Drug-induced Apoptotic Neurodegeneration in the Developing Brain

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NMDA	N-methyl-D-aspartate
Glu	glutamate
GABA	gamma-n-butyric acid
PCP	phencyclidine
CPP	carboxypiperazin-4-yl-propyl-1-phosphonic acid
HA/AD	hyperactivity/attention deficit

Physiological cell death (PCD), a process by which redundant or unsuccessful neurons are deleted by apoptosis (cell suicide) from the developing central nervous system, has been recognized as a natural phenomenon for many years. Whether environmental factors can interact with PCD mechanisms to increase the number of neurons undergoing PCD, thereby converting this natural phenomenon into a pathological process, is an interesting question for which new answers are just now becoming available. In a series of recent studies we have shown that 2 major classes of drugs (those that block NMDA glutamate receptors and those that promote GABAA receptor activation), when administered to immature rodents during the period of synaptogenesis, trigger widespread apoptotic neurodegeneration throughout the developing brain. In addition, we have found that ethanol, which has both NMDA antagonist and GABAmimetic properties, triggers a robust pattern of apoptotic neurodegeneration, thereby deleting large numbers of neurons from many different regions of the developing brain. These findings provide a more likely explanation than has heretofore been available for the reduced brain mass and lifelong neurobehavioral disturbances associated with the human fetal alcohol syndrome (FAS). The period of synaptogenesis, also known as the brain growth spurt period, occurs in different species at different times relative to birth. In rats and mice it is a postnatal event, but in humans it extends from the sixth month of gestation to several years after birth. Thus, there is a period in pre- and postnatal human development, lasting for several years, during which immature CNS neurons are prone to commit suicide if exposed to intoxicating concentrations of drugs with NMDA antagonist or GABAmimetic properties. These findings are important, not only because of their relevance to the FAS, but because there are many agents in the human environment, other than ethanol, that have NMDA antagonist or GABAmimetic properties. Such agents include drugs that may be abused by pregnant mothers (ethanol, phencyclidine [angel dust], ketamine [Special K], nitrous oxide [laughing gas], barbiturates, benzodiazepines), and many medicinals used in obstetric and pediatric neurology (anticonvulsants), and anesthesiology (all general anesthetics are either NMDA antagonists or GABAmimetics).

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Introduction

Transplacental exposure of the human fetus to ethanol can cause severe dysmorphogenic and neuropathological effects, including craniofacial malformations and reduced brain mass, which is associated with a variety of neurobehavioral disturbances, ranging from hyperactivity/attention deficit disorder and learning disabilities in childhood (60), to major depressive and psychotic disorders in adulthood (20). While this distinctive clinical picture, as originally described in its fully developed form (40, 41), has come to be known as the fetal alcohol syndrome (FAS), it is now recognized that the fetotoxic effects of ethanol can manifest as a partial syndrome comprised largely of neurobehavioral distubances ranging from mild to severe, unaccompanied by craniofacial malformations. Alcohol Related Neurodevelopmental Disorder (ARND) is a term recently recommended for referring to such partial syndromes (59), and a new term currently emerging to represent all clinicopathological manifestations of ethanol's fetotoxic effects is fetal alcohol spectrum disorder (FASD) (2). Regardless of terminology, disruption in brain development and consequent neurobehavioral disturbances comprise the most debilitating effects of ethanol on the developing human fetus.

Since the FAS was first described 25 years ago, there have been many efforts to reproduce various aspects of

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the syndrome in vivo in laboratory animals, to identify the stage(s) in development when the immature brain is most sensitive to the deleterious effects of ethanol and to gain insight into the underlying mechanism(s). Until recently, success toward meeting these goals was modest, the most reproducible and frequently reported neuropathological finding being that exposure of immature rats to ethanol during the perinatal period causes a significant, but not dramatic, reduction in the number of cerebellar Purkinje cells, and a generalized decrease in brain mass (3, 8, 54, 56), These findings, although promising, fell far short of explaining the brain changes associated with the human FASD. The human syndrome does entail loss of brain mass, but a modest loss of cerebellar neurons could not account for an overall reduction in brain mass, nor for the myriad neurobehavioral disturbances associated with the FASD. Thus, at the turn of the century (and millennium) the mechanism(s) underlying ethanol's injurious effects on the developing brain remained a mystery, and only limited progress had been made in developing suitable animal models for studying these effects.

Recently, we conducted a series of studies that led to the observation that during the developmental period of synaptogenesis, brief exposure to ethanol can trigger widespread apoptotic neurodegeneration in the in vivo mammalian brain (16, 32). Our evidence indicates that ethanol triggers apoptotic neurodegeneration by a dual mechanism-blockade of NMDA glutamate receptors and excessive activation of $GABA_A$ receptors (31, 32). While other mechanisms may play a role, the ability of ethanol to induce widespread apoptotic neurodegeneration throughout the forebrain during the synaptogenesis period, provides a more likely explanation than has heretofore been available for the reduced brain mass and lifelong neurobehavioral disturbances associated with the human FASD. The serendipidous path that led to our ethanol findings began with an inquiry into the relationship between excitotoxic and apoptotic cell death processes, and the role of glutamate signaling in either or both of these processes. Some background information will help place this inquiry in perspective.

Excitotoxic Versus Apoptotic Neurodegeneration

In recent years, there has been a great deal of interest in mechanisms of cell death that may contribute to human neurodegenerative diseases. In this context, considerable confusion has arisen over the relationship between excitotoxic and apoptotic neuronal cell death. Some researchers have reported that an excitotoxic stimulus triggers neuronal apoptosis, others that it does not and still others have claimed that it causes both apoptosis and necrosis. We decided to explore this issue using in vivo animal models of CNS apoptosis and excitotoxicity so that our results would be maximally relevant to neurodegenerative diseases. Thus, we performed a side by side ultrastructural comparison of a prototypic example of excitotoxic cell death (ECD) (glutamate-induced degeneration of neurons in the infant rat hypothalamus) and a prototypic apoptotic process, physiological cell death (PCD), the natural process by which biologically redundant or unsuccessful neurons are deleted from the developing brain. In this study (36), we demonstrated that the ultrastructural changes that characterize apoptotic cell death are strikingly different, in both type and sequence, from the changes that characterize excitotoxic cell death. Thus, ultrastructurally these 2 cell death processes are readily distinguishable. In addition, we found that DNA fragmentation analysis (either TUNEL staining or gel electrophoresis laddering pattern) is not a reliable means of identifying an apoptotic cell death process in that these tests were positive for both PCD and ECD. We concluded that heavy reliance on these methods and insufficient reliance on ultrastructural analysis has contributed significantly to the existing confusion over how apoptotic and excitotoxic cell death processes relate to one another. The conclusion that DNA fragmentation tests are not specific for apoptosis and do not reliably distinguish apoptosis from excitotoxic cell death is consistent with similar findings from several other laboratories (9, 24, 25), including the laboratory of JFR Kerr (11), who originally coined the term "apoptosis" (44).

The above results prompted us to undertake additional studies aimed at determining whether other examples of excitotoxic neurodegeneration in the in vivo mammalian brain could be distinguished from apoptosis, using PCD as a reference standard for recognizing apoptosis. The first example studied was a new model we developed for investigating concussive head trauma in infant rats (30, 55). Using this model, we found that concussive head trauma triggers in the infant rat brain both an excitotoxic (30) and an apoptotic (7) cell death process, and that these 2 cell death processes can be distinguished from one another quite readily by several criteria. They can be distinguished ultrastructurally in that the excitotoxic process meets all of the ultrastructural criteria we have described (36) for identifying ECD, and the apoptotic process is quite different and meets all of the ultrastructural criteria we have described for identifying PCD. In addition, the

ECD process occured only at the local site of concussive impact and transpired rapidly to end-stage necrosis in a 4 hour period, whereas the PCD (apoptotic) process occurred at disseminated sites and evolved more slowly over a 16 to 24 hour period. Finally, the 2 processes could be distinguished by their response to treatment in that NMDA antagonist drugs that block NMDA glutamate receptors protected against the ECD process and, instead of protecting, caused a worsening of the apoptotic cell death process (30. 55). This has important implications for the clinical management of pediatric head trauma in that a quantitative assessment of the numbers of neurons killed at the impact site by an excitotoxic mechanism compared to those killed at numerous distant sites by an apoptotic mechanism revealed that the magnitude of the apoptotic lesion (collectively) was at least 100 times greater than that of the excitotoxic lesion (6). Therefore, treatment of pediatric head trauma with an NMDA antagonist drug can be expected to rescue a small number of neurons at the risk of killing a much larger number.

Potential of NMDA Antagonists to Induce Apoptotic Neurodegeneration in the Developing Brain

The above findings raised a very interesting question: since NMDA antagonists promote the apoptotic neurodegenerative process induced in the immature brain by head trauma, is it possible that they might also promote the spontaneous apoptotic neurodegenerative process that occurs naturally (independent of head trauma) in the normal developing brain? We investigated this and found that indeed MK801, when administered to 7-day-old infant rats, triggers a massive apoptotic neurodegenerative response affecting many neurons in several major regions of the developing brain (31). In addition, PCP and ketamine (non-competitive NMDA antagonists) and CPP (competitive NMDA antagonist) were administered to 7-day-old infant rats and it was found that all 3 of these NMDA antagonists trigger a robust neurodegenerative response in the developing brain (31).

In additional experiments it was determined 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. This period in the rat is largely confined to the postnatal period; it begins one day before birth and terminates at approximately 14 days after birth, whereas in the human it spans the last 3 months of pregnancy and extends into the first several years postnatally (17). In these experiments it was also observed that within the brain growth spurt period different neuronal populations become sensitive at different times to the mechanism by which NMDA antagonists trigger apoptotic degeneration. Thus, depending on whether exposure occurs in the early, mid or late stage of the brain growth spurt period different combinations of neuronal groups will be deleted from the brain, from which it follows that this neurodevelopmental mechanism has the potential to produce a great variety of neurobehavioral disturbances.

Potential of GABAmimetics to Induce Apoptotic Neurodegeneration in the Developing Brain

Evidence that blockade of NMDA receptors during synaptogenesis triggers apoptotic neurodegeneration raised the important question of whether interference in other transmitter systems during synaptogenesis might also trigger apoptotic neurodegeneration. To explore this possibility we administered numerous agents that interact selectively with various transmitter receptor systems and found the following: We were unable to demonstrate an appreciable apoptotic response to agents that act as either agonists or antagonists at dopamine receptors, or that block kainic acid or muscarinic cholinergic receptors or that block voltage gated ion channels, but a robust apoptotic response was triggered by agents (benzodiazepines and barbiturates) that mimic or potentiate the action of GABA at $GABA_A$ receptors. The agents tested were diazepam, clonazepam, pentobarbital, and phenobarbital. These agents, in a dose-dependent manner, triggered widespread cell death in the infant rat brain which by ultrastructural analysis was apoptotic. The pattern of degeneration was similar for each GABAergic agent but this pattern differed in several major respects from that induced by NMDA antagonists (32).

Potential of Ethanol to Induce Apoptotic Neurodegeneration in the Developing Brain

Evidence that ethanol has NMDA antagonist properties (28, 46) and that it is also a positive modulator of $GABA_A$ receptors (26), prompted us to evaluate its ability to mimic the proapoptotic effects of NMDA antagonists and GABAmimetics. Administration of ethanol to 7-day-old infant rats revealed that it triggers a neurodegenerative response that is even more robust than the response to MK801 or GABAmimetics (32). Evaluation of the ethanol-induced degenerative response by electron microscopy revealed that it conforms to the criteria for apoptotic cell death (16, 32), and comparing the pattern of neurodegeneration induced by ethanol with that induced by NMDA antagonists or

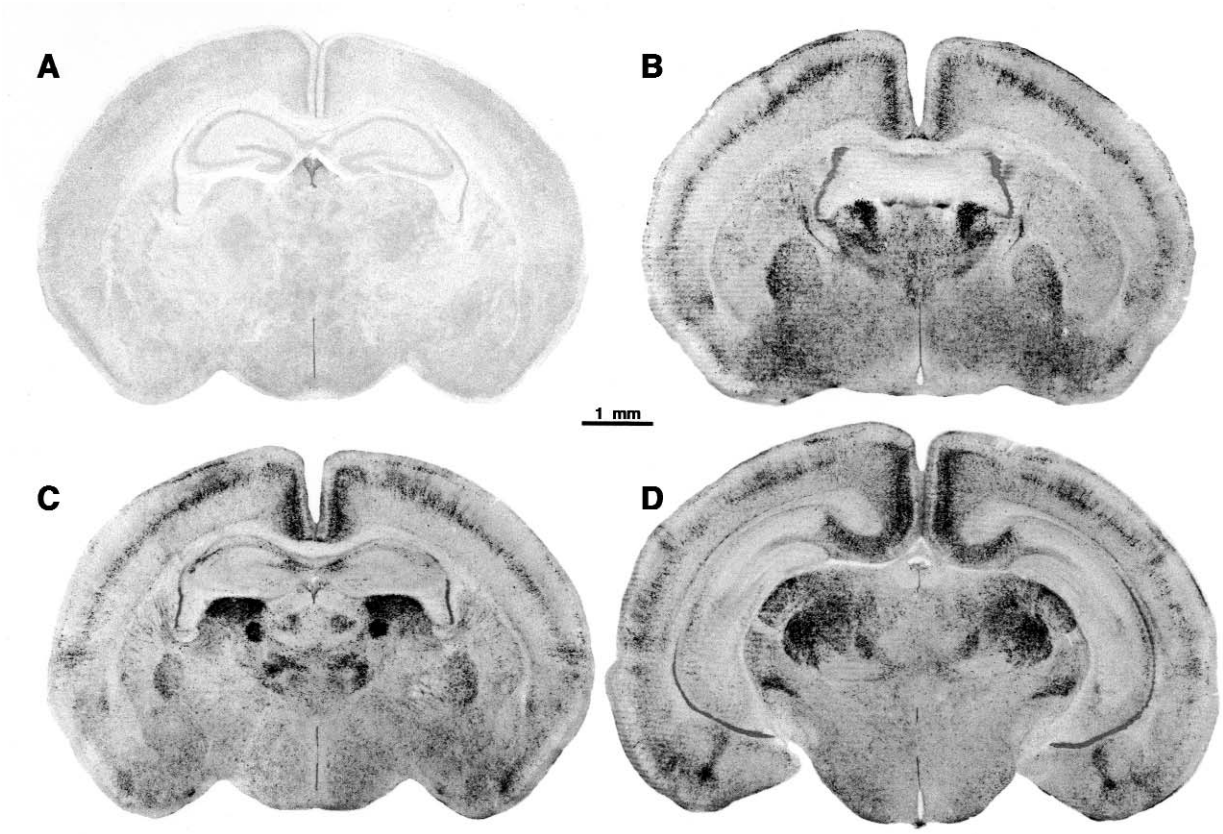

Figure 1. Pattern of ethanol-induced apoptotic neurodegeneration at three rostrocaudal levels of the brain as revealed by silver staining. These histological sections are from the 8-day-old C57BL/6 mouse brain 24 hours following subcutaneous treatment with saline (**A**) or ethanol (**B-D**). All sections are stained by the DeOlmos cupric silver method (13) which causes all neurons that are degenerating to be impregnated with silver. The photographs document that ethanol has triggered a robust neurodegenerative reaction throughout many regions of the mouse forebrain (each black speck is a degenerating neuron or fragment thereof), whereas saline has left the brain showing only a sparse pattern of apoptotic degeneration attributable to physiological cell death that occurs normally in the developing brain. The dying cells in the saline control are barely visible at low magnification because they are sparse in numbers, scattered in distribution and often shrunken and fragmented. Note the remarkable bilateral symmetry of the ethanol-induced neurodegenerative reaction (**B-D**). Reproduced by permission from Olney et al (52).

GABAmimetics revealed that the ethanol pattern comprised a composite of the NMDA antagonist and GABAmimetic patterns (32). The window of vulnerability to ethanol-induced apoptosis was found to be the same as that for NMDA antagonists and for GABAmimetics (coincides with the synaptogenesis/brain growth spurt period). In addition, we found that within the brain growth spurt period different neuronal populations become sensitive at different times to the mechanism by which ethanol triggers apoptotic degeneration. We also determined that maintaining blood ethanol concentrations at or above 200 mg/dl for 4 consecutive hours was the minimum condition for triggering extensive neurodegeneration, and if ethanol concentrations remained above 200 mg/dl for more than 4 hours the degenerative response became progressively more severe and more widespread in proportion to how long the concentrations remained above this level.

In anticipation that it may be advantageous to use transgenic/gene deletion technology to study genetic mechanisms relevant to ethanol-induced apoptotic neurodegeneration, we administered ethanol to 7-day-old C57BL/6 mice, a genetic strain that is frequently used for transgenic research, and determined (51, 52) that this mouse strain is exquisitely sensitive to ethanol-induced apoptotic neurodegeneration. The pattern of neurodegeneration induced in the 7 day old mouse brain by ethanol is illustrated in histological sections stained by the DeOlmos cupric silver method (13) in Figure 1. In ongoing studies we are examining the biochemical

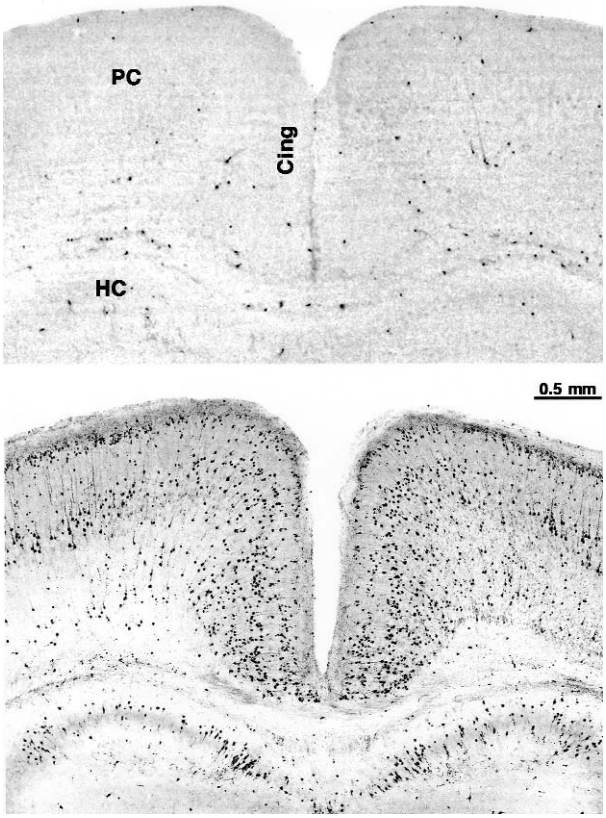

Figure 2. Pattern of ethanol-induced caspase-3 activation in the rostral forebrain. These histological sections depict the parietal cortex (PC), cingulate cortex (Cing) and rostral hippocampus (HC) of the 7-day-old C57BL/6 mouse 8 hours following subcutaneous treatment with saline (top) or ethanol (bottom). Both sections have been stained immunocytochemically with antibodies to activated caspase-3. The saline control brain shows a pattern of caspase-3 activation that occurs normally in the 7-day-old mouse brain, and is attributable to physiological cell death. These spontaneously degenerating neurons are more visible when stained by caspase-3 than by silver, because caspase activation occurs early while the neuron is still showing a well filled-out profile, and silver impregnation occurs later when the cell is condensed, shrunken and/or fragmented. Note the remarkable bilateral symmetry of the ethanol-induced caspase-3 activation pattern. Note also that the laminar pattern of caspase-3 activation in the cortex and hippocampus closely resembles the pattern of silver staining shown in Figure 1. The overall density of the caspase-3 activation pattern at 8 hours following ethanol is not as great as the density of silver staining at 24 hours following ethanol (Figure 1). The reason for this is that the silver stain marks all neurons that have degenerated over a 24 hour period, and caspase-3 immunocytochemistry marks only those neurons that are transiently undergoing caspase-3 activation at the 8 hour interval. Reproduced with permission from Olney et al (53).

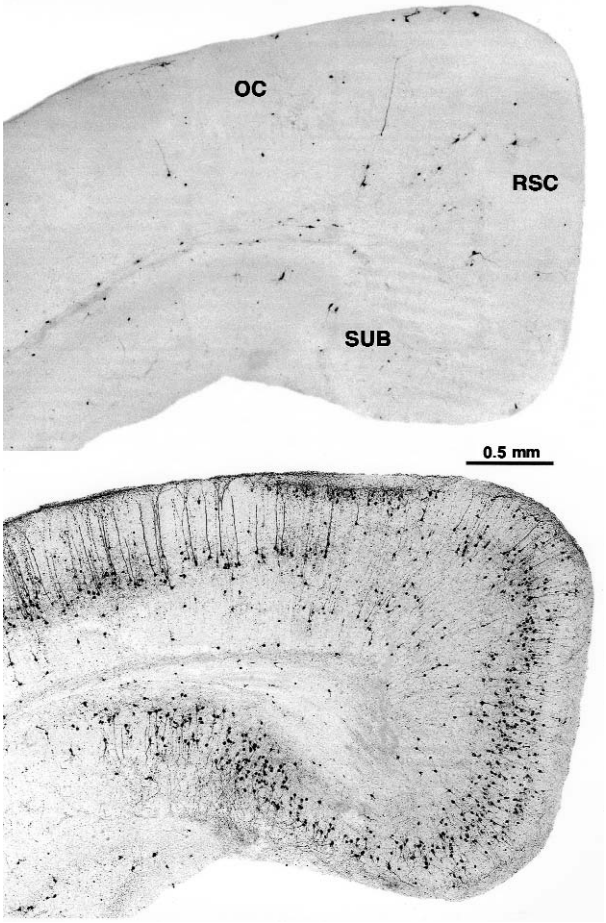

Figure 3. Pattern of ethanol-induced caspase-3 activation in the caudal forebrain. These histological sections illustrate the occipital cortex (OC), retrosplenial cortex (RSC) and subiculum (SUB) of the 7-day-old C57BL/6 mouse brain 8 hours following subcutaneous treatment with saline (top) or ethanol (bottom). Both sections have been stained immunocytochemically with antibodies to activated caspase-3. The caspase-3 activation pattern 8 hours following ethanol treatment is exceedingly robust in these caudal brain regions just as it is in more rostral regions (Figure 2), and at all other levels of the brain. Reproduced with permission from Olney et al (52).

pathways that mediate ethanol-induced apoptotic neurodegeneration. It is thought that caspase-3, a cysteine protease enzyme, plays an important role in the execution stage of apoptosis. Using immunocytochemical methods and antibodies to the activated form of caspase-3, we have found that a robust display of caspase-3 activation becomes evident in millions of neurons within a few hours following ethanol administration to infant mice (53), and the pattern of caspase-3 activation in the early period following ethanol administration (Figures 2, 3) closely resembles the pattern of silver degeneration observed 16 hours later. Thus, it appears that caspase-3 immunocytochemistry will prove useful as a

method for early detection of those neurons that are beginning to act upon a signal to commit suicide following ethanol administration.

Potential of Sodium Channel Blockers to Induce Apoptotic Neurodegeneration in the Developing Brain

The most obvious common denominator of NMDA antagonist and GABAmimetic drugs is that they both reduce neuronal activity. Prompted by this observation, we have begun testing other agents that reduce neuronal activity, namely anti-epileptic drugs such as valproate, phenytoin, carbamazepine and lamotrigine. These drugs block sodium channels, thereby reducing sustained repetitive neuronal firing. Our preliminary findings (33, 34, 15) indicate that at least 2 of these drugs, valproate and phenytoin, trigger apoptotic neurodegeneration in the developing mouse brain.

Drug-induced Apoptotic Neurodegeneration: Mechanistic Considerations

Because reports describing the apoptogenic effects of NMDA antagonists, GABAmimetics and Na+ channel blockers in the developing brain were only published very recently, research aimed at clarifying the underlying mechanisms has just begun. Thus, while general observations can be made, detailed mechanistic interpretations must await further research.

In general, it appears that during the developmental period of synaptogenesis, also known as the brain growth spurt period, neurons are very sensitive to specific disturbances in their synaptic environment. Abnormal increases in Glu stimulation trigger excitotoxic neurodegeneration, and abnormal inhibition of neuronal activity (via Glu, $GABA_A$, or sodium channels) triggers apoptotic neurodegeneration. While the most obvious common denominator of the three classes of drugs that have been found to trigger apoptotic neurodegeneration is that they all reduce neuronal activity, it may be questioned whether GABAmimetic agents trigger apoptosis by an inhibitory mechanism in that activation of $GABA_A$ receptors during certain developmental periods has a depolarizing, instead of hyperpolarizing, effect on neural membranes. However, Mennerick and Zorumski (48) have developed a hippocampal cell culture model in which GABAmimetics, NMDA antagonists and ethanol trigger neurodegeneration, and in this model depolarizing agents counteract the cell killing action of GABAmimetics. Moreover, various agents that suppress action potentials, including tedrodotoxin, mimic the cell killing action of GABAmimetics. Therefore, we tentatively propose that excessive depression of neuronal activity during synaptogenesis may constitute a generic signal for a developing neuron to commit suicide.

Age Dependancy of Ethanol-induced Apoptotic Neurodegeneration

There has been a great deal of speculation recently regarding the potential role of apoptosis in adult human neurodegenerative disorders, including acute conditions such as hypoxia/ischemia and head trauma, and chronic disorders such as Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis. The evidence supporting this speculation is not very compelling in that it has been generated either in cell culture systems or by application of non-specific cytochemical tests to histological brain sections. The only experimental model we are aware of for inducing apoptosis in the adult animal brain is a model described by Sloviter et al (58). These authors demonstrated that adrenocortical insufficiency following surgical adrenalectomy triggers apoptotic degeneration of hippocampal dentate granule neurons in the adult rat brain. Electron microscopic evaluation of the degenerating neurons revealed that they have the same appearance as neurons undergoing PCD in the developing rat brain. This provides evidence that neuronal apoptosis in the adult brain has the same appearance ultrastructurally as apoptosis in the immature brain, but we are not aware of any studies confirming that degenerating neurons in commonly occurring human adult neurological disorders display this PCD-like appearance.

Our studies suggest that in the developing brain physiological cell death (PCD) occurs transiently at an accelerated rate after neurons have differentiated, migrated to their final destination and have begun to form synaptic connections. At that stage there is a transient wave of apoptotic neurodegeneration involving approximately 1% of the neuronal population in a given brain region. Presumably these are neurons being deleted by apoptosis because they have failed to establish appropriate connections. It is during that time of ongoing physiological apoptosis that neurons become overly prone to commit suicide when external forces interfere with the synaptogenesis process. We have identified blockade of NMDA receptors and hyperactivation of $GABA_A$ receptors as 2 external forces that can trigger apoptotic neurodegeneration to such an extent that up to 30% of the neurons in particularly vulnerable brain regions are deleted (31, 32). These are neurons that would not otherwise be deleted from the developing

brain. Therefore, if many brain regions are involved it will result in a substantial reduction in the mass of the brain and in the functional capacity and modes of expression of the brain.

On the basis of these new findings we propose that apoptosis is a major mechanism by which neurodegeneration can be induced in the developing brain. However, it appears that heightened vulnerability of neurons to apoptosis is strictly confined to the synaptogenesis period. Once this period has terminated, external forces that would trigger apoptosis during synaptogenesis are no longer effective. Therefore, while we have identified apoptosis as a major mechanism that can contribute to neurodegenerative processes during development, our evidence does not support (nor does it refute) the interpretation that apoptosis also plays a role in adult neurodegenerative processes.

Before leaving this issue, an important but potentially confusing point should be discussed. We stated above that NMDA antagonist drugs do not trigger apoptotic neurodegeneration except during the synaptogenesis period. This is true, but NMDA antagonist drugs do trigger a neurodegenerative reaction in the adult rat brain which is non-apoptotic. Over the past decade it has become increasingly clear that low doses of NMDA antagonists, such as MK801 or phencyclidine (PCP), produce reversible pathomorphological changes in cerebrocortical neurons of the adult rat brain (49, 50), and that higher doses of these agents induce irreversible neuronal degeneration in several corticolimbic brain regions (12, 18, 29, 50, 63). The mechanism of cell death has been investigated in depth and has been shown to have characteristics consistent with excitotoxic but not apoptotic neurodegeneration. Interestingly, while the adult brain is highly vulnerable to excitotoxic NMDA antagonist neurotoxicity, the developing brain is insensitive to this mechanism (21) and, instead, is highly sensitive to apoptotic NMDA antagonist neurotoxicity (31). Furthermore, a singularly important observation is that GABAmimetic drugs in the adult brain have a very different effect than they have in the developing brain. GABAmimetic drugs in the adult brain protect against the excitotoxic neurotoxicity of NMDA antagonist drugs (36, 37, 45, 50), whereas in the developing brain GABAmimetic drugs act in concert with NMDA antagonist drugs to produce an additive (or perhaps superadditive) apoptotic neurodegenerative response (32).

The differences between the immature and mature brain discussed above have important implications in relation to societal attitudes and the human FASD. Because ethanol has both NMDA antagonist and GABAmimetic properties it is doubly damaging to the developing brain, as is witnessed in the human FASD. However, in the adult brain the neurotoxic potential associated with the NMDA antagonist properties of ethanol is cancelled out by its GABAmimetic properties (22). Thus, this neurotoxic potential of ethanol is not expressed in the adult brain and for this reason, human adults over the millennia have perceived ethanol as user-friendly and have used it as their euphoriant of choice, unfortunately to the detriment of their in utero fetuses.

Public Health Implications of the NMDA Antagonist/GABAmimetic/Ethanol Findings

Fetal alcohol spectrum disorders. The most disabling features of the FASD are the reduced brain mass and neurobehavioral disturbances ranging from hyperactivity and learning disabilities (including mental retardation) that manifest in childhood (10, 19, 43, 57, 61), to depression and psychotic illnesses that manifest in adulthood (20). The widespread pattern and high density of neuronal loss we have documented in ethanol treated infant rats can explain why exposure of the developing brain to ethanol causes an overall reduction of brain mass (microencephaly) in both rats and humans (56). In addition, it provides a credible explanation for the myriad lifelong neurobehavioral disturbances associated with the human FASD (20, 42). Finally, the observation that driving neurons to commit suicide (apoptosis) is the (or at least one) way ethanol disrupts brain development, and that it triggers cell suicide by interfering with glutamatergic and GABAergic neurotransmission, helps to demystify mechanisms underlying neuropathological and neurobehavioral aspects of the FASD.

Psychiatry. The psychiatric implications go beyond the context of hyperactivity/attention deficit disorder (HA/AD) and learning impairment for which the FAS is most well known. We have shown that, depending on whether exposure occurs during the early, mid or late phase of synaptogenesis, ethanol triggers different patterns of neuronal deletion, and it follows that each pattern has the potential to give rise to its own unique constellation of neurobehavioral disturbances. That this mechanism has the potential to contribute to a wide spectrum of neuropsychiatric disorders is documented in a recent study of FASD patients by Famy et al (20). These authors found that 72% of FASD patients, after having experienced HA/AD and/or learning disorders in

childhood, required psychiatric care for adult-onset disturbances, including a 44% incidence of major depression and 40% incidence of psychosis.

It is currently believed that major psychiatric disorders have a genetic predisposition that may or may not be expressed as a clinical illness, depending on the influence of relevant environmental factors. Intrauterine exposure to ethanol warrants consideration as a relevant environmental factor that could interact with genetic determinants to influence the expression of both childhood and adult psychiatric disorders. In addition, if ethanol and related drugs can delete neurons from the human brain by interferring with glutamatergic and GABAergic neurotransmission during a critical stage in development, it is likely that there are other aberrant circumstances, yet to be identified, that can similarly disrupt transmitter function during this critical period. The vulnerability is there—we must now identify all circumstances that can impinge upon it to disrupt development of the human brain.

Regarding the potential relevance of this neurodevelopmental mechanism to specific psychiatric disorders, it is important to recognize that the apoptogenic action of ethanol and related drugs, although widespread, consistently impacts upon certain neuronal populations more than others. For example, layer II non-pyramidal and layers IV and V pyramidal neurons in the cingulate and many other divisions of the cerebral cortex are severely affected. While most regions of the cerebral cortex of psychiatric patients have not been carefully evaluated, the cingulate cortex has been the subject of several quantitative histopathology studies in which the brains of both schizophrenic and manic depressive patients showed selective deficits of layer II non-pyramidal neurons and/or layers IV and V pyramidal neurons (4, 5). Also noteworthy is the extreme sensitivity of several thalamic nuclei to the apoptogenic action of ethanol and related drugs. A long-standing concept in schizophrenia research is that an inhibitory filter or gating mechanism is operative in sensorimotor circuits at a thalamic level that normally prevents receptive cerebrocortical centers from being flooded with unmodulated information. Andreason et al (1) have reported neuroimaging evidence of structural deficits in the thalamus of schizophrenic patients that might be viewed as morphological evidence for a damaged thalamic filter. Disruption of glutamatergic or GABAergic transmission during synaptogenesis by ethanol or by any other pathological vector is an excellent candidate mechanism to explain how the thalamic filter in the brain of a schizophrenic patient may have incurred damage during development.

Drug abuse. Ethanol is the most frequently abused drug in the world and in human history, but it is not the only drug of abuse that can transiently interfere with the glutamate or GABA transmitter systems during synaptogenesis, and thereby drive developing neurons to commit suicide. Various GABAmimetic drugs (barbiturates and benzodiazepines) and NMDA antagonists (phencyclidine [PCP, angel dust], ketamine [special K] and nitrous oxide [laughing gas]) are drugs of abuse that human fetuses are sometimes exposed to (by drug-abusing mothers) during the in utero brain growth spurt period.

Pediatric neurology. Some of the drugs that we have shown can trigger apoptotic neurodegeneration in the developing brain are currently used in relatively large doses as anticonvulsants for pregnant mothers and infants who suffer from seizures. Our animal studies suggest the possibility that this therapeutic regimen might cause reduced brain mass and cognitive impairment in fetuses or infants exposed during the synaptogenesis period. Reduced head circumference and learning impairment was recently reported (14) in children exposed in the pre and perinatal period to phenobarbital and phenytoin, 2 drugs that we have shown can trigger widespread apoptotic neurodegeneration in the infant rodent brain (15, 33, 34)

Pediatric anesthesia. The recent discovery that nitrous oxide (laughing gas) is an NMDA antagonist (38, 47) signifies that, without exception, all of the drugs currently used to induce general anesthesia are either NMDA antagonists or GABAmimetics. Typically, in pediatric anesthesia a cocktail of NMDA antagonists (ketamine, nitrous oxide) and GABAmimetics (benzodiazepines, barbiturates, isoflurane, propofol, etc.) is used. Since the goal is to render the patient unconscious and insentient to pain, sometimes for a period of many hours, the anesthetic agents are used in whatever doses are required to persistently occupy the relevant receptors. The question arises whether maintaining a surgical plane of anesthesia for many hours during the synaptogenesis period may be analogous to exposing the immature brain to solidly intoxicating blood levels of ethanol for many hours. We know from animal experiments that a single intoxication episode of this type can delete many neurons from the developing brain, and the human FAS serves as reminder that sen-

sitivity to this type of mechanism is not limited to animal brains. There do not appear to be any studies that have adequately investigated this issue, but clearly it is an issue that deserves attention.

Although our initial studies focused on both NMDA antagonists and GABAmimetics, most of the drugs we used were not those typically used clinically to induce general anesthesia. Therefore, we recently undertook experiments in which a clinically relevant combination of anesthetic drugs (midazolam, nitrous oxide and isoflurane) was administered to 7-day-old infant rats, using a dosing regimen that maintained a surgical plane of anesthesia for a 6-hour period. Blood gases and skin color were normal throughout this period, and the rat pups recovered rapidly from anesthesia and showed no detectable signs of neurological impairment. This anesthesia protocol resulted in a moderately robust apoptotic neurodegenerative reaction affecting several major regions of the brain (thalamus, hypothalamus, amygdala, caudate nucleus, frontal, parietal, temporal, occipital, cingulate cortices, hippocampus and subiculum), and treated animals showed significant learning deficits compared to controls when tested at 30 days of age (27, 39). These deficits appeared to be permanent, in that they were still present when retesting was performed at 160 days of age.

Concluding Remarks

Here we have summarized recent findings pertaining to several agents (ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, benzodiazepines, halothane, isoflurane, propofol, phenytoin, valproate) that have the potential to delete large numbers of neurons from the developing brain by a newly discovered mechanism involving interference in the action of neurotransmitters (glutamate and GABA) at NMDA and $GABA_A$ receptors during the synaptogenesis period, also known as the brain growth spurt period. Interference in the activity of these transmitters during the synaptogenesis period (last trimester of pregnancy and first several years after birth in humans) causes developing neurons to commit suicide (die by apoptosis). Many of these agents are drugs of abuse to which the human fetal brain may be exposed during the third trimester by drug abusing mothers. Ethanol, the most widely abused drug in the world, triggers massive apoptotic neurodegeneration in the developing brain by interfering with both the NMDA and $GABA_A$ receptor systems. This is a likely explanation for the reduced brain mass and lifelong neurobehavioral disturbances resulting from intrauterine exposure of the human fetus to ethanol (Fetal Alcohol Syndrome). Exposure of the immature brain in a medical treatment context is also of concern in that many of these agents are drugs used frequently as sedatives, tranquilizers, anti-convulsants or anesthetics in pediatric and/or obstetrical medicine. Since this is a newly discovered mechanism, much further research will be required to develop a full appreciation for the nature and degree of risk posed by exposure of the developing human brain to agents that have the potential to cause developing neurons to commit suicide.

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