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Research report

Intrinsic redox properties of *N*-methyl-D-aspartate receptor can determine the developmental expression of excitotoxicity in rat cortical neurons in vitro

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Abstract

The sensitivity of central neurons in culture to *N*-methyl-D-aspartate (NMDA) receptor-mediated cell death increases with development. In this study, we show that this phenomenon in vitro may be due, at least in part, to changes in the redox properties of the NMDA receptor itself. With increasing days in culture, NMDA-induced electrical responses in rat cortical neurons are less sensitive to dithiothreitol-induced potentiation and spontaneously oxidize less readily than in younger cells. These results imply that at earlier developmental ages NMDA receptors prefer a more oxidized state. Hence, in the presence of a reducing agent, NMDA-induced neurotoxicity was produced in normally resistant younger neurons. The observed changes in NMDA receptor properties with development could not be attributed to long-range diffusible redox endogenous factors. An oxidized NMDA receptor thus confers maturing neurons a protective mechanism against glutamate toxicity during development.

Keywords: *N*-Methyl-D-aspartate; Sulfhydryl; Rat cerebral cortex; Tissue culture; Glutamate; Neurotoxicity

1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor can be modulated by both exogenous and endogenous sulfhydryl redox reagents [1]. Reducing agents such as dithiothreitol (DTT) and dihydrolipoic acid potentiate NMDA-induced physiological responses [5,30,31]. Conversely, oxidizing agents such as 5,5′-dithio-bis-nitrobenzoic acid (DTNB), lipoic acid, reactive oxygen species, oxidized glutathione, and pyrroloquinoline quinone reverse the effects of reductants or depress native responses [2,3,5,12,29–31]. There is mounting evidence that all physiological and pathophysiological consequences of NMDA receptor activation can be dramatically altered by modification of its redox-sensitive sites [1]. For example, redox reagents have been shown to influence NMDA-stimulated neuronal death in vitro [2,4,17]. Hence, the addition of DTT to neurons during NMDA treatment can substantially increase neurotoxicity when compared to exposure to NMDA alone, whereas oxidizing agents usually attenuate cell death.

A hallmark of rapidly triggered NMDA receptor-mediated neurotoxicity of embryonic central nervous system in tissue culture is its developmental onset [9,11,24,34]. Initially, immature neuronal cultures from the cortex, hippocampus or cerebellum are resistant to NMDA-induced toxicity. However, as the neurons mature, their sensitivity to excitotoxicity increases. Changes in neuronal susceptibility to NMDA receptor overactivation also occurs in vivo at early postnatal stages [21]. Several mechanisms have been hypothesized to account for these observations, including changes in the NMDA receptor itself [11,24] or other cell-intrinsic factors that may allow neurons to handle the consequences of NMDA receptor stimulation differently as they mature [8,20,32]. This report demonstrates that immature neurons have the necessary cellular mechanisms in place to express NMDA-induced excitotoxicity but are normally protected due to the redox state of the receptor itself. Preliminary results of this study have appeared in abstract form [28].

2. Materials and methods

2.1. Cell culture

Neuronal cultures were prepared from enzymatically dissociated E16 rat cortices as described previously [10,25].

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Briefly, cells were plated onto poly-L-lysine-coated 12-mm glass coverslips at a density of 300 000–500 000 cells/ml of growth medium (v/v mixture of 80% Dulbecco's modified Eagle's minimum essential medium, 10% Ham's F12 nutrient mixture, 10% heat-inactivated iron-supplemented bovine calf serum, 25 mM HEPES, 24 U/ml penicillin, 24 μg/ml streptomycin, and 2 mM L-glutamine). Cells were maintained at 37°C in 5% CO_2. After 15 days in vitro (DIV), non-neuronal cell proliferation was inhibited with 2 μM cytosine arabinoside after which the growth medium used was devoid of F-12 and contained 2% serum. Partial media changes were performed on a Monday-Wednesday-Friday schedule.

2.2. Whole-cell recordings

Electrophysiological measurements were obtained from neurons using the whole-cell patch-clamp configuration. Methods for data acquisition and analysis have been previously described [4,30]. The external recording solution contained 150 mM NaCl, 2.8 mM KCl, 1.0 mM $CaCl_2$, 10 mM HEPES, 10 μM glycine and 0.5 μM TTX. Patch electrodes (2–4 MΩ) were filled with 140 mM CsF, 10 mM EGTA, 1 mM $CaCl_2$, and 10 mM HEPES. NMDA dissolved in extracellular solution, was applied onto cells for 3 s by close perfusion with a micropipette. Sulfhydryl reagents were added to the bath via a continuous perfusion system. Results are expressed as the mean ± standard deviation.

2.3. Toxicity assays

Cell survival was assessed using [^{3}H]ouabain binding method [2,19]. With this assay, the ligand binds to a Na^+,K^+-ATPase found more abundantly on viable neurons. Therefore, a decrease in specific binding reflects a loss in neurons. Briefly, cortical cells were plated on 12-mm coverslips contained in a chamber of culture plate. A treatment group consisted of four coverslips (three for total binding and one for non-specific binding). The cells were exposed to either the control or drug solution for 30 min after which the treatment was removed by two rinses with minimum essential medium. The ouabain assay was performed 18–24 h after drug exposure. Neurons were rinsed with binding buffer consisting of 100 mM Tris-HCl with 0.01% BSA. Cells were then incubated at 37°C for 30 min in 500 μl of the binding buffer with the inclusion of 5.0 mM ATP and 50 nM [^{3}H]ouabain. Non-specific binding was measured by the addition of 12.5 μM unlabeled ouabain and represented $<$10% of total binding. After the incubation, cells were rinsed three times with 1 ml of cold PBS, solubilized with 1 ml of a 10% SDS/10 mM EDTA solution and counted in 4 ml of scintillation fluid. Counting efficiencies between experiments did not vary greatly (H# values, a measurement by the scintillation counter assessing quenching variability, averaged to 86.4 ± 12.9); thus, results are expressed as mean counts per minute (cpm) ± standard deviation.

3. Results

3.1. Redox properties of cultured neurons during development

Whole-cell electrophysiological recordings were performed on rat cortical neurons in tissue culture. Neurons were categorized as either 'young' ($<$21 DIV) or 'mature' ($>$30 DIV) based primarily on their relative sensitivity to NMDA excitotoxicity. Under our culture conditions, neurons become fully sensitive to NMDA receptor-mediated cell death between 25 and 29 DIV [2]. To determine the resting redox state of NMDA receptors in young and

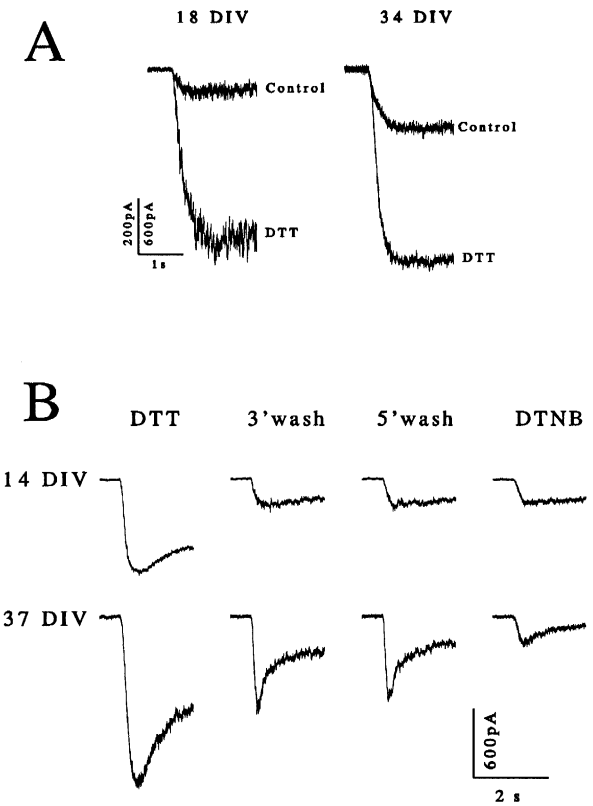


Fig. 1. Effects of DTT and DTNB on NMDA receptors: representative whole-cell responses (−60 mV) to 30 μM NMDA in rat cortical neurons at two different ages in vitro. A: NMDA-induced responses were measured from cortical neurons under control conditions and following treatment with 4 mM DTT for 3 min. The degree of potentiation by DTT was greater in the 18 DIV cell (8-fold) than in the 34 DIV cell (5-fold). B: consecutive responses were recorded following a 6-min incubation of 4 mM DTT, during wash with control solution, and following a 1-min treatment with 0.5 mM DTNB. A 3-min wash was sufficient to fully re-oxidize the NMDA receptors in the 14 DIV cell, with DTNB having little or no additional effect on the response amplitude. In contrast, spontaneous oxidation of the receptors in the 37 DIV cell is incomplete. In this cell, DTNB oxidation has a pronounced effect on the remaining current.

mature neurons, peak NMDA (30 μM)-induced whole-cell currents (-60 mV) produced under control conditions were compared to the potentiated currents that resulted from a 6-min incubation with 4 mM DTT (Fig. 1A and Fig. 2A). A significantly greater potentiation of the whole-cell currents was observed in young neurons (8.7-fold; $n = 10$) when compared to mature cells (5.3-fold; $n = 23$). Maximal current amplitudes (-60 mV) following the 6-min DTT treatment, however, were similar in both young (-1148.3 ± 202.5 pA; $n = 31$) and older (-1667.5 ± 152.2 pA; $n = 37$) neurons. The larger effect of DTT on younger neurons suggests that their NMDA receptors may normally rest at a more oxidized state.

Upon removal of DTT, potentiated NMDA-induced whole cell currents undergo spontaneous re-oxidation by a yet-undefined process [31]. We studied whether the rate and magnitude of this process also varied with development in culture. Following the DTT treatment, neurons were rinsed with control solution and NMDA-induced currents were measured every 30 s for a total of 5 min.

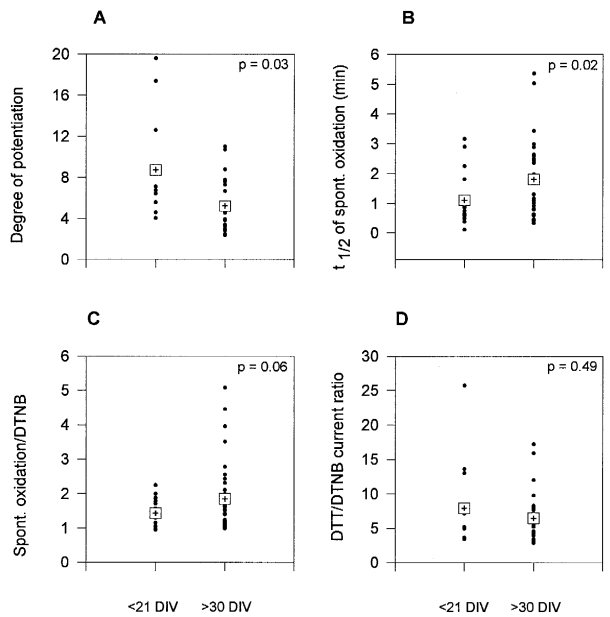


Fig. 2. Summary of the various parameters studied to assess the redox status of the NMDA receptors in all cells used in this study. The data plotted are the individual (●) and mean (+) values. A: DTT/control current ratios from a total of 33 cortical neurons, which is an indication of the fold-potentiation by a 4 mM DTT treatment. For cells < 21 DIV the mean ratio ($\pm$S.D.) was 8.7 ± 5.8 ($n = 10$) whereas the ratio for cells > 30 DIV was 5.3 ± 2.5 ($n = 23$). P value shown for this and subsequent panels was obtained by a non-parametric Mann-Whitney U test. B: rate of spontaneous oxidation as measured by $t_{1/2}$ values from a total of 50 cortical neurons. For neurons < 21 DIV, the $t_{1/2}$ was 1.2 ± 0.9 min ($n = 16$) while for older cells this value was 1.8 ± 1.2 min ($n = 34$). C: the final level of spontaneous oxidation/DTNB current ratio from a total of 61 cortical neurons. This ratio, which is indicative of the degree of spontaneous oxidation, was 1.4 ± 0.4 for younger cells ($n = 22$); and 1.9 ± 0.9 for cells > 30 DIV ($n = 39$). D: DTT/DTNB values from 41 cortical neurons, indicating the total redox range for these cells' receptors. The ratio for neurons < 21 DIV was 7.9 ± 6.5 ($n = 11$) whereas for cells > 30 DIV it was 6.5 ± 3.6 ($n = 30$).

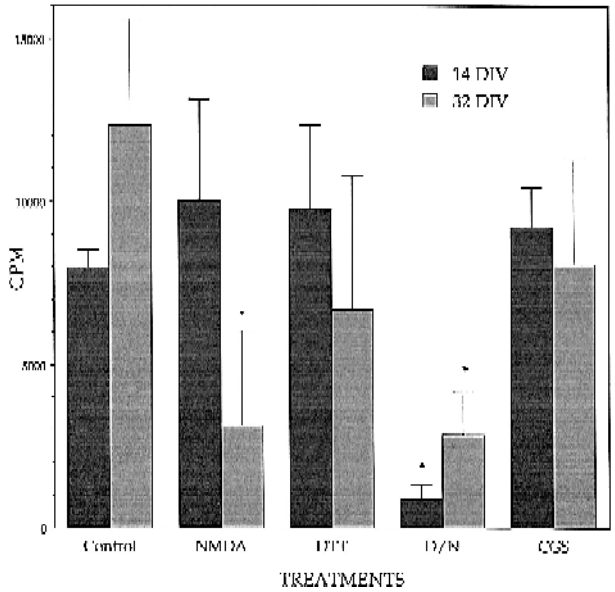
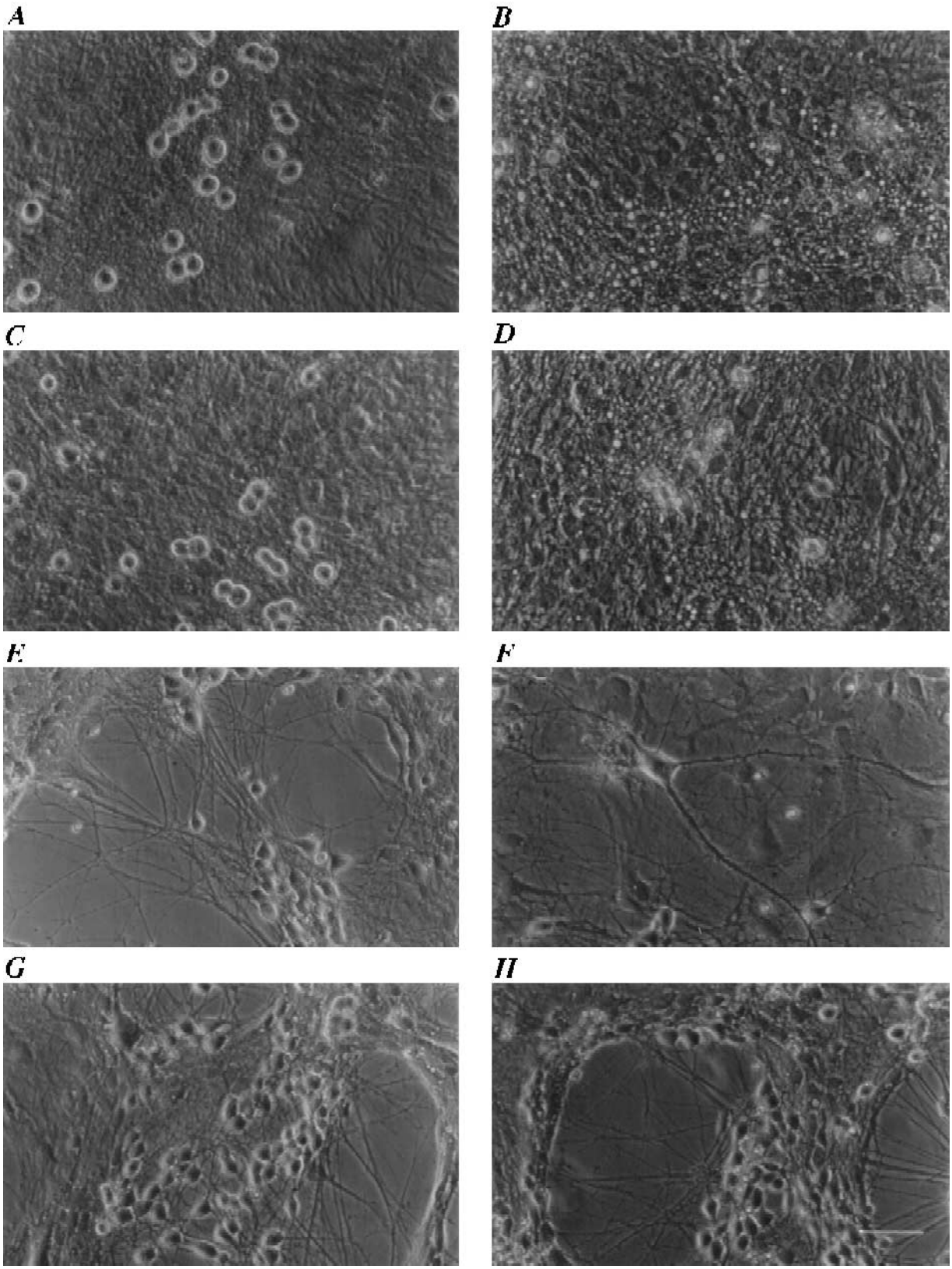


Fig. 3. Age-dependent changes in the sensitivity of cortical neurons to NMDA-induced excitotoxicity. A representative toxicity assay using sister cultures (i.e. from the same dissociation but utilized at different DIV). These neurons were treated for 30 min with either: (1) control solution, (2) NMDA (200 μM), (3) DTT (4 mM), (4) NMDA and DTT together (D/N), or (5) NMDA and DTT with CGS-19755 (100 μM; CGS). Cell survival was assessed using [^{3}H]ouabain binding method where the number of specific counts (cpm) is reflective of the number of living neurons. The younger neurons (14 DIV), normally resistant to NMDA toxicity, became susceptible to this drug in the presence of the reducing agent, but still could be rescued with co-exposure to the NMDA receptor antagonist. For each culture group, a one-way ANOVA revealed significant treatment effects. Post-hoc comparisons, using a Bonferroni correction for multiple tests, revealed a significant differences between the NMDA group and its control in the 32 DIV cells, and between the D/N group and their respective controls for both cell ages ($^* P < 0.05$). This experiment was performed in triplicate and repeated a total of four times with essentially identical results.

The $t_{1/2}$ of the decay of the response amplitudes was then measured to obtain the rate of spontaneous oxidation. The NMDA receptor in younger cells spontaneously oxidized faster ($t_{1/2} = 1.2$ min; $n = 16$) than in mature neurons ($t_{1/2} = 1.8$ min; $n = 34$; Fig. 1B and Fig. 2B). To measure the magnitude of spontaneous oxidation, the amplitude of the final response following this process was divided by the amplitude of the NMDA-stimulated current following a 1 min incubation of 0.5 mM DTNB. Thus, a ratio close to 1 would indicate that spontaneous oxidation was as complete as a DTNB treatment. The extent of spontaneous oxidation was slightly greater in younger cells (1.4 ratio; $n = 22$) than for mature neurons (1.9 ratio; $n = 39$; Fig. 1B and Fig. 2C). These findings suggest that not only is the redox site on NMDA receptors in younger neurons inherently more oxidized, but these receptors also return to this state more quickly and completely following DTT washout. Conversely, receptors in mature neurons appear to remain at an intermediate redox state following spontaneous oxidation and can only be fully oxidized after a treatment

with DTNB. Despite these differences, the ratio between the NMDA-induced current amplitudes following full reduction (4 mM DTT) and full oxidation (0.5 mM DTNB) was not very different for young ($n = 11$) versus mature neurons ($n = 30$; Fig. 2D), suggesting that these thiol-modifying reagents work similarly at all developmental



stages. The variability that was encountered in all the aforementioned comparisons is suggestive of a continuum of changes in redox properties with development. However, at the two ages we chose for our comparison there appears to be a biological difference in the redox properties of NMDA receptors.

3.2. Effects of redox state on susceptibility to NMDA-induced neurotoxicity

We next examined whether the observed changes in redox properties in vitro were linked to the developmental expression of sensitivity to NMDA-induced neurotoxicity. Since the redox state of the receptors in immature cortical neurons are more oxidized, an inherently neuroprotective state [3,4], reduction of the receptor should increase the susceptibility to NMDA-mediated neurotoxicity. That is, assuming that this process is solely dependent on receptor activation. To test this hypothesis, we exposed younger neurons, 11 to 13 DIV, to a concentration of NMDA (200 μM, 30 min) that is normally maximally toxic to mature neurons [26,27], and measured cell viability by [^{3}H]ouabain [2,19]. As expected, the immature neurons were resistant to this treatment. However, the presence of 4 mM DTT during the NMDA exposure was sufficient to induce widespread neuronal damage in these cells. This toxicity was mediated by increased NMDA receptor activity, because an antagonist specific for this receptor (CGS-19755 [23]; 100 μM) completely blocked cell death (Fig. 3). Finally, sister cultures were maintained until 25 to 32 DIV when their normal response to an excitotoxic stimulus was confirmed (Fig. 3). These results were also replicated once using a lactate dehydrogenase release assay and trypan blue exclusion (data not shown) to confirm the findings obtained with the ouabain assay.

3.3. Role of diffusible factors on NMDA receptor redox status

In order to elucidate the mechanism responsible for the changes in redox properties and excitotoxicity during in vitro development, we investigated whether a diffusible redox-active substance or substances could account for the aforementioned phenomena. A 12-mm glass coverslip containing either young (11–13 DIV) or mature (32–34 DIV) cultures was transferred to a culture dish which contained four coverslips with cultures of the alternate age. Thus a coverslip containing a 2-week-old culture was transferred to a 5-week-old culture dish, and vice versa. Twenty-four hours following this switch, the entire culture dish was exposed to 200 μM NMDA for 30 min. Twenty-four hours later, the cells were fixed with physiological buffer containing 2.5% glutaraldehyde and inspected qualitatively with a light microscope for widespread neuronal damage. Assessment of the cultures revealed that young neurons remained resistant to NMDA-induced excitotoxicity despite the proximity of mature neurons, while mature neurons perished even when co-cultured with immature neurons (Fig. 4). Long-range diffusible redox reagents are therefore unlikely to mediate the altering of the redox state of the NMDA receptor and influence the excitotoxic process. Similar results were obtained in a total of three separate experiments with different culture dates.

4. Discussion

The results from the present study show that the inherently oxidized state of the NMDA receptor in immature cortical neurons in vitro protects these cells from acute excitotoxicity. We further demonstrated that the NMDA receptor redox sites change from this oxidized state to a more reduced form with increasing age. Thus, addition of a thiol reducing agent during an NMDA exposure was in itself sufficient to trigger excitotoxicity in young neurons. This suggests that the level of NMDA receptor activity dictated by its redox status can dramatically influence cell survival during development following a potential excitotoxic insult. Redox modulation now joins several other properties of the NMDA receptor have been shown to change with development including Mg^{2+} [6,7,16,22], polyamine [33], glycine [16], and ethanol [18] sensitivity.

The cell culture environment for cells grown before and after 15 DIV were not identical. For example, the serum concentration was dropped, from 10% to 2% after treatment with AraC. It is thus conceivable that a factor present or absent in the serum may be responsible for maintaining a particular redox state. However, experiments modeled after classical conditioned media studies revealed that endogenous, stable, long-range diffusible factors were apparently not responsible for the observed differences in the redox state of the NMDA receptor with development. This was a somewhat surprising result in light of the large number of endogenous redox-active substances which have been reported to interact with the NMDA receptor [1]. However, our results do not rule out that the local microenvironment could influence the redox status of this receptor. In fact, recent studies by other investigators

Fig. 4. Age-dependent cell death takes place regardless of new environment. Representative micrographs of cultured neurons at 32 DIV (A–D) or 14 DIV (E–H). The lower panels for each developmental age (C, D, G and H) are photographs from coverslips which had been transferred to a culture dish containing the alternate developmental age. Cells were exposed to either control solution (left panels: A, C, E and G) or to 200 μM NMDA for 30 min (right panels: B, D, F and H). Note the widespread neuronal damage present in the 32 DIV cells despite the culture environment (B, D). In contrast, younger cells survive this treatment (F) even if co-cultured with older cells (H). Bar = 60 μm.

suggest that the NMDA receptor redox modulatory site can be physiologically altered during certain forms of long-term synaptic potentiation that are specifically expressed at this receptor [13,14]. These results argue for the existence of endogenous redox-active modulators, and do not rule out the possibility that the local redox microenvironment is also important is determining the native redox state of the receptor during development.

The most significant outcome of our studies is establishing a putative mechanism which underlies the insensitivity of immature cortical neurons in vitro to NMDA toxicity. This lack of excitotoxicity in younger neurons has previously been attributed to various possible scenarios including a lower number of receptors in young neurons. In support of this hypothesis, Frandsen and Schousboe [11] showed an increase in excitatory amino acid binding during the development of neurons in culture. Additionally, Peterson et al. [24] reported developmental increases in glutamate binding in vivo and correlated these changes to the onset of NMDA toxicity in cultured hippocampal neurons. In contrast to these findings, Xia et al. [34] reported that cultured cerebellar granule neurons had peak NR1 protein expression occurring long before the greatest sensitivity to NMDA toxicity had developed. Our results are suggestive that neurons have a sufficient number of receptors required for toxicity to occur at early developmental stages but that perhaps alterations in receptor properties occur with age. We demonstrate that for both developmental ages mean current amplitudes (following a 6-min exposure of DTT) were not very different. Assuming that single channel conductances and open times are similar for all receptors, and that frequency of channel opening has been maximized by DTT [31], these current values suggest, albeit indirectly, that younger cells contain about 70% of the number of receptors of older cells. This relatively small difference in the number of receptors is likely not sufficient to account for the total absence of toxicity in younger cells and the maximal toxicity exhibited by the older neurons, especially if one considers that older neurons have more elaborate processes. With this factored in, both cell types are likely to have similar current densities. Our findings thus strongly argue that immature cortical neurons in culture have the necessary number of receptors to express NMDA toxicity but are normally protected by an oxidized redox modulatory site. In earlier work, we have shown that oxidation of the NMDA receptor may provide a novel target for neuroprotective drugs [15]. Here, we show that a similar strategy may be normally employed by cortical neurons as a neuroprotective device at early developmental stages.

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