**Biomedical Reviews** 2002; 13: 49-61.

ISSN 1310-392X

# APOPTOSIS IN THE MAMMALIAN NERVOUS SYSTEM: DEVELOPMENTAL AND CLINICAL IMPLICATIONS

***Krikor Dikranian***
*Department of Anatomy and Neurobiology and Department of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA*

*Among the many regulatory steps in brain development is the process of elimination of differentiating neurons at certain stages of maturation through an intrinsic suicide program now widely known as apoptosis. Apoptosis may thus describe a cell death pathway utilized by many developing cells in the nervous system, but may also be activated as a consequence of acute or chronic pathological impulses. Such pathological impulses may include brain injury, cerebral hypoxia-ischemia and the potentials of selected drugs such as N-methyl D-aspartate (NMDA) receptor antagonists, GABA mimetics and ethanol. In recent years, there has been a great interest in mechanisms of cell death in the nervous system and apoptotic cell death has been implicated in many neurodegenerative diseases such as Alzheimer's disease, amiotrophic lateral sclerosis, Parkinson's disease and other central and peripheral nervous system disorders. Recent findings have evaluated the contribution of programmed cell death and specific gene products involved in each of these cases. A deeper understanding of these processes may lead to the discovery of strategies for slowing, reducing or arresting neuronal and glial cell death induced by injury, aging or disease.*
***Biomed Rev 2002; 13: 49-61.***

## INTRODUCTION

Massive cell death characterizes the ontogeny and turnover of virtually all mammalian tissues and has been identified even among plants and bacteria. Spontaneous cell death as a physiological event has been described as soon as histologic stains became available. First Flemming in 1885, while studying ovarian follicles in mammals, and later Strobe in 1892 while studying breast cancer, observed regressing cells at a stage of, what we would now call, apoptosis (1,2). They named the process "*chromatolyis*", referring to the fact that the broken up nucleus ultimately disappears. In 1914 Ludwig Gräper published a paper entitled "A new point of view regarding elimination of cells" (3). In 1934, V. Hamburger described significant hypoplasia in dorsal root ganglia (DGR) and lateral motor columns, following the extirpation of limb buds in chick embryos (4). In the early 1940s, Rita Levi-Montalcini repeated Hamburger's experiment and suggested that the hypoplasia might result from the death of young differentiated neurons. In a joint reinvestigation published in 1949, large numbers of degenerating neurons were described in brachial DRG, following wing bud extirpations (5). In the cervical and thoracic DRG of the same embryos, Levi-Montalcini observed massive neuronal death, which had not been affected by the operation. This in fact marked the discovery of naturally occurring cell death in the nervous system. The German anatomists M. Ernst and A. Glucksmann also deserve credit for the discovery of widespread cell death in embryonic nervous tissues (6). In

Received 7 November 2002 and accepted 10 December 2002.
Correspondence and reprint requests to Dr Krikor Dikranian, Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 S. Euclid Ave, Box: 8108, Saint Louis, MO, 63110 USA. Tel.: +1 314 362 3548, E-mail: kdikrani@pcg.wustl.edu

1972 Kerr and colleagues introduced the term "*apoptosis*" and defined the phenomenon in terms of its ultrastructural appearance (7). Although the main focus of their work was on non-neuronal tissue, they cited a single example of neuronal apoptosis, namely physiological cell death, a suicidal process by which redundant or unsuccessful neurons are eliminated from the developing brain (7,8). Several years earlier, however, before the term "apoptosis" was introduced, Sabatini *et al* (9), and later Levi-Montalcini *et al* (10), in sympathetic ganglion neurons of nerve growth factor (NGF)-deprived animals, described ultrastructural changes, remarkably similar to those provided later by Kerr and Wyllie.

## CELL DEATH DURING THE DEVELOPMENT OF THE NERVOUS SYSTEM

Among the many regulatory steps in brain development, and probably one of the most intensely studied in the past two decades, is the process of elimination of differentiating neurons at certain stages of maturation known to occur through an intrinsic suicide program now widely known as apoptosis. Although "*apoptosis*" is by far the most extensively used and often "misused" term to describe examples of genetically regulated cell death processes, perhaps it is best to use the term "physiological" or "programmed" cell death (PCD) to describe the spatially and temporally reproducible and ordered death of cells during embryonic, fetal and early postnatal development. We intend to use "*apoptosis*" in a broader aspect to describe in addition death processes induced by pathophysiological stimuli during development as well as in adulthood. Apoptosis may thus describe a cell death pathway utilized by many, if not all, developing cells in the nervous system, but may also be activated at the same time as a consequence of acute or chronic pathological impulse (11-13). For detailed discussion on the controversial and sometimes confusing issues surrounding the use of terminologies see references 2,14-19.

In virtually all neuronal populations cell numbers are substantially greater at early development stages than in maturity and the development of the nervous system requires the selective elimination of a vast number of neurons and glia. Another fundamental principle of brain development is the removal of early phylogenetic patterns of neuronal organization. For example neurons generated early in the development of the neocortex are situated in a transient zone known as "cortical subplate". These neurons interconnect with layer I neurons and participate in important cell to cell interactions with migrating neurons, and also with incoming afferent and efferent projections. This zone disappears during the course of neurogenesis by activating a process of PCD. According to studies in the human telencephalon two distinct types of PCD have been observed: embryonic, which is synchronous with proliferation and migration of neuronal cells, and fetal, which coincides with differentiation and synaptogenesis (20).

Neuronal PCD has been described virtually everywhere in the developing nervous system and across species from fish to humans (11,21). Although the magnitude of cell death varies depending on the specific brain region and postnatal period (22), generally 50% of the cell population in the central and peripheral nervous systems will be eventually eliminated until animals reach maturity. For example, on postnatal day 8 (P8) various cortical regions of the rat and mouse brain undergo substantial PCD accounting in some layers of the cingulate, frontal and parietal cortex for more than 1% of the total cell population (22,23). By P21 virtually no cortical PCD has been detected. In the cerebellum, however, a number of cells in the granule cell layer and white matter exhibit morphological and immunohistochemical features consistent with PCD at P21 (Dikranian *et al*, unpublished data). In the visual system, more than half of neuronal projections from the retina to other visual structures are also lost during development. The deletion of up to 50% of motoneurons is a normal feature of the developing spinal cord too. Moreover, PCD involves not only projection neurons innervating skeletal muscles and those of the dorsal root ganglia, but also intrinsic interneurons (11,24).

While it has been accepted that large-scale neuronal death is a regular feature of the developing vertebrate nervous system, much less attention has been given to glial cell apoptosis. However Barres *et al* (25) have shown that about 50% of newly formed oligodendrocytes normally die in the developing rat optic nerve. During the first and second postnatal week Schwann cells of the developing chick embryo spinal cord also die in large numbers (26). Krueger *et al* (27) reported evidence (including electron microscopy) for massive (50%) astrocytic PCD in the developing rat cerebella during the first and second week of development. In addition, about 2% of apoptotic cells belong to the oligodendrocyte lineage (27). These findings suggest that naturally occurring glial cell death can be just as substantial as naturally occurring neuronal cell death. They represent also a major advance in our understanding of cell elimination in the developing nervous system. Finally, these data suggest that as axon number regulates the survival of myelinating glial cells (25,26), the normal PCD of these cells reflects a competition for limited amounts of axon-derived growth factors (11). Therefore, just as neuronal PCD adjusts the number of neurons, PCD in Schwann cells and oligodendrocytes adjusts the glial cell number to the number of axons that require myelination.

Current theories about the regulation of neuronal survival hold that most, if not all, neurons in the mammalian nervous system are dependent on specific growth factors at certain developmental stages (11,21,28-30). The experimental evidence gathered so far strongly suggests that developing neurons are initially overproduced and then later compete for limited amounts of population-specific and target-derived trophic factors. Those that fail to do so undergo orderly cell suicide. Survival

dependence during development is thought to be most acute around the time axons reach their target fields (synaptogenesis), the principal source of growth factors. PCD mostly affects relatively mature neurons that have either established connections with afferent or efferent targets, or are about to establish them (11,31). Deprivation of NGF results in apoptotic cell death of virtually all neurons in the peripheral nervous system which express its signaling receptor (TrkA). Target deprivation by axotomy during the perinatal period also results in massive cell death in all populations of neurons presumably due to interruption of supply of known and as yet unknown growth/trophic factors. Of interest is the fact that early spinal motor neuron PCD may be determined either by intrinsic programs or local factors within the spinal cord, whereas later stages of motor neuron death may be dominated by trophic factor influence related to interaction with the target muscle (11). While most sensory neurons require nerve growth factor (NGF) for their survival, other populations require different neurotrophins such as neurotrophin (NT)-3, NT-4/5, brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF). For more information on the diverse role of neurotrophins and their receptors see references 11,32-35 as well as *Volume 10* (1999) of this journal dedicated to NGF and the 90$^{th}$ anniversary of Dr Rita Levi-Montalcini.

There are also other key regulatory molecules that contribute to different stages of PCD and many of them are being used lately as specific diagnostic hallmarks. Cytochrome C release from mitochondria and subsequent activities of Apaf-1, caspase-9 and caspase-3 have been shown to play a critical role in cell elimination during neurogenesis (21,36-38). Caspases are a family of cysteine proteases that have been identified as key regulators and effectors of apoptotic response in a variety of species. Following their activation by a specific signal, either through cells surface receptors or through intracellular signaling pathways, caspases act concertedly in a cascade cleaving a number of proteins, followed by cell disassembly and destruction (39). Animals deficient in any of these proteins exhibit dramatic neural pathology due to supernumerary neuronal progenitor cells. Complementary to these findings, Bcl-2 family members have been shown to significantly affect the death of postmitotic neuroins in response to trophic factor withdrawal or to the addition of apoptotic stimuli (11,28). Bax and Bcl-2 have been shown to respectively promote and prevent cell death in response to the absence of trophic stimulation (40). In a comprehensive study on the developing rat brain Mooney and Miller (41) reported that the timing of the subsequent expression and ratio of different members of Bcl family of proteins and caspase-3 elevation remarkably coincided with episodes of developmental cell death. The survival of DRG neurons is dependent on the availability of NGF for a transient period early in development after which these neurons become independent of NGF and survival seems to occur *via* control of the relative levels of expression of members of the *bcl-2* gene family (42).

## APOPTOTIC CELL DEATH INDUCED BY VARIOUS STIMULI

### *Traumatic brain injury: head trauma*

Children younger than 6 years of age sustain traumatic brain injury more frequently than any other age group. Clinical observations suggest that age decidedly influences morbidity and mortality after head injury in children, with those less than 4 years of age developing worst outcomes (43-45). Brain damage resulting from mechanical trauma can be classified into primary damage occurring shortly after impact, and secondary or delayed damage, which may appear several hours or even days later (46). It has been recently found that concussive head trauma in infant rats can trigger both excitotoxic and apoptotic cell death process (46-48). These two cell processes can be distinguished from one another quite readily by several criteria that were developed in a separate study where ultrastructural evaluation of the degenerative process proved to be of strategic value for determining the specific nature of cell death (16,49). Excitotoxic cell death occurs only at the local side of concussive impact and transpires rapidly in a 4 hour period. Apoptotic cell death process occurs in a disseminated manner and evolves on a more delayed schedule over 6 to 24 hour period and at more distant sites. It affects substantially larger number of neurons. Ultrastructurally, the sequence and types of morphological changes that characterize excitotoxic cell death are strikingly different from the sequence and types of changes that characterize apoptotic cell death (49). The two processes could be readily distinguished also by their response to treatment. N-methyl D-aspartate (NMDA) glutamate receptor antagonists protected against the excitotoxic cell death process (47) but exacerbated the apoptotic cell death process (48). Activation of caspase-3 appears also to play a role (50). Although apoptosis may have contribution to post-traumatic deficits, excitotoxic cell death is considered to have a primary role (18). Importantly, apoptotic damage shows a clear-cut dependency from age in that the younger animals (3-14 postnatal days) demonstrate increased vulnerability compared to 30-day old animals where virtually no apoptotic lesions are detected after the same amount of impact force. It raises also the intriguing question for prevailance of an apoptotic cell death process after traumatic injury in the developing CNS *versus* the prevailance of a non-apoptotic cell death process in the adult CNS (46). The above findings may have important implications for the clinical management of pediatric head trauma in that apoptotic cell death is by far the dominant cell mechanism contributing to the deletion of neurons and anti-apoptotic therapy in such cases may prove to be beneficial. It questions also the practice of use of ketamine (a proven NMDA antagonist) during the stabilization period immediately following head trauma (46). On the other hand,

the delayed nature of apoptotic neurodegeneration following head trauma suggests that there may be a wide time window for therapeutic intervention.

### *Traumatic brain injury: spinal cord trauma.*

Apoptotic cells have been identified by immunostaining for activated caspase-3 and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) in neurons and oligodendrocytes in adult mice after spinal cord contusion (51). It has also been suggested that caspase protease activities may be involved in delayed neuronal and glial cell apoptosis and this increased activity can be reduced by specific inhibitors. However, there are also arguments that such a therapeutic stimulus may not be effective in improving functional and histopathological outcomes following spinal cord injury. Ozawa *et al* (52) documented no significant differences in the BBB locomotor scores and contusion volumes between animals receiving specific caspase inhibitors and animals receiving vehicle alone. Further studies including electron microscopic observation of characteristic features of apoptosis suggested that peroxynitrite is capable of inducing neuronal apoptosis indicating its role especially in secondary spinal cord lesions (53). The study suggests that removal of peroxynitrite should be considered as a therapeutic approach. Similar to cortical neurons, it has been suggested that motoneurons, which die following neonatal nerve injury do so through an excitotoxic mechanism. Lawson and Lowrie (54), based on quantitative analysis and TUNEL (later proven to be a non-selective apoptosis marker) after peripheral nerve injury, discussed the possibility that excitotoxicity induces motoneuron death by apoptosis. They concluded that all motoneuronal death induced during the first 12 days after injury occurs by apoptosis, the active process being recognizable only for 2 hours. Treatment with a NMDA receptor antagonist has reduced the level of cell death. However, ultrastructural data demonstrating neuronal cells with features characteristic for apoptosis have not been presented to verify the nature of the cell death process.

### *Hypoxia/ischemia (HI)*

The results of morphological, histochemical, and molecular studies indicate that in neonatal animal models apoptotic mechanisms may account for neuronal death after cerebral HI (55-59). A human study demonstrated evidence for apoptotic forms of cell death after hypoxic injury, similar to those found in brains from stillbirths (60). In addition *in vitro* evidence indicated to an increased susceptibility to apoptosis in immature cortical neurons (61). Nakadjima *et al* (56) provided a detailed light microscopic (cells with activated caspase-3) and also electron microscopic data for a contribution of apoptotic cell death process to the post-ischemic lesions in a P7 *in vivo* rat model. Western blot analysis showed that activation of caspase-3 protein persists for a protracted period after HI injury, suggesting an extended therapeutic window in which caspase inhibitors may reduce or prevent brain damage after neonatal asphyxia. These findings are consistent with other studies showing a delayed caspase-3 activation and neuroprotection with caspase-3 inhibitors (62). Olney *et al* (59) induced HI (by means of carotid artery ligation) in a 7 day old infant rat CNS and found that it caused a primary wave of excitotoxic degeneration that transpired in 4 hours and destroyed retinal neurons and cells in several brain regions on the ipsilateral side. This was followed by a delayed wave of apoptotic neurodegeneration (confirmed by electron microscopy) within 16-24 hours and affected in addition specific contralateral brain regions such as the lateral geniculate nucleus and superior colliculus, areas that are heavily innervated by retinal ganglion cells destroyed by the excitotoxic process. These data imply that when HI occurs during synaptogenesis, it can destroy various developing neurons both by excitotoxic and apoptotic cell death process (55,59). However, the primary and direct effect is excitotoxic and mediated by the excessive activation of glutamate receptors leading to the destruction of neurons (59). Loss of these neurons causes deafferentation-mediated apoptotic cell death at synaptic targets.

In the adult mammalian brain apoptotic cell death in HI has been suggested by several research groups. In 1993 MacManus *et al* (63) and in 1994 Kihara *et al* (64), mainly on the basis of evidence from DNA laddering, concluded that there is an apoptotic component in the selective neuronal death following global ischemia in rat and gerbil brain. Later it was shown that delayed neuronal death after transient cerebral ischemia may be mediated, in part, by the induction of apoptosis-regulatory gene products (62). At 8-72 hour interval after ischemia, caspase-3 mRNA and protein were induced in the hippocampus and caudate-putamen accompanied by increased caspase-3-like protease activity in degenerating CA1 neurons. Double-label experiments have detected DNA fragmentation in the majority of neurons that overexpressed caspase-3. Application of Z-DEVD-FMK, a caspase-3 inhibitor, decreased caspase-3 activity and significantly reduced cell death and DNA fragmentation. Similar data were reported in the adult mouse brain after temporary middle cerebral artery occlusion (65). Caspase-like enzyme activity was elevated in brain homogenate shortly after reperfusion and reached a peak within 30 to 60 min. Activated caspase-3 immunoreactivity became prominent within the middle cerebral artery territory at the time of reperfusion and 1-12 hours later. Although no electron microscopic evidence for apoptotic morphology was presented and DNA laddering and TUNEL were the only reference standards, both groups pointed to the existence of a time-dependent evolution of the ischemic injury characterized by the close correspondence between caspase-like enzyme activation and an associated increase in activated caspase-3

immunoreactivity. On the other hand, it has been shown that other cysteine proteases such as calpain and cathepsin may also have a role in delayed ischemic neuronal cell death that is not represented by morphological features of apoptosis (66,87). Given the fact that caspase-3 and calpain share common substrates in neurons, and calpains appear to be involved in the activation of caspase-3 in the immature CNS (67), a mutual communication during the execution of the cell death process after ischemia has been predicted.

The effect of perinatal asphyxia in the spinal cord in the neonatal animals has also been investigated (68). A substantial increase in TUNEL and caspase-3 positive cells identified as microglia and oligodendrocytes has been established in laminae I-III of the lumbar spinal cord of rats at different postnatal days following ischemia. In the adult rat, the association of motor neuron cell death with activation of caspases after transient ischemia of the spinal cord was reported (69).

In conclusion, despite emerging data for a possible contribution of an apoptotic mechanism of cell death in selected brain regions after ischemic injury in the adult CNS, it appears that post HI apoptosis may play a more extensive role in the neonatal mammalian brain.

### *Adrenal hormones and neuronal cell survival*

Searching the literature for evidence of apoptotic neurodegeneration in the adult mammalian CNS, the only well characterized example was found in a paper published by Sloviter *et al* in 1993 (70). The authors induced degeneration of hippocampal granule neurons in the adult rat brain by surgical removal of the adrenal glands. The morphological (including electron microscopy) features of this process were virtually identical to those classically described by Kerr *et al* (7). The authors concluded also that adult hippocampal granule neurons require circulating adrenal hormones for survival. This finding puts forward another important question: do mature neurons require survival signals to maintain their viability? Since cell death during aging, neurodegenerative disease or injury could result from disturbances of the same cell-to-cell interactions in vogue during development it is important to know how trophic requirements change throughout aging. As shown by Sloviter *et al* (70), it appears that with limited exceptions developing neurons express an age-dependent decline in trophic signal dependence (11).

## DRUG-INDUCED APOPTOTIC NEURODEGENERATION

### *Potential of NMDA antagonists as inducers of apoptotic neurodegeneration during development*

As glutamate promotes certain aspects of neuronal development such as migration, differentiation, survival and plasticity, evidence that even transient inactivation of NMDA glutamate receptors can be lethal has been provided. In 1999 Ikonomidou and colleagues reported that during a specific stage in ontogenetic development (between E21 and P14) coinciding with the period of NMDA receptor hypersensitivity, transient blockade of NMDA receptors with MK801 triggers a huge wave of apoptotic neurodegeneration in the cortex and brainstem (almost 40 fold in laterodorsal thalamus at P7)(22). In addition, phencyclidine and ketamine (NMDA antagonists) were also shown to be capable of triggering a robust neurodegenerative response (22). Non-NMDA antagonists, antagonists to the cholinergic muscarinic receptors and dopamine receptor antagonists failed to do so. Distinct patterns of neurodegeneration were observed at different developmental ages which suggested that the time window of vulnerability to the apoptosis-inducing action of NMDA antagonists coincides with the period of synaptogenesis, also known as the brain growth spurt period. In the human it spans the last three months of pregnancy and lasts until the first several years of postnatal life (71). These studies also suggested that as different combinations of neuronal groups become susceptible to apoptotic cell demise depending on the period of NMDA receptor deprivation, this neurodegenerative mechanism has the potential to induce a variety of neurobehavioral disturbances in later life.

### *Potential of GABA mimetics as inducers of apoptotic neurodegeneration during development*

The fact that blockade of NMDA receptors during synaptogenesis was capable of inducing a substantial apoptotic neurodegeneration raised the intriguing question of whether interference in other transmitter systems during the same period might also trigger apoptotic cell death in the CNS. To explore this possibility Ikonomidou *et al* (72) administered numerous agents that interact selectively with various transmitter receptor systems in the *in vivo* developing rodent brain. They were able to show that only agents that mimic or potentiate the action of GABA or GABA-A receptors (benzodiazepines and barbiturates) such as phenobarbital, pentobarbital, diazepam and clonazepam, all in a dose-dependent manner, triggered widespread cell death in the infant rat brain which by various analyses including ultrastructural, was apoptotic. NMDA antagonists and GABA mimetic drugs both reduced neuronal activity. The authors also tested drugs that block sodium channels thereby reducing sustained repetitive neuronal firing and found that valproate and phenytoin were capable of triggering widespread apoptosis in the infant rodent brain (72,73).

### *Antiepileptic drugs and apoptotic neurodegeneration*

Some of the drugs that were shown to cause massive apoptotic cell death in the developing brain are currently used in large doses as anticonvulsants for pregnant mothers and infants that suffer from seizures. Epilepsy is the most common neurological disorder of youth and antiepileptic drugs are excessively used worldwide as a treatment of choice. Unfortunately, they

have been reported to cause cognitive impairment (74-76), microcephaly (77-79) and other birth defects. The cause of these side effects of therapy with these drugs is unknown. Because depression of synaptic transmission is the common denominator in the action of antiepileptic drugs Bittigau *et al* (80) postulated that drugs currently used to prevent or interrupt seizures such as phenytoin, valproate, phenobarbital, pentobarbital, diazepam, clonazepam and vigabatrin may stimulate apoptotic cell death during the brain growth spurt. Indeed, the results showed that at plasma concentrations relevant for seizure control in humans, each drug induced wide-spread apoptotic neurodegeneration in the rat brain which in some regions such as the thalamus reached up to 33-fold increase compared to vehicle controls (80). In addition, these drugs caused decrease in brain weight. Interestingly, combinations of antiepileptic drugs (eg. phenobarbital and diazepam) caused more pronounced neurotoxic effect. This finding is especially valuable as it offers one possible explanation for the increased risk for cognitive impairment associated with drug polytherapy. Another important finding in these studies was the ability of antiepileptic drugs to depress endogenous neuroprotective systems such as BDNF and NT-3. Neuronal death was associated with reduced expression of neurotrophins and decreased concentrations of survival-promoting proteins. Beta-estradiol, which stimulates pathways that are activated by neurotrophins, ameliorated this apoptotic neurodegeneration. Estrogen injected *in vivo* also diminished the detrimental effects of phenobarbital and phenytoin. These findings present one possible mechanism to explain cognitive impairment and reduced brain mass associated with prenatal or postnatal exposure of humans to antiepileptic therapy. They also imply that measures that promote neurotrophin signaling and β-estradiol replacement may offer a novel adjunctive neuroprotective approach in the prophylaxis of antiepileptic drug-related brain defects.

### *Potential of common anesthetics as inducers of widespread apoptotic neurodegeneration in the developing brain*

Anesthetics currently used in pediatric surgery act by two principal mechanisms: an increase in inhibition *via* GABA-A receptors (benzodiazepines, barbiturates, propofol, isofurane, halotane)(81) and a decrease in excitation through NMDA receptors (ketamine, nitrous oxide, xenon)(82). As it was shown that such agents, applied during a vulnerable period of brain synaptogenesis may induce a widespread apoptotic neurodegeneration, Jevtovic-Todorovic *et al* (83) addressed the potential risk in the developing brain by administering the most commonly used anesthetic cocktails in pediatric anesthesia to 7 day old infant rats. They found that anesthetic drugs either alone, but most impressively in combination (midazolam, nitrous oxide and isofurane), in doses sufficient to maintain a surgical plane of anesthesia for several hours, caused wide-spread apoptotic cell death, deficits in hippocampal synaptic function, and persistent memory and learning impairment.

### *Potential of ethanol to induce apoptotic neurodegeneration in the developing brain*

Ethanol has NMDA antagonist properties and is also a positive modulator of GABA-A receptors (84-86). This evidence prompted the evaluation of the ability of ethanol to mimic the proapoptotic effects of NMDA antagonists and GABA mimetics. As postulated, ethanol administration in rats and mice during the first and second postnatal weeks leads to an even more robust response than that triggered by NMDA antagonists or GABA mimetics alone (17,72). It also showed a substantial decrease in cingulate cortical mass and *corpus callosum* size (17). Electron microscopy revealed that ethanol-induced degenerative response conforms to the criteria of apoptotic cell death developed by Kerr *et al* (7) and Ishimaru *et al* (49). In fact, the ability of ethanol to trigger a massive wave of apoptosis in the developing mammalian brain provided the opportunity to study the *in vivo* apoptotic degeneration in fine detail not only in the cortex, but also in the brain stem, cerebellum and spinal cord (16,88). It also enabled us to compare our ultrastructural findings with those described by others in neuronal regions such as sympathetic and dorsal root ganglia, hippocampus, and granule cells in the cerebellum (9,15,16,70,89). The electron microscopic evidence provided by these sources under experimental conditions that were quite different from ours, revealed remarkable similarities to the sequence and types of changes described by us. A comparison of the pattern of neurodegeneration induced by alcohol with that induced by NMDA antagonists or GABA mimetics revealed that the ethanol pattern comprised a composite of the NMDA antagonist and GABA mimetic patterns (17,23). Moreover, within the brain growth spurt period different neuronal populations became sensitive at different times to the mechanism by which ethanol triggers apoptotic degeneration.

The ethanol findings are of particular interest in that exposure of the human fetus to ethanol causes a dysmorphogenic neurological syndrome known largely as Fetal Alcohol Syndrome (FAS), Fetal Alcohol Effects (FAE)(90,91) or Fetal Alcohol Spectrum Disorders (FASD)(92). Alcohol Related Neurodevelomental Disorder (ARND) is a term recently recommended to describe partial syndromes not fully developed as FAS (93). The most disabling features of FAS/FAE/FASD are neurobehavioral disturbances, learning disabilities, depression and psychosis (23). While other mechanisms may also play a role, the fact that ethanol may cause a widespread and large-scale apoptotic neurodegeneration throughout the entire neuraxis during the synaptogenesis period, provides a more likely explanation than has been available so far for the reduced brain mass and major neurobehavioral disturbances associated

with FASD (17).

## APOPTOSIS IN NEURODEGENERATIVE DISEASES

It is perhaps natural to expect that the nervous system's ability to conduct graceful and orderly removal of cells during development might reemerge during injury or the onset and progression of certain neurodegenerative diseases. Despite the fact that in the recent neuroscience literature, it is increasingly being suggested that an apoptotic mechanism may underline a wide variety of disease processes affecting the human central and peripheral nervous systems (11,13,21,24,94-96), to date the role of apoptotic cell death in neurodegenerative disorders still remains controversial. Concluding evidence supporting the involvement of apoptosis in the pathogenesis of any of the chronic neurodegenerative diseases is less than compelling. Many agree that there are several important reasons for this state of affairs:

1. Contrary to animal studies, it has been a particular problem for human studies that almost all brain tissues are obtained postmortem and are thus harvested late in the course of the disease. Persons dying late in the course of their illness have a large scale of perimortem morbidity and various uncontrolled factors such as hypoxia, dehydration, sepsis, *etc*, may affect the results of a postmortem examination especially by pathomorphological methods. Additionally, in a recent review on apoptosis in Alzheimer's disease Roth (13) suggested that "considering the relatively brief period during which a cell appears apoptotic and the chronicity of the neurodegenerative disease, the number of apoptotic neurons in AD brain at any given time may be quite low".

2. Another problem is still the lack of validated methods or agreed upon criteria for identifying neuronal apoptosis and distinguishing this cell death process from myriad of others that occur in the *in vivo* mammalian brain. The term "*apoptosis*" as introduced by Kerr and colleagues in the early 70s (7) refers to a cell death process that exhibits specific morphological ultrastructural features. On the basis of these features it was hypothesized that all cell death processes can be placed in two categories - apoptosis and necrosis. However, based on similar strict morphological and ultrastructural criteria, Olney and colleagues have shown that excitotoxic cell death does not conform to the Kerr and Wyllie criteria for either of these two cell death processes. Other scientists have described excitotoxic cell death as a necrotic process, as an apoptotic process or as a hybrid mixture of the two. It has not yet been clarified (at least in the nervous system) how these phenomena match the ultrastructural description given by the original coiners of the term apoptosis. To add to the existing confusion, various other terms such as "excitotoxic apoptosis" or "pathological apoptosis" have been introduced.

3. It is only recently that molecular genetics, molecular biology and physiology have started to provide more reliable complex criteria for diagnosing apoptosis. For example, it is now recognized that the TUNEL staining is not specific for apoptosis; it labels also cells that are degenerating by non-apoptotic mechanism (13,49,87). However, until the mid-90$^{ies}$, the majority of studies in this field heavily relied on TUNEL staining and DNA-laddering and only few have applied ultrastructural morphological methods.

4. Much of the research pertaining to CNS apoptosis has been conducted in cell cultures and despite the wealth of information that they have provided especially on the mechanisms of apoptotic cell death, these studies are of uncertain relevance to the *in vivo* adult disease process.

5. In each particular disease model of substantial neuronal or glial cell loss that leads to clinically expressed neurodegenerative syndrome, it is strategically important to ask whether the timing and the number of cells that are involved in each particular model is sufficient to consider apoptosis a key element of a disease process.

Eight years ago in this journal, Timmers and Kremer (95), while discussing the controversies linked to the role of apoptosis concluded, that "absence of proof is not proof of absence". Probably we still navigate in similar territories. However, our knowledge has vastly improved and new evidence for the involvement of apoptotic cell death in neurodegenerative diseases has generated renewed attention and is widely discussed in the literature.

### *Potential for apoptosis in amyotrophic lateral sclerosis*

ALS or Lou Gehrig's disease represents a neurodegenerative disorder accompanied by a progressive loss of upper and lower motor neurons. It results in a severe disability, progressive paralysis and death. The discovery of superoxide dismutase (SOD) mutations as a cause for some cases of familial ALS (97,98) has supported a role of apoptosis since the effect of the mutation is to convert an antioxidant defense enzyme into a proapoptotic compound (21,24,99). Transgenic mice have been generated expressing different *mSOD1* genes identified in ALS patients (100). Like humans with ALS, these mice develop an adult-onset progressive motor deterioration (101,102). Although the mechanism leading to motor neuron loss are not thoroughly understood it has been proposed that caspases -1 and -3 play an instrumental role in the neurodegeneration process (103,104) suggesting that caspase inhibition may have a therapeutic role.

### *Potential for apoptosis in Parkinson's disease (PD).*

Apoptosis and oxidative stress have been implicated in the progressive loss of dopaminergic neurons of *substantia nigra* in PD (24,105-107). Data from two postmortem studies have shown contradicting results. Marshal *et al* (108) reported that Bcl-2 is significantly raised in the basal ganglia regions of PD patients as compared to age-matched controls. A similar trend was also found in Lewy Body disease and proposed that Bcl-

2 increases in some brain regions as an early event and that these brain regions are under a stress for many years before any symptomatic changes occur. On the other hand, Vyas *et al* (109) reported that the expression of Bcl-2 protein by Western blot analysis and immunohistochemistry was unaltered in the nucleus basalis of Meynert and *substantia nigra*. However, the same group provided morphological evidence for apoptosis in melanized neurons of the *substantia nigra* in PD patients (110). Mogi *et al* (111) reported that the soluble form of Fas molecule is elevated in the tissues homogenates from nigro-striatal dopaminergic regions of PD brains.

### *Potential for apoptosis in Alzheimer's disease (AD)*

Do neuronal cells degenerate via apoptotic or non-apoptotic pathway in AD patients? Extensive neuronal loss occurs in AD brains and some authors have speculated that deregulation of apoptotic death pathways is etiologically responsible for the disease development (see ref. 13 for an extensive review). It has been suggested that apoptosis operates as a mechanism of cell death for at least some neurons in AD and that this process may not be restricted to neurons, but may also occur in microglia (112). Lack of morphologically convincing apoptotic neurons in the vast majority of AD brains has led to the revised hypothesis that apoptosis-associated molecular events cause neuronal dysfunction in the absence of, or prior to, neuronal death (13). Proof of apoptotic cell death mechanism in AD brain tissue has been based mainly on the demonstration of TUNEL-positive staining (113,114) and DNA laddering and only lately on correlations with apoptotic markers such as Bcl-2, Bax, c-Jun, Fos (24,112). Activated caspase-3 immunoreactivity has been reported only in single hippocampal neurons undergoing granulovacuolar degeneration but not in senile plaques or neurofibrillary tangles. (13,115). Based on this and evidence from AD mouse models, it was suggested that activated caspase-3 does not appear to have a significant role in the widespread neuronal cell death that occurs in AD, but may contribute to the specific loss of hippocampal neurons involved in learning and memory. Ultrastructural evidence has been even less compelling as much of the information on cell death in AD has been gathered from the endpoints of the disease process when various other factors may have intervened, most notably the process of dying. To overcome this, over the past decade it has been of crucial importance for neuroscientists to model the beginning of AD pathology and, particularly, the subclinical pathology that exists in patients at an early age. The way to circumvent this problem has been the creation of *in vitro* and *in vivo* models of AD pathology in animals and human-derived tissue. At least 7 different caspases (including caspases -3, -8 and -9) have been implicated in regulating neuronal cell death in response to amyloid-beta exposure *in vitro*, in animal models of neurodegenerative diseases, and in AD brain. The data from such studies have not been encouraging despite the impressive flood of information implicating caspases and apoptosis as contributing etiologic factors. For example, although it has been suggested that presenilin genes and amyloid precursor protein (APP) mutations may be involved in the apoptotic process (116), amyloid-beta deposition in the APP transgenic mouse model of AD did not result in caspase-3 activation despite the ability of amyloid-beta to induce caspase-3 activation and neuronal apoptosis *in vitro* (115). There is little evidence to date for the involvement of an apoptotic neurodegenerative process in genetic animal models based on similar mutations (24,117). Thus the direct involvement of caspase-dependent neuronal apoptosis in AD pathogenesis is still uncertain. On the other hand, based on evidence stemming from culture experiments, neuronal apoptosis might be induced by a variety of stimuli and many of these conditions are known to be present in the AD brain. For example, application of amyloid-beta has resulted in apoptosis-like morphology (118,119) and the induction of immediate early gene proteins (120). Other potential inducers of apoptotic neuronal death include oxidative insults and damage, sensitizing the cell to trophic factor deficiency, altering APP metabolism, protein trafficking and G-protein related mechanisms (see also 24,113). However there is yet no firm evidence to support apoptotic cell death with *APOE* allele 4 (associated with a high risk for AD) although *in vitro* evidence suggests that APOE and APP can interact to produce increased oxidative damage to neurons (121). Despite these discouraging and controversial developments in AD apoptology, Roth (13) suggested the possibility that apoptotic neuronal death may contribute to neuronal loss only in a tertiary fashion. He speculated that there is a sub-lethal increase in pro-apoptotic molecules and if pro-death stimuli persist, cell death will ultimately occur and "caspases may be involved in the last stage of the disease process". If so, caspase inhibition offers some hope for extending neuron survival so that other agents, targeting upstream events, may delay or possibly reverse AD pathology.

### *Potential for apoptosis in spinal muscular atrophy (SMA)*

In this severe human disorder PCD that occurs in spinal motoneurons during normal development continues unabated in SMA patients resulting in massive motoneuron death and subsequent respiratory failure. SMA or Werdig-Hoffmann disease as it is otherwise known is probably the most severe form in the human neurodegenerative disease family. It leads almost certainly to death by the age of three. In approximately 70% of young infants the first two coding exons of the gene for the neuronal apoptosis inhibitory protein (NAIP) at chromosome 5 are deleted (11,24,122). This implies that failure to inhibit PCD because of genetic deficits at an appropriate time during development may be responsible for the massive

cell loss as seen in SMA patients. It shows also that absence of apoptotic cell death in the mature nervous system is likewise dependent on active genetic mechanisms that are anti-apoptotic in nature.

### *Potential for apoptosis in Battens's disease*

The most convincing case for an involvement of apoptosis in human disease reported so far pertains to Battens disease, also termed neuronal ceroid lipofuscinosis (24). The disease is characterized by massive neuronal cell death and, in most cases, death of photoreceptors in the retina with a resultant blindness. Recent studies have shown that cell deletion is accompanied by elevation of Bcl-2 and ceramide levels, implicating a close relationship to the regulation of apoptotic cell death process. The discovery of the responsible gene *CLN3* on chromosome 16 shows that a product of this gene, a 438-aminoacid protein is operative in a novel antiapoptotic pathway. The human disease is associated with deletions of the *CLN3* gene, which apparently results in loss of function.

## CONCLUSION

The last case in the present review illustrates the sheer power of characterizing the precise molecular mechanisms that control the survival of neurons during development and disease. PCD is regulated by key molecular mechanisms that initiate and execute cell death. PCD and induced apoptotic neuronal cell death share common molecular steps. The recent characterization of such mechanisms has resulted in the identification of various molecules that may arrest disease-related cell loss. A deeper understanding of these processes may lead to the discovery of multiple strategies for slowing, reducing or even arresting neuronal and glial cell death induced by injury, aging or disease.

## ACKNOWLEDGMENTS

I wish to thank Dr John Olney and Dr David Wozniak from the Department of Psychiatry at Washington University School of Medicine, St. Louis for their insights into the diverse aspects of neuronal cell death and for their stimulating discussion in preparing the manuscript.

## REFERENCES

1. Flemming W. Uber die Bildung von Richtungsfiguren in Saugethieriern beim Untergang Graafscher Follikel. *Arch Anat Entw Gesch* 1885; 221-244.
2. Mayno G, Joris I. Apoptosis, oncosis and necrosis. A review of cell death. *Am J Pathol* 1995; 146: 3-15.
3. Gräper L. Eine neue Anschaung über physiologische Zellausschaltung. *Arch Zellfoesch* 1914; 12: 373-394.
4. Hamburger V. The effects of wing bud extirpation on the development of the central nervous system in chick embryos. *J Exp Zool* 1934; 68: 449-494.
5. Hamburger V, Levi-Montalcini R. Proluferation, differentiation, and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J Exp Zool* 1949; 111: 457.
6. Glucksman A. Cell death in normal vertebrate ontogeny. *Biol Rev* 1951; 26: 59-86.
7. Kerr J, Wyllie A, Currie A. Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239-257.
8. Wyllie A, Kerr J, Currie A. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; 68: 251-306.
9. Sabatini M, De Iraldi A, De Robertis E. Early effects of antiserum (AS) against the nerve growth factor (NGF) on the structure of sympathetic neurons. *J Exp Neurol* 1965; 12: 370-383.
10. Levi-Montalcini R, Caramia F, Angeletti PU. Alterations in the fine structure of nucleoli in sympathetic neurons following NGF-antiserum treatment. *Brain Res* 1969; 12: 54-73.
11. Burek M, Oppenheim R. Programmed cell death in the developing nervous system. *Brain Pathol* 1996; 6: 427-446.
12. Narayanan A. Cell death in the developing nervous system. In: Hannun Y and Boustany R, editors. *Apoptosis in Neurobiology*. CRS Press. 1999; 13-24.
13. Roth K. Caspases, apoptosis, and Alzheimer disease: causation, correlation, and confusion. *J Neuropathol Exp Neurol* 2001; 60: 829-838.
14. Farber E. Ideas in Pathology. Programmed cell death: necrosis versus apoptosis. *Modern Pathol* 1994; 7: 605-609.
15. Clarke P. Apoptosis versus necrosis: how valid a dichotomy for neurons? In: Koliatsos VE and Ratan RR, editors. *Cell Death and Diseases of the Nervous System*. Humana Press, Totowa, NJ, 1999.
16. Dikranian K, Ishimaru M, Tenkova T, Labuyere J, Qin Y, Ikonomodou C, *et al*. Apoptosis in the in vivo mammalian forebrain. *Neurobiol Dis* 2001; 8: 359-379.
17. Olney JW, Tenkova T, Dikranian K, Labruyere J, Qin Y, Ikonomidou C. Ethanol-induced apoptotic neurodegeneration in the developing C57BL/6 mouse brain. *Dev Brain Res* 2002; 133: 115-126.
18. Olney JW. Excitotoxicity, apoptosis and neuropsychiatric disorders. *Cur Oppin Pharmacol* 2003; 3: 1-9.
19. Sloviter R. Apoptosis: a guide for the perplexed. *Trends Pharmacol Sci* 2002; 23: 19-24.
20. Rakic S, Zecevic N. Programmed cell death in the human telencephalon. *Eur J Neurosci* 2000; 12: 2721-2734.
21. Bredesen D. Neural apoptosis. *Ann Neurol* 1995; 38: 839-851.

22. Ikonomidou C, Bosch K, Miksa F, Bittigau M, Vockler P, Dikranian K, *et al*. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999; 283: 70-74.
23. Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, *et al*. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* 2000; 287: 1056-1060.
24. Schmechel, D. Apoptosis in neurodegenerative disorders. In: Hannun Y and Boustany R, editors. *Apoptosis in Neurobiology*. CRS Press. 1999; 23-47.
25. Barres B, Hart I, Coles H, Burne J, Voyvodic J, Richardson W, *et al*. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 1992; 70: 31-46.
26. Ciutat D, Caldero J, Oppenheim RW, Esquerda JE. Schwann cell apoptosis during normal development and after axonal degeneration induced by neurotoxins in the chick embryo. *J Neurosci* 1996; 16: 3979-3990.
27. Krueger BK, Burne JF, Raff MC. Evidence for large-scale astrocyte death in the developing cerebellum. *J Neurosci* 1995; 15: 3366-3374.
28. Deckwerth TL, Johnson EM Jr. Neurotrophic factor deprivation-induced death. *Ann NY Acad Sci* 1993; 28: 679: 121-131.
29. Johnson EM Jr, Deckwerth TL. Molecular mechanisms of developmental neuronal death. *Annu Rev Neurosci* 1993; 6: 31-46.
30. Deshmikh M, Johnson EM Jr. Programmed cell death in neurons: focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. *Mol Pharmacol* 1997; 51: 897-906.
31. Cowan WM, Fawcett JW, O'Leary DM, Stanfield BB. Regressive events in neurogenesis. *Science* 1984; 262: 689-695.
32. Bennet, MR, Gibson WG, Lemon G. Neuronal cell death, nerve growth factor and neurotrophic models: 50 years on. *Autonomic Neurosci: Basic and Clinical* 2002; 95:1-23.
33. Chang LK, Putcha GV, Deshmukh M, Johnson EM Jr. Mitochondrial involvement in the point of no return in neuronal apoptosis. *Biochimie* 2002; 84: 223-231.
34. Baloh RH, Enomoto H, Johnson EM Jr, Milbrandt J. The GDNF family ligands and receptors - implications for neural development. *Curr Opin Neurobiol* 2000; 10: 103-110.
35. Oppenheim RW, Houenou LJ, Parsadanian AS, Prevette D, Snider WD, Shen L. Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: regulation of programmed cell death among motoneuron subtypes. *J Neurosci* 2000; 20: 5001-5011.
36. Bredesen D. Apoptosis: overview and signal transduction pathways. *J Neurotrauma* 2000; 17: 801-810.
37. Kuida K, Haydar T, Kuan C, Gu Y, Taya C, Karasyama H, *et al*. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase-9. *Cell* 1998; 94: 325-337.
38. Mesner PW, Kaufmann S. The role of proteases in neuronal apoptosis. In: Hannun Y and Boustany R, editors. *Apoptosis in Neurobiology*. CRS Press. 1999; 73-103.
39. Thornberry N, Lazebnik Y. Caspases: enemies within. *Science* 1998; 281: 1312-1316.
40. Deckwert T, Elliot J, Knudson C, Johnson EM Jr, Snider W, Korsmeyer S. Bax is required for neuronal cell death after trophic factor deprivation during development. *Neuron* 1996; 17: 401-411.
41. Mooney S, Miller M. Expression of bcl-2, bax and caspase-3 in the brain of the developing rat. *Dev Brain Res* 2000; 123: 103-117.
42. Vogelbaum A, Tong J, Rich K. Developmental regulation of apoptosis in dorsal root ganglion neurons. *J Neurosci* 1998; 18: 8928-8935.
43. Adelson P, Kochanek P. Head injury in children. *J Clin Neurol* 1998; 13: 2-15.
44. Brink J, Garret A, Hale W. Recovery of motor and intellectual function in children sustaining severe head injuries. *Dev Med Child Neurol* 1970; 12: 565-571.
45. Diamond C. Brain injury in the Commonwealth of Virginia: an analysis of central registry data 1980-1993. *Brain Injury* 1996; 10: 413-419.
46. Bittigau P, Sifringer M, Pohl D, Stadthaus D, Ishimaru M, Shimizu H, *et al*. Apoptotic neurodegeneration following trauma is markedly enhanced in the immature brain. *Ann Neurol* 1999; 45: 724-735.
47. Ikonomidou C, Qin Y, Labruyere J, Kirby C, Olney JW. Prevention of trauma-induced neurodegeneration in infant rat brain. *Pediatr Res* 1996; 39: 1020-1027.
48. Pohl D, Bittigau P, Ishimaru M., Stadthaus D, Hubner C, Olney J, *et al*. N-Methyl-D-Aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain. *Proc Natl Acad Sci USA* 1999; 96: 2508-2513.
49. Ishimaru M, Ikonomidou C, Tenkova T, Dikranian K, Sesma M, Olney J. Distinguishing excitotoxic from apoptotic neurodegeneration in the developing brain. *J Comp Neurol* 1999; 408: 461-476.
50. Yakovlev AG, Knoblach SM, Fan L, Fox GB, Goodnight R, Faden AI. Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. *J Neurosci* 1997; 17: 7415-7424.
51. Takagi T, Takayasu M, Mizuno M, Yoshimoto M, Yoshida J. Caspase activation in neuronal and glial apoptosis following spinal cord injury in mice. *Neurol Med Chir* 2003; 43: 20-29.
52. Ozawa H, Keane RW, Marcillo AE, Diaz PH, Dietrich WD. Therapeutic strategies targeting caspase inhibition following spinal cord injury in rats. *Exp Neurol* 2002;

177: 306-313.
53. Bao F, Liu D. Peroxynitrite generated in the rat spinal cord induces apoptotic cell death and activates caspase-3. *Neuroscience* 2003; 116: 59-70.
54. Lawson SJ, Lowrie MB. The role of apoptosis and excitotoxicity in the death of spinal motoneurons and interneurons after neonatal nerve injury. *Neuroscience* 1998; 87: 337-348.
55. Johnston MV, Nakajina W, Hagberg H. Mechanisms of hypoxic neurodegeneration in the developing brain. *The Neuroscientist* 2002; 8: 212-220.
56. Nakajima W, Ishida A, Lange M, Gabrielson K, Wilson Mary A, *et al*. Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. *J Neurosci* 2000; 20: 7994-8004.
57. Macaya A. Apoptosis in the nervous system. *Rev Neurol* 1996; 24: 1356-1360.
58. Mehmet H, Yue X, Squier MV, Lorek A, Cady E, Penrice J, *et al*. Increased apoptosis in the cingulate sulcus of newborn piglets following transient hypoxia-ischemia is related to the degree of high energy phosphate depletion during the insult. *Neurosci Lett* 1994; 181: 121-125.
59. Olney JW, Price M, Labuyere J, Tenkova T. Perinatal hypoxia/ischemia kills neurons directly by excitotoxic necrosis and indirectly by deafferentation apoptosis. *Soc Neurosci Abs* 2000; 26: 1180.
60. Scott RJ, Hegyi L. Cell death in perinatal hypoxic-ischemic brain injury. *Neuropathol Appl Neurobiol* 1997; 4:307-314.
61. McDonald JW, Behrens MI, Chung C, Bhattacharyya T, Choi DW. Susceptibility to apoptosis is enhanced in immature cortical neurons. *Brain Res* 1997; 759: 228-232.
62. Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, *et al*. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci* 1998; 18: 4914-4928.
63. MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston E. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett* 1993; 164: 89-92.
64. Kihara S, Shiraishi T, Nakagawa S, Toda K, Tabuchi K. Visualization of DNA double strand breaks in the gerbil hippocampal CA1 following transient ischemia. *Neurosci Lett* 1994; 175: 133-136.
65. Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, *et al*. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *Neuroscience* 1998; 18: 3659-3668.
66. Tontchev A, Yamashima T. Ischemic delayed neuronal death: role of the cysteine proteases calpain and cathepsins. *Neuropathology* 1999; 19: 356-365.
67. Bloomgren K, Zhu C, Wang X, Karlsson JO, Leverin AL, Bahr BA, *et al*. Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia: a mechanism of "pathological apoptosis"? *J Biol Chem* 2001; 276: 10191-10198.
68. de Louw AJ, de Vente J, Steinbusch HP, Gavilanes AW, Steinbusch HW, Blanco CE, *et al*. Apoptosis in the rat spinal cord during postnatal development; the effect of perinatal asphyxia on programmed cell death. *Neuroscience* 2002; 112: 751-758.
69. Hayashi T, Sakurai M, Abe K, Sadahiro M, Tabayashi K, Itoyama Y. Apoptosis of motor neurons with induction of caspases in the spinal cord after ischemia. *Stroke* 1998; 29:1007-1012.
70. Sloviter R, Dean E, Neubort S. An electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat; apoptosis in the adult central nervous system. *J Comp Neurol* 1993; 330: 337-351.
71. Dobbing J, Sands J. The brain growth spurt in various mammalian species. *Early Human Dev* 1979; 3: 79-84.
72. Ikonomidou C, Genz K, Engelbrechtren S, Dikranian K, Olney J, Bittigau P. Antiepileptic drugs which block sodium channels cause neuronal apoptosis in the developing rat brain. *Soc Neurosci Abs* 2000; 26: 323.
73. Dikranian K, Tenkova T, Bittigau P, Ikonomidou C, Olney JW. Histological characterization of apoptotic neurodegeneration induced in the developing rat brain by drugs that block sodium channels. *Soc Neurosci Abs* 2000; 26: 323.
74. Thorp J, O'Connor M, Jones M., Hoffman E, Belden B. Does perinatal phenobarbital exposure affect developmental outcome at age 2? *Am J Perinatol* 1999; 16: 51-60.
75. Sulzbacher S, Farwell J, Tenkin N, Lu A, Hirtz D. Late cognitive effects of early treatment with phenobarbital. *Clin Pediatr (Philadelphia)* 1999; 7: 387-394.
76. Dessens A, Cohen-Kettenis P, Mellenebrg G, Koppe J, van de Poll N, Boer K. Association of prenatal phenobarbital and phenytoin exposure with small head size and with learning problems. *Acta Paediatrica* 2000; 89: 533-541.
77. Speidel B, Meadow S. Maternal epilepsy and abnormalities of the fetus and newborn. *Lancet* 1972; 2: 839-843.
78. Strickler S, Dansky L, Miller M, Seni M, Andermann E, Spielberg S. Genetic predisposition to phenytoin-induced birth defects. *Lancet* 1985; 2: 746-749.
79. Buehler B, Delimont D, Van Waes M, Finnel R. Prenatal prediction of risk of the fetal hydantoin syndrome. *N Engl J Med* 1990; 322: 1567-1572.
80. Bittgau P, Sifringer M, Genz K, Reith E, Pospishil D, Govoindarajalu S, *et al*. Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc Natl Acad Sci USA* 2002; 99: 15089-15094.
81. Franks N, Lieb W. Molecular and cellular mechanisms of general anesthesia. *Nature* 1994; 367: 607-614.
82. Franks N, Dickinson R, deSausa S, Hall A, Lieb W. How

does xenon produce anesthesia? *Nature* 1998; 396: 324.
83. Jevtovic-Todorovic V, Hartman R, Izumi Y, Benchoff N, Dikranian K, Zorumski Ch, *et al*. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing brain and persistent learning deficits. *J Neurosci* 2003; 23: 876-882.
84. Hoffman PL, Rabe CS, Moses F, Tabakoff B. N-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J Neurochem* 1989; 6: 1937-1940.
85. Lovinger DM, White G, Weight FF. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science* 1989; 243: 1721-1724.
86. Harris RA, Proctor WR, McQuilkin SJ, Klein RL, Mascia MP, Whatley V, *et al*. Ethanol increases GABA-A responses in cells stably transfected with receptor subunits. *Alcohol Clin Exp Res* 1995; 19: 226-232.
87. Yamashima T. Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog Neurobiol* 2000; 62: 273-295.
88. Dikranian K, Labuyere J, Qin Y, Olney JW. Ethanol-induced apoptotic neurodegeneration in the developing rodent cerebellum and brainstem. *Soc Neurosci Abs* 2002: 28:105.
89. Lafarga M, Lerga A, Andres MA, Polanco JI, Calle E, *et al*. Apoptosis induced by methylazoxymethanol in developing rat cerebellum: organization of the cell nucleus and its relationship to DNA and rRNA degradation. *Cell Tissue Res* 1997; 289: 25-38.
90. Jones K, Smith D, Ulleland C, Stressguth A. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1973; 1267-1271.
91. Clarren S, Alford A, Sumi S, Stressguth A, Smith D. Brain malformations related to prenatal exposure of ethanol. *J Pediatr* 1978; 92: 64-67.
92. Barr HM, Streissguth AP. Identifying maternal self-reported alcohol use associated with fetal alcohol spectrum disorders. *Alcohol Clin Exp Res* 2001; 25: 283-287.
93. Stratton K, Howe C, Battaglia F. *Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention and Treatment*. National Academy Press, Washington DC, 1996.
94. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995; 267: 1456-1462.
95. Timmers H, Kremmer B. Apoptosis in neurodegenerative diseases: to be or not to be? *Biomed Rev* 1995; 4: 103-108.
96. Martin LJ. Neuronal cell death in nervous system development, disease and injury. *Int J Mol Med* 2001; 7: 455-478.
97. Rosen DR. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993; 364:362.
98. Deng H, Hentati A, Tainer J. Iqbal Z, Cayabyab A, Hung W, *et al*. Amyotrophic lateral sclerosis and structural defects in Cu/Zn superoxide dismutase. *Science* 1993; 261: 1047-1051.
99. Rabizadeh S, Gralla E, Borchelt D, Gwinn R, Valentine J, Sisodia S, *et al*. Mutations associated with amyotrophic lateral sclerosis convert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: studies in yeast and neural cells. *Proc Natl Acad Sci USA* 1995; 92: 3024-3030.
100. Borchelt D, Wong P, Becher M, Pardo C, Lee M, Xu Z, *et al*. Axonal transport of mutant superoxide dismutase 1 and focal axonal abnormalities in the proximal axons of transgenic mice. *Neurobiol Dis* 1998; 5: 27-35.
101. Gurney M, Pu H, Chin A, Dal Cant M, Polchow C, Alexander D, *et al*. Motor neuron degeneration in mice that express a human Cu/Zn superoxide dismutase mutation. *Science* 1994; 264: 1772-1775.
102. Gurney M, Fleck T, Himes C, Hall E. Riluzole preserves motor function in a transgenic model of familial amyotrophic lateral sclerosis. *Neurology* 1998; 50: 62-66.
103. Martin LJ, Sieber FE, Traystman RJ. Apoptosis and necrosis occur in separate neuronal populations in hippocampus and cerebellum after ischemia and are associated with differential alterations in metabotropic glutamate receptor signaling pathways. *J Cereb Blood Flow Metab* 2000; 20: 153-167.
104. Li M, Ona V, Guegan C, Chen M, Jackson-Lewis V, Andrews L, *et al*. Functional role of caspase-1 and caspase-3 in ALS transgenic mouse model. *Science* 2000; 288: 335-338.
105. Simonian NA, Coyle JT. Oxidative stress in neurodegenerative diseases. *Annu Rev Pharmacol Toxicol* 1996; 36: 83-106.
106. Stern G. Parkinson's disease. The apoptosis hypothesis. *Adv Neurol* 1996; 69: 101-117.
107. Tatton WG, Chalmers-Redman R, Brown D, Tatton N. Apoptosis in Parkinson's disease: signals for neuronal degradation. *Ann Neurol*. 2003; 53 Suppl 3: S61-70.
108. Marshall KA, Daniel SE, Cairns N, Jenner P, Halliwell B. Upregulation of the antiapoptotic protein Bcl-2 may be an early event in neurodegeneration: studies on Parkinson's and incidental Lewy body disease. *Biochem Biophys Res Commun* 1997; 240: 84-87
109. Vyas S, Javoy-Agid F, Herrero MT, Strada O, Boissiere F, Hibner U, *et al*. Expression of Bcl-2 in adult human brain regions with special reference to neurodegenerative disorders. *J Neurochem* 1997; 69: 223-231.
110. Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, *et al*. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histol Histopathol* 1997; 12: 25-31.

111. Mogi M, Harada M, Kondo T, Mizuno Y, Narabayashi H, Riederer P, *et al*. The soluble form of Fas molecule is elevated in Parkinsonian brain tissues. *Neurosci Lett* 1996; 220: 195-198.
112. Cotman C, Su J. Mechanisms of neuronal death in Alzheimer's disease. *Brain Pathol* 1996; 6: 493-506.
113. Su J, Anderson A, Cummings B, Cotman C. Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* 1994; 5: 2529-2533.
114. Smale G, Nichols NR, Brady DR, Finch CE, Horton WE, Jr. Evidence for apoptotic cell death in Alzheimer's disease. *Exp Neurol* 1995; 133: 225-230.
115. Selznick LA, Holtzman DM, Han BH, Gokden M, Srinivasan AN, Johnson EM Jr, *et al*. In situ immunodetection of neuronal caspase-3 activation in Alzheimer disease. *J Neuropathol Exp Neurol* 1999; 58: 1020-1026.
116. Vito P, Wolozin B, Ganjei JK, Iwasaki K, Lacana E, D'Adamio L. Requirement of the familial Alzheimer's disease gene PS2 for apoptosis. Opposing effect of ALG-3. *J Biol Chem* 1996; 271: 31025-31028.
117. Irizarry M, Soriano F, McNamara M, Page K, Schenk D, Games D, *et al*. A-beta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse. *J Neurosci* 1997; 17: 7053-7065.
118. Watt J, Pike C, Walencewicz-Wasserman A, Cotman C. Ultrastructural analysis of beta-amyloid-induced apoptosis in cultured hippocampal neurons. *Brain Res* 1994; 661: 147-156.
119. Loo D, Copani A, Pike C, Whittemore E, Walencewicz A, Cotman C. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci USA* 1993; 90: 7951-7955.
120. Anderson AJ, Pike CJ, Cotman CW. Differential induction of immediate early gene proteins in cultured neurons by beta-amyloid (A beta): association of c-Jun with A beta-induced apoptosis. *J Neurochem* 1995; 65: 1487-1498.
121. Miyata M, Smith J. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nat Genet* 1996; 14: 55-61.
122. Liston P, Roy N, Katsuyuki T, Lefebvre C, Baird S, Cherton-Horvat G, *et al*. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 1996; 379: 349-353.