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# Glutamate-mediated selective vulnerability to ischemia is present in organotypic cultures of hippocampus

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Ischemic damage to the brain, whether induced experimentally or observed clinically, often produces a pattern of delayed selective cell death in subfield CA1 of hippocampus which has been associated with significant neurologic deficits. The present study demonstrates that this selective vulnerability of CA1 neurons to ischemia, with relative preservation of their neighbors, is expressed in organotypic tissue culture and is prevented by the *N*-methyl-D-aspartate (NMDA) receptor blocker, MK-801. These data provide conclusive evidence that this selective cell death does not have a vascular etiology but is mediated by factors intrinsic to the hippocampal neurons and/or local circuitry. This model system provides an opportunity both to examine mechanisms of ischemic cell death in an avascular environment and to study methods of prevention in the absence of systemic variables.

It has long been recognized that discrete cell groups in the brain are particularly vulnerable to transient ischemia. Perhaps the most dramatic pattern of selective vulnerability to ischemia is observed in hippocampus. In this structure the pyramidal cells of subfield CA1 can be completely destroyed while adjacent CA3 neurons and dentate granule cells remain apparently healthy [7, 19]. Clinically, a similar pattern of damage is commonly seen in the hippocampus of individuals with temporal lobe epilepsy, closed head injury [14] and following stroke involving this structure [1, 24]. The prevalence of hippocampal selective vulnerability and the occurrence of adverse neurologic consequences specifically associated with this loss, including memory deficits [18, 24] is a significant health concern. It may also be amenable to timely therapeutic intervention since, experimentally, it does not become maximal until several days post-ischemia [7, 19]; an understanding of the mechanisms of this selective vulnerability, and their temporal sequence post-insult, may allow the development of

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specific treatments which take advantage of the window between ischemia and hippocampal cell lysis.

Compelling evidence suggests that the critical step in hippocampal cell death with ischemia is an influx of calcium primarily through *N*-methyl-D-aspartate (NMDA) type glutamate-gated channels [5, 8, 12, 20, 22]. Examining the mechanisms and treatment of selective vulnerability in vivo, however, remains problematic. Intrinsic alterations in selective regions of hippocampus with ischemia may interact with features of the microvasculature to alter local perfusion selectively. For example, changes in extracellular calcium concentration alters the diameter of capillaries; selective calcium influx into CA1 pyramidal cells and the resulting products of neuronal breakdown released back into the neuropil could produce localized vascular effects during ischemia or in the reflow period which contribute to the specific vulnerability of this region [15, 16]. Systemic variables may also cloud the interpretation of therapeutic effects. The hippocampal protection observed with the specific NMDA receptor blocker, MK-801 [12], may be related to its ability to lower body temperature [3], a manipulation which alone prevents ischemic damage [4]. Similarly, dextromethorphan, another NMDA receptor blocker which has been shown to be neuroprotective in cortical tissue [11], may attenuate ischemic damage by altering post-ischemic perfusion [23]. Studies examining ischemia in dissociated cell culture have addressed these issues and demonstrated that calcium mediated, NMDA sensitive cell death occurs in vitro [6, 13, 20]. These studies in dispersed cultures cannot, however, examine the mechanisms, or even demonstrate the occurrence, of selective hippocampal subfield vulnerability. In order to isolate the cellular and structural mechanisms of selective cell death in the absence of vascular and systemic factors we examined ischemia-induced necrosis in organotypic hippocampal cultures prepared by the roller tube method of Gähwiler [9]. The present work indicates that (1) CA1 subfield vulnerability develops in culture and, (2) MK-801 is effective in decreasing ischemic damage in CA1 independent of systemic variables.

Two-week-old cultures were placed in Hank's balanced salt solution (an equimolar concentration of mannitol replaced glucose) and exposed to 85% $N_2$, 10% $H_2$ and 5% $CO_2$ for 30–90 min. Each culture was placed in a 35 mm polystyrene Petri dish and covered with 1.5 ml balanced salt solution. Artificial ischemia (AI) was induced in a glass desiccator by multiple exchanges of room air using a mild vacuum and replacing it with the gas mixture; the desiccator was then sealed and warmed to 37°C. A palladium catalyst was used to remove residual oxygen and anaerobic conditions were confirmed using a BBL Gas-pak methylene blue indicator. At the termination of AI the cultures were returned to their roller tubes in growth medium and maintained for 48 h. Control cultures were inspected, but not exposed to AI conditions, and then further incubated for 48 h. The cultures were then fixed in 10% buffered formalin and stained with Cresyl-violet for demonstration of Nissl substance. Two independent observers unaware of culture treatments examined each culture and scored the morphologic appearance according to the following 5 point lesion index: (1) presence of complete pyramidal and granule cell layers; (2) incomplete, or patchy, pyramidal cell loss in CA1; (3) complete pyramidal cell loss in CA1; (4) severe pyra-

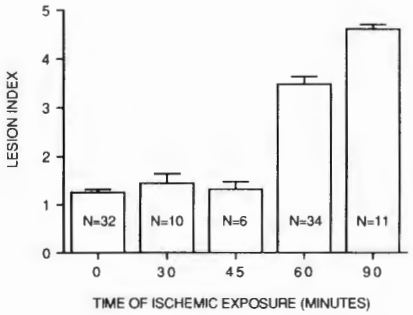


Fig. 1. The severity of ischemic damage to hippocampal slice cultures shown as a function of ischemic exposure time (mean ± S.E.M.). The lesion index is described in the text. Significant damage was observed with 60 min of artificial ischemia ($t_{64} = 13.38$; $P < 0.001$, relative to control tissue). Significantly greater damage was observed following 90 min of ischemia ($t_{43} = 3.85$; $P < 0.001$, relative to 60 min).

midal cell damage in all subfields with dentate gyrus preservation; (5) degeneration of pyramidal cells and dentate. Fig. 1 summarizes these results. Up to 45 min of AI had little gross effect on the morphologic integrity of the cultures. When AI lasted for 60 min, however, there was a significant increase in the incidence of tissue damage

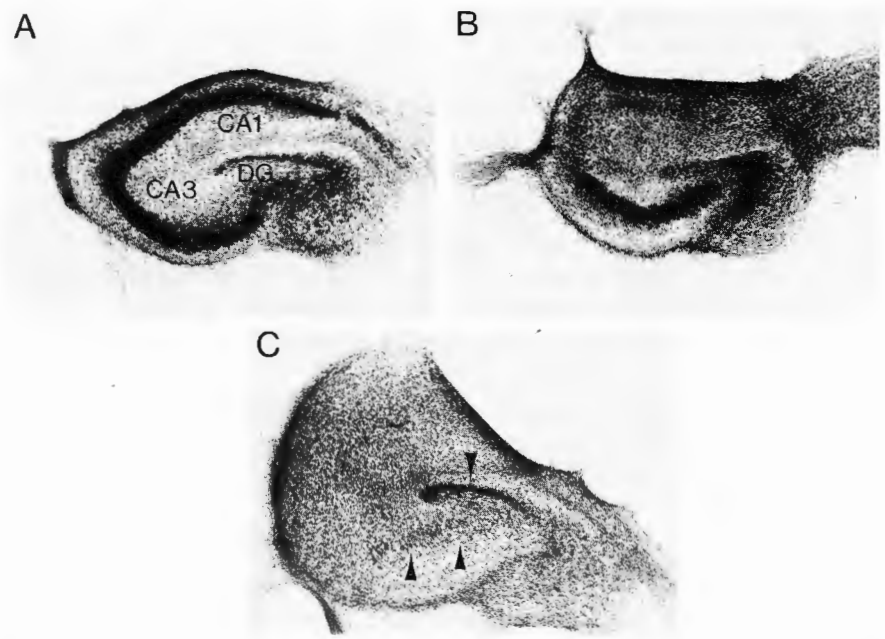


Fig. 2. Representative examples of a control culture (A), and cultures exposed to 60 (B) or 90 min (C) of ischemia. In B there is a selective loss of much of subfield CA1. With longer ischemic exposure (C), CA1 is entirely destroyed; some neurons are still apparent in the relatively resistant CA3 subfield (double arrowhead) and the dentate gyrus (arrowhead). Cresyl violet stain, ×90. CA1, CA1 pyramidal cell layer of hippocampus; CA3, CA3 pyramidal cell layer of hippocampus; DG, dentate gyrus granule cell layer.

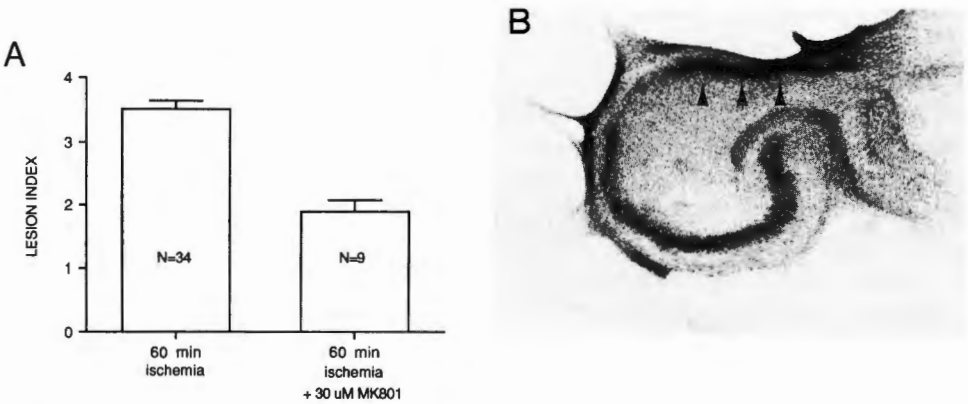


Fig. 3. The presence of 30 $\mu$M MK-801 during 60 min ischemia, and in the 48 h post-ischemia survival time, significantly reduced the mean ($\pm$S.E.M.) lesion index ($t_{41} = 5.24$; $P < 0.001$). The representative culture exposed to ischemia with MK-801 demonstrates significant preservation of the CA1 pyramidal cell layer. Cresyl violet stain, $\times 90$.

primarily restricted to subfield CA1 (mean lesion index = 3.5; $t_{64} = 13.38$, $P < 0.001$, relative to control). Further increasing AI exposure to 90 min produced significantly greater damage involving all subfields of the pyramidal cell layer but often sparing portions of CA3 and the dentate gyrus (mean lesion index = 4.6; $t_{43} = 3.85$, $P < 0.001$, relative to 60 min). Representative micrographs of a normal culture (A), and cultures exposed to 60 (B) or 90 min of AI (C) are presented in Fig. 2. The unexposed culture appears much as would a hippocampal slice, with a complete pyramidal cell layer inserting into the dentate gyrus. After 60 min of AI, and 48 h recovery, there is a clear selective loss of pyramidal cells in CA1; subfield CA3 and the dentate gyrus are still apparent and appear grossly healthy. With 90 min of AI, much of the entire pyramidal cell layer is gone; a small portion of CA3 is still evident as it inserts into the dentate hilus (double arrowhead). The infrapyramidal blade and the taper of the dentate gyrus are also damaged, but a portion of the suprapyramidal granule cell layer is still apparent (arrowhead).

In a second experiment a subset of cultures was exposed to 60 min of AI in the presence of 30 $\mu$M MK-801. The same concentration of MK-801 was present in the growth medium during the 48 h prior to fixation and staining. As shown in Fig. 3A, MK-801 significantly attenuated the damage produced by AI (mean lesion index = 1.9; $t_{41} = 5.24$, $P < 0.001$, relative to 60 min alone). The representative culture, exposed to both AI and MK-801, shown in Fig. 3B, demonstrates considerable preservation of neurons in the CA1 region relative to the damage observed without NMDA receptor blockade (Fig. 2B).

Studies to date suggest that many characteristics of normal hippocampus are present in these organotypic cultures [10, 17]. Ischemia in vivo produces an increase in extracellular glutamate, an influx of calcium into all hippocampal neurons and, notably, a second, delayed, calcium increase in CA1 pyramidal cells [21]. Damage to CA1

neurons, moreover, appears to depend on an intact Schaffer collateral projection from CA3, most likely as a source of glutamate, since selective destruction of this region prevents ischemic damage [2]. The present data indicate that the factors conferring subfield CA1 selective vulnerability to ischemia also develop 'correctly' in organotypic cultures and that neither this vulnerability, nor neuroprotection by MK-801, depends on differential vascular or systemic factors.

The presence of selective vulnerability to ischemia in organotypic hippocampal cultures provides a unique, well controlled, system to study mechanisms of cell loss and the efficacy of neuroprotectants. Sampling of chemical factors in the tissue and medium, direct placement of drugs and recording electrodes and manipulation of intrinsic circuitry can be accomplished with ease. These features make this an attractive model worthy of significant further study.

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