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# Neurosteroids and Glutamate Toxicity in Fibroblasts Expressing Human NMDA Receptors

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**We characterized glutamate receptor-mediated toxicity in mouse fibroblasts expressing the human NR1a/2A or NR1a/2B NMDA receptor. After induction of NMDA receptors, cells in both lines died over a 24 h time period. This toxicity was associated with a progressive increase in the glutamate content of the media. Cell death could be prevented by including either the non-competitive NMDA receptor antagonist ketamine or the competitive antagonist D,L-AP-5. Cells expressing NR1a/2A receptors were maximally protected by 0.5 mM D,L-AP-5, while those expressing NR1a/2B receptors required 2 mM D,L-AP-5 for maximal protection. The neurosteroid pregnanolone sulfate, which negatively modulates NMDA receptor function, partially protected fibroblasts containing NR1a/2A or NR1a/2B NMDA receptor constructs. However, the neurosteroid pregnenolone sulfate, which has been reported to act as a positive allosteric modulator of the NMDA receptor, had no effect on the toxicity caused by endogenous glutamate. Our results on cells expressing human NMDA receptors suggest that certain neurosteriods may protect against NMDA induced toxicity while having low neurotoxic liabilities of their own.**

*Keywords*: NMDA receptor; Glutamate; Toxicity; Neurosteroids

## INTRODUCTION

L-Glutamate (glutamate) is the principal excitatory neurotransmitter in the central nervous system and is also capable of acting as an excitotoxin (Olney, 1969). Intriguingly, both actions of glutamate are mediated by ionotropic (NMDA, AMPA, kainate) or metabotropic receptors (Frandsen *et al.*, 1989; Koh *et al.*, 1990; Bruno *et al.*, 1995). While activation of these receptors during normal neurotransmission is non-toxic, prolonged activation and excitation leads to neurotoxicity (Pawley *et al.*, 1996). This toxicity is prevented by the glutamate receptor antagonists that target NMDA and AMPA receptors (Jhamandas *et al.*, 2000).

The NMDA receptor is of particular interest in the study of excitotoxicity since it functions as a glutamate gated ion channel, permitting calcium ion entry into the neuron. Because a derangement in calcium ion homeostasis is paramount in the progression of events leading to excitotoxic cell death (Tymianski *et al.*, 1994; Schinder *et al.*, 1996), the NMDA receptor presents itself as an important regulatory site in the excitotoxic process. For these reasons, the study of structure and function of the NMDA receptor has attracted much attention.

The NMDA receptor is composed of subunits which are drawn from two different gene families. In the rat, the NR1 subunit can exist as eight alternatively spliced isoforms from a single gene product (Moryoshi *et al.*, 1991; Durand *et al.*, 1993; Hollman *et al.*, 1993), whereas four distinct genes encode the NR2 subunits (2A–2D) (Ishii *et al.*, 1993). Recently, recombinant human NR1 and NR2 subunits have been synthesized, and show different levels of homology with cloned rat NR subunits (Le Bourdellès *et al.*, 1994). While the NR1 subunits differ by only eight amino acid substitutions, the NR2A and 2B subunits exhibit 21 and 81 amino acid substitutions, respectively. The glutamate and glycine binding sites of recombinant receptors composed of NR1a and NR2A or 2B subunits have been well characterized (Grimwood *et al.*, 1996; Varney

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*et al.*, 1996). Other modulatory sites associated with human recombinant NMDA receptor subunits such as the polyamine binding site (Kashiwagi *et al.*, 1997) have not been characterized. It is possible that these modulatory sites on human recombinant NMDA receptors may differ from those of rodent NMDA receptors, as they are associated with the NR2 subunit. Recent studies suggest modulation of NMDA receptors by steroids however, the steroid modulation of human recombinant receptors has yet to be characterized, and it may differ from that of rodent NMDA receptors. The putative steroid binding sites on the rodent NMDA receptor differ from other modulatory sites and these sites are thought to involve both the NR1 and NR2 subunits (Park-Chung *et al.*, 1997; Yaghoubi *et al.*, 1998). The steroids which modulate the NMDA receptor belong to a class of compounds termed "neurosteroids" (Corpechot *et al.*, 1981). Apart from genomic effects, these steroids have been shown to modulate membrane bound neurotransmitter receptors. Specifically, the two neurosteroids pregnenolone sulfate and pregnanolone sulfate have been shown to modulate NMDA receptor function. Thus, pregnenolone sulfate positively modulates the NMDA receptor (Bowlby, 1993, Guarneri *et al.*, 1998; Cascio *et al.*, 2000), while pregnanolone sulfate negatively modulates the receptor (Park-Chung *et al.*, 1994). The NR2 subunit appears to determine the efficacy of pregnenolone sulfate modulation of the NMDA receptor, while the presence of the NR1 subunit alone is sufficient for pregnanolone sulfate to exert a modulatory effect on the NMDA receptor current. The role of neurosteroid sites in the NMDA receptor-mediated excitotoxic response has not been sufficiently elucidated.

Recently, novel *in vitro* approaches have been developed to examine toxicity. Specifically, non-neuronal cell lines have been employed to investigate the role of individual glutamate receptor subtypes and subunits in the progression of toxicity. Thus, when recombinant rat NMDA receptors are expressed in Chinese hamster ovary (CHO) or in human embryonic kidney (HEK) cells, these cells undergo toxic cell death (Anegawa *et al.*, 1995; Boeckman and Aizenman, 1996; Raymond *et al.*, 1996; Garcia-Gallo *et al.*, 1999). The observed toxicity can be prevented with NMDA receptor antagonists. Permanently transfected cells represent themselves a novel model in which to investigate NMDA receptor-dependent toxicity because, unlike transiently transfected cells, the degree of receptor expression between cells and experiments can be controlled to a greater extent. Recently, it has been demonstrated that mouse fibroblasts expressing NMDA receptors can undergo toxic cell death if NMDA receptor antagonists are absent from the culture medium (Grimwood *et al.*, 1996). Thus, this model of spontaneous NMDA receptor-mediated toxicity may be useful to study steroid modulation of toxicity. Here we describe endogenous glutamate—induced cell death in permanently transfected mouse fibroblasts expressing NMDA receptors composed of human recombinant NR1a/2A or NR1a/2B subunits. We also examined the ability of two neurosteroids reported to modulate NMDA receptor function, on NMDA receptor mediated cell death.

## MATERIAL AND METHODS

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Canada (Oakville, ON) and were of tissue culture grade. Tissue culture 24 and 96 well plates were purchased from Fisher Scientific Canada (Mississauga, ON). Tissue culture media filters were obtained from Starstedt (Montreal, PQ). All non-sterile solutions, other than tissue culture media, were filtered using 0.2 μm syringe filters from Fisher Scientific, Canada. All solutions were prepared using deionized water obtained through a Nanopure water purification system (Barnstead Sybron, Boston, MA, USA). Pregnenolone sulfate and pregnanolone sulfate were purchased from Steraloids Incorporation (Wilton, NH).

### Cell Culture of Transformed Cell Lines

Frozen cell stocks of mouse L($tk^-$) fibroblasts containing cDNA encoding human NR1a and NR2B or NR1a and NR2A NMDA receptor subunits were obtained from Dr Paul Whiting at Merck Sharpe and Dohme (UK). Stocks were thawed and grown in 20% FBS/DMEM in 100 mm tissue culture dishes for 2 weeks. Cells containing the transgene were selected by using the antibiotic G418 at 5 mg $ml^{-1}$ for 7 days and were grown in 100 mm culture dishes until a full monolayer developed. The cultures were then dissociated using trypsin at 0.5 mg/ml in HBSS. Cells ($40 \times 10^3$ per well) were plated in 10% FBS/DMEM on a 96-well plate and utilized in toxicity assays. The remaining cells were plated on new 100 mm tissue culture plates and grown in 10% FBS/DMEM. This cycle was repeated every 4–5 days, when cells in the 100 mm culture plates grew to form a monolayer.

### Toxicity Assay in NR1a/2A and NR1a/2B Containing Fibroblasts

In order to investigate toxicity mediated by the NMDA receptor, expression of the NMDA receptor subunits is necessary. Expression of the NMDA receptor subunits in these cells is under the control of a dexamethasone response element. Thus, after 1 DIV, 1 μM dexamethasone in 10% FBS/DMEM was

added to the culture plate wells for 6 h to induce NMDA receptor expression. During the induction phase, 0.50 mM ketamine was also present in the medium, because it has been previously determined that NMDA receptor antagonism prevents toxicity from endogenous excitatory amino acids in the cell culture medium (Priestley *et al.*, 1995). After the 6-h induction period, wells were washed twice with DMEM base and then were incubated with either a neurosteroid or a NMDA receptor antagonist in DMEM containing 1% DMSO. After 8 or 18 h, cell viability was assessed using the acid phosphatase assay (see below). To determine the time-course of cell death, cell viability was measured at varying times after NMDA receptor induction.

### Acid Phosphatase Assay

The acid phosphatase assay was conducted according to the method of Connoly *et al.* (1986). Cells were washed with 0.1 M phosphate buffered saline (PBS) and incubated at 37°C for 1 h in 100 μl of a 0.1 M sodium acetate buffer containing 10 mM Sigma 104 phosphatase substrate and 15 μl of triton-X100. The reaction was stopped by adding 10 μl of 1 M sodium hydroxide. The absorbance resulting from the formation of reaction product was measured at 405 nm using a Spectramax multiwell plate scanner. A linear relationship was obtained between optical density and cell number in the range of 5000–8000 cells per well.

### Glutamate Production by Transformed Fibroblasts

Production of glutamate by viable transformed fibroblasts was monitored during incubation in salt solution containing 146 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, 5 mM KCl, 10 mM glucose and 0.1 mM glycine buffered at pH 7.4. At set time intervals, the incubation solution was sampled from sister cultures and analyzed for the presence of glutamate using a reverse-phase high performance liquid chromatographic (HPLC) procedure (Vrooman *et al.*, 1993). A 200-μl sample of the incubation solution was reacted with 200 μl of *o*-phthalaldehyde (OPA) and β-mercaptoethanol (β-ME). The reaction mixture (100 μl) was injected onto an octadecylsilane reverse phase column (Supelcosil LC-18, 4.6 mm interior diameter × 150 mm length, 5 μm particle size) using a Shimadzu SIL-9A diluter/autosampler. Mobile phase consisted of 50 mM sodium acetate/methanol (72/28, v/v) at pH 7.5 and was pumped through the reverse phase column at a flow rate of 1.5 ml $min^{-1}$. The derivatized amino acids were detected using a Shimadzu Model RF-530 fluorescence detector operating at excitation and emission wavelengths of 345 and 470 nm, respectively. Quantitation of derivatized amino acids was performed using a Shimadzu C-R3A Chromatopac integrator.

## RESULTS

### Toxic Response

Toxic cell death in non-neuronal cells has been reported previously in CHO and HEK cells transiently expressing rat NMDA receptors. Here we characterized toxic cell death in permanently transfected fibroblasts expressing either human NR1a/2A or NR1a/2B receptors. Fibroblasts exhibited a time-dependent cell death, as indicated by the loss of alkaline phosphatase activity, which reached a maximum at 18–24 h after NMDA receptor induction (Fig. 1). The viability of NR1a/2A expressing cells appeared to be greater than that of NR1a/2B expressing fibroblasts. Thus, whereas 50% of the NR1a/2A cells survived at 12 h following removal of the NMDA receptor antagonist, less than 5% of cells containing NR1a/2B receptors survived at this time period. However, both cell types were lost at 24 h.

### Release of Glutamate

The onset of toxicity following removal of the NMDA antagonist suggested that the cells may be killed by excitatory amino acids present in the culture medium. To determine if the loss of cells was related to the accumulation of glutamate, the amount of glutamate present in the medium at different time points was measured. As shown in Fig. 2, low levels of glutamate were present in the growth media at time periods immediately after removal of the NMDA antagonist (time = 0). However, there was a progressive increase in glutamate levels at subsequent time periods. While the same number of cells were plated in each well, there was a greater amount of glutamate present in wells containing the NR1a/2B cell line. The difference between the two

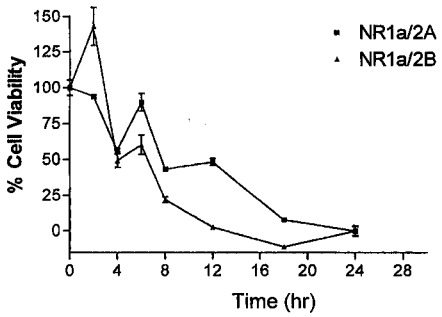


FIGURE 1 Decrease in viability of mouse fibroblasts expressing either human NR1a/2A or NR1a/2B NMDA receptor constructs with respect to time. Cell viability was determined using the acid phosphatase assay. Data presented as mean ± SEM of two separate cultures, each assayed in triplicate.

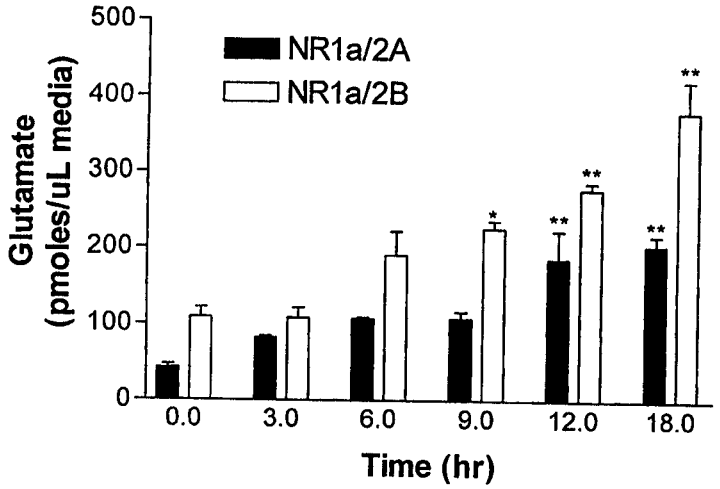


FIGURE 2 Accumulation of glutamate in culture wells of fibroblasts expressing either human NR1a/2A or NR1a/2B receptor constructs with respect to time. Glutamate was quantified using reverse-phase HPLC. Data presented as mean ± SEM of two separate cultures assayed in triplicate. $*P < 0.05$; $**P < 0.01$ versus Time (0 h).

cell lines was significant ($p < 0.01$) at $t = 9$, 12, and 18 h. In both cell types, the level of glutamate at 18 h was three-fold above the baseline level measured at time 0. The baseline release of glutamate in the medium containing NR1a/2A cells, which showed a slower rate of cell death (see Fig. 1) following removal of ketamine from the media, was almost 50% of that detected in media in which cells with the NR1a/2B receptor were cultured. Thus, the population of cells that showed an accelerated rate of death also released more glutamate into the growth medium; however, the relative change with time was similar in the two populations.

### Action of NMDA Receptor Antagonists

The competitive NMDA receptor antagonist D,L-AP-5 was employed to determine whether the toxicity indeed was NMDA receptor-dependent. In each case, three concentrations of the antagonist were tested. Additionally, in separate experiments, the cells were also exposed to the non-competitive antagonist ketamine. The results of these experiments are represented in Figs. 3 and 4. In NR1a/2A expressing cells, the increase in cell viability was apparent after exposure to 0.1 mM D,L-AP-5 and a maximum effect (100% viability) was achieved with a dose of 0.5 mM D,L-AP-5 (Fig. 3). However, in NR1a/2B expressing cells, the increase in viability was apparent at 0.5 mM and a maximal protective effect was achieved at 2 mM (Fig. 4). Thus, the two cell populations showed differential sensitivity to the action of the competitive NMDA receptor antagonist. In both cases, however, maximum protection from toxic cell death with D,L-AP-5 approximated protection seen with the non-competitive NMDA receptor antagonist ketamine, at a concentration (0.5 mM) which had previously been shown to prevent cell

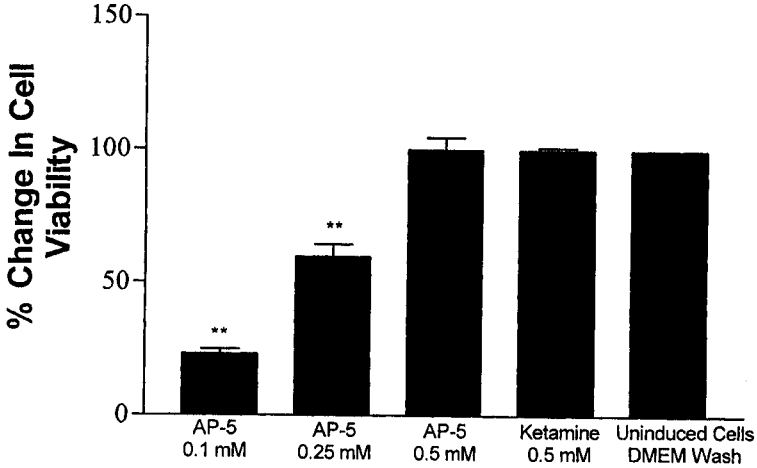


FIGURE 3 The effect of D,L-AP-5 on the viability of NR1a/2A expressing fibroblasts. Data presented as mean ± SEM (three cultures assayed in triplicate) of % increase in cell viability relative to DMEM treatment alone, which resulted in 100% cell death 24 h after induction of NMDA receptors. $**P < 0.01$ versus Uninduced cells.

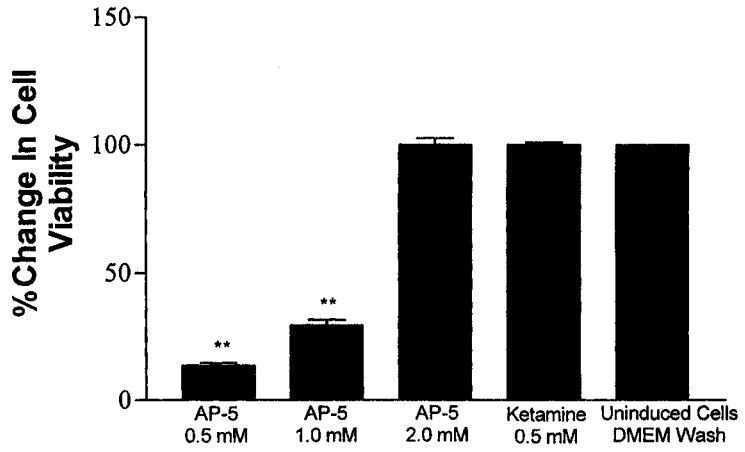


FIGURE 4 The effect of D,L-AP-5 on the viability of NR1a/2B-expressing fibroblasts. Data presented as mean ± SEM (three cultures assayed in triplicate) of % increase in cell viability relative to DMEM treatment alone, which resulted in 100% cell death 24 h after induction of NMDA receptors. * * $P < 0.01$ versus Uninduced cells.

death in both cell lines (Priestley *et al.*, 1995). In both NMDA receptor-expressing cell lines, complete antagonism of toxicity resulted in cell viability similar to that observed in the non-NMDA receptor expressing mouse fibroblast cultures (uninduced cells) which had been exposed only to DMEM (Figs. 3 and 4).

### Neurosteroid Modulation of Toxicity

The effects of the neurosteroids pregnanolone sulfate and pregnenolone sulfate, which exert anti-NMDA and pro-NMDA receptor activity in hippocampal neurons, respectively (Bowlby, 1993; Park-Chung *et al.*, 1994), on the viability of fibroblasts following removal of the NMDA antagonist from the culture medium are presented in (Figs. 5 and 6). Pregnanolone sulfate, at a concentration of 0.1 mM, negatively modulated toxicity mediated by NMDA receptors composed of NR1a/2A and NR1a/2B subunits 18 h after removal of ketamine (Fig. 5). Thus, cell viability increased relative to that obtained without neurosteroid in NR1a/2A expressing cells. Similarly, cell viability in NR1a/2B expressing fibroblasts increased on incubation with 0.1 mM pregnanolone sulfate. There was no subunit dependence observed with respect to the antagonism of toxicity by the neurosteroid.

The effect of pregnenolone sulfate, which has previously been shown to positively modulate the NMDA receptor (Bowlby, 1993), was examined 8 h after the initiation of toxicity. At this time point, cell death was determined to be submaximal (see Fig. 1). Addition of pregnenolone sulfate to the medium, however, failed to modulate NMDA toxicity in the assay system (Fig. 6). The use of higher concentrations of both neurosteroids was precluded by the lack of solubility in the culture medium.

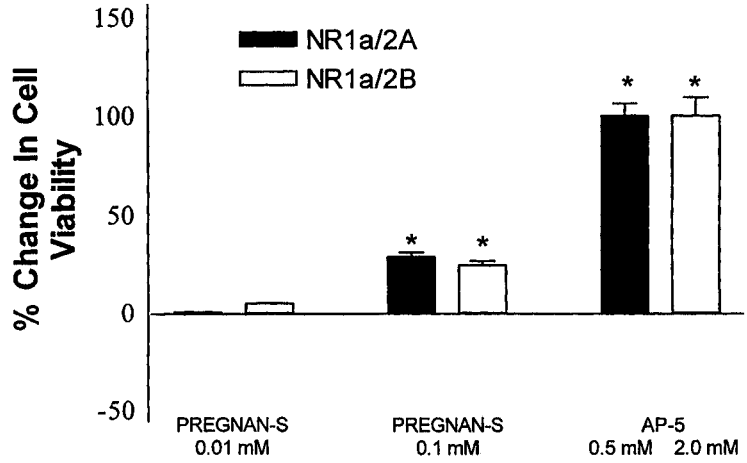


FIGURE 5 The effect of pregnanolone sulfate (PREGNAN-S) on NMDA receptor-mediated toxicity in NR1a/2A and NR1a/2B expressing fibroblasts. Data presented as mean ± SEM (three cultures assayed in triplicate) of % increase in cell viability relative to DMEM treatment, which was determined to result in 100% cell death. *$P < 0.05$ versus NMDA receptor expressing cells washed only with DMEM.

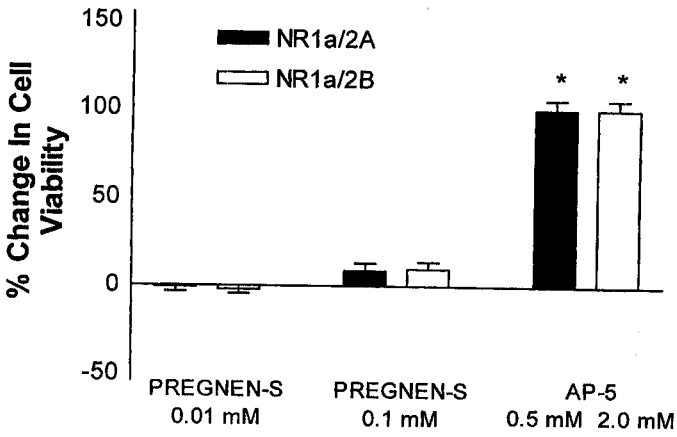


FIGURE 6 The effect of pregnenolone sulfate (PREGNEN-S) on NMDA receptor-mediated toxicity in NR1a/2A and NR1a/2B expressing fibroblasts. Data presented as mean ± SEM (three cultures assayed in triplicate) of % increase in cell viability relative to DMEM treatment, which was determined to result in 100% cell death. $*P < 0.05$ versus NMDA receptor expressing cells washed only with DMEM.

## DISCUSSION

Grimwood *et al.* (1996) reported that mouse fibroblasts expressing human NMDA receptors undergo toxic cell death when NMDA receptor antagonists are absent from the culture medium. In the present study, the NR1a/2A and NR1a/2B subunit-expressing fibroblast cell lines were both shown to exhibit spontaneous cell death when the NMDA receptor antagonist, ketamine, was excluded from the culture medium. The death of these cells is likely due to overactivation of NMDA receptors by glutamate in the culture medium. This study showed an increase in the level of glutamate over the 18 h incubation period. Although both cell populations were lost in 18–24 h, they differed with respect to: the rate at which cells lost viability, the efflux of the amino acid glutamate and sensitivity to the protective action of the competitive NMDA receptor antagonist, D,L-AP-5. Thus, in the 0–12 h incubation period, the NR1A/2B cells suffered a faster decline in viability and showed reduced sensitivity to the protective action of D,L-AP-5.

The reduced sensitivity to the antagonist may be due to a lower affinity of the NR1A/2B subunit to the competitive antagonist. This explanation is favored by previous studies of Priestley *et al.* (1995) involving pharmacological characterization of these subunits in electrophysiological experiments. They observed that antagonism of the NMDA receptor conductance by D,L-AP-5 was dependent on subunit composition. For example, the dissociation constant ($K_D$) for D,L-AP-5 at the NR1/2A-containing receptors was two-fold lower than that observed at the NR2B-expressing receptors, indicating a higher affinity for the antagonist. Interestingly, ketamine, the non-competitive NMDA receptor antagonist, exhibited no difference in $K_D$ between the two receptor populations (Grimwood *et al.*, 1996). In the present study, the two cell populations expressed similar sensitivity to the protective effects of ketamine. Thus, differences in the ligand recognition site or receptor number rather than in the channel site could be responsible for the differential loss of viability in the two cell populations. Another factor contributing to the more rapid loss of NR1a/2B cells may be the higher efflux of glutamate, the amino acid most likely responsible for the activation of NMDA receptors and the subsequent toxic response. The higher efflux of glutamate in these cells could explain accelerated cell death and the relative resistance to the inhibitory action of the competitive antagonist. Thus, both differences in receptor affinity and agonist availability could contribute to the differences in the pattern of toxicity observed in the two cell populations.

It should be noted that in the present study glutamate efflux and toxicity experiments were carried out under different conditions. The former included an NMDA receptor antagonist, while the latter involved removal of the antagonist to produce toxicity. It would be of interest to determine whether reductions in the synthesis or efflux of glutamate can modulate the toxic response in this model.

The cells expressing NR1a/2A and NR1a/2B receptors did not differ with respect to the actions of neurosteroids. While pregnanolone sulfate exhibited a significant reduction in toxicity at the maximal soluble concentration of steroid (0.1 mM) in 1% DMSO, pregnenolone sulfate at 0.1 mM exhibited no modulatory effect on excitotoxicity in both cell populations. These results are not in accordance with what may be predicted from electrophysiological experiments employing rat recombinant NR1a/2A receptors (Yaghoubi *et al.*, 1998). The $EC_{50}$ for pregnanolone sulfate to inhibit NMDA currents was 0.041 mM, while the $EC_{50}$ for pregnenolone sulfate to potentiate NMDA currents was 0.037 mM. As the steroid concentrations used in this study exceeded these concentrations, one would

expect to observe a modulation of toxicity by both steroids. Pregnanolone sulfate, at a concentration of 0.1 mM in the rat recombinant receptor model, reduced the NMDA receptor current by 50%. In the present study, antagonism of toxicity by pregnanolone sulfate is consistent with its previously demonstrated ability to inhibit NMDA receptor-mediated excitotoxicity. Exposure of cells to pregnenolone sulfate did not influence toxicity even though it was expected to increase the toxicity in our fibroblast model as it behaves as an agonist at the NMDA receptor (Cascio *et al.*, 2000). This failure does not appear to be due to the concentration used in our study as it was higher than the concentration (0.02 mM) reported to potentiate the NMDA current by more than 200% in cells expressing rat recombinant receptors (Yaghoubi *et al.*, 1998). A possible explanation for this discrepancy is that the steroid binding domain for pregnenolone sulfate on the human recombinant receptor is different from that on the rat recombinant receptor. Since it is the NR2 subunit that is responsible for determining the efficacy of pregnenolone sulfate modulation of NMDA receptor current (Yaghoubi *et al.*, 1998), differences in NR2 subunit sequence may lead to differences in the modulatory site and, thus, the action of pregnenolone sulfate.

The NR1 subunit contains the modulatory site for pregnanolone sulfate and there is little difference between the rodent NR1 subunit and human recombinant NR1 subunit, suggesting there would be less variation in pregnanolone sulfate modulation of the NMDA receptor composed of human or rodent subunits. This indeed was observed when comparing the relative effects of pregnanolone sulfate and pregnenolone sulfate modulation of excitotoxicity and ion conductance in the human and rodent receptor models. In the rodent system, pregnenolone sulfate produces a modulatory effect which was several-fold greater than that of pregnanolone sulfate (Yaghoubi *et al.*, 1998), while the human recombinant receptors showed pregnanolone sulfate to produce a greater modulatory effect, compared to pregnenolone sulfate. Further confirmation of this difference would be gained by examining pregnenolone sulfate modulation of toxicity in a system expressing rodent recombinant NMDA receptors.

In summary, transformed mouse fibroblasts expressing human recombinant NMDA receptors undergo toxic cell death. Furthermore, this toxicity is mediated by endogenously produced glutamate and is prevented in a concentration-dependent manner by D,L-AP-5 or by ketamine. The neurosteroid pregnanolone sulfate was shown to reduce this toxicity, while pregnenolone sulfate had no effect. The fibroblast model may be useful in determining post receptor mechanisms underlying toxic cell death and these are currently under investigation.

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