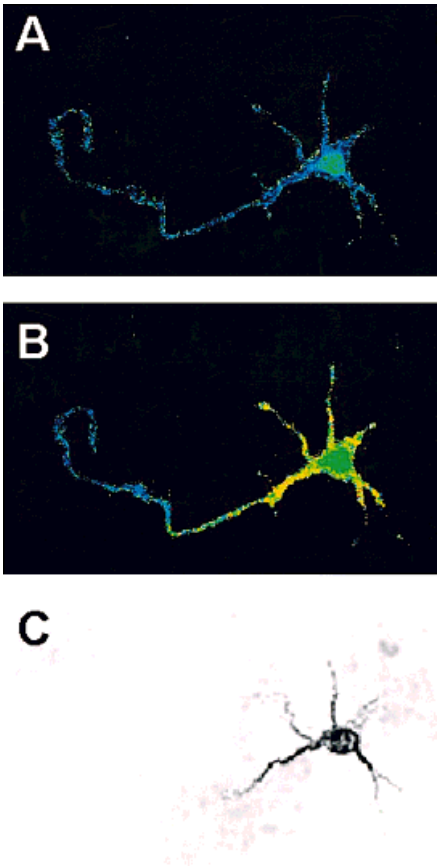

Fig. 3. Spatial restriction of cytosolic [Ca^{2+}] responses to glutamate. A single hippocampal neuron grown in culture was loaded with a fluorescent dye that allows spatially resolved measurements of [Ca^{2+}]. A computer-generated mapping has assigned different colors to different ranges of [Ca^{2+}]: low concentrations are indicated with cool colors (blue to green), and high concentrations are indicated with hot colors (yellow to red). Images are shown before (A) and 30 s after (B) application of 100 μM glutamate. (C) After imaging, the cell was fixed, and immunocytochemical methods were used to label MAP2, a protein found in dendrites but not in axons. Note that the axon (characteristically the longest extension of the cell) shows little calcium response to glutamate.

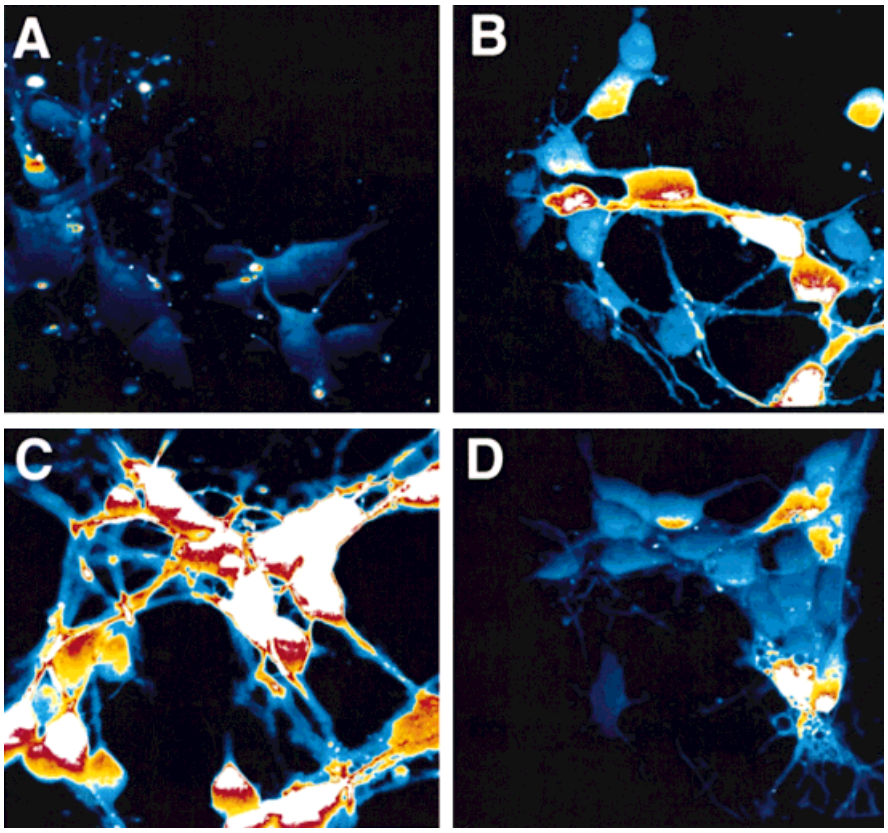

Fig. 4. Elevation of reactive oxygen species by calcium. Primary cultures of hippocampal neurons were loaded with a fluorescent dye (dichlorofluorescein) which fluoresces in the presence of peroxide. The cells were imaged by confocal laser scanning microscopy in an unstimulated state (A) or 2 min after the application of calcium ionophore (B) or glutamate (C). The cells in D were exposed to glutamate in the absence of extracellular calcium. A computer-generated pseudocolor representation of the fluorescence intensity was rendered, similar to Fig. 2 (white represents the maximal reading). Note the apparent increase in cellular peroxide in conditions that elevate cytosolic [Ca^{2+}].

of formation, high cytosolic [Ca^{2+}] certainly generates reactive oxygen species (Fig. 4).

It has been known for some time that certain leucocytes deliberately generate free radicals to destroy infectious agents. A better understanding of how these cells avoid damage themselves may lead us to an appreciation of how neurons could be coaxed to better manage their free radicals. In addition, the inappropriate production of free radicals by these leucocytes appears to contribute to the negative effects of inflammation and related reactions.

Of course, injuries that break blood vessels also can expose neurons and glia to the free-radical-forming catalyst iron. The Fenton reaction, through which iron catalyzes the production of $OH^{\bullet}$, often is initiated experimentally by exposing neurons to ferric iron.

Another clinically relevant class of compounds that can lead to increases in free-radical production within neurons is the amyloid peptides. Free-radical elevation is a shared effect of the prion protein implicated in bovine spongioform encephalopathy ("mad cow disease"), the Alzheimer β-amyloid peptide, and amylin. The peptide β-amyloid is the main constituent of plaques that are the hallmarks of Alzheimer disease. It has been shown to directly generate free radicals in aqueous solution even in the absence of cellular components.[14] The stable by-products of reactive oxygen species (oxidized lipids and modified proteins) can be detected in Alzheimer disease as well as other neurodegenerative diseases.

Hypoglycemia and Neuronal Death

In the neuron, as in other cells, ATP is produced with low efficiency by glycolysis in the cytoplasm. Mitochondria import the pyruvate end product of glycolysis, and within this organelle the citric acid cycle and oxidative phosphorylation combine to produce virtually all of the ATP used by the cell for energy-requiring functions. In particular, nerve cells must maintain membrane potential by pumping ions against either a concentration gradient and/or an electrical gradient. Further, neurons with long axons have the additional load of metabolic support of a huge surface area which involves pumping necessary metabolites over long distances.

Therefore, loss of glucose leads to a rapid depletion of the energy reserves of the cell. Since the cell's stock of ATP is turned over every 2 min on average, it does not take long to completely deplete the cell's ATP stores.

Mitochondria must maintain a proton gradient between the inner and outer mitochondrial spaces for oxidative phosphorylation-mediated energy production to occur. The maintenance of this gradient requires energy as

well. As the ATP stocks are depleted, the mitochondria lose the ability to maintain a potential difference (designated $\Delta\Psi_m$).[15] The collapse of mitochondrial membrane potential is due to the opening of protein channels in the inner mitochondrial membrane called the mitochondrial permeability transition pores (MPT pores).[15] The opening of these pores is a self-reinforcing process: the opening of MPT pores causes a collapse of mitochondrial membrane potential, leading to further pore opening.

When MPT pores open, several death-dealing blows to the neuron result[15]: (1) the respiratory electron transport chain is uncoupled, and the mitochondrion is no longer able to produce ATP; (2) the reduced forms of glutathione, NADPH, and NADH are depleted; (3) generation of the harmful $O_2^{\bullet-}$ (superoxide anion) is increased; (4) calcium is released from the mitochondrial matrix where it is normally sequestered; 5) cytochrome c is released from the mitochondria and initiates a signal transduction pathway that ultimately induces enzymes which trigger apoptosis.

When all these observations are taken together and the central role of mitochondria in calcium buffering, free-radical production and glucose utilization is considered, it seems that mitochondria are the primary effector organelles of apoptotic cell death. However, mitochondria also play a role in necrotic death. A unique relationship between energy depletion and necrosis is found in the brain. Although only 0.0012% of the ionic gradient is depleted every time an action potential is fired, repeated electrical potential changes ultimately lead to ionic imbalances in the neuronal cell. Normally, balance is restored by ATP-dependent mechanisms (such as the Na^+/K^+ pump, which transports these ions across the cell membrane), but in the absence of ATP the neurons' membrane potential slowly equilibrates with the extracellular fluid. This depolarization results in the inappropriate release of neurotransmitter. Because glutamate is such a common neurotransmitter, brain ischemia, or loss of blood supply, involves a considerable degree of glutamate toxicity.

All the routes to apoptotic death described above have the mitochondrion as a common feature. Recently, investigators have proposed a critical role for mitochondria in a variety of neurological diseases. Mitochondria as the root cause of neuronal death or damage has been implicated in pathologies as diverse as those of Huntington's chorea, Parkinsonism, and Alzheimer disease.[16,17]

MOLECULAR MECHANISMS OF APOPTOTIC CELL DEATH

Table 1 lists the known pro- and antiapoptotic factors which interact to either set a cell death program into motion or to hold it in check. The balance between pro- and antiapoptotic factors appears to be the critical element in deciding whether a cell will live or die.

Mitochondria as the root cause of neuronal death or damage has been implicated in pathologies as diverse as those of Huntington's chorea, Parkinsonism, and Alzheimer disease.

Proapoptotic Factors

The discovery of the genes responsible for programmed cell death in the nematode *Caenorhabditis elegans* led to a revolution in our understanding of the molecular basis for apoptotic cell death. There are 1,090 cell births and 131 cell deaths in the development of the worm, exclusive of the germ line; this gives a total of 959 nongerm cells in the adult worm.[6] Because of its simplicity, *C. elegans* has provided a treasure trove of genetic markers for programmed cell death, of which the best known is the *C. elegans* death, or *ced,* class of genes.

Ced-3/ICE and *ced-4*

It has been shown that the combination of *ced-3* and *ced-4* expression is necessary and sufficient for activating the cell death program in *C. elegans* development.[6] It was therefore of obvious interest when *ced-3* was found to have considerable homology to the gene for the already characterized interleukin 1 converting enzyme (ICE), a protein which cleaves and therefore activates the cytokine interleukin 1, leading to inflammation. The homolog of *ced-4* is still a wanted criminal. While it is not entirely certain that ICE plays a pivotal role in mammalian apoptosis[18] as some have claimed, it is clear that a related family of proteins called the caspases are the outlaw family responsible for both appropriate and inappropriate neuronal cell death.

Caspases

ICE is the prototype of a family of related enzymes collectively called caspases; they contain a cysteine residue at the active site, they cleave substrates at the carboxyl side of an aspartate residue, and they are proteases or peptidases.[6] Caspases cleave two major categories of protein: homeostatic or repair proteins (e.g., poly[ADP-ribose] polymerase, DNA-dependent protein kinase, protein kinase Cδ) and cytoskeletal or structural proteins (e.g., lamin A, fodrin, G-actin).

Caspases are thought to be present throughout the neuron's lifetime. They reside in the cytoplasm in an inactive, proenzyme form and are activated by proteolysis, perhaps by other family members. In one scenario, a key enzyme in oxidative phosphorylation, cytochrome c, is released from stressed mitochondria. Released cytochrome c combines with dATP and a protein called Apf-1 to activate caspase-9; caspase-9 then acts on caspase-3 to initiate the apparently irreversible phases of apoptosis.[19] Other triggers could also activate the latent caspases which are constantly carried by the cell. Neurons carry the dynamite with them at all times, and if someone supplies the blasting caps, suicide will result.

Bad

The aptly named proapoptotic factor Bad is an example of a class of neuronal death facilitators which includes the factors Bax, Bak, Bik/Nbk, Bim, Hrk, and Bid. These proapoptotic factors act by sequestering or binding the good antiapoptotic factors such as Bcl-2 (discussed below) and therefore

TABLE 1. Current Lineup of the Nematode Programmed Cell Death Genes and Their Mammalian Homologs[a]

C. elegans death gene	Mammalian homolog(s)	Function or mechanism
Proapoptotic		
ced-3	ICE[b] Caspases[c]	Cleavage of proteins responsible for other cell homeostasis, cell repair, or cytoskeletal integrity
ced-4	Unknown	With ced-3, necessary and sufficient to initiate a cell death program
—	Bad	Binds Bcl-2 or Bcl-x_L, blocking their antiapoptotic effects; phosphorylated on serine
—	Bax Bak Bcl-x_S	
—	Bik/Nbk Bim Hrk	
—	Bid	
Antiapoptotic		
ced-9	Bcl-2 Bcl-x_L Bcl-w Mcl-1 A1	Binds Bax, other proapoptotic molecules; inserted in ER and outer mitochondrial membranes; role in mitochondrial permeability transition
—	Bag-1	Partner/positive effector of Bcl-2; interacts with HGF and PDGF receptors

[a]Genes are designated by lower-case italics (e.g., *bax*). The protein gene product is designated by the same term in Roman text with an initial capital (Bax).
[b]ICE, interleukin-1β converting enzyme, also called caspase-1.
[c]Caspases, cysteine-containing aspartate-cleaving proteases, generally cleave on the carboxy side of aspartate residues which are part of a four peptide consensus sequence. ICE is the prototypical caspase. To date, ten have been described and are designated caspase-1 through caspase-10. Caspase-3 is the enzyme with the greatest sequence similarity to Ced-3.

allow programmed cell death to proceed unimpeded. Bax has also been shown to localize to the mitochondria and trigger the release of cytochrome c, which eventually results in caspase activation.[20]

Guido Kroemer[15] has called this balance between pro- and antiapoptotic factors the "death-life rheostat." It is intriguing to think of the system described here as a dial, where a sharp counterclockwise turn will activate caspases and the *ced-4* analog. Meanwhile, the Bad family keeps rescuers such as Bcl-2 at bay. Conversely, a sharp clockwise turn will ensure caspases remain in their harmless proenzyme forms and keep Bad and its related desperados safely behind bars.

Antiapoptotic Factors

Bcl-2 and related proteins

Bcl-2 and the related protein Bcl-x_L appear to be the primary factors responsible for keeping cells from choosing the apoptotic pathway. This family of molecules is found inserted into the mitochondrial membrane and the endoplasmic reticulum; it is capable of binding proapoptotic factors such as Bax and thereby keeping them from activating the apoptotic program. Bcl-2 has pleotropic effects (for review, see Kroemer[15]) including decreasing generation of reactive oxygen species and maintaining the mitochondrial membrane potential. As described above, the collapse in mitochondrial membrane potential is a key event in apoptosis. In fact, Kroemer has argued that loss of mitochondrial membrane potential and the resulting opening of MPT pores is the "final common pathway" in apoptotic cell death.[15] However, Bcl-2 also appears capable of blocking apoptosis by acting downstream of the MPT pore opening, interfering with the ability of cytochrome c to activate caspases.[20]

Bag-1

One of the surest ways to evoke apoptosis in cultured cells is to remove serum growth factors from their medium.[21] The antiapoptotic factor Bag-1 may serve as a link between growth factors in serum and maintenance of cell integrity and prevention of apoptosis.

A MODEL OF NEURONAL DAMAGE IN ISCHEMIC STROKE

Figure 5 shows a proposed flowchart for the series of events in ischemic stroke that leads to cell death. The details of the model are discussed in the text above. The positive feedback loops that exist at several levels of the model are significant. The presence of these feedback loops explains how neuronal damage starts out bad and gets worse. It also gives us hope for the treatment of neuronal death: if one or more of these feedback loops can be interrupted, it might help protect neurons from extensive damage.

CAN WE CONVINCE NEURONS TO GO ON LIVING?

NMDA Receptor Antagonists

Drugs which block glutamate-triggered entry of calcium into neurons have been shown to exert a neuroprotective effect, both in tissue culture studies and in studies on experimental animals. For example, the drug MK-801, which blocks the NMDA receptor-gated channel, reduces the neuronal death following glutamate application to neuronal cultures. Similarly, in rodent and primate studies, MK-801 has been shown to reduce the neuronal

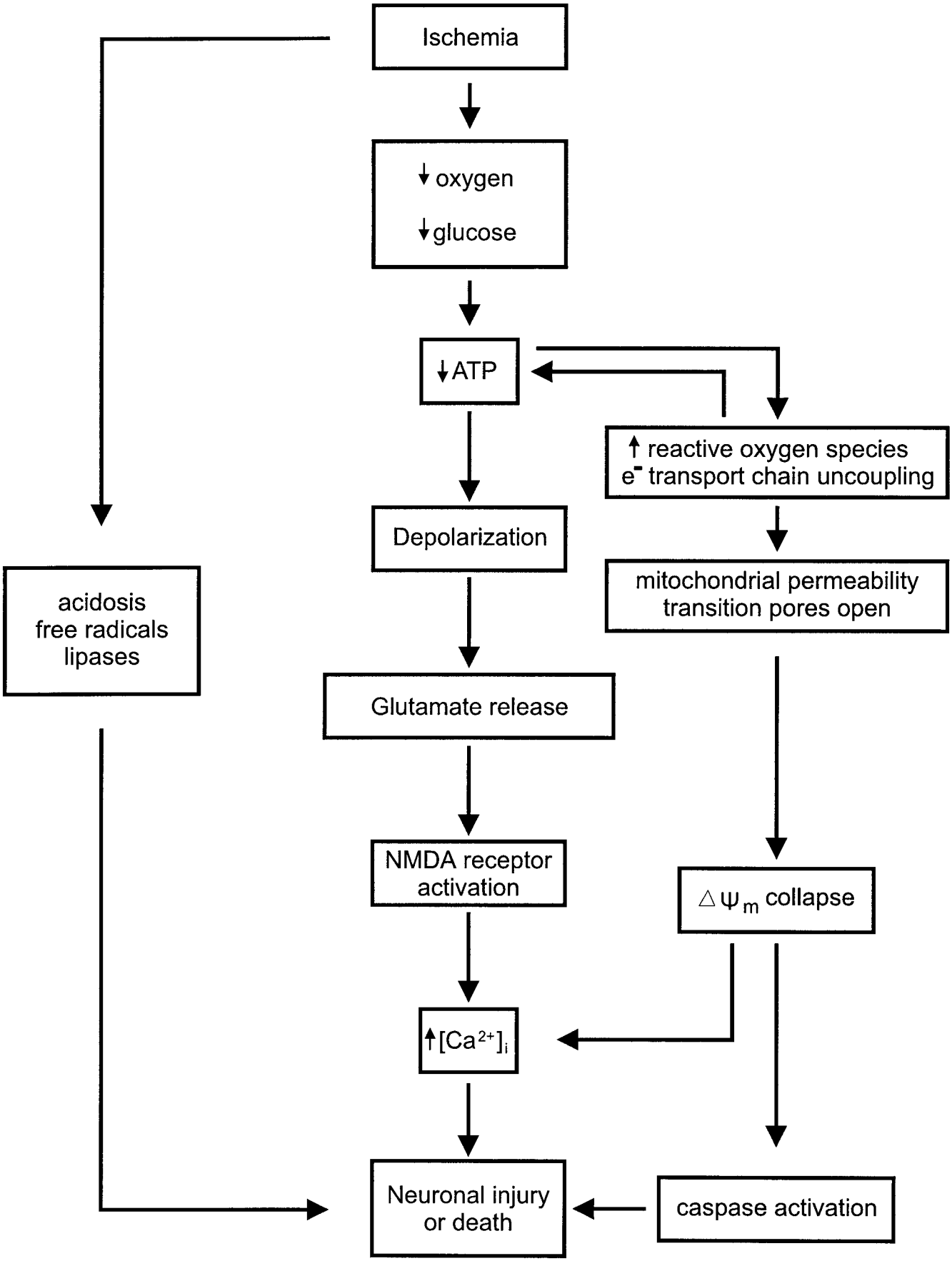

Fig. 5. Flowchart of the events leading to inappropriate cell death. Adapted from Clarke and Sokoloff.[5] Abbreviations: ATP, adenosine triphosphate; $[Ca^{2+}]_i$, concentration of free calcium ion inside the cell; e^-, electron; $\Delta\Psi_m$, mitochondrial membrane potential difference between the matrix and intermembranous space.

cell death following experimental ischemia. Other glutamate receptor blockers, particularly the NMDA receptor antagonists, have been shown to have similar effects.[22] The clinical utility of this category of drug has been limited by severe side effects, including psychosis and hallucinations.

Growth Factors

Another line of investigation has been to use growth factors to block neuronal death triggered by increased glutamate, oxidative damage from iron metabolites, or experimental hypoglycemia.[23] Work from a number of different laboratories has shown that growth factors may protect neurons from premature death, particularly apoptotic cell death.[24]

Platelet-derived growth factor (PDGF) has been shown by Cheng and Mattson[23] to exert a neuroprotective effect on cultured neurons. In vivo, glucose is virtually the only source of energy for neurons. When embryonic cortical or hippocampal neurons are maintained in primary culture, glucose is required for neuronal survival (typically 4.5 g/L or 25 mM, about five times higher concentration than in mammalian serum). Not surprisingly, when neurons are cultured in the absence of glucose, massive cell death occurs[23] beginning several hours after glucose deprivation. What *is* surprising is that growth factors such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF), or platelet-derived growth factor (PDGF) can prevent neuronal cell death from glucose deprivation.[23] Of these, PDGF is by far the most effective agent. When rat or mouse hippocampal or cortical neurons are cultured in the absence of glucose, all neurons die within 3 days of glucose starvation. However, if PDGF is added to the culture medium, about half the neurons survive even in zero glucose. This is about twice as many neurons as survive if NGF or bFGF is added.[23]

How can neurons survive without glucose? Do the cells treated with PDGF reduce their energy requirements, or are they capable of switching to another energy source? It seems important to understand how growth factors protect neurons from death. It is conceivable that they reduce the rate of electrophysiological activity in neurons such that their metabolic requirements are eased. In this case, the therapeutic utility of growth factors would be limited; a brain full of live neurons is worthless if they are not capable of the normal communicative abilities.

The Future of Neuroprotection

The delineation of discrete protein-protein cascades in apoptotic cell death has provided attractive targets for therapeutic intervention. Necrosis may also be amenable to protein agents such as antioxidant enzymes. Gene therapy—injecting viral vectors, genetically altered cells, or liposome-encapsulated DNA—may seem a logical way to manipulate proteins that can attenuate inappropriate neuronal death of any type. However, a multitude of conceptual and technical obstacles remain to be scaled before gene therapy for promoting neuronal survival is even attempted. Only by fully understanding the complex dance of molecules involved in triggering apoptosis will we know which gene products should be blocked and which should be stimulated. Further, delivery of drugs to the brain has always been hampered by the blood-brain barrier; delivery of gene-based therapeutics is no exception. One ameliorating factor may be the breakdown of the blood-brain barrier in ischemic stroke, which will allow therapeutic substances to be specifically targeted to areas of damage without further intervention. The rational manipulation of cell death (and life) genes is a promising dream but remains a fantasy for now. In the short term, more gains may be made by tweaking the structures of existing pharmacological agents or applying them in novel combinations or situations. Regardless of the ultimate therapeutic strategies, their design will be the product of research into the basic elements of the question of why neurons die.

It seems important to understand how growth factors protect neurons from death.

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