0022-3565/99/2912-0785$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 291:785–792, 1999

Vol. 291, No. 2
Printed in U.S.A.

A Critical Role of the *N*-Methyl-D-aspartate (NMDA) Receptor Subunit (NR) 2A in the Expression of Redox Sensitivity of NR1/NR2A Recombinant NMDA Receptors[1]

JESSICA C. BRIMECOMBE, WILLIAM K. POTTHOFF, and ELIAS AIZENMAN

Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Accepted for publication July 8, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

In recombinant *N*-methyl-D-aspartate (NMDA) receptors, two redox modulatory sites are thought to exist, one formed by Cys744 and Cys798 on NMDA receptor subunit (NR) 1, and a second one, not yet localized, on NR2A. Reductants increase the open dwell-time and opening frequency of NR1/NR2A channels. In contrast, NR1/NR2B and NR1/NR2C channels exhibit changes only in opening frequency after redox treatments. Here, we evaluated whether the two redox sites act independently of each other, with the NR1 site affecting the opening frequency and the NR2A site altering open dwell-time. Unitary and whole-cell currents mediated by NMDA receptors composed of a cysteine-mutated NR1 subunit, NR1(C744A, C798A) were thus investigated. Dithiothreitol increased the open dwell-time and opening frequency of NR1(C744A, C798A)/NR2A receptors in a manner indistinguishable from that previously seen in wild-type channels. Marginal redox-induced changes in opening frequency of NR1(C744A, C798A)/NR2B receptors were noted. Redox modulation was completely abolished in NR1(C744A, C798A)/NR2C channels. Whole-cell recordings confirmed the single-channel results. Sulfhydryl reagents modulated NR1(C744A, C798A)/NR2A receptors identically to wild-type NR1/NR2A channels, whereas NR1(C744A, C798A)/NR2C receptors were insensitive to redox modulation. The oxidant 5,5′-dithio-bis-(2-nitrobenzoate) attenuated NR1(C744A, C798A)/NR2B receptor-mediated responses in a dithiothreitol-reversible manner. We conclude that cysteines 744 and 798 on the NR1 subunit are not involved in the redox modulation of NR1/NR2A receptors, but are crucial for the modulation of NR1/NR2C-containing receptors. This suggests that the NR2A subunit is necessary and sufficient for the expression of redox sensitivity in NR1/NR2A channels. The slight, but measurable residual redox sensitivity of the mutant NR1(C744A, C798A)/NR2B receptors suggests the existence of an additional redox-sensitive site on NR2B.

N-Methyl-D-aspartate (NMDA) receptor activation has been linked to many important cellular processes in the brain, including neuronal development, synaptic plasticity and excitotoxic cell death (Michaelis, 1998). It is therefore not surprising that the function of the NMDA receptor itself is tightly regulated by a variety of endogenous factors such as magnesium, protons, polyamines, and zinc (McBain and Mayer, 1994; Dingledine et al., 1999). Redox-active agents can also modify NMDA receptor activity, with reducing agents potentiating the response to NMDA, and oxidizing agents reversing the actions of reductants and sometimes attenuating the response to the agonist (Aizenman et al., 1989; Tang and Aizenman, 1993a). A physiological role for this modulatory site was suggested following the discovery of a number of redox-active substances of endogenous origin, which could alter the redox state of the receptor in dissociated mammalian neurons (Aizenman, 1994; Sinor et al., 1997). In fact, alteration of the redox site by endogenously derived factors has been associated with certain forms of long-term potentiation and long-term depression in hippocampal slices (Gozlan et al., 1995). The redox-active substances responsible for these effects in more intact brain tissue remain to be identified.

In recombinant receptors, two separate redox sites on the NMDA receptor have been proposed to exist, each on separate subunits. Using site-directed mutagenesis, Sullivan et al. (1994) determined that two cysteines on the NMDA receptor subunit (NR) 1, Cys744 and Cys798, were largely responsible for redox modulation of receptors composed of NR1/NR2B, NR1/NR2C, or NR1/NR2D subunit combinations. As the sensitivity of NR1/NR2A receptors to thiol agents was only slightly diminished when these two cysteine residues were mutated, an additional redox site, unique to NR1/NR2A receptors, was suggested. Using chimeric constructs, Köhr et al. (1994) concluded that the amino terminus of the NR2A subunit contained such site. However, to date,

Received for publication March 25, 1999.
[1] This work was supported by National Institutes of Health Grant NS29365.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CHO, Chinese hamster ovary; cys, cysteine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; F, opening frequency; GFP, green fluorescent protein; NR, NMDA receptor subunit; OT, open time.

the amino acid residues constituting the redox site on NR2A have not been identified. Mutating various cysteines located in the amino terminus of this subunit, either individually (Sullivan et al., 1994; Köhr et al., 1994), or in tandem (Choi et al., 1997), does not totally abolish the redox sensitivity of NR2A-containing receptors. Although the existence of a separate redox site on NR2A has been questioned (Paoletti et al., 1997), the recent discovery of an NR2A-selective reducing agent strongly argues for the presence of such a site (Arden et al., 1998).

Single-channel studies have revealed that reducing agents potentiate NMDA-induced responses of NR1/NR2A receptors by increasing both the open dwell-time of the channel and increasing the frequency of channel opening relative to the oxidized state (Brimecombe et al., 1997). A sulfhydryl agent-induced alteration in NR1/NR2B- or NR1/NR2C-mediated channel activity, however, is manifested only as a change in opening frequency (Brimecombe et al., 1997). The present study attempted to determine whether the two proposed redox sites on NR1 and on NR2A could act independently of each other in affecting channel function. Because only NR1/NR2A receptors display an increase in open dwell-time in the presence of reductants, we hypothesized that the putative NR2A redox site alone could affect this channel property. Furthermore, because redox agents influence the frequency of channel opening in all receptor configurations, and all receptors contain the NR1 subunit, we further hypothesized that cysteines 744 and 798 of NR1 were critical for this type of channel alteration. We have therefore determined the extent of redox modulation of receptors composed of NR2A, NR2B, or NR2C when coexpressed with an NR1 subunit in which Cys744 and Cys798 have been mutated.

Materials and Methods

Transfection Protocol. Chinese hamster ovary (CHO)-K1 cells were grown in Ham's F-12 nutrient medium with 10% fetal bovine serum and 1 mM glutamine and passaged (<30 times) at a 1:10 dilution when 80% confluent, approximately every 2 days. The cDNAs for NR1a (hereafter referred to simply as NR1; a gift from Dr. S. Nakanishi, Kyoto University, Kyoto, Japan), NR2A, and NR2C (gifts from Dr. P. Seeburg, Max Planck Institute for Medical Research, Heidelberg, Germany) were previously subcloned into a mammalian expression vector (Boeckman and Aizenman, 1994, 1996). NR1(C744A, C798A) was a kind gift from Dr. S. Traynelis, Emory University, Atlanta, GA. The mouse clones ϵ1 (NR2A) and ϵ2 (NR2B) were previously subcloned into pRK7 (Meguro et al., 1992; Gallagher et al., 1996) and were generous gifts from Dr. D. Lynch, University of Pennsylvania, Philadelphia, PA, and Dr. M. Mishina, University of Tokyo, Tokyo, Japan. NR2A and ϵ1 were used interchangeably. For this study we used ϵ2 exclusively rather than NR2B (Boeckman and Aizenman, 1996), because functional expression of receptors with this latter vector was unexplainably lost. Although we noted an increase in the mean open dwell-time of NR1/ϵ1 and NR1/ϵ2 receptors when compared with those previously reported for NR1/NR2A and NR1/NR2B channels (Brimecombe et al., 1997) all other receptor properties, including redox sensitivity, were virtually identical between the rat and mouse subunits. The vector for a positive transfection marker, green fluorescent protein (pCI/GFP; a gift from Dr. M. Chalfie, Columbia University, New York, NY), was also generated previously (Brimecombe et al., 1997). CHO cells were seeded at 2.5×10^5 cells/well in 6-well plates approximately 24 h before transfection with 1.3 μg of total DNA and 5 μl of lipofectAMINE (Gibco-BRL) in 1 ml of serum-free CHO media per 35-mm dish. The ratio of pCI/GFP to total other DNA was 1:4.3, and the ratio of NR1 to NR2 was always 1:3 (Cik et al., 1993). After a 4- to 5-h incubation at 37°C, cells were refed with CHO medium containing 300 μM ketamine to prevent cell death which ensues following functional receptor expression (Boeckman and Aizenman, 1996). Cells were used for recording 40 to 50 h after transfection.

Patch-Clamp Recordings and Analysis. The extracellular solution contained: 150 mM NaCl, 2.8 mM KCl, 1.0 mM $CaCl_2$, 10 mM HEPES, and 0.01 mM glycine 0.01, pH adjusted to 7.2 with 0.3 N NaOH. The pipette solution contained: 14 mM CsF, 10 mM EGTA, 1.0 mM $CaCl_2$, and 10 mM HEPES, pH adjusted to 7.2 with CsOH. Whole-cell measurements were performed at -60 mV with 2 MΩ electrodes; methods for acquisition and analysis of whole-cell data have been described previously (Tang and Aizenman, 1993a). NMDA (30 μM), dithiothreitol (DTT; 4 mM) and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB; 0.5 mM) were applied to the cells by a multibarrel fast perfusion system (Warner Instruments, Hamden, CT). Single-channel recordings were performed at -60 mV with the same recording solutions on outside-out patches using 10 to 15 MΩ electrodes. Drugs were applied to the patch by complete bath exchange (Brimecombe et al., 1997).

Outside-out patches were exposed to 1 mM DTT and 0.5 mM DTNB for approximately 1 to 2 min for each single treatment, initiated in random order across patches. Patches were exposed to the reductant and oxidant repeated times, each seeing both conditions two to three times, on average. Under each treatment, NMDA (10 μM)-activated events were recorded and later analyzed. Unitary conductances were amplified with an Axopatch 200 (Axon Instruments, Foster City, CA), filtered at 2 kHz, stored on videotape, and later replayed and digitized at 10 kHz (Digidata 1200; Axon Instruments). Single-channel analysis was performed using pClamp6 software (Axon Instruments) using a 50% threshold detection criteria. A large number of patches from CHO cells positive for GFP had no NMDA channel activity. In total, data were gathered from 17 patches obtained from approximately 20 separate transfection experiments. Data from patches lost before the completion of a treatment protocol were not used, because each patch served as its own control. Normally, 200 to 500 events were analyzed per single treatment. Amplitude histograms were most commonly fit with single Gaussians. Most open dwell-time histograms were best fit with a single exponential function using a simplex maximum likelihood routine on log-transformed binned data (6 bins/decade). A χ-squared test was used to determine the simplest fit of the data. When an open dwell-time histogram was better fit by the sum of two exponentials (e.g., see Fig. 1B), the weighted mean open time was used for the comparison of means (Brimecombe et al., 1997). Events briefer than 180 μs (twice the rise time of the filter) were ignored. The single-channel amplitude and open dwell-time values were calculated and averaged for all similar treatments for each patch (usually 2–3/patch), and then averaged across patches. Opening frequency in sequential DTT and DTNB treatments were used to calculate the F_{DTT}/F_{DTNB} value (see Table 1) for a single patch. These were then averaged across all patches, which are the values shown in Table 1. As an example, one NR1(C744A, C798A)/NR2A patch had the following opening frequency values (in events per second) DTT = 7.5, DTNB = 0.5, DTT = 5.5, DTNB = 1.6, and DTT = 19. These values resulted in the following F_{DTT}/F_{DTNB} values: 15, 11, 3.4, and 11.9, which averaged 10.3. This procedure was done for all five patches with this subunit configuration. We thought it was important to perform several treatments per patch to determine whether the opening frequency changed as a result of a redox treatment. A F_{DTT}/F_{DTNB} ratio of 1 would suggest no treatment effect (i.e., the null hypothesis). Results are expressed as mean ± S.E.

Results

Single-Channel Redox Properties of NR1-Mutated NMDA Receptors. NMDA (10 μM)-activated channels were first recorded from patches excised from cells transfected

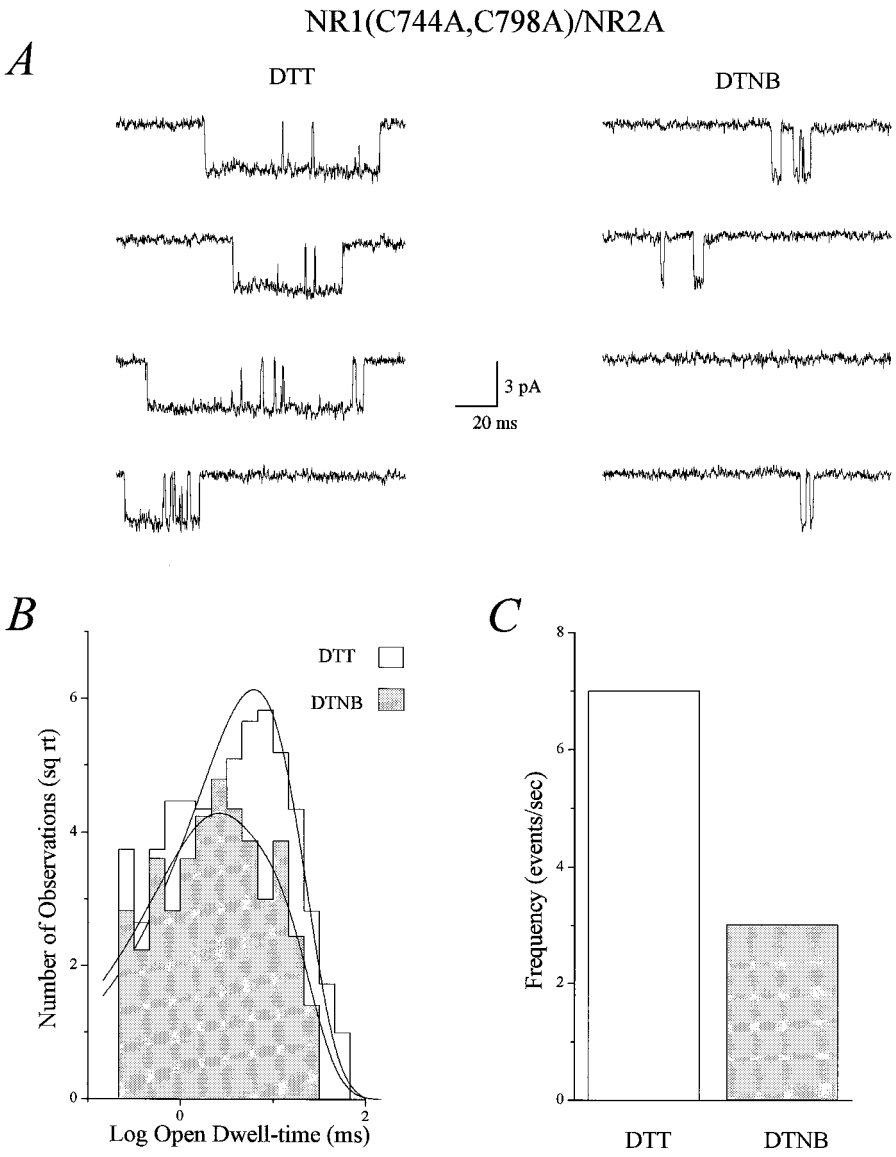

Fig. 1. Mutating Cys744 and Cys798 on the NR1 subunit does not alter the single-channel redox properties of NR2A-containing receptors. A, representative 10 μM NMDA-induced single channel events from an outside-out membrane patch excised from a CHO cell transfected with NR1(C744A, C798A)/NR2A in the continuous presence of either 1 mM DTT or 0.1 mM DTNB. B, open dwell-time histograms for the events from the patch represented in A. The exponential functions fitting both distributions denote a change in time constants between the reduced (7.5 ms) and oxidized (5.4 and 6.3 ms) conditions. C, frequency of NMDA channel opening during the DTT and DTNB treatments for the same patch (hence the lack of error bars). The average change in open channel frequency between the two redox treatments (F_{DTT}/F_{DTNB}) for the five patches obtained from cells transfected with this subunit combination are shown in Table 1.

TABLE 1
Redox properties of single recombinant NMDA receptor channels

	OT_{DTT}	OT_{DTNB}	F_{DTT}/F_{DTNB}
	ms		
NR1(c744,798a)/NR2A (n = 5)	5.0 ± 0.5	3.3 ± 0.6*	5.0 ± 1.6**
NR1(c744,798a)/NR2B (n = 7)	4.8 ± 0.6	4.5 ± 0.6	1.6 ± 0.5
NR1(c744,798a)/NR2C (n = 5)	1.1 ± 0.1	1.3 ± 0.1	0.8 ± 0.1

OT_{DTT}, Open dwell-time of NMDA-activated events under reduced conditions; OT_{DTNB}, Open dwell-time of NMDA-activated events under oxidized conditions; F_{DTT}/F_{DTNB}, ratio of the frequency of channel opening of NMDA activated events in reduced over-oxidized conditions.

*, significantly different from the open dwell-time obtained in the reduced conditions ($p < .01$, paired t test).

**, significantly different from unity ($p < .05$, one-sample t test).

with the NR1(C744A, C798A)/NR2A subunit combination (n = 5; Fig. 1A). The single-channel amplitudes of the unitary currents mediated by these channels were not altered by redox treatments (−3.4 ± 0.1 pA in DTT, −3.5 ± 0.1 pA in DTNB; $p > .05$, paired t test). As seen in wild-type NR1/NR2A receptors (Brimecombe et al., 1997), the open dwell-time of the events increased significantly in the presence of DTT when compared with DTNB (5.0 ± 0.5 ms in DTT, 3.3 ± 0.6 ms in DTNB; $p < .01$, paired t test; Fig. 1B and Table 1). The mean change in open dwell-time, which is the difference in the open time between reduced and oxidized conditions for each patch, averaged across all patches [Δ open time (OT) in Brimecombe et al., 1997] was 1.9 ± 0.2 ms for this receptor configuration. Unexpectedly, DTT increased the frequency of channel opening 5.0- ± 1.6-fold (n-fold = F_{DTT}/F_{DTNB}, where F is opening frequency), when compared with the oxidized state (Fig. 1C and Table 1). These values are very similar to those previously obtained with wild-type NR1/NR2A channels (ΔOT = 1.6 ms, F_{DTT}/F_{DTNB} = 5.7; Brimecombe et al., 1997). In fact, there are no significant differences in ΔOT or F_{DTT}/F_{DTNB} between wild-type and mutant receptors ($p > .05$, unpaired t tests).

The observed changes in opening frequency in NR1(C744A, C798A)/NR2A channel seem to refute the hypothesis that the NR1 site mediates the actions of redox agents for inducing this type of alteration of channel properties. Therefore, we analyzed our data by a different method to ensure that that these changes were indeed reflective of a biologically significant phenomenon. We took advantage of the fact that un-

treated control patches have consistent frequency of openings for periods of up to at least 8 to 10 min, which is sufficient to change DTT and DTNB treatments two to three times in a single patch (Brimecombe et al., 1998). We averaged all opening frequencies for a single treatment per patch and performed a pairwise comparison. The average frequency (in events per second) for DTNB across all NR1(C744A, C798A)/NR2A patches ($n = 5$) was 9.8 ± 6.1 and 22.7 ± 8.2 for DTT. These values are significantly different ($p < .05$; one-tailed paired t test). Therefore, our results clearly indicate that redox modulation of NR1/NR2A receptors is not dependent, to any extent, on the cysteines that form the redox site on the NR1 subunit.

Similar to NR2A-containing channels and to wild-type NR1/NR2B receptors (Brimecombe et al., 1997), the single-channel amplitudes of NR1(C744A, C798A)/NR2B-mediated events were unaffected by redox treatments (−3.5 ± 0.1 pA in DTT, −3.4 ± 0.2 pA in DTNB; $n = 7$; $p > .05$, paired t test). In addition, there was no significant difference in the mean open dwell-time of the channels between the reduced and oxidized states (Fig. 2A and Table 1): 4.8 ± 0.6 ms in DTT and 4.5 ± 0.6 in DTNB ($p > .05$, paired t test; ΔOT = 0.6 ± 0.3 ms). This was not unexpected as the open dwell-times of wild-type NR1/NR2B receptor channels are similarly unaffected by redox treatments (ΔOT = 0.3 ms; Brimecombe et al., 1997). The frequency of channel opening, however, appeared to have been moderately increased 1.6- ± 0.5-fold in the presence of DTT, when compared with DTNB (Fig. 2A and Table 1). In fact, this F_{DTT}/F_{DTNB} value for the mutated channels is not significantly different from that previously recorded from wild-type receptors (2.0; Brimecombe et al., 1997; $p > .05$, unpaired t test). This implies that the muta-

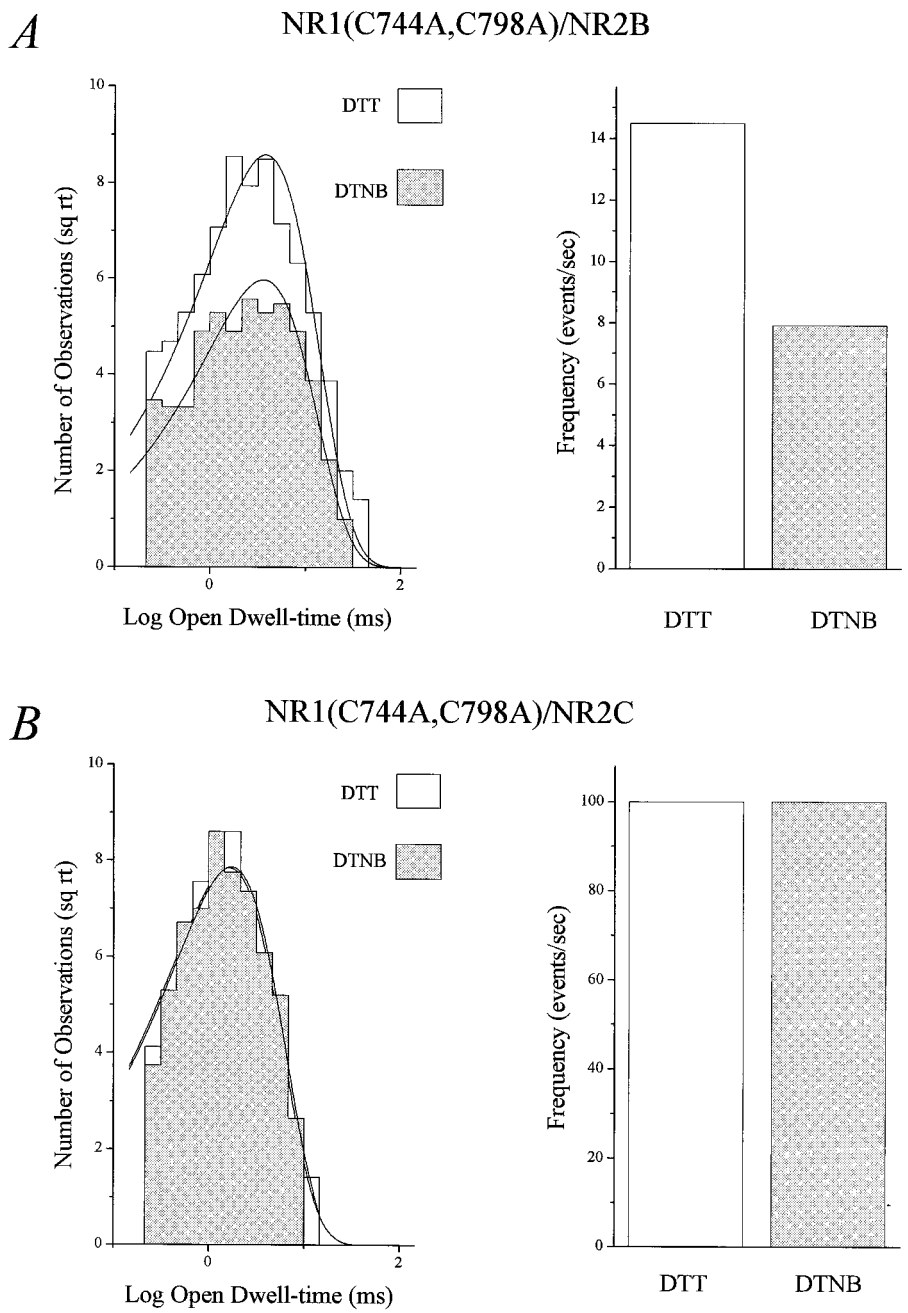

Fig. 2. Single channel redox properties of the cysteine-mutated NR1 subunit coexpressed with either NR2B or NR2C. A, open dwell-time histograms of NMDA-activated events obtained from a representative NR1(C744A, C798A)/NR2B-containing patch (left). The open dwell-time constants did not differ between the reduced and oxidized conditions (4.1 and 3.9 ms, respectively). In contrast, the frequency of channel opening was decreased in the presence of the oxidant when compared with the open channel frequency in the presence of the reductant (right). The average values from the seven patches obtained from cells transfected with this receptor combination are shown in Table 1. B, NR1(C744A, C798A)/NR2C-containing receptors were insensitive to redox agents. There was no change in the open dwell-time between the reduced and oxidized conditions (1.7 and 1.8 ms, respectively; left). Furthermore, there was no change in the frequency of channel opening between the two redox states (right). The average values from the five patches obtained from cells expressing NR1(C744A, C798A)/NR2C are summarized in Table 1.

tion does not affect the ability of redox agents to modulate receptor activity. However, F_{DTT}/F_{DTNB} in the mutant channel is also not significantly different from unity ($p > .05$, one sample t test), which suggests no change in the parameter between the two treatments. Obviously, the open-channel frequency data obtained with the NR1(C744A, C798A)/NR2B receptors are inconclusive at this point. However, they are suggestive of an incomplete abolition of redox sensitivity of NR1/NR2B receptors after mutation of the redox site on NR1. This issue will be revisited below with the whole-cell recording data.

Mutating cysteines 744 and 798 on the NR1 subunit appeared to be sufficient to eliminate the redox sensitivity of NR1(C744A, C798A)/NR2C receptors ($n = 5$). As observed in wild-type NR1/NR2C receptors (Brimecombe et al., 1997), redox agents did not alter the single-channel amplitudes (-2.1 ± 0.1 pA in DTT, -2.1 ± 0.1 pA in DTNB) or the open dwell-times of the mutated channels (1.1 ± 0.1 ms DTT, 1.3 ± 0.1 ms DTNB, $\Delta OT = -0.1 \pm 0.0$ ms; Fig. 2B and Table 1). Furthermore, there was no effect of redox agents on opening frequency in the NR1(C744A, C798A)/NR2C subunit configuration (Fig. 2B and Table 1). F_{DTT}/F_{DTNB} for NR1(C744A, C798A)/NR2C receptors was 0.8 ± 0.1, a value not significantly different from unity ($p > .05$, one sample t test). By comparison, the frequency of channel opening in wild-type NR1/NR2C channels was previously noted to increase 2.2-fold after reduction (Brimecombe et al., 1997).

Whole-Cell Measurements Suggest Presence of an Additional Redox Site on NR2B. NMDA (30 μM)-induced whole-cell responses were obtained from cells transfected with NR1 or NR1(C744A, C798A) together with either NR2A, NR2B, or NR2C. Current amplitude measurements were obtained after a 3-min incubation in 4 mM DTT and after a 1-min incubation with the oxidizing agent DTNB (0.5 mM). Mutating cysteines 744 and 798 on the NR1 subunit did not alter the extent of redox modulation of NR1/NR2A receptors (Fig. 3). The I_{DTT}/I_{DTNB} peak current ratio was 3.6 ± 0.3 for wild-type NR1/NR2A receptors and 4.0 ± 0.3 for the NR1(C744A, C798A)/NR2A subunit configuration (Table 2). These current ratios were not significantly different from each other ($p > .05$, unpaired t test), and, as such, this finding indicates that cysteines 744 and 798 are not required for the redox modulation of NR1/NR2A receptors, similar to what the single-channel data revealed.

Sullivan et al. (1994) reported that mutating cysteines 744 and 798 on the NR1 subunit rendered responses mediated by NR1/NR2B receptors insensitive to potentiation by DTT. Our results suggest that, in fact, NR1(C744A, C798A)/NR2B receptors are slightly redox-sensitive. Whole-cell current amplitudes obtained from cells transfected with the subunit combination revealed that there was no significant potentiation of NMDA-induced responses following a 3-min DTT treatment, at least when compared with the initial, control responses in chemically naive cells (Fig. 4). This is in contrast

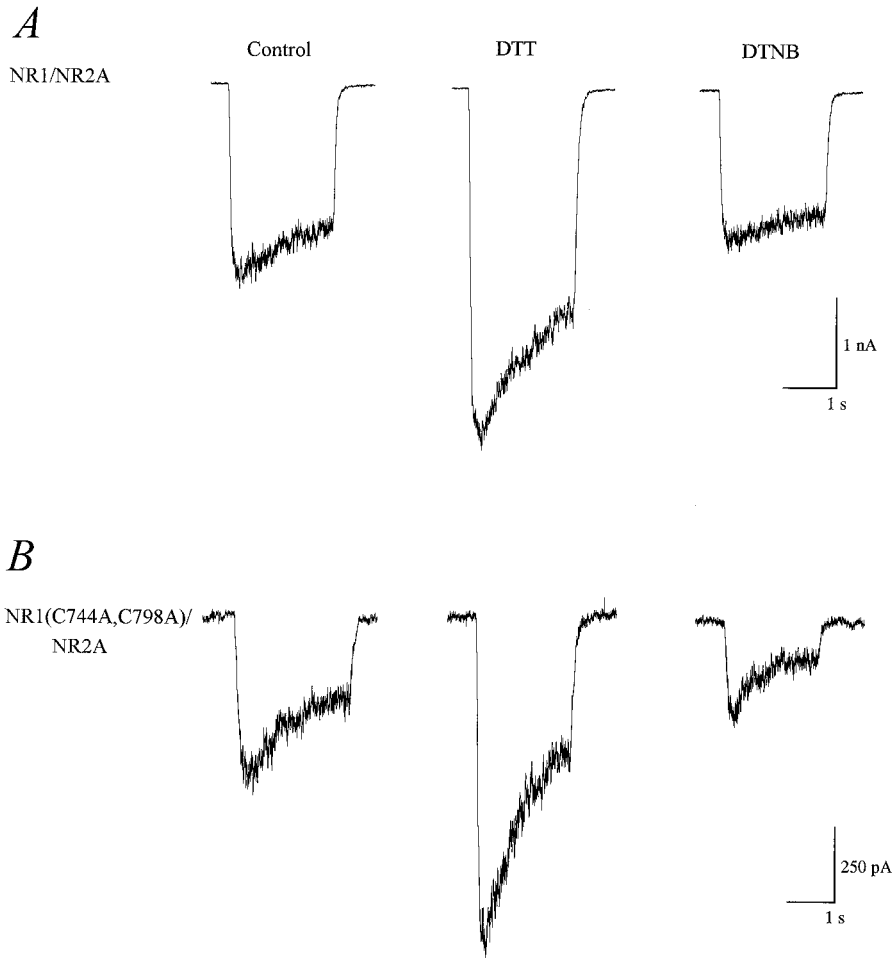

Fig. 3. Redox modulation of whole-cell currents mediated by NR2A-containing receptors. A, representative whole-cell responses recorded from a CHO cell expressing wild-type NR1/NR2A receptors during application of 30 μM NMDA. Currents were measured during control conditions, following a 3-min incubation with 4 mM DTT and after a 1-min incubation with 0.5 mM DTNB. B, similar measurements were obtained from another CHO cell transfected with NR1(C744A, C798A)/NR2A subunits. Responses were obtained from a total seven cells transfected with the wild-type NR1, and from five cells transfected with the cysteine mutated subunit. Peak current amplitudes from traces such as these ones were used to obtained the values shown in Table 2.

TABLE 2
Redox properties of NMDA-induced whole-cell currents

	$I_{DTT}/I_{CONTROL}$	I_{DTT}/I_{DTNB}
NR1/NR2A ($n = 7$)	1.8 ± 0.3*	3.6 ± 0.3*
NR1(c744,798a)/NR2A ($n = 5$)	2.4 ± 0.1*	4.0 ± 0.3*
NR1/NR2B ($n = 7$)	2.6 ± 0.4*	4.8 ± 0.9*
NR1(c744,798a)/NR2B ($n = 5$)	1.2 ± 0.1	1.4 ± 0.1*
NR1/NR2C ($n = 4$)	2.6 ± 0.3*	3.6 ± 0.4*
NR1(c744,798a)/NR2C ($n = 4$)	1.1 ± 0.1	1.1 ± 0.1

$I_{DTT}/I_{Control}$, ratio of the peak whole-cell current amplitude response after a 3-min incubation in 4 mM DTT to the initial control peak current; I_{DTT}/I_{DTNB}, ratio of the peak whole-cell current amplitude response after a 3-min incubation in 4 mM DTT to the peak current amplitude response after a 1-min incubation in 0.5 mM DTNB.
*, significantly different from unity ($p < .05$, one-sample t test).

to what is seen in wild-type NR1/NR2B-transfected cells, as well as in cells expressing wild-type NR1/NR2A, NR1/NR2C channels, or mutated NR1(C744A, C798A)/NR2A channels (Table 2). Nonetheless, we noted that currents mediated by NR1(C744A, C798A)/NR2B were depressed by DTNB in a DTT-reversible fashion (Fig. 4). The I_{DTT}/I_{DTNB} peak current ratio for this receptor configuration was 1.4 ± 0.1 (Table 2). This ratio value is significantly different from unity ($p < .01$, one sample t test), implying that this subunit combination is redox sensitive. Yet, this I_{DTT}/I_{DTNB} ratio was also significantly different from the I_{DTT}/I_{DTNB} ratio of wild-type NR1/NR2B receptors (4.8 ± 0.9; $p < .01$, unpaired t test). Therefore, as suggested earlier, mutating cysteines 744 and 798 of NR1 diminishes, but does not abolish, the redox sensitivity of NR1/NR2B receptors.

Whole-cell recordings confirmed the finding that mutating cysteines 744 and 798 on the NR1 subunit completely abolished the redox sensitivity of NR1/NR2C. Whereas wild-type NR1/NR2C receptors had a I_{DTT}/I_{DTNB} ratio of 3.6 ± 0.4, NR1(C744A, C798A)/NR2C receptors had a I_{DTT}/I_{DTNB} ratio of 1.1 ± 0.1 (Table 2). These two values were significantly different from each other ($p < .01$, unpaired t test). Furthermore, the I_{DTT}/I_{DTNB} ratio of NR1(C744A, C798A)/NR2C receptors was not significantly different from unity ($p > .05$, one sample t test). Thus, cysteines 744 and 798 are necessary and sufficient for full redox modulation of NR1/NR2C receptors.

Discussion

The experiments presented here were aimed at determining the functional effects of thiol modification of two putative redox sites on the NMDA receptor. We have observed that Cys744 and Cys798 in the NR1 subunit are critical for redox modulation of NR2C-containing receptors, and that these residues contribute to some, but not all, of the redox sensitivity of the NR1/NR2B subunit combination. These findings are mostly in agreement with the results of Sullivan et al. (1994), although the residual redox sensitivity of NR1(C744A, C798B)/NR2B receptors had not been reported previously and is suggestive of yet an additional redox site on NR2B. The most surprising result obtained in the present study, however, is that the redox site on NR1 appears not to contribute to the actions of DTT and DTNB on NR1/NR2A receptor function, at least within the parameters examined here.

Evidence suggesting a redox sensitive in the extracellular amino terminus of NR2A was obtained by Köhr et al. (1994) using chimeric subunits. In that study, it was shown that the redox properties of NR1/NR2A receptors were substantially different from those observed in all other receptor configurations tested (NR1/NR2B, NR1/NR2C, NR1/NR2D). First, although the effects of DTT on whole-cell currents mediated by all other subunits combinations were relatively slow to develop (minutes), NR1/NR2A responses were enhanced very quickly (seconds) after exposure to the reducing agent. This rapid effect of DTT on NR1/NR2A was followed by a subsequent slow rise in current amplitude with a time course reminiscent of the effects of this substance on the other subunit configurations. Second, the actions of the reductant on NR1/NR2B, NR1/NR2C, and NR1/NR2D receptors were very long-lasting and only fully reversible following treatment with DTNB. In contrast, the rapid current-enhancing actions of DTT on NR1/NR2A receptors were also quick to reverse, leaving behind a DTNB-sensitive, more persistent component, which, again, was very similar to what was observed with the other receptors. The rapidly reversibly DTT effect on NR1/NR2A was abolished in receptors composed of a NR2A chimeric construct in which the amino terminus of the latter subunit was substituted for the analogous segment of NR2C (NR2[CA]). This suggested to the authors that an NR2A redox-sensitive site was localized to this region. Third, all receptor configurations, except for NR1/NR2A, could be permanently potentiated (that is, be rendered insensitive to subsequent DTNB oxidation) when treatment with an alkylating agent immediately followed the DTT exposure. Alkylation has also been produced in native neuronal receptors (Tang and Aizenman, 1993a), and it has been a useful tool for establishing redox modulation as the mechanism of action of endogenous redox-active substances (Aizenman et al., 1992; Tang and Aizenman, 1993b). Whether alkylation can be produced in an NR1/NR2[CA] receptor remains to be established.

Paoletti et al. (1997) recently suggested that some of the effect of DTT on NR1/NR2A-mediated currents are due to zinc chelation by the reductant itself, with the ensuing removal of a high-affinity tonic receptor block by background contamination levels of this metal. These investigators demonstrated that rapid potentiating effects of DTT were indistinguishable from those produced by more conventional metal chelators. Hence, the authors concluded that the amino terminus of NR2A likely contained a Zn^{2+}-sensitive site, rather than a true redox-sensitive site. Yet, in spite of these findings, there are two lines of evidence that still favor the existence of a unique redox site on NR2A. One, mutation of Cys744 and Cys798 in NR1, dramatically reduces the affinity of NR1/NR2A receptors for zinc (Zheng et al., 1998; Traynelis et al., 1998). In fact, Zheng et al. (1998) observed virtually no potentiation of NR1(C744A, C798A)/NR2A responses with concentrations of a metal chelator that were very effective in enhancing NR1/NR2A-mediated currents. As is evident in the present report, the effects of DTT on wild-type and mutant NR2A-containing receptors are essentially indistinguishable. Second, recent experiments conducted in our laboratory (Arden et al., 1998) demonstrated that cyanide can selectively potentiate NMDA-induced currents mediated by NR1/NR2A receptors. The cyanide-induced current enhancement occurred slowly (minutes) and was readily reversible by DTNB. Mutation of Cys744 of NR1 alone, sufficient to abolish DTT potentiation in NR1/NR2B receptors (Sullivan et al., 1994), and decrease high-affinity zinc block in NR1/NR2A (Traynelis et al., 1998), did not block

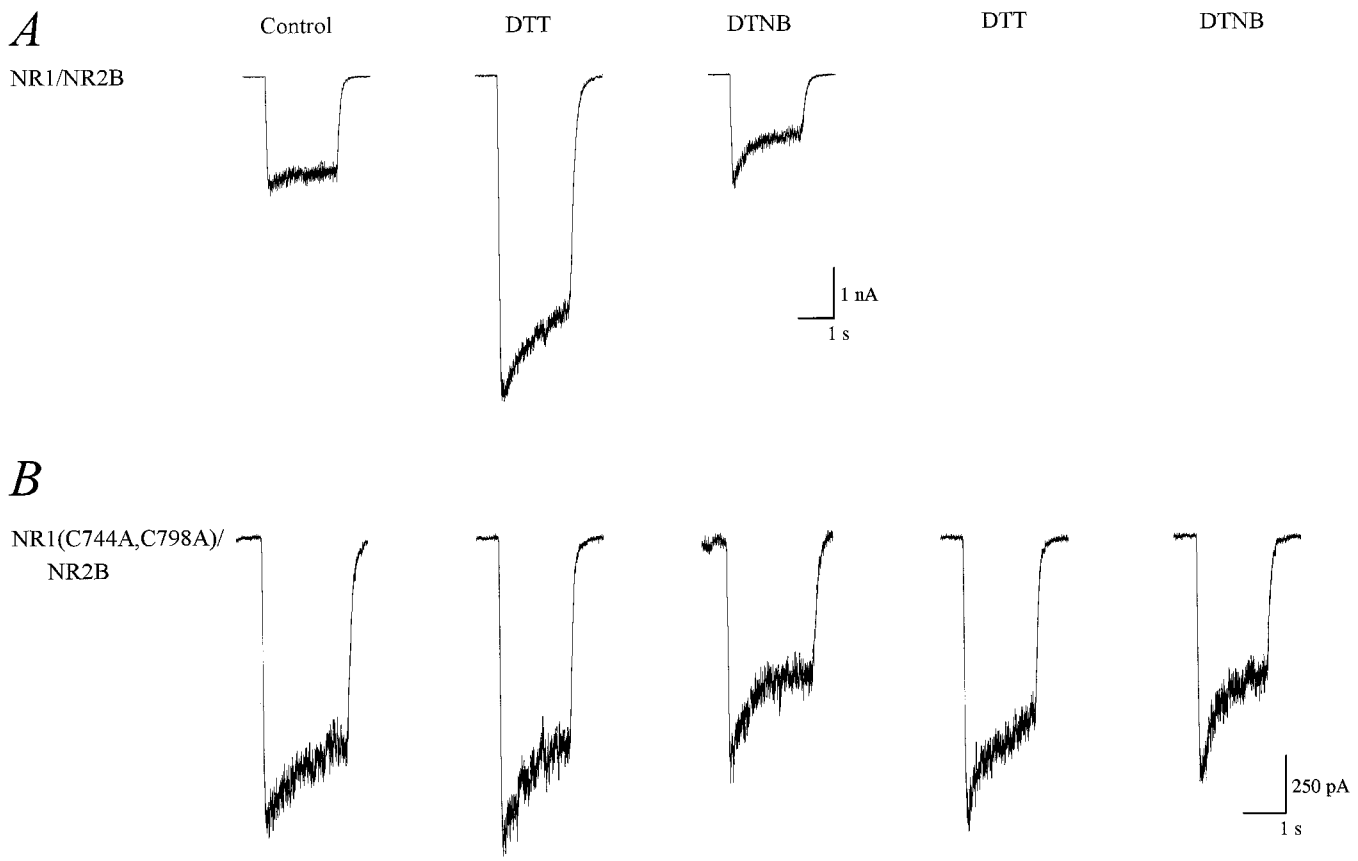

Fig. 4. Residual redox sensitivity of NR1(C744A, C798A)/NR2B receptors. A, representative whole-cell responses recorded from a CHO cell expressing NR1/NR2B receptors during application of 30 μM NMDA. Currents were measured during control conditions, following a 3-min incubation with 4 mM DTT and after a 1-min treatment with 0.5 mM DTNB. Similar measurements were obtained in six additional cells. B, responses obtained from a CHO cell transfected with NR1(C744A, C798A)/NR2B subunits. Note that although the DTT treatment does not substantially enhance the amplitude of the control current, DTNB depresses the response in a DTT-reversible fashion. Similar recordings were obtained in total of five cells transfected with this subunit configuration. Data are summarized in Table 2.

the actions of cyanide or DTT on NR2A-containing receptors. Interestingly, the mitochondrial poison *depressed* NR1/NR2B-mediated currents, reinforcing the notion that the redox sensitivity of NR1/NR2A is indeed unique. Finally, cyanide was able to potentiate NR1/NR2A responses in the presence of a metal chelator. It is noteworthy that in our laboratory we have not reliably seen potentiation by zinc chelators in wild-type NR1/NR2A receptors (Arden et al., 1998). This likely indicates low background levels of contaminating metals in our solutions, or that endogenous tyrosine kinase Src activity is high in CHO cells, as NMDA receptor phosphorylation by this enzyme reduces tonic zinc inhibition in NR1/NR2A receptor channels (Zheng et al., 1998). The fact that NR1/NR2A receptors cannot be alkylated (Köhr et al., 1994) is further suggestive that the redox site on NR1 does not mediate the effects of thiol substances on this subunit combination.

The single-channel experiments in the present investigations were initially designed to test the hypothesis that the redox-sensitive site on NR1 would influence the frequency of channel opening and that the NR2A redox site would modify the open dwell-time of the channel. Namely, we were interested in investigating whether the two separate redox sites acted independently of each other. However, our data strongly suggest that Cys744 and Cys798 on the NR1 subunit are not necessary for the redox modulation of NR1/NR2A receptors. What other residues may be involved in the thiol sensitivity of this receptor configuration? In a preliminary report (Choi et al., 1997), the DTT sensitivity of NR1/NR2A receptors in which five cysteines were mutated simultaneously (Cys744 and Cys798 on NR1 and Cys87, Cys231, and Cys320 in the amino terminus of NR2A) was evaluated. Even after these mutations, NMDA-activated currents were still potentiated by DTT, implying that additional cysteines are involved in redox modulation of this receptor. Second, logical guesses are the cysteines in NR2A corresponding to Cys744 and Cys798 in the NR1 subunit, which would provide a "redundant" redox site in NR1/NR2A receptors. By analogy, the homologous cysteines in NR2B may impart the residual redox sensitivity to NR1(C744A, C798A)/NR2B receptors. Although these cysteines are located on all the NR2 subunits, including NR2C, there may be structural limitations in an NR1/NR2C receptor, which do not allow for these two cysteines to be accessible to redox-active substances. In fact, there are no instances where the NR2A or NR2B subunits contain homologous cysteines that are not present in NR2C in regions that face the extracellular milieu (Monyer et al., 1992). Clearly, additional work is required to resolve these issues. Determining the manner in which individual redox sites alter channel function will likely aid in elucidating how the various domains of NMDA receptor subunits modify channel gating.

Acknowledgments

We thank Drs. John Horn, Pat Levitt, Jon Johnson, Michael Cascio, Stuart Arden, and Jeroo Sinor for helpful comments and discussions, Karen Hartnett for technical assistance, and Dr. Stephen Traynelis for sharing unpublished data.

References

Aizenman E (1994) Redox modulation of the NMDA receptor, in *Direct and Allosteric Control of Glutamate Receptors* (Palfreyman MG, Reynolds IJ and Skolnik P eds) pp 95–104, CRC Press, Boca Raton, FL.

Aizenman E, Hartnett KA, Zhong C, Gallop PM and Rosenberg PA (1992) Interaction of the putative essential nutrient pyrroloquinoline quinone with the *N*-methyl-D-aspartate receptor redox modulatory site. *J Neurosci* **12:**2362–2369.

Aizenman E, Lipton SA and Loring RH (1989) Selective modulation of NMDA responses by reduction and oxidation. *Neuron* **2:**1257–1263.

Arden SR, Sinor JD, Potthoff WK and Aizenman E (1998) Subunit-specific interactions of cyanide with the NMDA receptor. *J Biol Chem* **273:**21505–21511.

Boeckman FA and Aizenman E (1994) Stable transfection of the NR1 subunit in Chinese hamster ovary cells fails to produce a functional *N*-methyl-D-aspartate receptor. *Neurosci Lett* **173:**189–192.

Boeckman FA and Aizenman E (1996) Pharmacological properties of acquired excitotoxicity in Chinese hamster ovary cells transfected with *N*-methyl-D-aspartate receptor subunits. *J Pharmacol Exper Ther* **279:**515–523.

Brimecombe JC, Boeckman FA and Aizenman E (1997) Functional consequences of NR2 subunit composition in single recombinant *N*-methyl-D-aspartate receptors. *Proc Natl Acad Sci USA* **94:**11019–11024.

Brimecombe JC, Gallagher MJ, Lynch DR and Aizenman E (1998) An NR2B point mutation affecting haloperidol and CP101,606 sensitivity of single recombinant *N*-methyl-D-aspartate receptors. *J Pharmacol Exp Therap* **286:**627–634.

Choi YB, Chen HS and Lipton SA (1997) Multiple cysteine residues on different subunits are involved in redox modulation of NR1/NR2A receptors. *Soc Neurosci Abstr* **23:**932.

Cik M, Chazot PL and Stephenson FA (1993) Optimal expression of cloned NMDAR1/NMDAR2A heteromeric glutamate receptors: A biochemical characterization. *Biochem J* **296:**877–883.

Dingledine R, Borges K, Bowie D and Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* **51:**7–61.

Gallagher MJ, Huang H, Pritchett DB and Lynch DR (1996) Interactions between ifenprodil and the NR2B subunit of the *N*-methyl-D-aspartate receptor. *J Biol Chem* **271:**9603–9611.

Gozlan H, Khazipov R and Ben-Ari Y (1995) Multiple forms of long-term potentiation and multiple regulatory sites of *N*-methyl-D-aspartate receptors: Role of the redox site. *J Neurobiol* **26:**360–369.

Köhr G, Eckardt S, Luddens H, Monyer H and Seeburg PH (1994) NMDA receptor channels: Subunit-specific potentiation by reducing agents. *Neuron* **12:**1031–1040.

McBain CJ and Mayer ML (1994) *N*-methyl-D-aspartic acid receptor structure and function. *Physiol Rev* **74:**723–760.

Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K and Mishina M (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature (Lond)* **357:**70–74.

Michaelis EK (1998) Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Prog Neurobiol* **54:**369–415.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B and Seeburg PH (1992) Heteromeric NMDA receptors: Molecular and functional distinction of subtypes. *Science (Wash DC)* **256:**1217–1221.

Paoletti P, Ascher P and Neyton J (1997) High-affinity zinc inhibition of NMDA NR1/NR2A receptors. *J Neurosci* **17:**5711–5725.

Sinor JD, Boeckman FA and Aizenman E (1997) Intrinsic redox properties of *N*-methyl-D-aspartate receptor can determine the developmental expression of excitotoxicity in rat cortical neurons in vitro. *Brain Res* **747:**297–303.

Sullivan JM, Traynelis SF, Chen HS, Escobar W, Heinemann SF and Lipton SA (1994) Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor. *Neuron* **13:**929–936.

Tang LH and Aizenman E (1993a) The modulation of *N*-methyl-D-aspartate receptors by redox and alkylating reagents in rat cortical neurones in vitro. *J Physiol (Lond)* **465:**303–323.

Tang LH and Aizenman E (1993b) Allosteric modulation of the NMDA receptor by dihydrolipoic and lipoic acid in rat cortical neurons in vitro. *Neuron* **11:**857–863.

Traynelis SF, Burgess MF, Zheng F, Lyuboslaversusky P and Powers JL (1998) Control of voltage-independent zinc inhibition of NMDA receptors by the NR1 subunit. *J Neurosci* **18:**6163–6175.

Zheng F, Gingrich MB, Traynelis SF and Conn PJ (1998) Tyrosine kinase potentiates NMDA receptor currents by reducing tonic zinc inhibition. *Nature Neurosci* **1:**185–191.

Send reprint requests to: Dr. Elias Aizenman, Department of Neurobiology, E1456 BST, University of Pittsburgh School of Medicine, 3500 Terrace St., Pittsburgh, PA 15261. E-mail: redox+@pitt.edu