Newman-Keuls tests established that brain concentrations at 30 minutes were significantly higher than those at 15 minutes for both pregnant and nonpregnant mice. A further increase was observed at 60 minutes for pregnant but not nonpregnant animals, indicating that peak brain concentrations may have occurred later for pregnant mice. The maximum concentration obtained, however, was similar for both groups.
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Synaptic Activity Mediates Death of Hypoxic Neurons

Abstract. *Cultured hippocampal neurons, when exposed to cyanide or an anoxic atmosphere in the early stages of differentiation, were not visibly affected. However, neurons in mature cultures died when exposed to cyanide or anoxia. Cell death could be prevented by treatment with magnesium, which eliminates synaptic activity. These observations suggest that damage in hypoxic neurons is mediated by synaptic activity.*

A number of clinical disorders, including stroke, perinatal asphyxia, and shock, result in an insufficient supply of oxygen to the brain. Lack of oxygen leads to a cessation of cerebral function that becomes irreversible if the oxygen supply is not replenished. The pathological concomitant of irreversible cerebral dysfunction is extensive death of neurons. The precise mechanism responsible for the death of hypoxic neurons is unknown (*1*). Most studies of brain hypoxia in intact animals are difficult to interpret because other variables, such as hypotension, hypercarbia, and acidosis, contaminate the experimental design. The problem of studying hypoxia as an isolated variable can be partly circumvented by using cultured neurons. Cultures of central neurons undergo structural changes within 15 minutes of exposure to low oxygen tension (*2*).

We used cultures of rat hippocampus to define more precisely the factors responsible for the death of hypoxic neurons. The cultures were grown from dissociated hippocampal neurons obtained from fetal rats in the 18th day of gestation. A suspension of 1.5×10^5 cells was plated into poly-L-lysine–coated culture dishes (diameter, 35 mm) containing 1.5 ml of synthetic medium supplemented with 10 percent human serum (*3*). Over the first 2 weeks in vitro these cells form an extensive network of processes and develop spontaneous synaptic activity (*4*, *5*). Intracellular recording has demonstrated physiological and pharmacological properties similar to those of hippocampal neurons in other preparations in vivo and in vitro (*5*).

Addition of 1 m*M* NaCN (to duplicate the effects of hypoxia) had no effect on cultures less than 2 days old. Cells that were added to media containing NaCN attached normally to the culture dish and began extending processes. Cyanide has been reported to decrease neuronal adhesion to polylysine at higher concentrations, but did not affect adhesion in this system (*6*). Cell loss was the same in control and NaCN-containing cultures (Fig. 1).

In contrast, NaCN had marked effects on cultures older than 2 weeks. Within 1 hour the neurons became swollen and vacuolated, and by 4 hours they had started to disintegrate. After 1 day they had been replaced by debris (Fig. 2A).

Similar results were obtained when

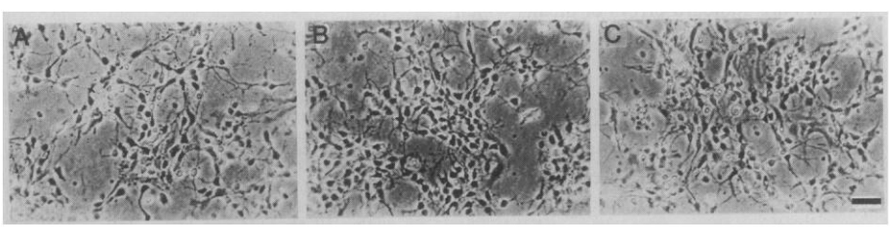

Fig. 1. (A) Phase-contrast photomicrograph of control hippocampal cells after 2 days in vitro. (B) Two-day-old cells that were plated and grown in medium containing 1 m*M* NaCN. (C) Two-day-old cells that were placed in the anoxic atmosphere 5 hours after plating. They remained in the anoxic environment for the next 43 hours. Scale bar, 50 μm.

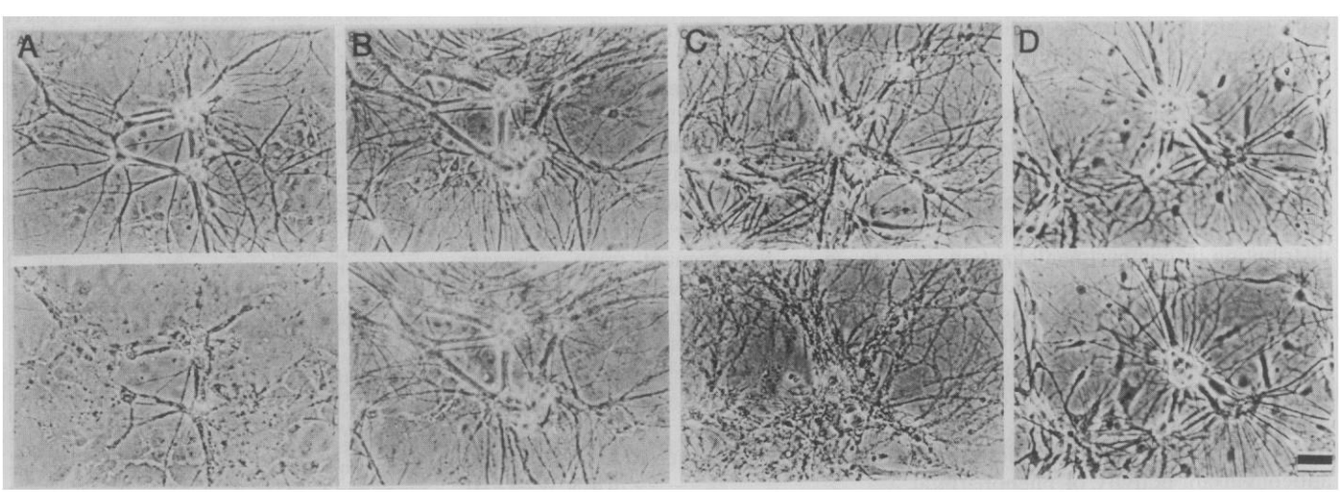

Fig. 2. (A) Phase-contrast photomicrographs of hippocampal neurons after 23 days in vitro. The same field is shown before (top) and after (bottom) 21 hours of exposure to 1 m*M* NaCN. The neurons died and were replaced by debris. (B) Hippocampal neurons (23 days in vitro) before and after 21 hours of exposure to 1 m*M* NaCN, in which the culture was first treated with 10 m*M* $MgCl_2$. There is virtually no change in the neurons. (C) Neurons (18 days in vitro) before and after exposure to the anoxic atmosphere. As in (A), the neurons have died, leaving only debris. (D) Neurons (18 days in vitro) before and after exposure to the anoxic atmosphere for 14 hours, in which the culture was first treated with 10 m*M* $MgCl_2$. The neurons are still intact. Scale bar, 50 μm.

cultures were exposed to an anoxic atmosphere of 95 percent nitrogen and 5 percent CO_2. Cultures deprived of oxygen within 1 day of plating continued to grow normally and were indistinguishable from control cultures for the next 2 days (Fig. 1). In 2-week-old cultures, however, virtually all neurons were dead after 14 hours in the anoxic environment (Fig. 2C).

Older cultures of neurons contain large cells whose processes sometimes exceed 1 mm in length. It is possible that the metabolic requirements of these neurons cannot be met by anaerobic metabolism. However, because neurons in young cultures are actively extending processes and doubling or tripling in size, their metabolic requirements must also be high. A more cogent explanation for the differences in vulnerability is based on differences in synaptic activity between young and mature cultures. Although scanning electron micrographs of developing hippocampal cultures show contacts between cells less than 1 day after plating, terminal swellings resembling synaptic boutons are seen only in older cultures (*4*). While the development of electrical activity has not been studied in hippocampal cultures, serial recordings from cultures of developing mouse spinal cord show that less than 20 percent of the neurons in 1-day-old cultures are spontaneously active, compared to almost all the neurons in 2-week-old cultures (*7*). It is likely that synaptic activity develops along a similar time course in hippocampal cultures.

To test the hypothesis that synaptic activity is responsible for the sensitivity of mature cultures to anoxia, we blocked synaptic transmission by adding 10 m*M* $MgCl_2$ to cultures before exposing them to NaCN or the anoxic atmosphere. Treatment with $MgCl_2$ protected cultures from both insults. Sequential photographs of the same microscope field showed almost no neuronal loss in $MgCl_2$-treated cultures by the time all control neurons had died (Fig. 2, B and D). In addition, intracellular recordings from neurons in a culture treated with $MgCl_2$ and exposed to NaCN showed normal resting membrane potentials and action potentials after the $MgCl_2$ and NaCN were removed (*8*). This is further indication of neuronal integrity.

Glucose concentration and *p*H did not differ between $MgCl_2$-protected and control cultures, making it unlikely that magnesium was only preventing hypoglycemia or lactic acidosis. Cyanide had no obvious effect on the morphology of the glial cells. A small number of astrocytes appeared to be swollen after prolonged anoxia.

Magnesium may exert its protective effect through three mechanisms. First, by blocking transmitter release, magnesium eliminates activity dependent on synaptic transmission. Second, by elevating the threshold for action potential generation, magnesium decreases spontaneous activity independent of synaptic transmission. Third, by blocking transmitter release, magnesium may prevent neurotransmitters from exerting direct toxic effects on postsynaptic cells. There is evidence that the excitatory hippocampal transmitter—probably glutamate—allows entry of calcium, which could lead to neuronal damage if the cell was incapable of maintaining calcium homeostasis in the anaerobic state (*1, 9, 10*). Although the events mediating neuronal loss may occur at the synapse, there is no evidence of any selective damage at that location.

Further evidence that magnesium works by decreasing synaptic activity comes from experiments with tetrodotoxin, which blocks sodium-dependent action potentials. Addition of 3 μM tetrodotoxin before NaCN also protected cultures from cyanide toxicity.

Although these results were obtained in vitro, they may be relevant clinically. The relative resistance of the neonatal brain to hypoxia may be due to decreased synaptic activity, especially in the cerebral cortex, rather than to different metabolic requirements between relatively undifferentiated and differentiated neurons, as was suggested by others (*11*). The ability of barbiturates to protect the mature brain from hypoxic and ischemic injury may be due to their ability to decrease synaptic activity (*12*). Brain damage occasionally associated with prolonged seizures may be due to excess synaptic activity despite normal availability of oxygen (*13*).

While it may be unreasonable to suggest that magnesium, in doses capable of blocking synaptic release, will ever prove to be useful clinically, alternative methods of decreasing transmitter release and neuronal activity may be discovered. Such techniques could be used to help protect the human brain from irreversible hypoxic injury.

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8. The culture medium containing the NaCN and $MgCl_2$ was replaced with Hanks balanced salt solution (*p*H 7.4). Magnesium concentration was decreased to 1 m*M* and calcium was increased to 5 m*M*. Six randomly selected neurons had an average resting potential of 74 mV and an average action potential of 76 mV. The cells also showed spontaneous synaptic activity. When 10 m*M* $MgCl_2$ was perfused near two synaptically connected neurons, the postsynaptic potential almost completely disappeared while a normal presynaptic action potential could still be elicited.
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