Applications of quantitative measurements for assessing glutamate neurotoxicity
(survival function/2-amino-5-phosphonovaleric acid/glycine/magnesium/N-methyl-D-aspartate receptor)

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ABSTRACT The role of the N-methyl-D-aspartate receptor channel in glutamate neurotoxicity was investigated in cultured hippocampal neurons of the CA1 region. An equation, the survival function, was developed to quantify the effects of putative modulators of neurotoxicity. 2-Amino-5-phosphonovaleric acid (30 μM) reduced the neuronal sensitivity to glutamate by a factor >20, whereas glycine (1 μM) enhanced it by a factor of 7.5 ± 2.5. Neurons were protected by increasing Mg²⁺ concentrations in a predictable way based on the ion’s ability to block the N-methyl-D-aspartate channel. These findings provide a quantitative basis for the assessment of various neuroprotective agents and add further support to the hypothesis that the N-methyl-D-aspartate channel is central to glutamate neurotoxicity.

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METHODS

Hippocampal Cultures. The most sensitive area of the brain to periods of anoxia is the CA1 section of the hippocampus (21, 22). This region is also the site of greatest N-Me-D-Asp receptor density (22–24). For these reasons and because of the greater accessibility afforded by tissue culture, in vitro dissociated hippocampal neurons from the CA1 region were selected as the model system.

These neurons were obtained from 1- to 3-day-old rats (Long-Evans) by the methods of Huettner and Baugman (25). Hippocampi were dissociated and cultured according to published methods, with minor modifications (26). Kynurenic acid (1 mM) and MITO Serum Extender (per manufacturer’s instructions; Collaborative Research, Bedford, MA) were used to supplement the medium. Cultures were allowed to mature for 15–35 days before use in neurotoxicity studies.

Measurement of Neurotoxicity. The device built for the study consisted of two chambers. A photoetched coverslip (Belco Glass) formed the floor of the central chamber and an adapter held the circle of cells in place on the photoetched grid. Inlet and outlet ports were positioned to maximize mixing and to facilitate aspiration. A second chamber surrounding the central chamber functioned as a water bath.

For a dose–response experiment, a circle of cells, etched in two locations along its edge, was transferred to the central chamber. This chamber contained 3 ml of warmed (37°C) control salt solution (120 mM NaCl/5.4 mM KCl/0.8 mM MgCl₂/1.8 mM CaCl₂/25 mM Tris Cl, pH 7.4/15 mM glucose). With a phase-contrast microscope, by use of the grid and the two etches on the circle, a relative position was recorded. Ten to twenty fields of neurons (totaling 50–500 distinct neurons depending on plating density) were counted per circle, with the grid coordinates providing a reference on each field. After 20 min of counting, a warmed (37°C) test solution was injected by syringe in four 2.5-ml aliquots. Before each of the four additions, 2.5 ml of the central solution was aspirated to produce a final solution that approximated the test solution by 99.8%. The cells were allowed to bathe for 5 min after the last addition and then the solution was replaced with control solution by the same serial exchange method. Finally, the coverslip was returned to the incubator in its original medium.

After 24 hr, the circle was incubated in 0.4% trypan blue (Sigma) for 5 min. The treated circle was placed back into the central chamber with control salt solution, and the surviving

Abbreviations: N-Me-D-Asp, N-methyl-D-aspartate; APV, 2-amino-5-phosphonovaleric acid.

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neurons were counted. Survivors excluded trypan blue, whereas those that were damaged or dead did not.

RESULTS

Dose–Response Normalization. Many of the observations made by Choi et al. (4), who used similar experimental conditions to study the neocortex, are confirmed here. Some acute swelling was observed soon after glutamate application; however, accurate detection of neuronal death required staining. Reaggregated neuronal clusters were more glutamate-sensitive than single cells. Background cell death, as detected by controls, varied from 5% to 15% and did not differ significantly for control treatments with Mg2+uro, APV, or glycine added. Maximum cell death ranged from 85% to 95% in most cases. To facilitate comparison, data were normalized to produce dose–response curves spanning 0–100%. This entailed subtracting the control death rate from associated test rates and dividing all the values by the highest test value. This procedure would produce a typical normalized dose–response curve (Fig. 1A).

The nature of the curve prompted us to see whether it could be described mathematically. Because the number of surviving neurons decreases monotonically toward zero as glutamate concentration is increased, a natural expression for the fraction of surviving cells $S(G)$, where $G$ is the glutamate concentration, is $S(G) = 1/[1 + f(G)]$; here $f(G)$ is some as-yet-undetermined function of glutamate concentration.

We anticipate, however, that the construction of $f(G)$ will yield a useful approximation to the observed $S(G)$ when $f(.)$ is expanded in a power series and only a few terms are retained. By the way $S(G)$ is measured, the zeroth-order term in the expansion must vanish. To lowest order, then,

$$S(G) = 1/[1 + (G/k)],$$

where $k$ represents the glutamate concentration used for a particular trial, $k$ is the glutamate concentration for which half of the neurons survive, and $S(G)$ represents the normalized fraction of neurons that survive for the same trial. We call $S(G)$ the survival function and $k$ the survival constant.

In Fig. 1B, observed survival data are plotted on an abscissa defined by Eq. 1 so that, were the equation an accurate expression of the survival data, a straight line would result. As seen in the figure, the function provides a good fit for data from four dose–response curves with $k$ values ranging from 3.4 μM to 74.5 μM. The important point is that the survival data are completely specified, within experimental error, by a single number, the survival constant $k$. Fitting our survival function $S(G)$ to the dose–response data showed that the survival constant $k$ depends on culture conditions in that it varied in an orderly fashion with factors such as cell density, synaptic density, and neuronal age measured as days in vitro. Nevertheless, as Fig. 1B demonstrates, the survival curves for cultures with widely varying glutamate sensitivity all were described well by the equation. However, such variation made it apparent that meaningful interpretation of the glycine and Mg2+ data to be described below would require comparison to a control glutamate dose–response curve performed on cells from the same plating.

APV Blocks Glutamate Neurotoxicity. We initially tested the $N$-Me-D-Asp hypothesis directly by adding 30 μM APV, a competitive $N$-Me-D-Asp-receptor antagonist, to the test solutions. The dose–response curve (not shown) shifted considerably from $k = 48$ μM to $k > 1000$ μM. Low glutamate doses (10 μM) were rendered nontoxic and high doses (1 mM) killed only 25% of the cells. Hence, competitive blockade of the $N$-Me-D-Asp receptor appears to provide significant protection to the neurons.

Glycine Potentiates Glutamate Neurotoxicity. Johnson and Ascher (ref. 27; see also ref. 28) showed that glycine, in concentrations up to about 1 μM, greatly increases the sensitivity of the $N$-Me-D-Asp receptor to glutamate: a particular nonsaturating dose of glutamate will produce a much larger conductance change if low (micromolar) concentrations of glycine are present than if they are absent. Similarly, Reynolds et al. (29) showed that glycine potentiates $N$-Me-D-Asp-stimulated Ca2+ conductances into spinal neurons. If the mechanism of glutamate neurotoxicity involves operation of the $N$-Me-D-Asp-receptor channel, then the survival curve should be shifted significantly along the glutamate concentration axis by the addition of 1 μM glycine.

An example of the glycine effect can be seen in Fig. 2, where the survival curve was shifted to a lower concentration range. For the purpose of quantitative comparison, the $k$ survival curve is divided by the $k'$ value from the associated glycine survival curve. The resulting ratio was $7.5 \pm 2.5$ ($n = 5$) over a fairly wide range of control survival constants. Thus, the predicted shift of the survival constant by glycine was consistently large. In other experiments, the neurotoxic

![Fig. 1.](image-url)
effects of glutamate or of glutamate and glycine were entirely negated by removal of Ca^2+ (data not shown).

Mg^2+ Protects Neurons in a Dose-Dependent Fashion. Extracellular Mg^2+ ions block N-Me-D-Asp-receptor channels in a voltage-dependent manner (30, 31). If current flow through these channels is responsible for glutamate neurotoxicity, increasing the extracellular Mg^2+ concentration should increase the survival constant. Survival functions for various Mg^2+ concentrations are presented in Fig. 3; as predicted, the survival curves are shifted to the right (higher glutamate concentrations are required to kill half of the neurons) by increasing Mg^2+ concentrations. The survival constants are plotted as a function of Mg^2+ concentration in Fig. 4. As predicted, then, increasing extracellular Mg^2+ concentrations increase the survival constant—they protect against glutamate neurotoxicity.

As the results reported above confirm the predictions based on the hypothesis that N-Me-D-Asp-receptor activation is an important link in glutamate neurotoxicity, they add further support to the hypothesis that this channel is important in the delayed type of neurotoxicity in the CA1 section of the hippocampus.

**DISCUSSION**

Several researchers noted that the ionic conditions used during a glutamate-neurotoxicity study may primarily pro-

**Fig. 3.** Four dose–response experiments testing the effect of 0.0, 0.8, 1.8, and 3.5 mM Mg^2+ on glutamate neurotoxicity. Higher Mg^2+ concentration correlates with greater cell survival.

**Fig. 4.** Survival constants from Fig. 3 are plotted against Mg^2+ concentration. The smooth curve fitted to the data is the one predicted by a simple theory for the Mg^2+ block effect. The theory, based partly on an empirical relationship between Mg^2+ concentration, transmembrane voltage, and N-Me-D-Asp channel conductance (C. E. Jahr and C. F. S., unpublished data), predicts a decrease in Ca^2+ conductance with increasing Mg^2+. The sharp rise in survival at high Mg^2+ concentrations also reflects the saturating nature of the glutamate dose–response curve; incremental glutamate doses produce smaller conductance changes at high glutamate levels than at more moderate levels.

duce either early neurotoxicity or the delayed type (11, 13). Low Ca^2+ (0.5 mM) and high Mg^2+ (4.5–5 mM) seem to produce the early type, whereas high Ca^2+ (1.8–2.5 mM) and low Mg^2+ (0.8–1.0 mM) favor the delayed type. These results are consistent with the hypothesis that the N-Me-D-Asp channel plays a role in excitotoxicity. Low Ca^2+ and high Mg^2+ reduce Ca^2+ conductance across the N-Me-D-Asp channel, and the inverse condition produces greater conductances. Greater Ca^2+ conductances would lead to an increase in the toxicity of a given glutamate dose.

However, some question remains as to the relevance of these results to other regions of the brain. The Mg^2+ antagonism of excitotoxicity has been observed in anoxia studies using hippocampal slices (32) and culture neurons (33, 34), but appears to show little antagonism in the neocortex (11). Nevertheless, neocortical neurons were protected by Mg^2+ when Na+ ions were replaced by impermeant monovalent cations. Likewise, APV, an N-Me-D-Asp antagonist, protects hippocampal CA1 cells against glutamate neurotoxicity, whereas higher doses of APV did not protect neocortical neurons very well (11). Given the different distribution of glutamate-sensitive N-Me-D-Asp and non-N-Me-D-Asp receptors in the hippocampus and neocortex, the precise pathophysiology may involve a relationship between the receptor types that depends on their relative numbers.

The significant potentiation by glycine is interesting for several reasons. Pharmacologically, this dose–response approach provides a method for evaluating possible functional antagonists to the glycine site on the N-Me-D-Asp channel. Clinically, a properly designed glycine antagonist might down-regulate the conductance of the N-Me-D-Asp channel, preserving its important physiological role while reducing the pathological effects. Since the concentrations of free glycine in the human central nervous system are probably greater than 1 μM (35), even at postsynaptic membranes, a pathological role for glycine in excitotoxicity is certainly a possibility. In addition to this potential candidate, glutamine protected cells from hypoxia in a recent study using hippocampal slices (36). The quantitative approach described above may be one route to a clearer understanding of the
mechanisms, the relationships, and the relative importance of putative agonists and antagonists in excitotoxicity.

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