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Glycine Causes Increased Excitability and Neurotoxicity by Activation of NMDA Receptors in the Hippocampus

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Glycine is an inhibitory neurotransmitter in the spinal cord and also acts as a permissive cofactor required for activation of the *N*-methyl-D-aspartate (NMDA) receptor. We have found that high concentrations of glycine (10 m*M*) cause marked hyperexcitability and neurotoxicity in organotypic hippocampal slice cultures. The hyperexcitability, measured using intracellular recording in CA1 pyramidal neurons was completely blocked by the NMDA receptor antagonist MK-801 (10 μ*M*), but not by the AMPA receptor antagonist DNQX (100 μ*M*). The neurotoxicity caused by glycine occurred in all regions of hippocampal cultures but was most marked in area CA1. There was significant CA1 neuronal damage in cultures exposed to 10 m*M* glycine for 30 min or longer ($P < 0.01$) or those exposed to 4 m*M* glycine for 24 h compared to control cultures ($P < 0.01$). The NMDA antagonists MK-801 (10 μ*M*) and APV (100 μ*M*) significantly reduced glycine-induced neuronal damage in all hippocampal subfields ($P < 0.01$). The AMPA antagonists CNQX, DNQX, and NBQX (100 μ*M*) had no effect on glycine-induced neuronal damage. High concentrations of glycine therefore appear to enhance the excitability of hippocampal slices in an NMDA receptor-dependent manner. The neurotoxic actions of glycine are also blocked by NMDA receptor antagonists.

INTRODUCTION

Glycine is an inhibitory neurotransmitter in the brain stem and spinal cord (7, 9, 26, 30, 55). Glycine inhibits spinal neurons by acting on receptors that are blocked by strychnine (8, 9, 30). Glycine also binds to a specific site on the *N*-methyl-D-aspartate (NMDA) receptor complex, and low concentrations of glycine are required for activation of the NMDA receptor (20, 21, 23, 28, 36, 38, 47). This cofactor site is normally fully saturated at 1 μ*M* glycine (21), which is close to the physiological concentration of glycine in the extracellular space of brain tissue (15). The glycine site on NMDA receptors is not blocked by strychnine and is referred to as the "strychnine-insensitive" glycine site (28, 47, 57).

It has recently been reported that glycine, at much higher concentrations than those required to fully saturate the glycine cofactor site on the NMDA receptor, can cause NMDA mediated electrophysiologic changes resulting in marked hyperexcitability and also excitotoxicity in forebrain regions (37, 42, 43, 53). In acutely prepared hippocampal slices, Wallis *et al.* observed that 10 m*M* glycine increased the amplitude of stimulation-evoked population spikes, followed by a subsequent loss of synaptic transmission in area CA1 (53). This electrophysiologic evidence of excitotoxicity in hippocampal slices was also partially blocked by NMDA antagonists.

The action of low concentrations of glycine as a cofactor on the NMDA glycine site can be blocked by indole-2-carboxylic acid (19) and also by quinoxalinedione derivatives such as 7-chlorokynurenic acid and Acea 1021 (5, 22, 24, 33). These compounds are termed glycine site NMDA antagonists and inactivate the NMDA receptor by blocking the cofactor site on the receptor complex. Similar to other NMDA antagonists, glycine site NMDA antagonists have recently been reported to reduce ischemic neuronal damage (33, 54, 56). We reported that the addition of 1 m*M* glycine reversed the protective effects of the glycine site NMDA antagonists ACEA 1021 and 7-chlorokynurenic acid against damage caused by oxygen and glucose deprivation in hippocampal slice cultures (33). Curiously, when we performed an extended dose–response relationship for glycine, we observed that 10 m*M* glycine alone was neurotoxic when added to hippocampal slice cultures for 24 h.

The present study was performed to examine the physiologic and neurotoxic properties of high concentrations of glycine in hippocampal slice cultures. These cultures retain much of the intrinsic synaptic circuitry and neurotransmitter receptor distribution of the intact hippocampus (12, 18, 48). In our laboratory, hippocampal slice cultures have proved to be an excellent *in vitro* model system for analyzing CA1-specific neuronal damage resulting from oxygen and glucose deprivation

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(31, 34). Slice cultures have also demonstrated a similar pattern of vulnerability to neurotoxins and ischemic damage as seen *in vivo* (31, 32, 50). Our present study demonstrated that glycine produced neurotoxicity that showed preference for area CA1 and that the physiologic and neurotoxic properties of glycine involve activation of NMDA receptors.

METHODS

Preparation of Cultures

Organotypic slice cultures of the hippocampus were prepared using the method described by Stoppini *et al.* (46). Slices of the hippocampus were prepared from 4- to 7-day-old neonatal rats (Sprague–Dawley, Bantin and Kingman Inc. Fremont, CA) by removing the brain, dissecting the hippocampal formation, and making transverse slices (400–500 μm) using a McIlwain tissue slicer. Slices were then placed in Gey's balanced salt solution supplemented with glucose (final concentration 6.5 mg/ml), for 1 h at 4°C. Individual slices were then transferred to 28-mm sterile transparent Anocel membranes (Whatman Inc. Clifton NJ). Membranes containing two slices each, were placed in 6-well culture trays with 1.5 ml growth medium consisting of 50% MEM (Gibco laboratories Grand Island, NY) supplemented with Hepes and sodium bicarbonate, 25% Hanks' balanced salt solution (HBSS), 25% horse serum, and glucose to a final concentration of 6.5 mg/ml. The slice cultures were then grown at 36.5°C, 90–100% humidity, 5% CO_2. The culture medium was changed every 3 days. All cultures used in this study were grown for 10–14 days *in vitro,* and only those cultures with all hippocampal subfields (dentate, CA1, and CA3) were used. Animals were cared for according to the guidelines of the University of Washington animal care committee.

Preparation of Drugs

The glutamate receptor antagonists MK-801, APV, CNQX, and DNQX were obtained from Research Biochemicals Inc. (Natick, MA). NBQX was kindly provided by Novo Nordisk Pharmaceuticals (Copenhagen, Denmark). Glycine was obtained from Sigma Chemical (St. Louis, MO). MK-801 and APV were prepared as 10 m*M* solutions and CNQX, DNQX, and NBQX were prepared as 5 m*M* solutions in glucose-free HBSS. Glycine was prepared as a 1 *M* solution in glucose-free HBSS.

Incubation of Cultures with Glycine and Glutamate Receptor Antagonists

Glycine was added as small aliquots in HBSS to fresh growth medium containing the cultures to achieve the final concentrations described. An equivalent amount of HBSS was added to control cultures. During the addition of compounds, care was taken to maintain the temperature of the cultures at 36.5°C. The glutamate receptor antagonists were added to the culture medium containing the cultures as small aliquots to give the final concentrations described, 30 min before the addition of glycine. The cultures were then placed in the CO_2 incubator at 36.5°C for the described time intervals. Following the exposure to glycine, the cultures were then placed in fresh growth medium containing propidium iodide (PI) (Sigma chemical Co.) and returned to the incubator. The cultures were then assessed for cell death by either the PI fluorescence method or the histologic assessment method, 2 days (40–48 h) after the initiation of the glycine exposure.

Histologic Analysis of Glycine Effect on Slice Cultures

Histological analysis of glycine-induced neuronal damage was performed on a group of cultures exposed to either no insult (control, $n = 10$) or 10 m*M* glycine for 24 h (glycine treated, $n = 10$). Following glycine exposure for 24 h, the cultures were placed in fresh growth medium for an additional 24 h and then fixed and stained. Cultures were fixed in 5% paraformaldehyde in phosphate-buffered saline, transferred to glass coverslips, and then stained using cresyl violet.

The CA1, CA3, and dentate regions of each culture were examined using light microscopy. Dead neurons were identified by characteristic pyknotic nuclei and disappearance of the normal cytoplasm and cellular architecture as seen in ischemic damage (3). A quantitative analysis of cell death in all three regions of each culture was performed using a modification of the sampling method described by Bolender *et al.* (2). Multiple high-power fields (40×) were examined and internal cursors were used to delineate the cells to be counted in multiple samples of each region. The number of dead cells was divided by the number of dead and live cells and multiplied by 100 to yield a percentage of dead cells in each sample. This process was repeated for each of the cultures and the results were averaged for each region within each group.

Assessment of Cell Death by Image Analysis Using PI

The intensity of PI fluorescence in each described subfield of the cultures was used as an index of cell death. We have previously found a close correlation between this method and cell counts in histologically prepared specimens to quantitate ischemia-induced cell death in slice cultures (34). Propidium iodide, which rapidly enters cells with damaged membranes and becomes brightly fluorescent after binding to nucleic acids, was added (0.5 μg/ml) to the culture medium used in the experiments. By itself, PI is nontoxic to neurons and has been used as an indicator of neuronal

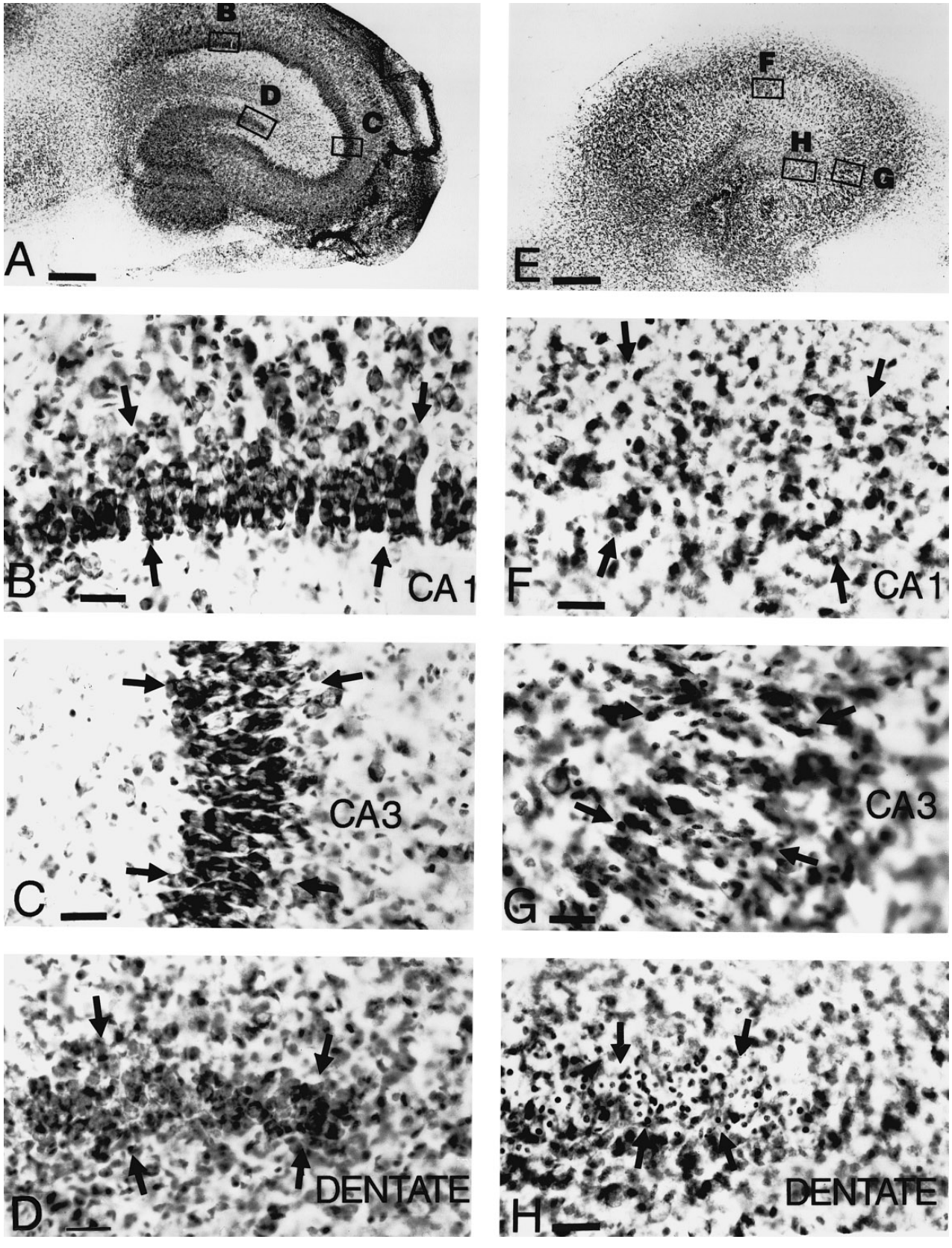

FIG. 1. Cresyl violet-stained hippocampal slice culture illustrates neuronal damage in all subfields caused by exposure to 10 m*M* glycine for 24 h. (A) Control culture not exposed to glycine showed intact neuronal cell layers in all subfields. Higher magnification photomicrographs of the cell layers (arrows) of area CA1 (B), CA3 (C), and dentate (D) in the same culture. Glycine-treated cultures show extensive neuronal destruction in all hippocampal subfields (E). Cultures were allowed to survive for an additional 24 h after removal of glycine before being fixed. Higher magnification photomicrographs of the cell layers (arrows) of area CA1 (B), CA3 (C), and dentate (D) in the same culture. Bars, 210 μm (A, E), 21 μm (B–D and F–H).

membrane integrity and cell viability (27). The cultures were examined using an inverted microscope to verify that the cultures were healthy prior to insult exposure. The first measurement of fluorescent intensity was performed between 40 and 48 h following the initial insult, using a Nikon Diaphot (Nikon Corp., Tokyo, Japan) inverted fluorescent microscope with a digital camera (Dage 72 CCD, Michigan City, IA) attached. Fluorescent images were obtained and digitized using Optimas image analysis software (Bio-Scan, Inc., Edmonds, WA) running under Microsoft windows. Forty to forty-eight hours after the glycine or control exposure, the remaining neurons were killed by exposing the cultures to 3 h of anoxia. The fluorescent intensity, 24 h after 3 h of anoxia, was set equal to 100% damage and was then compared to the fluorescent intensity following the initial insult. The integrated gray value (the numerical value for the fluorescent intensity) in the region of interest (ROI) was calculated by the computer and was used as an index of 100% toxicity. Computer analysis of each image was performed by creating an ROI around each hippocampal subfield 24 h after anoxia exposure. The images from the same culture taken at 40–48 h following the insult were then recalled and analyzed by superimposing the ROI which had been created from that identical region following the 3-h anoxia exposure. The integrated gray values (fluorescent intensity) from each subregion following the initial insult were then expressed as a percentage of the value obtained following 3 h of anoxia. The values were averaged for each group and compared.

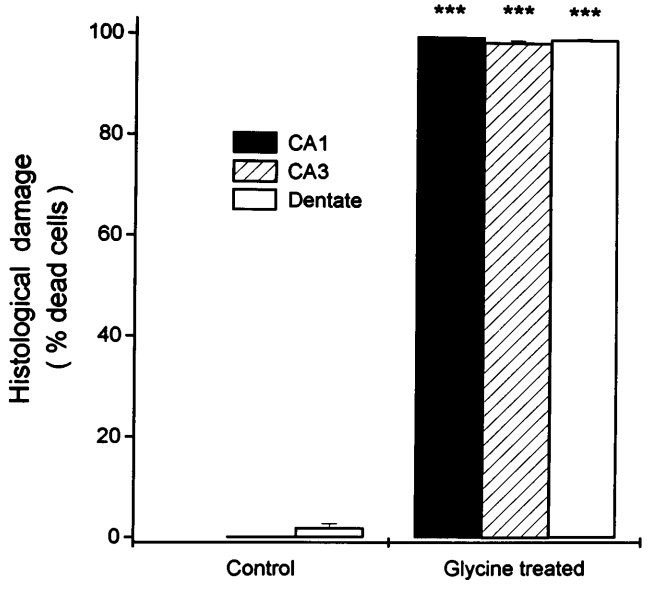

FIG. 2. Quantitative analysis of cell counts in cresyl violet-stained control cultures and cultures treated with 10 m*M* glycine. There was extensive neuronal loss in all subfields, as indicated by marked pyknosis and loss of cytoplasm. Neuronal toxicity was quantified by counting the neurons with these characteristic morphological changes and compared to the total number of neurons in multiple high-power fields (see Methods). The number of dead neurons was significantly higher in all subfields in the glycine-treated than in control cultures (***$P < 0.001$ by Mann–Whitney, $n = 10$).

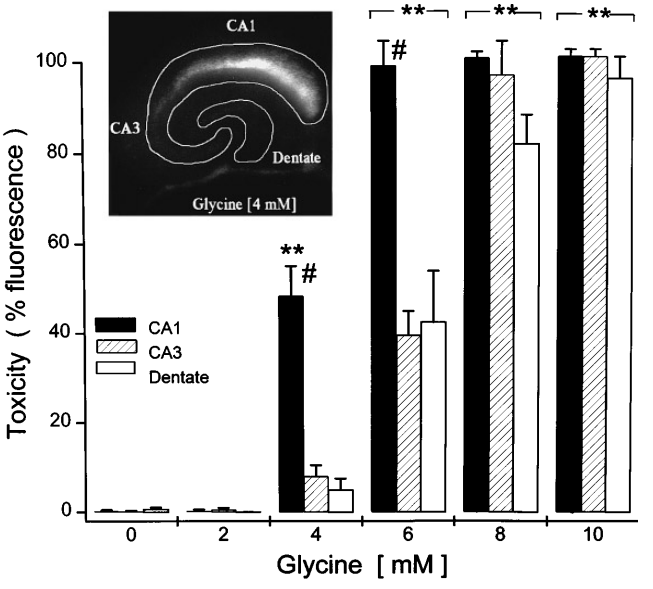

FIG. 3. Dose–response of glycine neurotoxicity in different subfields of hippocampal cultures. Neuronal toxicity was quantitated using propidium iodide fluorescence (see Methods). There was significant neuronal damage induced by glycine at concentrations of 6 m*M* and higher in all subfields ($P \leq 0.001$, Kruskal–Wallis test, **$P < 0.01$ for all subfields compared to controls Mann–Whitney test for posthoc analysis with Bonferroni correction for multiple comparisons). There was also significant damage caused by 4 m*M* glycine in CA1 (**$P < 0.01$ compared to control, Kruskal–Wallis test with Tukey's test for posthoc comparison). The neuronal damage caused by 4 and 6 m*M* glycine was significantly greater in CA1 than in other subfields (#$P < 0.01$ CA1 compared to CA3 or dentate, Kruskal–Wallis test with Tukey's test for posthoc comparison). Inset shows photomicrograph of propidium iodide fluorescence, demonstrating specific neurotoxicity to area CA1. Higher concentrations of glycine (8 and 10 mM) caused equivalent damage in all subfields ($n = 8$ in each group).

Electrophysiologic Studies

Physiological experiments were performed using standard intracellular recording techniques. Intracellular electrodes (70–120 Mohm) were filled with 4 *M* potassium acetate. The cultures were placed in a small-volume recording chamber attached to the stage of an inverted microscope (Olympus IMT-2). Only one cell was recorded from each culture; therefore, n = number of cells and cultures. The artificial cerebral spinal fluid (ACSF) recording solution consisted of (in m*M*): NaCl, 124; KCl, 3, $MgSO_4$, 2; $NaHCO_3$, 26; dextrose, 10; $CaCl_2$, 2. Slices were perfused with ACSF at 1 ml/min maintained at 35°C. A manifold allowed selection of the perfusing solution from one of four reservoirs containing the various drug solutions. Intracellular signals were amplified using an Axoclamp 2A amplifier. The

timing and the duration of all intracellular current pulses were controlled by a Neurodata PG4000 digital stimulator. All signals, current records, and calibration pulses were recorded on videotape with a Neurodata Neuro-corder DR-484. Analysis was conducted off-line with an IBM-compatible 486 personal computer, running pClamp and Axoclamp software (Axon Instruments, Foster City, CA). Records were printed on a laser printer or using a Gould EasyGraf portable chart recorder.

All values were expressed as means ± SEM. Statistical analysis of the results was performed using the Mann–Whitney test with Bonferroni correction for multiple comparisons where appropriate and also the Kruskal–Wallis test with Tukey's test for posthoc comparison. Significant differences were considered at the P value <0.05.

RESULTS

Examination of cresyl violet-stained slice cultures using light microscopy revealed extensive cytopathological changes consistent with neuronal death after 24 h of glycine exposure (10 mM), followed by 24 h of incubation in growth medium (Figs. 1E and 1F). These cytopathological changes included pyknosis, early karyorexis, and karyolysis as well as loss of cytoplasm and normal cellular architecture. Control cultures showed none of these changes (Figs. 1A–1D). Cell counting was performed as described under Methods by counting multiple samples in each subfield (CA1, CA3, dentate). Comparison of the percentage dead cells between control and glycine-treated cultures revealed highly significant increases in the percentage of dead cells in all subfields of the glycine-treated cultures ($P < 0.001$ Mann–Whitney test, $n = 10$ in each group) (Fig. 2).

The dose–response for glycine neurotoxicity was performed by adding 0, 2, 4, 6, 8, and 10 mM glycine to the cultures for 24 h and analyzing the amount of damage in the each hippocampal subfield at each glycine concentration. Significant destruction in the CA1 subfield ($P \leq 0.001$, Kruskal–Wallis test, $P < 0.01$ with Mann–Whitney test for posthoc analysis with Bonferroni correction for multiple comparisons) occurred after exposure to 4 mM glycine for 24 h (Fig. 3). Quantitative analysis of PI fluorescence revealed that glycine caused significantly greater damage to CA1 relative to the other subfields at concentrations of 4 and 6 mM ($P < 0.01$, Kruskal–Wallis test with Tukey's test for posthoc comparison, CA1 versus CA3 or dentate at both concentrations). Equivalent damage to all subfields occurred at glycine concentrations of 8 and 10 mM (Fig. 3).

Glycine (10 mM) was added to hippocampal slice cultures between 30 min and 8 h to determine the time required for toxicity to occur. Since glycine-induced neurotoxicity was found to be most marked in CA1, evaluation of damage to the CA1 subfield was used to determine the duration of glycine exposure which produced complete destruction in this subfield. Quantitative analysis of PI fluorescence revealed that significant toxicity occurred at 30 min of exposure ($P \leq 0.001$, Kruskal–Wallis, $P < 0.01$, Mann–Whitney test with Bonferroni correction for multiple comparisons) and reached maximal levels after 4 h (Fig. 4).

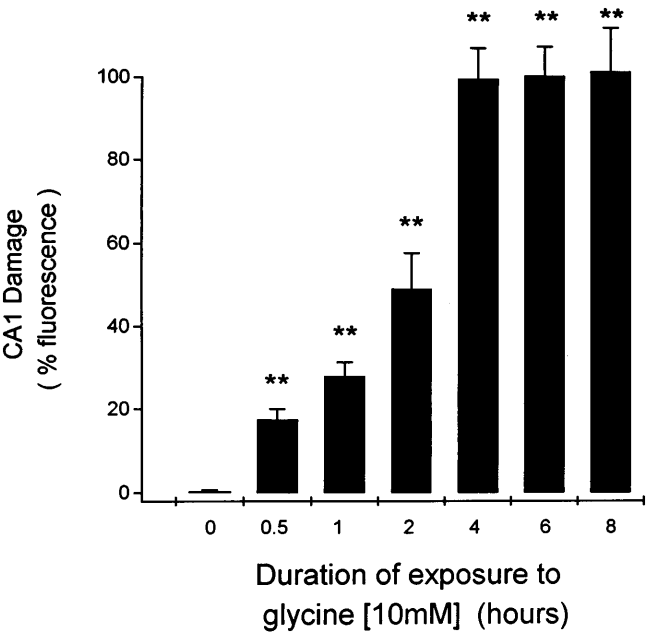

FIG. 4. Analysis of neurotoxicity in area CA1 with increasing duration of exposure to 10 mM glycine. Neurotoxicity was quantitated using analysis of propidium iodide fluorescence (% toxicity) in the CA1 region of cultures. There was significant damage in CA1 in cultures exposed to glycine for 30 min or longer, compared to control cultures that were not exposed to glycine ($P \leq 0.001$ by Kruskal–Wallis, $^{**}P < 0.01$ by Mann–Whitney with Bonferroni correction for multiple comparisons for posthoc analysis, $n = 8$ in each group). Cultures exposed to 10 mM glycine for 4 h or longer showed complete loss of CA1 neurons.

The effect of NMDA and AMPA receptor antagonists on glycine-induced neurotoxicity in hippocampal subfields CA1, CA3, and dentate was evaluated. Cultures were either pretreated with buffer or with glutamate receptor antagonists 30 min before the addition of 10 mM glycine for 24 h. After 24 h, the glycine containing medium was removed and the cultures were transferred to growth medium containing PI for 24 h and then imaged. Quantitative analysis of PI fluorescence was used to evaluate neuronal damage.

Glycine (10 mM) for 24 h caused significant neurotoxicity in all subfields in the slice cultures as quantified by PI fluorescence ($P \leq 0.001$, Kruskal–Wallis test, $P < 0.01$ with Tukey's test for posthoc comparison). This neurotoxicity was completely blocked by the competitive NMDA antagonist APV (100 μM) and the

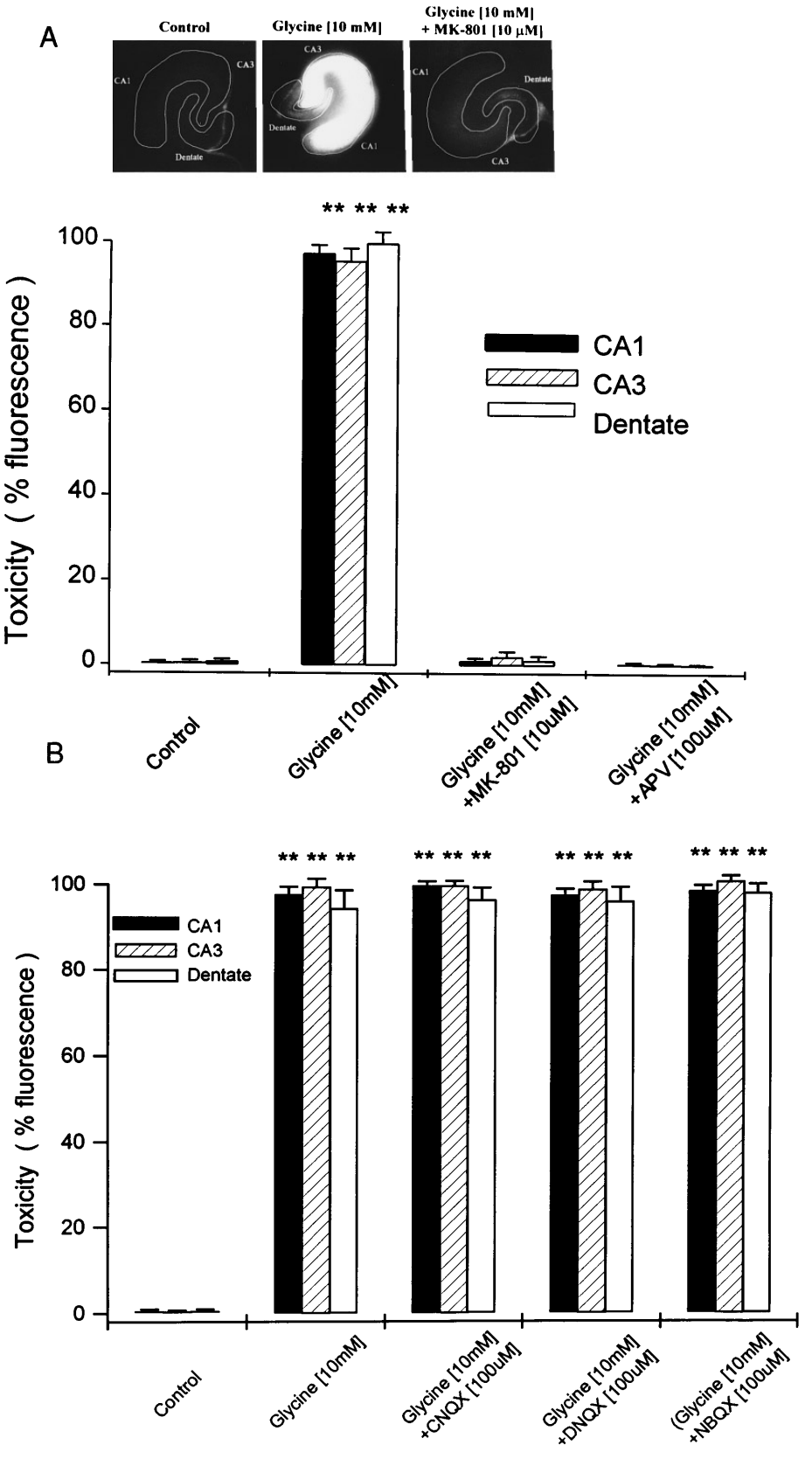

FIG. 5. Effects of NMDA and AMPA receptor antagonists on glycine-induced neurotoxicity in hippocampal slice cultures. (A) There was significant damage to all hippocampal subfields by exposure to 10 m*M* glycine for 24 h relative to untreated control cultures ($p \leq 0.001$ Kruskal Wallis test, **$p < 0.01$ by Tukey's for posthoc analysis). The NMDA antagonists MK-801 (10 μ*M*) and APV (100 μ*M*) significantly

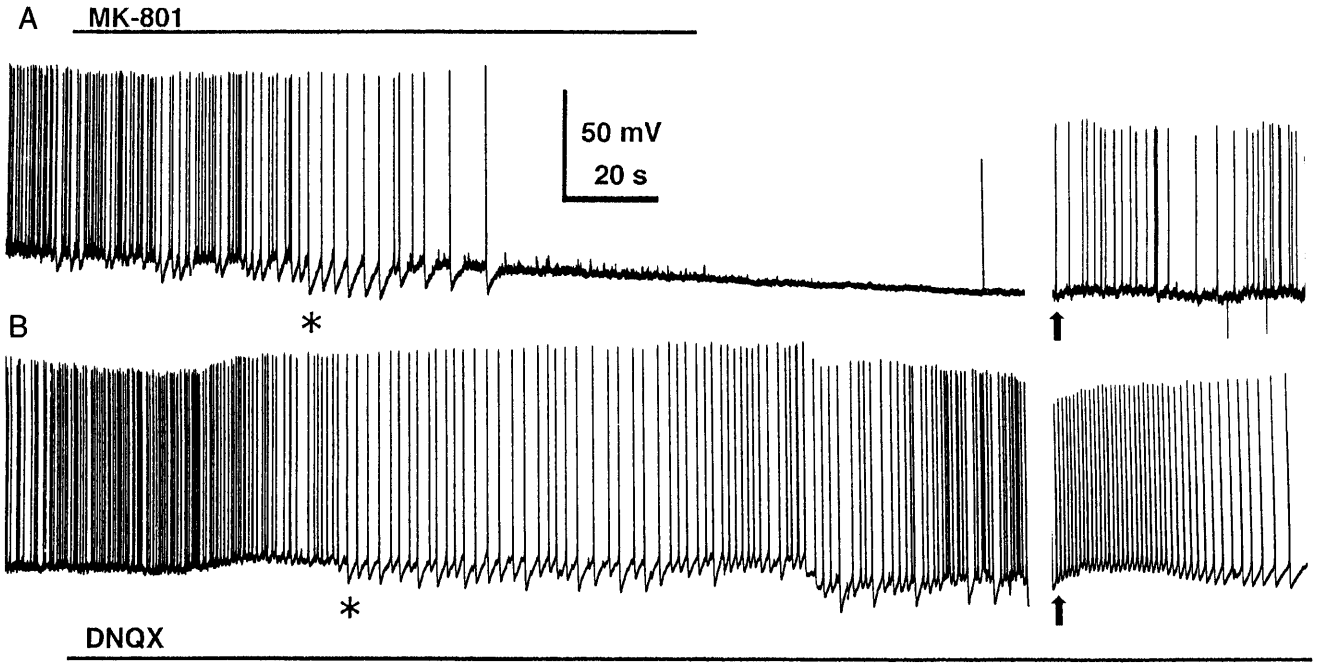

FIG. 6. Intracellular recordings from CA1 pyramidal neurons showed spontaneous EPSPs and IPSPs, but no spontaneous action potentials prior to glycine application (not shown, mean V_m (resting potential) = −64.5 mV). Continuous bath application of 10 m*M* glycine initially caused a transient hyperpolarization in 8 of 12 cells (not shown, mean hyperpolarization = −9.3 ± 1.3 mV). An increase in spike frequency was observed during continuous application of 10 m*M* glycine in 12/12 cells, as shown at the beginning of traces in 6A (V_m = −60 mV) and 6B (V_m = −61 mV). The increase in spike frequency was followed by burst activity in 8 of 12 cells. Bursts can be identified by large burst after hyperpolarizations (asterisks). (A) When the buffer was switched from one containing 10 m*M* glycine alone to a buffer containing 10 m*M* glycine plus 10 μ*M* MK-801, the glycine-induced increase in neuronal excitability was completely and rapidly blocked (n = 3). Note that during glycine plus MK-801 application, the spontaneous activity returns close to values observed after applying glycine alone, but prior to the appearance of hyperexcitability, i.e., hyperpolarized membrane potential and no spontaneous action potentials. The glycine-induced increase in spike frequency partially returned 36 min after the start of MK-801 washout (arrow). (B) In contrast, the glycine-induced increase in neuronal excitability was not blocked by the AMPA receptor antagonist DNQX (100 μ*M*, n = 3). The glycine-induced increase in spontaneous action potential firing rate was still apparent 10 min after the start of DNQX application (arrow).

noncompetitive NMDA antagonist MK-801 (10 μ*M*), as demonstrated by a significant reduction in the PI fluorescence in CA1, CA3, and dentate in the treated groups, compared to the control group ($P < 0.01$, Tukey's test for posthoc comparison) (Fig. 5A, and inset). In contrast, AMPA receptor antagonists [CNQX, DNQX, and NBQX (100 μ*M*)] did not significantly block glycine-induced neurotoxicity (Fig. 5B).

Intracellular recording was used to examine the acute effects of glycine on the electrophysiological properties of CA1 pyramidal neurons (n = 12 cells, 1 cell/culture). Prior to glycine application, all cells exhibited spontaneous excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs), but no spontaneous action potentials (not shown). Continuous bath application of 10 m*M* glycine initially caused a transient hyperpolarization in 8 of 12 cells (not shown, mean hyperpolarization = −9.3 ± 1.3 mV), followed by a sustained increase in the frequency of spontaneous action potentials from 0 to 15 Hz in all cells (n = 12 cells). This increase in spontaneous action potential firing rate is shown at the beginning of traces in Figs. 6A and 6B. Eight of the 12 cells also exhibited repetitive bursts of action potentials and large burst afterhyperpolarizations (asterisks, Fig. 6). The noncompetitive NMDA receptor antagonist MK-801 (10 μ*M*)

reduced glycine-induced damage in all hippocampal subfields quantitated by propidium iodide (PI) fluorescence ($P < 0.01$, Tukey's test for posthoc analysis comparing glycine-treated to glycine + MK-801 or APV). PI measurements of glycine-induced damage in MK-801- and APV-treated cultures were not significantly different from those of untreated control. (Inset) Photomicrographs of PI fluorescence in a control culture (left), a culture exposed to 10 m*M* glycine for 24 h (center), and a culture exposed to 10 m*M* glycine + 10 μ*M* MK-801 for 24 h (right). (B) AMPA antagonists did not protect hippocampal neurons from glycine-induced neurotoxicity. Cultures exposed to 10 m*M* glycine alone for 24 h or to glycine in the presence of 100 μ*M* CNQX, DNQX, or NBQX all produced neurotoxicity that was significantly different from that observed in untreated control cultures (**p < 0.01 by Kruskal–Wallis test with Tukey's test for post-hoc analysis). However, there was no significant difference in neurotoxicty observed in glycine treated vs. cultures treated with glycine plus either CNQX, DNQX, or NBQX. (n = 8 for each group).

completely and rapidly blocked glycine-induced hyperexcitability ($n = 3$, Fig. 6A). In contrast, the AMPA receptor antagonist DNQX (100 μ*M*) did not block glycine-induced bursting or the continued increase in spontaneous action potential firing rate (Fig. 6B, $n = 3$).

DISCUSSION

These results demonstrate that glycine can produce increased excitability and neurotoxicity in cultured hippocampal slices. Glycine-induced neurotoxicity and excitability appear to be mediated by activation of NMDA receptors, since the neuronal damage caused by glycine was completely blocked by NMDA antagonists but not by AMPA receptor antagonists. Although glycine has previously been reported to produce an apparent nonreversible deterioration of synaptic transmission in acute hippocampal slices (53), this report clearly demonstrates that glycine caused neuronal cell death.

Glycine, acting as a cofactor, is required for glutamate activation of NMDA receptors (21, 23, 28, 47, 49). The cofactor site for glycine is reported to be fully occupied at concentrations of 1 μ*M* or less (21). Glycine is normally present in the brain interstitial space at a concentration of approximately 10 μ*M* (15), suggesting that the glycine cofactor site on the NMDA receptor is fully saturated under normal physiological conditions. At concentrations up to 3 m*M*, application of glycine alone does not appear to activate NMDA receptors (23, 28). However, our results show that 4 m*M* glycine alone for 24 h or 10 m*M* glycine for at least 30 min produces significant neurotoxicity. Similarly, Wallis *et al.* reported that the EC_{50} concentration of glycine required to produce loss of CA1 orthodromic population spikes in acutely prepared hippocampal slices was 7.1 m*M* (53). Therefore, glycine may have two separate actions on the NMDA receptor. At low concentration, glycine acts as a permissive cofactor for glutamate activation of NMDA receptors, but micromolar concentrations of glycine do not appear to increase excitability or produce neurotoxicity. In contrast, 4–10 m*M* glycine appears to lead to the activation NMDA receptors, resulting in an increase in excitability and neurotoxicity.

It is unclear from these results whether high concentrations of glycine can directly activate NMDA receptors or whether high concentrations of glycine increase the sensitivity of NMDA receptors to endogenous glutamate. Glycine could enhance glutamate activation of NMDA receptors by reducing the voltage-dependent magnesium blockade of the NMDA receptor, making this receptor more easily activated at resting membrane potentials. This latter explanation is supported by the observation that 10 m*M* magnesium provided partial protection against glycine-induced excitotoxicity in acutely prepared hippocampal slices (53). Tetrodotoxin, which blocks synaptic transmission, also did not block glycine-induced CA1 dysfunction, suggesting that presynaptic glutamate release was not the primary mechanism responsible for the glycine-induced excitotoxicity (53). Further studies will be required to determine the precise mechanisms of glycine-induced NMDA receptor activation and neurotoxicity.

Glycine's acute electrophysiological effects on CA1 pyramidal neurons are consistent with an excitotoxic mechanism leading to the observed toxicity seen in whole cultures. Sustained spiking and bursting in pyramidal neurons could cause increased influx of calcium, leading to cell death (17, 29, 44, 45). Blockade of the electrophysiological and neurotoxic effects of glycine by MK-801 suggest that these affects were mediated by NMDA receptors.

The high numbers of NMDA receptors in area CA1 may also explain the partially selective damage to this area by 4 and 6 m*M* glycine. CA1 pyramidal neurons are among the most sensitive neurons in the brain to damage induced by energy deprivation and ischemia (31, 41, 45), and this sensitivity of CA1 neurons has been attributed to a high density of NMDA receptors. Dentate granule cells also have a high density of NMDA receptors, but are typically less sensitive to excitotoxic damage than CA1 neurons. Therefore, glycine and ischemic insults produce a similar pattern of injury.

Glycine neurotoxity may be a physiologically relevant phenomenon, even though very high concentrations are required to activate NMDA receptors. Glycine-induced hyperexcitability and neurodegeneration may occur in several disorders. For example, nonketotic hyperglycinemia is a rare inborn error in metabolism of glycine degradation, in which markedly elevated glycine levels are found in the plasma and spinal fluid (10). The clinical course is characterized by progressive severe mental deficiency and continuous low-grade convulsions. Glycine is also found in elevated concentrations in epileptic foci and may play a role in neuronal hyperexcitability seen in epilepsy (25, 39). Glycine may also contribute to excess NMDA receptor activation leading to neuronal damage produced by ischemia (14, 15, 38, 53).

The concentrations of glycine which have been reported in brain tissue during ischemia (30–60 μ*M* assuming an 18% recovery rate from microdialysis (15)) are lower than the concentrations which we observed to be neurotoxic (4–10 m*M*). However, specific glycine uptake systems exist in spinal cord and hippocampus (53, 58), and these glycine uptake system may increase the concentration of exogenous glycine needed to produce neurotoxicity. These uptake mechanisms are similar to those used to remove synaptically released glutamate. Glutamate, which has been implicated in ischemic neuronal damage (4, 6, 29, 40), also is required in high concentrations to produce neurotoxicity in organotypic hippocampal cultures. We have found that 10 m*M*

glutamate for 30 min is required to produce neuronal damage in slice cultures (34). These cultures, like brain tissue, appear to have an efficient glutamate uptake system, which is primarily present in glia. These glial uptake mechanisms appear to reduce glutamate-mediated neurotoxicity. For example, dissociated cultures of murine cortical neurons are 10-fold less sensitive to glutamate neurotoxicity when glia are present versus when they are absent (11). Glutamate uptake mechanisms appear to be partially inactivated under ischemic conditions (13), thereby reducing the concentration at which glutamate can produce neurotoxicity. Glutamate may also be toxic at lower concentrations during ischemia because neurons become depolarized (51, 52). If inhibition of glycine uptake also occurs under certain pathological conditions, glycine may produce neurotoxicity at lower (more physiological) concentrations. Alternatively, if glycine acts by enhancing glutamate's neurotoxic properties, then much lower concentrations of glycine may be required to produce neurotoxicity during ischemic insults when decreased glutamate uptake leads to a rise in extracellular glutamate. Indeed, Globus *et al.* have suggested that extracellular glutamate levels which are reached during ischemia are insufficient by themselves to account for the neuronal damage seen in ischemia, and indicate that elevated glycine may act in combination with glutamate to enhance excitotoxicity (14, 15).

These results demonstrate that glycine can produce neuronal excitation and neurotoxicity that is associated with the activation of NMDA receptors. Under these *in vitro* conditions, glycine-mediated neuronal excitation and neuronal damage occur at glycine concentrations that are higher than those required for its role as a permissive cofactor for activation of the NMDA receptor. Glycine-induced/NMDA receptor-mediated neurotoxicity occurs in all hippocampal subfields, but shows a slight selectivity for area CA1. These previously unrecognized neurotoxic effects of glycine may have relevance in a variety of pathologic processes including abnormalities in glycine metabolism, epilepsy, and ischemia.

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