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Differentiation renders susceptibility to excitotoxicity in HT22 neurons[☆]

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Abstract

HT22 is an immortalized mouse hippocampal neuronal cell line that does not express cholinergic and glutamate receptors like mature hippocampal neurons *in vivo*. This in part prevents its use as a model for mature hippocampal neurons in memory-related studies. We now report that HT22 cells were appropriately induced to differentiate and possess properties similar to those of mature hippocampal neurons *in vivo*, such as becoming more glutamate-receptive and excitatory. Results showed that sensitivity of HT22 cells to glutamate-induced toxicity changed dramatically when comparing undifferentiated with differentiated cells, with the half-effective concentration for differentiated cells reducing approximately two orders of magnitude. Moreover, glutamate-induced toxicity in differentiated cells, but not undifferentiated cells, was inhibited by the N-methyl-D-aspartate receptor antagonists MK-801 and memantine. Evidently, differentiated HT22 cells expressed N-methyl-D-aspartate receptors, while undifferentiated cells did not. Our experimental findings indicated that differentiation is important for immortalized cell lines to render post-mitotic neuronal properties, and that differentiated HT22 neurons represent a better model of hippocampal neurons than undifferentiated cells.

Key Words

neural regeneration; brain injury; HT22; differentiation; N-methyl-D-aspartate receptor; glutamate; excitatory neurotoxicity; mitosis; hippocampus; neurons; MK-801; memantine; grants-supported paper; neuroregeneration

Research Highlights

- (1) HT22 cells have been shown to demonstrate properties of cholinergic neurons, and express active cholinergic markers, making them a good cell model of Alzheimer's disease.
- (2) HT22 cells were investigated in the undifferentiated and differentiated state.
- (3) Differentiated HT22 cells expressed N-methyl-D-aspartate receptors and became more sensitive to excitotoxicity. Therefore, these cells are a better model of hippocampal neurons.

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INTRODUCTION

Glutamate is an excitatory neurotransmitter involved in learning and memory^[1-2]. Under pathological conditions, such as brain injury and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and Huntington's disease, glutamate may be neurotoxic *via* excitotoxicity or oxidative stress^[3-8].

Glutamate-induced excitotoxicity is mainly mediated by N-methyl-D-aspartate (NMDA) receptors^[4, 9] *via* downstream changes including calcium influx^[10], nitric oxide generation^[11], calpain/poly(ADP-ribose) polymerase-1/apoptosis inducing factor^[12], free radicals and mitochondria^[13], and caspase-mediated apoptosis^[14-15]. NMDA receptor antagonists, such as MK-801, memantine or amantadine, can effectively prevent glutamate-induced excitotoxicity, with memantine even being approved as an Alzheimer's disease medication^[16-21].

Immortalized cell lines are valuable tools for mechanistic studies. If used properly, they can provide a rapid, inexpensive, and simple means to identify and test molecular and cellular mechanisms. HT22 is one such cell line subcloned from its parent line, HT4, which are immortalized mouse hippocampal neuronal precursor cells^[22-24]. Because of their tissue origin, HT22 cells have been used as a hippocampal neuronal cell model in numerous studies^[25-30].

Previous studies^[31-32] show that one important feature of this cell line was that it did not express NMDA receptors, thus it is resistant to excitotoxicity. However, high concentrations of glutamate can be toxic to HT22 cells. Scholars^[32-33] put forward a theory that glutamate and cystine compete for cystine transport in HT22 cells, which inhibit cystine uptake and lead to intracellular cystine exhaustion, glutathione depletion, and ultimately oxidative stress. Indeed, increasing studies replicated the original experiments and validated this hypothesis in HT22 cells^[31, 34-41]. It has almost become consensus that HT22 cells do not possess excitatory properties because of the lack of NMDA receptors.

Nonetheless, lack of certain essential properties of mature hippocampal neurons in this cell model can be troublesome, and has prompted efforts to overcome this problem. One recent study^[42] reported that the differentiated HT22 cells possessed more post-mitotic neuronal characteristics, such as neurite outgrowth and

expression of functional cholinergic markers and receptors, while the undifferentiated HT22 cells did not possess cholinergic neuronal properties. This drastic transformation before and after differentiation in HT22 cells prompted us to question whether or not differentiation can also induce the cell line to become a glutamate-receptive excitatory hippocampal neuronal model. The relevant findings are described in this report.

RESULTS

Differentiation rendered HT22 cells more susceptible to glutamate toxicity

Previous studies have found that HT22 cells were resistant to excitotoxicity because of the lack of NMDA receptor expression in these cells^[31-32]. Nonetheless, when the concentration of glutamate increased to millimolar levels, glutamate was toxic to HT22 cells, though the underlying mechanisms were oxidative stress rather than NMDA receptor-mediated excitotoxicity^[16, 32-33, 38, 43-45]. Consistent with these previous observations, we were able to replicate the aforementioned findings using undifferentiated HT22 cells.

Results showed that glutamate was toxic to undifferentiated HT22 cells with a half-effective concentration (EC_{50}) of approximately 2.5 mmol/L, as determined by the lactate dehydrogenase assay (Figure 1A). However, when HT22 cells were differentiated, the half-effective concentration of glutamate-induced toxicity reduced to 0.03 mmol/L, and the sensitivity reduced nearly two orders of magnitude (Figures 1A, C).

Similar results were observed with the methyl thiazolyl tetrazolium (MTT) cell viability assay (Figure 1B), which revealed the EC_{50} concentration of glutamate toxicity as 1.8 mmol/L and 0.12 mmol/L for undifferentiated and differentiated HT22 cells, respectively. This dramatic change of cell susceptibility to glutamate-induced toxicity inferred that the differentiation process may have induced a significant alteration in cellular receptiveness to glutamate.

Oxidative stress mediated glutamate-induced toxicity in undifferentiated cells, but not differentiated HT22 cells

As previously demonstrated, millimolar concentrations of glutamate can be toxic to undifferentiated HT22 cells *via* oxidative stress^[16, 32-33, 38, 43-45]. One such experiment used the antioxidant dithiothreitol to block glutamate toxicity in undifferentiated HT22 cells^[33]. Dithiothreitol (250 μ mol/L)

reduced glutamate toxicity in undifferentiated HT22 cells by 28.34% ($P < 0.05$; Figure 2A) as determined by the lactate dehydrogenase assay. The MTT assay also revealed that dithiothreitol reduced glutamate toxicity in undifferentiated HT22 cells, with cell viability increasing from 26.19% to 78.85% ($P < 0.05$; Figure 2B). It is worth noting that toxicity in undifferentiated HT22 cells was induced by 1.8 mmol/L glutamate, which is the EC_{50} value shown in Figure 1B. For the purpose of parallel

comparisons, a near EC_{50} value of glutamate in differentiated HT22 cells of 50 $\mu\text{mol/L}$ was used to induce a comparable level of toxicity^[45]. When dithiothreitol (250 $\mu\text{mol/L}$) was tested in differentiated HT22 cells, dithiothreitol significantly reduced glutamate toxicity ($P < 0.05$), and the toxicity was reduced by 13.34%, as detected by the lactate dehydrogenase assay (Figure 2A). Dithiothreitol was less effective in differentiated cells than in undifferentiated HT22 cells (28.34%).

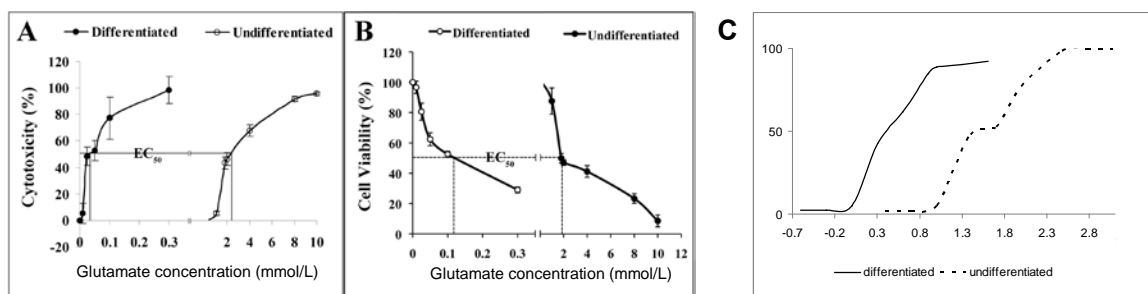


Figure 1 Dose-dependent glutamate cytotoxicity and cell viability in differentiated and undifferentiated HT22 cells.

Cells were administered with different doses of glutamate. Cell toxicity and viability were estimated using the lactate dehydrogenase (A) and methyl thiazolyl tetrazolium (B) assays, respectively. After dose concentration and effect (lactate dehydrogenase percentage) was obtained, dose concentration values were changed to log values. The dose-effect curves were generated and the half-effective concentration (EC_{50}) concentration was confirmed by graph construction (C). (C) Y-axis: Cytotoxicity (%); X-axis: log glutamate concentration (log C).

All quantitative data were expressed as mean \pm SEM, and analyzed using analysis of variance, followed by *post hoc* comparison of the means using Fisher least significant difference test and Bonferroni correction. Lactate dehydrogenase and methyl thiazolyl tetrazolium results consistently showed that differentiated HT22 cells were more sensitive to glutamate than undifferentiated cells, and differentiated cell survival curves shifted to the left. The dose-effect relationship curves showed different EC_{50} concentrations for differentiated and undifferentiated HT22 cells. The curves obviously shifted to the left after differentiation. Lactate dehydrogenase and methyl thiazolyl tetrazolium assays showed that the EC_{50} concentration of differentiated HT22 cells was about 0.03 mmol/L and 0.12 mmol/L, while that of undifferentiated HT22 cells was 2.5 mmol/L and 1.8 mmol/L, respectively.

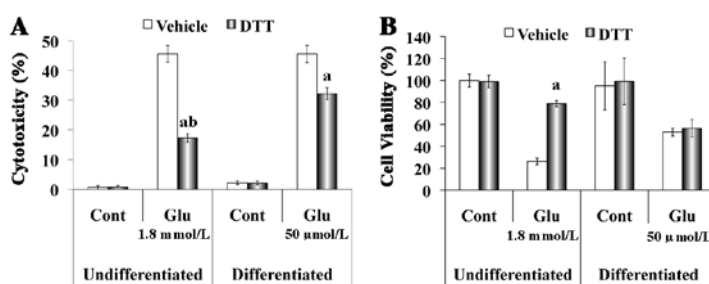


Figure 2 Effect of antioxidant on glutamate toxicity in differentiated and undifferentiated HT22 cells.

Dithiothreitol (DTT; 250 $\mu\text{mol/L}$) was added as an antioxidant to reduce cytotoxicity in HT22 cells by oxidative stress. 1.8 mmol/L and 50 $\mu\text{mol/L}$ glutamate (Glu) was added to undifferentiated and differentiated HT22 cells separately to induce basic cytotoxicity. Cell toxicity and viability were estimated using the lactate dehydrogenase (A) and methyl thiazolyl tetrazolium (MTT; B) assays, respectively. Sterile PBS is marked by 'cont'.

All quantitative data were expressed as mean \pm SEM, and analyzed using appropriate analysis of variance, followed by *post hoc* comparison of the means using Fisher least significant difference test and Bonferroni correction. After DTT was added, undifferentiated HT22 cells were rescued from oxidative stress as determined by both the lactate dehydrogenase (cytotoxicity from 45.57% to 17.23%; $^aP < 0.05$) and MTT assay (cell viability from 26.19% to 78.85%; $^aP < 0.05$). DTT showed more protective effects on undifferentiated rather than differentiated HT22 cells with glutamate ($^bP < 0.05$). DTT showed less protective effects on differentiated cells, as determined by the lactate dehydrogenase assay (from 45.55% to 32.21%; $^aP < 0.05$). No effect was observed using the MTT assay (from 52.93% to 56.53%; $P = 0.687$). Vehicle means untreated differentiated and undifferentiated HT22 cells.

However, with respect to the MTT assay, dithiothreitol was found to be ineffective in increasing the viability of differentiated HT22 cells (Figure 2B). Therefore, despite the minor inconsistency between the two assays, it is fair to conclude that oxidative stress is the dominant mechanism underlying glutamate toxicity in undifferentiated HT22 cells, but this is not the case for differentiated HT22 cells.

Excitotoxicity mediated glutamate toxicity in differentiated cells, but not undifferentiated HT22 cells

To prevent the drastically increased sensitivity of differentiated cells to glutamate toxicity in HT22 cells, two

different NMDA receptor antagonists, MK-801 and memantine^[46-47], were tested. The lactate dehydrogenase assay showed that MK-801 at 20 $\mu\text{mol/L}$ effectively reduced glutamate toxicity in differentiated HT22 cells by 27.46% ($P < 0.05$; Figure 3A), but had no effect on undifferentiated HT22 cells. Similarly, memantine at 12 $\mu\text{mol/L}$ reduced glutamate toxicity in differentiated HT22 cells by 37.73% ($P < 0.05$; Figure 4A), but had no effect on undifferentiated HT22 cells. Consistent with the results of the lactate dehydrogenase assay, the MTT assay revealed that MK-801 and memantine were significantly protective against glutamate toxicity in differentiated, but not undifferentiated HT22 cells ($P < 0.05$; Figures 3B, 4B).

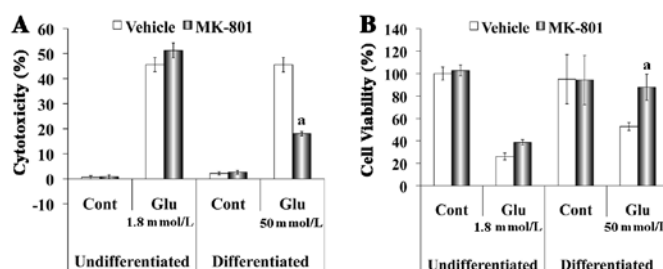


Figure 3 Effect of MK-801 on glutamate toxicity in differentiated and undifferentiated HT22 cells.

MK-801 (20 $\mu\text{mol/L}$) was added as the N-methyl-D-aspartate (NMDA) receptor antagonist to prevent excitotoxicity induced by the NMDA pathway. Cells were exposed to the NMDA pathway antagonist combined with 1.8 mmol/L and 50 $\mu\text{mol/L}$ glutamate (Glu). Cell toxicity and viability were estimated using the lactate dehydrogenase (A) and methyl thiazolyl tetrazolium (MTT; B) assays, respectively. Sterile PBS is marked as 'cont'.

All quantitative data were expressed as mean \pm SEM, and analyzed using appropriate analysis of variance, followed by *post hoc* comparison of the means using the Fisher least significant difference test and Bonferroni correction. The lactate dehydrogenase assay found that MK-801 showed no protective effect on undifferentiated HT22 cells (cytotoxicity from 45.57% to 51.27%; $P = 0.184$), but did have an effect on differentiated cells (from 45.55% to 18.09%; $^aP < 0.05$). The MTT assay found that MK-801 showed no protective effect on undifferentiated HT22 cells (cell viability from 26.19% to 38.72%; $P = 0.05$), but did have an effect on differentiated cells (from 52.93% to 87.87%; $^aP < 0.05$). Vehicle means untreated differentiated and undifferentiated HT22 cells.

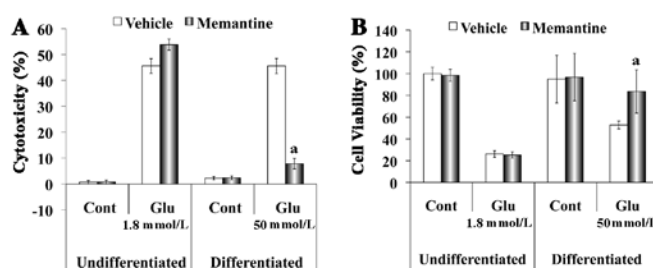


Figure 4 Effect of memantine on glutamate toxicity in differentiated and undifferentiated HT22 cells.

Memantine (12 $\mu\text{mol/L}$) was added as another N-methyl-D-aspartate (NMDA) receptor antagonist. 1.8 mmol/L and 50 $\mu\text{mol/L}$ glutamate (Glu) was added to undifferentiated and differentiated HT22 cells separately. Cell toxicity and viability were estimated using the lactate dehydrogenase (A) and methyl thiazolyl tetrazolium (MTT; B) assays, respectively. Sterile PBS is marked by 'cont'.

All quantitative data were expressed as mean \pm SEM, and analyzed using appropriate analysis of variance, followed by *post hoc* comparison of the means using the Fisher least significant difference test and Bonferroni correction. Results were similar to MK-801, with a minor difference: no protective effect was observed on undifferentiated HT22 cells (cytotoxicity from 45.57% to 53.83%; $P = 0.34$, by lactate dehydrogenase assay; cell viability from 26.19% to 25.29%; $P = 0.835$, by MTT assay), while memantine showed prominent protective effects on differentiated cells, as determined by the lactate dehydrogenase (from 45.55% to 7.82%; $^aP < 0.05$) and MTT assay (from 52.93% to 83.73%; $^aP < 0.05$). Vehicle means untreated differentiated and undifferentiated HT22 cells.

MK-801 and memantine prevented glutamate toxicity in differentiated cells, while dithiothreitol prevented undifferentiated HT22 cell injury

In addition to the lactate dehydrogenase and MTT assays, we also attempted to visualize the differential glutamate toxicity in differentiated and undifferentiated HT22 cells using propidium iodide/Hoechst staining in living cells. The representative examples of glutamate toxicity and the effects of dithiothreitol, MK-801 and memantine in these cells are shown in Figure 5, which revealed the same findings as indicated by the lactate dehydrogenase and MTT assays. Therefore, glutamate toxicity in differentiated HT22 cells is most likely mediated by excitotoxicity *via* NMDA receptors, which is different from undifferentiated HT22 cells that are mediated *via* oxidative stress.

Differentiated HT22 cells expressed NMDA receptor subunit 1 at both the mRNA and protein levels

It still remains unclear if HT22 cells are resistant to excitotoxicity because they do not express NMDA receptors^[31-32]. The experimental findings here showed that only undifferentiated cells, but not differentiated HT22 cells, were resistant to glutamate excitotoxicity at the 'mmol/L' level. Does this mean that the differentiation process may lead to expression of NMDA receptors in the same cell line? To answer this question, we chose NMDA receptor subunit 1 as an example and performed reverse transcription-PCR and western blot analysis in differentiated and undifferentiated HT22 cells. As shown in

Figure 6, although weakly, the differentiated HT22 cells clearly revealed the presence of NMDA receptor subunit 1 at both the mRNA and protein levels, while the undifferentiated HT22 cells showed no sign of NMDA receptor subunit 1. Therefore, together with the positive effects of NMDA antagonists in differentiated HT22 cells, we believe that differentiation triggers NMDA receptor expression in HT22 cells, which changes the cell line from excitotoxic-resistant to sensitive.

DISCUSSION

Excitatory neurotransmission is important for normal memory formation and memory loss in Alzheimer's disease^[1-4, 9, 48-51]. Yet our knowledge regarding the memory process in normal and abnormal circumstances is limited and there is almost no effective treatment to prevent memory loss in Alzheimer's disease. Therefore, the need for deepening our understanding of memory formation and loss is compelling.

Among various investigational tools, neuronal cell lines are the most basic and most commonly used *in vitro* model for relevant mechanistic and pharmaceutical studies. With particular concerns for memory and Alzheimer's disease-related studies, hippocampal neuronal cell lines are very limited, of which HT22 appears to be one of the most commonly used^[31, 52-53].

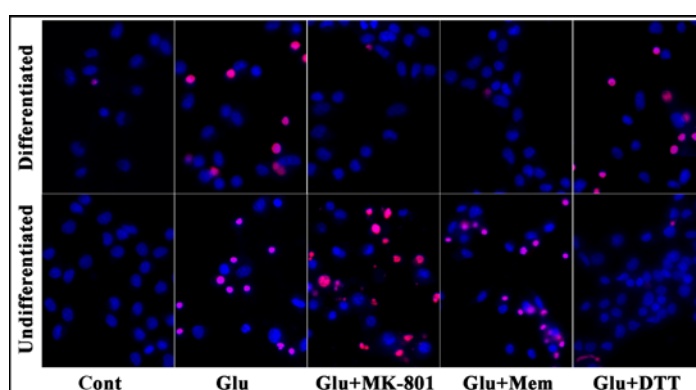


Figure 5 Morphological analysis on the effect of N-methyl-D-aspartate (NMDA) receptor antagonists and antioxidants on glutamate toxicity in undifferentiated and differentiated HT22 cells.

Propidium iodide (red) and Hoechst 33342 (blue) staining were used as apoptosis markers on undifferentiated and differentiated HT22 cells following exposure to glutamate (Glu) half-effective concentrations (1.8 mmol/L and 50 μ mol/L, respectively) and interfering drugs (MK-801 at 20 μ mol/L, memantine (Mem) at 12 μ mol/L and dithiothreitol (DTT) at 250 μ mol/L). Apoptotic cells were stained with propidium iodide.

Staining revealed that glutamate-induced cytotoxicity on undifferentiated HT22 cells could be prevented by dithiothreitol (an antioxidant), while dithiothreitol exhibited little effect on differentiated cells. Meanwhile, MK-801 and memantine (as NMDA receptor antagonists) could protect differentiated cells from glutamate toxicity, but had no effect on undifferentiated HT22 cells.

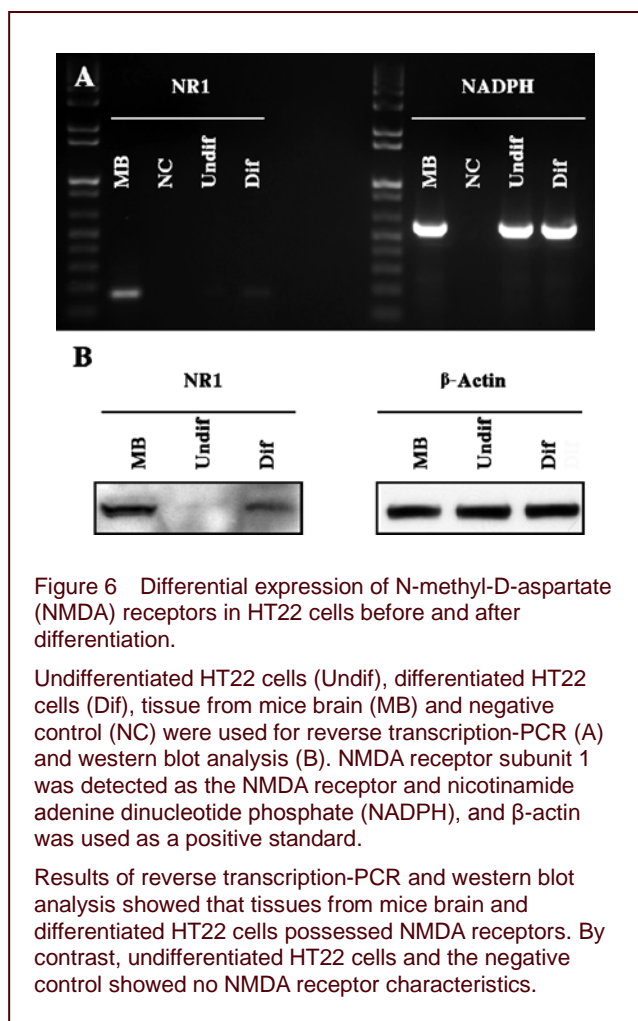


Figure 6 Differential expression of N-methyl-D-aspartate (NMDA) receptors in HT22 cells before and after differentiation.

Undifferentiated HT22 cells (Undif), differentiated HT22 cells (Dif), tissue from mice brain (MB) and negative control (NC) were used for reverse transcription-PCR (A) and western blot analysis (B). NMDA receptor subunit 1 was detected as the NMDA receptor and nicotinamide adenine dinucleotide phosphate (NADPH), and β -actin was used as a positive standard.

Results of reverse transcription-PCR and western blot analysis showed that tissues from mice brain and differentiated HT22 cells possessed NMDA receptors. By contrast, undifferentiated HT22 cells and the negative control showed no NMDA receptor characteristics.

Therefore, for accurate data interpretation, it is essential to understand what the cell line really models for, and under what circumstances. Mature neurons are known to be post-mitotic cells, which have distinct cellular properties as opposed to their mitotic precursor cells^[54-57]. However, it is surprising to see how many articles use undifferentiated mitotic neuronal precursor cells to model the biology and pathology of mature neurons. Therefore, it is a challenge to interpret these previously published findings that use cell models in ambiguous conditions. For example, there is a long list of publications that studied glutamate toxicity in HT22 cells^[31, 44-53]. These studies used glutamate at dosages varying from 1 mmol/L to 10 mmol/L to induce HT22 cell death at a rate varying from 10% to 90% following 24-hour treatment^[32-33, 44, 52]. Based on the dosages of glutamate in these studies, we speculate that they may have used undifferentiated HT22 cells, and therefore their findings most likely represent the cellular properties of immature hippocampal neuronal precursor cells rather than post-mitotic hippocampal neurons.

On the other hand, glutamate excitotoxicity in

differentiated primary neurons requires a much lower glutamate dosage^[10, 45, 58]. Fang *et al.*^[45] revealed dose-dependent injury in primary cultured retinal neurons following glutamate exposure ranging from 25 μ mol/L to 300 μ mol/L. They showed that the EC₅₀ concentration of glutamate required to induce cytotoxicity in primary retinal neurons was approximately 50 μ mol/L at 24 hours, which is very close to our findings in differentiated HT22 cells using the lactate dehydrogenase assay (30 μ mol/L; Figure 1A). In agreement with this observation, previous studies have shown increased vulnerability of mature neurons vs. immature neurons in primary cultures to glutamate toxicity^[10, 58]. Therefore, differentiation appears to be an essential step for immature neuronal precursor cell transition to mature neuronal cells^[59-61].

Increasing evidence supports the theory that differentiation causes cellular property changes in HT22 cells, and this study found that the NMDA receptor antagonists MK-801 and memantine could effectively block the glutamate toxicity in the differentiated, but not the undifferentiated, cells. Moreover, reverse transcription-PCR and western blot analysis provided direct evidence that differentiation triggered cells to express NMDA receptor subunit 1. Therefore, differentiation renders the susceptibility of HT22 cells to glutamate excitotoxicity *via* newly expressed NMDA receptors. Differentiation is an essential process for HT22 cells to possess the cellular properties of mature hippocampal neurons, with undifferentiated and differentiated HT22 cells better resembling immature hippocampal neuronal precursor cells and mature hippocampal neurons, respectively.

From a technical perspective, the present study employed the biochemical assays of lactate dehydrogenase and MTT, and the morphological method of propidium iodide/Hoechst staining to evaluate glutamate toxicity. The results from all methods were in good agreement, despite some minor inconsistencies in the EC₅₀ values of glutamate toxicity. The inconsistency is mostly likely due to the biochemical nature and sensitivities of the methods, which cannot affect the main conclusions. To verify whether the differentiation process triggers expression of glutamate receptors, we used both reverse transcription-PCR and western blot analysis, which confirmed the same findings. There are multiple subtypes of glutamate receptors, with each containing various subunits^[4]. We demonstrated newly expressed NMDA receptor subunit 1 in differentiated, but not undifferentiated, HT22 cells. It may be interesting to map all other subtypes and subunits of glutamate receptors

before and after differentiation in HT22 cells in future studies, but the results will not affect the essential conclusion of this study that differentiation triggers cellular property changes in HT22 cells and renders them to possess more cellular properties similar to mature hippocampal neurons, including susceptibility to excitotoxicity.

MATERIALS AND METHODS

Design

An *in vitro* study regarding molecular biology.

Time and setting

The experiments were carried out in the Laboratory for Alzheimer's Disease & Aging Research (Veterans Affairs Medical Center, Kansas, MO, USA) from December 2010 to March 2011.

Materials

The HT22 cell line was a generous gift from the Salk Institute (La Jolla, CA, USA).

Methods

HT22 cell culture and differentiation

Details of cell maintenance and differentiation were described previously^[53]. Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C and 5% (v/v) CO₂. Cells were differentiated in NeuroBasal medium (Invitrogen) containing 2 mmol/L glutamine and 1 × N₂ supplement (Invitrogen) for 24 hours before use^[42].

Determination of glutamate-induced cell cytotoxicity

Glutamate, MK-801, memantine and dithiothreitol (Sigma, St. Louis, MO, USA) were used as interventional agents. MK801 and memantine were used as antagonists of NMDA receptors and dithiothreitol was used as an antioxidant. The concentrations were used as follows: MK-801 at 20 µmol/L, memantine at 12 µmol/L and dithiothreitol at 250 µmol/L. The following glutamate concentrations were used for toxicity curves: 1, 2, 4, 8 and 10 mmol/L for undifferentiated HT22 cells; 10, 25, 50, 100 and 300 µmol/L for differentiated HT22 cells. Following pilot studies, the glutamate concentrations used were 1.8 mmol/L for undifferentiated HT22 cells and 50 µmol/L for differentiated cells, respectively. Cell toxicity and viability were estimated using the lactate

dehydrogenase and MTT, respectively. HT22 cells were grown on 96-well plates at a density of 2 500 cells per well^[44]. Lactate dehydrogenase release estimate was performed according to the manufacturer's instructions for the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA). The absorbance was measured using the Synergy HT Multi-Mode Microplate Reader (Bio-tek, Winooski, VT, USA) at 490 nm. Sterile PBS was used as a blank. Untreated differentiated and undifferentiated HT22 cells were regarded as positive controls (100% cell survival), which were marked as 'vehicle'. The results were expressed as a percentage of the maximum lactate dehydrogenase release. For the MTT assay, the CellTiter 96[®] AQueous Assay Kit (Promega) was used according to the manufacturer's instructions. The absorbance was measured at 570 nm. The cell viability was expressed as a percentage of untreated cells. After the dose concentration and effect (lactate dehydrogenase percentage) was obtained, dose concentration values were changed to log values. The dose-effect curves were generated and the EC₅₀ concentration was confirmed.

Propidium iodide and Hoechst 33342 staining

Beyond the cytochemical assays, propidium iodide (Invitrogen) and Hoechst 33342 (Invitrogen) staining was also performed for each treatment group. Glutamate, MK-801, memantine and dithiothreitol concentrations were used as determined from cytotoxicity assays. At the end of each treatment, the cells were stained with the dyes. Propidium iodide (1.5 µmol/L) and Hoechst 33342 (5 µg/mL) were incubated with the cells at 37°C for 10 and 20 minutes, respectively. After rinsing, the cells were observed and photographed using the Leica DMI 6000 B microscope (Leica Microsystems Inc, Buffalo Grove, IL, USA).

Semi-quantitative reverse transcription-PCR

Total RNA was extracted from differentiated and undifferentiated HT22 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples from HT22 cells and brain tissue from mice (Kansas City Veterans Affairs Medical Center^[62]) were adjusted to a A_{260/280} ratio of 2. All procedures using animal tissue were approved by the Kansas City Veterans Affairs Medical Center Institutional Animal Care and Use Committee (Kansas, MO, USA). RNA concentrations were determined using the formula: A₂₆₀ × dilution × 40 = µg RNA/mL, and were controlled at 0.5 µmol/L. First-strand cDNA was synthesized with 0.5 µg of total RNA using SuperScript[™] II First-Strand Synthesis System for reverse transcription-PCR,

following the manufacturer's instructions. cDNA (1 μ L) was then amplified in a 20 μ L reaction volume containing 1.25 U of Flexi DNA polymerase (Promega), 125 μ mol/L dNTPs, 0.625 mmol/L $MgCl_2$, and 500 pmol of the respective forward and reverse primers. DNA polymerase chain reaction on total RNA extracted from C57/BL6 mouse brain (mainly from brainstem) was used as a positive control, and a vial containing all the components except for template DNA was used as a negative control.

For NMDA receptor type 1 (GeneTex, San Antonio, TX, USA), the amplification protocol entailed 32 cycles of denaturation at 94°C for 30 seconds, annealing at 66°C for 30 seconds and extension at 72°C for 30 seconds, followed by a 5-minute final extension at 72°C. Meanwhile, nicotinamide adenine dinucleotide phosphate (NADPH; Sigma) was used as an internal control, and the amplification entailed 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds, followed by a 5-minute final extension at 72°C.

The gene specific primer pairs used for PCR were: NMDA receptor subunit 1, forward primer 5'-CAG CGT CTG GTT TGA GAT GAT GC-3', reverse primer 5'-AGC AGA GCC GTC ACA TTC TTG GT-3'; NADPH, forward primer 5'-CGT ATT GGG CGC CTG GTC ACC AG-3', reverse primer 5'-GAC CTT GCC CAC AGC CTT GGC AGC-3'. PCR products (10 μ L) were analyzed and visualized on a 1.5% (w/v) agarose gel.

Western blot analysis

The hippocampal tissue from C57/BL6 mice, and differentiated and undifferentiated HT22 cells were lysed with the appropriate amounts of boiling denaturing lysate buffer (1% (w/v) sodium dodecyl sulfate, 1 mmol/L sodium orthovanadate, 10 mmol/L Tris-Cl, pH 7.4) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Total protein quantification and semi-quantitative western blot procedures were performed routinely as previously described^[53]. NMDA receptor subunit 1 (1:200) and β -actin (Sigma, St. Louis, MO, USA) were used as the primary antibodies. Donkey anti-rabbit (1:500; Jackson Laboratory, Bar Harbor, ME, USA) and donkey anti-mouse (1:400; Jackson Laboratory) were used as the secondary antibodies. The primary antibodies were incubated at 4°C overnight and secondary antibodies were incubated at room temperature for 1 hour.

Statistical analysis

All quantitative data were expressed as mean \pm SEM,

and analyzed using appropriate analysis of variance, followed by *post hoc* comparison of the means using Fisher least significant difference test and Bonferroni correction where appropriate. Significant difference was set at $P < 0.05$. Statistical analysis was performed using SPSS 11.0 software (SPSS, Chicago, IL, USA).

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Author contributions: Minchao He performed the research and wrote the paper. Jun Liu put forward the scientific concepts and assisted with cell culture. Shaowu Cheng organized the figures and analyzed the data. Yigang Xing assisted with immunohistochemistry, PCR and western blot analysis. William Z Suo obtained funds, designed the research and supervised the writing and organization process. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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