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Redox modulation of NMDA receptor-mediated toxicity in mammalian central neurons

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Acute neurological injury from hypoxia-ischemia, hypoglycemia, and trauma is thought to be predominantly mediated by activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor in the brain and the subsequent influx of calcium ions through receptor-operated channels. Several chronic degenerative diseases, such as Huntington's disease and the amyotrophic lateral sclerosis-Parkinsonism-dementia complex found on Guam, may share a similar pathogenesis due to a glutamate-like toxin. This laboratory recently reported that exposure to a reducing agent, such as dithiothreitol (DTT), selectively increases ionic current flow through NMDA-activated channels in several types of central neurons; conversely, oxidizing agents reverse this effect. To investigate the novel influence of redox modulation on NMDA neurotoxicity, in the present in vitro study we monitored survival of an identified central neuron, the retinal ganglion cell, ~24 h after a brief exposure to DTT. To determine the degree of killing specifically related to activation of the NMDA receptor, 2-amino-5-phosphonovalerate (APV, a selective NMDA antagonist) was added to sibling cultures. APV-preventable, glutamate-induced death was increased 70 ± 9% with DTT treatment. This effect was totally blocked by the concomitant addition of an oxidizing agent, 5,5-dithiobis-2-nitrobenzoic acid (DTNB). These findings suggest that the enhanced killing following chemical reduction with DTT is mediated at the NMDA receptor site, and that the redox state of the NMDA receptor is crucial for the survival of neurons facing glutamate-related injury. Since an altered reducing state has been found in cerebral infarcts, these results have implications for the treatment of stroke and possibly other forms of NMDA receptor-mediated neuronal death.

Excessive stimulation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor is thought to mediate acute neurological insults due to hypoxia-ischemia, hypoglycemia and trauma, and possibly chronic dementing illnesses such as Huntington's disease [3, 7, 8, 10, 12, 18, 19, 21, 22, 24, 25]. Calcium ion influx through NMDA receptor-operated channels appears to be responsible for the predominant form of this type of neurotoxicity [5, 6, 13, 14]. Recently, using patch-clamp recordings of single central neurons, Aizenman et al. [2] showed that oxidizing and reducing

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agents can profoundly and selectively regulate the magnitude of the ionic current response to NMDA. We now report that modulation of the redox state can strikingly alter NMDA receptor-mediated injury.

Fig. 1 illustrates whole-cell recordings of currents elicited by NMDA in rat neonatal retinal ganglion cell neurons before and after superfusion in disulfide reducing or oxidizing agents. Exposure to the reducing agent dithiothreitol (DTT; 500 μM) resulted in a marked increase in the response to NMDA compared to control (cf. ref. 2). In contrast, when the same concentration of DTT was oxidized by an excess of 5,5-dithiobis-2-nitrobenzoic acid (DTNB; 1 mM), the NMDA-evoked current was somewhat smaller instead of larger than the control. In addition, rat cortical neurons were influenced in a similar fashion [2], proving that these findings can be generalized to different types of central neurons. The effect of redox modulation was selective for the NMDA subtype of glutamate receptor since responses to kainate and quisqualate were unaffected [2]. Moreover, neither DTT nor DTNB by itself had any direct effect on the ionic currents [1].

To investigate next the effect of redox agents on neurotoxicity mediated at the NMDA receptor, we studied an identified population of central neurons in culture. For this purpose, the survival of neonatal rat retinal ganglion cells was assayed ~24 h after a 5–10 min exposure to DTT or to DTT plus DTNB in the presence of micro-

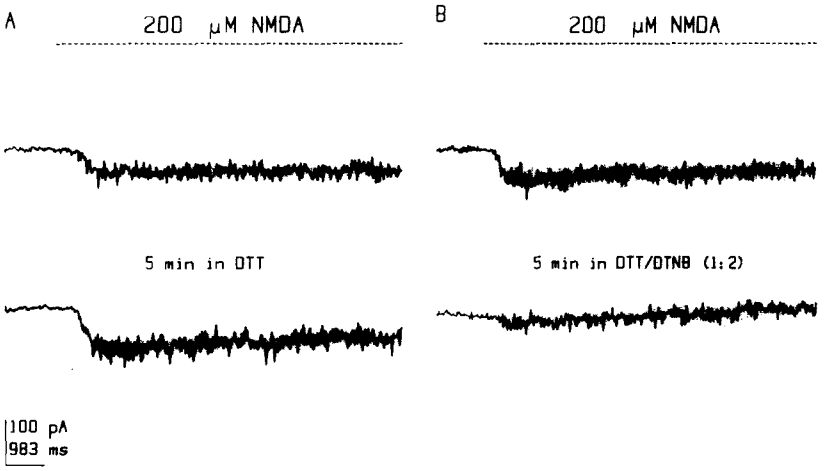
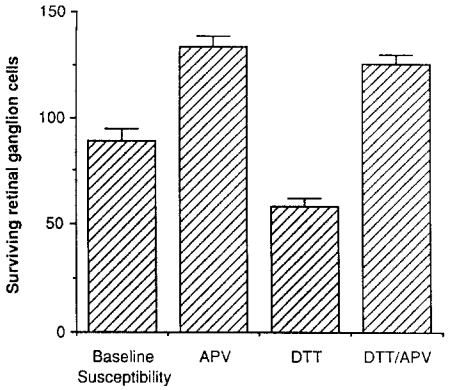


Fig. 1. Chemical reduction and oxidation modulate the response to NMDA in rat retinal ganglion cell neurons. A: whole-cell currents were recorded with a patch pipette from a retinal ganglion cell in culture as previously described [2, 16]. Control responses were initially elicited with 200 μM NMDA applied with a pneumatic pipette. Next, the cells were superfused with a solution containing the reducing agent DTT (500 μM) for 5 min. The subsequent response to 200 μM NMDA (labeled '5 min in DTT') was substantially increased in magnitude. B: in another retinal ganglion cell, following superfusion with a mixture of DTT (500 μM) and DTNB (1 mM), the NMDA-evoked current was somewhat smaller than the control response. For these recordings the holding potential of the voltage-clamped neurons was −60 mV. The bath solution contained a physiological saline based upon Hanks' salts (see text for composition) with a $CaCl_2$ concentration of 2.5 mM, glycine of 1 μM, and was nominally free of magnesium. The patch pipette solution contained (in mM): KCl 140, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 2.25, HEPES 10, adjusted to pH 7.2 with NaOH.

molar concentrations of glutamate. Retinal ganglion cells from 7- to 12-day old Long–Evans rats were labeled and enzymatically dissociated as described previously [15]. Following rinse of the dissociated retinal cells with a physiological saline based on Hanks' salts (composition in mM: NaCl 137, $NaHCO_3$ 1, Na_2HPO_4 0.34, KCl 5.36, KH_2PO_4 0.44, $CaCl_2$ 1.25, $MgSO_4$ 0.5, $MgCl_2$ 0.5, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 5, dextrose 22.2, Phenol red 0.001% v/v; adjusted to pH 7.2 with 0.3 M NaOH), the cells were mechanically dispersed and aliquoted to treatment vials. These vials contained control saline solution, or saline solution with either 0.5–2 mM DTT, or 0.5 mM DTT in the presence of 1 mM DTNB. Treatment groups with DTNB were readjusted to pH 7.2 with 0.3 M NaOH. Cells were agitated gently over a 5–10 min period. After treatment, 100 μl volumes of the cell suspension were plated onto glass coverslips coated with poly-L-lysine in 35 × 10 mm tissue-culture dishes containing 2 ml of culture medium. The cell culture medium was based on Eagle's minimum essential medium (MEM), modified so that it was nominally free of magnesium and contained 10 mM $CaCl_2$ to enhance NMDA receptor-mediated neurotoxicity in this preparation as shown previously [14]. The medium was also supplemented with methylcellulose 0.7% w/v, gentamicin 1 μg/ml, dextrose 16 mM, and rat serum 5% v/v. Glutamate levels in the culture medium were monitored by HPLC analysis. The concentration of exogenous glutamate added to the medium of each experiment to induce cell killing was 10 μM (day-to-day variability in neuronal cell death was most probably due to slight variations in the amount of endogenous glutamate or a glutamate-like substance in these cultures [14]). The 100 μl aliquots of cell suspension diluted into the 2 ml of MEM produced a final concentration of 50 μM Mg^{2+} as well as levels of DTT and DTNB at 5% of their initial treatment concentrations. The cell cultures were incubated for ~24 h at 37°C in an atmosphere of 5% CO_2/95% humidified air. Neuronal survival was then scored by counting retinal ganglion cells displaying uptake and cleavage of fluorescein diacetate to fluorescein, as described previously (for color photographs, see ref. 14). To assess specifically the degree of NMDA receptor-mediated neuronal death, the selective antagonist 2-amino-5-phosphonovalerate (APV; 200 μM) was added to half of the culture dishes in any one treatment group.

Experiments in the present study demonstrate that NMDA receptor-mediated death of retinal ganglion cells is markedly potentiated by a brief treatment with the reducing agent DTT. As shown in Fig. 2, addition of APV (column 2) increased survival above baseline in cultures exposed to 10 μM glutamate (column 1) by $30 \pm 6\%$ (mean $\pm$ S.E.M., $n = 33$), indicating that approximately one-third of the neurons succumbed to NMDA-induced toxicity under these conditions. In contrast, pretreatment of cultures with DTT for 5–10 min enhanced neuronal killing by $70 \pm 9\%$ (comparing columns 1 and 3 to column 2). Since the augmented neuronal death associated with DTT was completely prevented with APV (column 4), this form of toxicity appeared to be mediated through activation of the NMDA receptor.

In contrast to the findings obtained with the reduced form of DTT, oxidized DTT did not effect neuronal cell injury. We produced oxidized DTT from the reduced form using the oxidizing agent DTNB and following the chemical conversion spec-



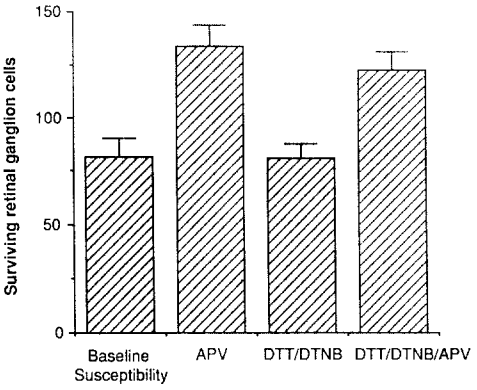


Fig. 2 (left). Transient exposure to the disulfide reducing agent dithiothreitol (DTT) exacerbates NMDA receptor-mediated neurotoxicity. Retinal ganglion cell neurons were labeled in situ, dissociated from the retina, and cultured. Prior to plating, the cells were rinsed for 5–10 min with either a control saline solution or a saline solution containing DTT (0.5–2.0 mM). After 1 day in culture retinal ganglion cell survival was assayed by the uptake and cleavage of fluorescein diacetate to fluorescein [14]. Experiments were conducted in triplicate and repeated on 11 separate days. In order to compare results obtained on different days, data were normalized such that the total number of surviving neurons in a given experiment divided by the number of treatment categories (i.e. 4) equalled 100. Values shown are mean+S.E.M. Each of the treatment categories was significantly different from baseline susceptibility ($P<0.01$) by an analysis of variance followed by a Scheffé multiple comparison of means performed on the raw data or on the pooled data. 2-Amino-5-phosphonovalerate (APV; 200 μM) prevented the death of retinal ganglion cells exposed to glutamate in the culture medium (compare columns 1 and 2). APV incubated in cultures with normal (1.8 mM Ca^{2+}) had no effect on neuronal survival [17]. On the other hand, transient exposure to DTT substantially increased death (column 3). Vulnerability of the neurons could be blocked with 200 μM APV despite DTT treatment (compare columns 2 and 4 to column 3). This finding strongly suggests that the increased susceptibility of neurons following treatment with the reducing agent was mediated via the NMDA receptor.

Fig. 3 (right). Unlike the reduced form, oxidized dithiothreitol (DTT) does not significantly affect NMDA receptor-mediated neurotoxicity. Retinal cells were cultured as in Fig. 2. In these experiments transient exposure to the reduced form of DTT (500 μM) occurred in the presence of the oxidizing agent 5,5-dithiobis-2-nitrobenzoic acid (DTNB; 1 mM). The experiments were conducted in triplicate and repeated on four separate days; survival in cultures treated with APV (columns 2 and 4) was significantly greater than baseline (column 1) by an analysis of variance followed by a Scheffé multiple comparison of means ($P<0.01$). In contrast, exposure to the combination of DTT/DTNB (column 3) did not affect neuronal survival compared to baseline (column 1).

trophotometrically. For example, Fig. 3 shows that concomitant exposure of the cultures to the oxidizing agent DTNB (1 mM) could completely prevent the increased vulnerability of neurons engendered by the reducing form of DTT (500 μM). Spectrophotometric analysis of the redox state [11] showed that this concentration of DTNB produced virtually total (>90%) oxidation of the DTT, rendering it ineffective as a sulfhydryl reducing agent. Thus, APV-preventable neuronal death was enhanced only when cultures were exposed to the reduced form of DTT. Moreover, following incubation with the DTT/DTNB mixture, APV could still prevent the death of approximately 30% of the retinal ganglion cells; this finding indicated that susceptibi-

lity to NMDA receptor-mediated toxicity continued at its baseline level, as observed prior to treatment with redox agents (cf. effect of APV in columns 1 and 2 of Figs. 2 and 3). In some culture dishes the combination of DTT/DTNB in a 1:2 ratio was found to increase neuronal survival substantially above baseline susceptibility to glutamate in the absence of DTT ($P<0.01$), but averaged across many experiments these data failed to reach statistical significance. A further complication was brought to light in other experiments in which a high concentration of DTNB by itself was shown to be somewhat toxic to neurons (1 mM producing a $27\pm6\%$ increase in cell death, $n=7$); this lethal effect could not be totally prevented by APV, indicating a deleterious action not mediated at the NMDA receptor. To avoid the harmful action of DTNB itself, in the experiments described here DTNB was always titrated against DTT. Taken together, these data suggest that a delicate balance between oxidizing and reducing agents is necessary in order to optimize survival of neurons.

In conjunction with the patch-clamp electrophysiological data, this study of neuronal survival suggests that glutamate neurotoxicity mediated by NMDA receptor activation can be substantially modulated by altering the redox state. For example, exposure to an exogenous reducing agent nearly doubled the degree of NMDA receptor-mediated neuronal killing (Fig. 2). Moreover, cytosolic levels of *endogenous* reducing agents can change dramatically in tissues under varying physiological conditions [4]. In fact, Tanaka et al. have shown that reducing equivalents in brain increase markedly with mild to moderate strokes resulting in a net reduced state [23], and endogenous reducing equivalents appear capable of modulating the redox state of the NMDA receptor-channel complex. For instance, patch-clamp recordings have shown that the redox state of the *native* NMDA receptor-channel complex varies widely among neurons obtained from each of 3 different central preparations including mammalian retina and cerebral cortex [2]. This finding strongly suggests that there is an in vivo system of endogenous substances that regulates NMDA-activated functions by modulating the redox site [2].

As neurons die due to NMDA-receptor activation following hypoxic-ischemic insult, additional glutamate or related substances are thought to be released from intracellular compartments resulting in a positive feedback loop of glutamate-induced neurotoxicity [20]. It might be anticipated therefore that the increased reducing equivalents released from the cytosol would also contribute to this process by increasing the number of NMDA receptors in the chemically reduced, more active state. Thus, based upon our observations, we propose that redox modulation of the NMDA receptor could be an important factor contributing to neuronal death following a cerebral infarct. In the future, therapeutic strategies may be developed to control the redox state of patients suffering from cerebral ischemia or other forms of NMDA receptor-mediated injury in order to limit the extent of damage to neurons.

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