Chronic glutamate toxicity in mouse cortical neuron culture

Jong Seong Ha,1, Chul-Sang Lee, Jin-Soo Maeng,1, Ki-Sun Kwon,1, Sung Sup Park⁎

ARTICLE INFO

Article history:
Accepted 18 March 2009
Available online 1 April 2009

Keywords:
Glutamate toxicity
Acute exposure
Chronic exposure
Ionotropic glutamate receptor
Metabotropic glutamate receptor

ABSTRACT

Two pathways for glutamate toxicity have been described, receptor-mediated excitotoxicity and non-receptor mediated oxidative glutamate toxicity. Here, we show that two distinct forms of receptor-mediated primary cortical neuronal death exist, chronic and acute glutamate toxicity, and that these depend on exposure time. In vitro, neuronal sensitivity to chronic glutamate exposure increased as neurons matured and the initial plating medium contributed as well. In immature neurons, high concentrations of glutamate induced neuronal death. The chronic glutamate toxicity was independent of neuronal density, whereas increased density potentiated acute glutamate toxicity. Activation of ionotropic glutamate receptors (iGluRs) contributed to induction of chronic and acute glutamate toxicity at similar rates at DIV14. Inactivation of the metabotropic glutamate receptors (mGluRs) by AIDA increased neuronal sensitivity to chronic glutamate exposure but not to acute exposure. Neuronal death by acute toxicity was much faster than by chronic toxicity in which activation of mGluRs was involved. These results suggest that acute glutamate toxicity is quite different from chronic toxicity, in which activation of mGluRs is associated with resistance to glutamate toxicity.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Glutamate is the major fast excitatory neurotransmitter in the mammalian central nerve system. However, when excessively released and accumulated in the extracellular space of the brain, endogenous glutamate is associated with neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and Huntington’s disease (Choi, 1988; Simonian and Coyle, 1996). Thus far, two distinct glutamate-induced cell death pathways have been identified. The excitotoxic pathway relies on hyper-activation of glutamate receptors (Choi, 1992; Rothman, 1985), whereas the oxidative pathway involves breakdown of the glutamate/cystine anti-porter without glutamate receptor-mediation (Murphy et al., 1989; Sato et al., 1999). Recently, two pathways have been identified in a primary neuron culture, which suggests oxidative glutamate toxicity is a component of the excitotoxicity cascade (Schubert and Piasecki, 2001). Neuronal cell death mediated by prolonged exposure to NMDA and non-NMDA glutamate receptor agonists has been observed in cortical neuron culture (Dugan et al., 1995; Koh et al., 1990). However, neurotoxicity by chronic glutamate exposure may be considered a slow variant of acute glutamate toxicity (excitotoxicity) because the sensitivity to glutamate and receptor-specific agonists is similar in chronic and in acute toxicity. Generally, chronic glutamate toxicity has been overlooked in primary neuron culture, presumably because high glutamate concentrations (mM range) are required in cultures of immature neurons as in NMDA

⁎ Corresponding author. Fax: +82 42 860 4598.
E-mail address: sspark@kribb.re.kr (S.S. Park).
1 Present address: Aging Research Center, Korea Research Institute of Bioscience and Biotechnology (KRRBB), 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea.
0006-8993/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2009.03.050
receptor-deficient HT22 cells, an excellent model for oxidative glutamate toxicity (Maher and Davis, 1996). However, the expression of iGluRs at early culture stages (DIV3–5) (Lesuisse and Martin, 2002; Sagara and Schubert, 1998) indicates that iGluR receptor-mediated toxicity possibly occurs by chronic and acute glutamate exposure. In this study, we analyzed neuronal responses to glutamate by comparing the sensitivities observed in chronic and acute glutamate exposure.

2. Results

2.1. Glutamate sensitivity in chronic exposure

First, to determine the sensitivity to glutamate during chronic exposure, cytotoxicity was monitored 24 h after exposure in cortical neuron culture at DIV7 and 14. When neurons were initially cultured in MEM with 10% serum, the neuronal sensitivity to glutamate was higher than in NB medium at DIV7 (Fig. 1A) but similar at DIV14 (Fig. 1B). In the NB medium, serum did not significantly alter neuronal glutamate sensitivity. In all tested culture mediums, neuronal sensitivity to chronic glutamate exposure increased as the neurons matured from DIV7 to DIV14. Neurons cultured in MEM supplemented with glucose showed increased sensitivity (data not shown). Along with these results, we suggest differential sensitivity to glutamate depending on culture conditions. Interestingly, neurons initially cultured in NB medium were highly resistant to chronic glutamate exposure at DIV7 and required more than 2 mM glutamate to achieve 80% cell death (Fig. 1A). To investigate whether neuronal glutamate resistance affects acute glutamate toxicity

<table>
<thead>
<tr>
<th>Table 1 – Toxicity of glutamate and NMDA.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIV7</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>Acute Glutamate</td>
</tr>
<tr>
<td>NMDA</td>
</tr>
<tr>
<td>Chronic Glutamate</td>
</tr>
<tr>
<td>NMDA</td>
</tr>
</tbody>
</table>

The EC50 concentrations (μM) were derived from concentration–cytotoxicity curves.

DIV7 (Fig. 1A) but similar at DIV14 (Fig. 1B). In the NB medium, serum did not significantly alter neuronal glutamate sensitivity. In all tested culture mediums, neuronal sensitivity to chronic glutamate exposure increased as the neurons matured from DIV7 to DIV14. Neurons cultured in MEM supplemented with glucose showed increased sensitivity (data not shown). Along with these results, we suggest differential sensitivity to glutamate depending on culture conditions. Interestingly, neurons initially cultured in NB medium were highly resistant to chronic glutamate exposure at DIV7 and required more than 2 mM glutamate to achieve 80% cell death (Fig. 1A). To investigate whether neuronal glutamate resistance affects acute glutamate toxicity

Fig. 1 – Neuronal sensitivity to chronic glutamate exposure depends on the initial plating medium and time in culture. Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (MEM-F) or B27 neurobasal medium alone (NB) or containing 10% fetal bovine serum (NB-F) were used for 3 days and replaced with serum-free B27 neurobasal medium (NB) media. Neuronal (1×10⁵ cells per well) cytotoxicity in chronic exposure was measured by LDH release at DIV7 (A) and DIV 14 (B). Results of triplicate experiments are shown. The values were expressed as means±SD. *p<0.05, **p<0.01 and ***p<0.001 as compared with both NB and NB-F.

Fig. 2 – Neuronal sensitivity to glutamate depends on cell density in chronic and acute exposure at DIV7. Neurons were plated and cultured in serum-free NB medium at 2×10⁴ and 5×10⁴ cells per well. At DIV7, cytotoxicity was observed in chronic (A) and acute (B) glutamate exposure. The values were expressed as mean±SD. *p<0.01 and ***p<0.001 versus 2×10⁴ cells.
(excitotoxicity), we analyzed neuronal excitotoxicity induced by acute glutamate exposure. Serum-free B27 (Minus AO)-NB medium was used to avoid effects of uncharacterized materials and antioxidants on differences between chronic and acute toxicity. During acute glutamate exposure, the neuronal cytotoxicity was induced by glutamate with an EC_{50} of ∼42 and ∼14 μM at DIV7 and 14, respectively (Table 1). These results suggest that although at DIV7, low sensitivity to glutamate was observed during chronic exposure (EC_{50} of ∼685 μM), acute glutamate toxicity (excitotoxicity) was clearly induced, and the neuronal sensitivity to acute glutamate exposure increased moderately from DIV7 to DIV14.

2.2. Glutamate sensitivity depends on neuronal density

Next, we examined whether glutamate tolerance was correlated with cell density in glutamate exposure. Neuronal cells were plated at 2×10^4 and 5×10^5 cells per well, and cultured in NB medium until DIV7. In very high cell density cultures, the sensitivity to glutamate during chronic exposure did not increase (Fig. 2A), while it increased significantly during acute exposure (the EC_{50} decreased from ∼490 μM to ∼20 μM; Fig. 2B). These results suggest that glutamate sensitivity is correlated with neuronal density and that high density neuron cultures are more sensitive to glutamate in acute than in chronic exposure.

2.3. Glutamate receptor contribution in neurotoxicity

To compare the contributions of iGluRs in chronic and acute glutamate toxicity, cytotoxicity was determined after treatment with the glutamate receptor agonists NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate. NMDA receptors largely contributed to chronic and acute glutamate toxicity at DIV7 and DIV14, whereas AMPA and kainate receptors contributed partly to glutamate toxicity (Figs. 3A and B). The sensitivity to NMDA increased slightly as neuronal maturation progressed, but glutamate sensitivity at DIV7 was significantly distinguishable from that at DIV14 (Table 1). The receptor-mediated cytotoxicity was confirmed by using receptor-specific antagonists to inhibit cell death induced by each agonist (data not shown). These results suggest that neuronal resistance to chronic glutamate exposure is independent of iGluRs activation at DIV7. To examine whether mGluRs contribute to chronic glutamate toxicity, we measured LDH release after the neurons were exposed to glutamate for 24 h or 10 min in the presence or absence of 0.5 mM (R,S)-1-aminoindan-1,5-dicarboxylic acid (AIDA).
(AIDA), a potent competitive antagonist of group I mGluRs. Application of AIDA significantly increased the sensitivity to glutamate during chronic toxicity, the EC\textsubscript{50} decreased more than 2-fold at DIV7 and 14. However, the presence of AIDA did not alter the sensitivity to glutamate during acute exposure at DIV7 and 14 (Figs. 3C and D). In the presence of (R,S)-3,5-dihydroxyphenylglycine (DHPG a group I mGluR agonist), reduced neuronal sensitivity was observed in chronic exposure (data not shown). These results indicate that low sensitivity to glutamate in chronic exposure is largely dependent on activation of mGluRs.

### 2.4. Temporal requirement for glutamate toxicity

At DIV14, neuronal sensitivity to chronic exposure to glutamate in the presence of AIDA was similar with that to acute exposure. Lastly, we compared the temporal requirement for glutamate toxicity between chronic and acute exposure. Neurons at DIV14 were exposed to 200 μM glutamate in the presence or absence of 0.5 mM AIDA, followed by measurement of released LDH for cytotoxicity at various times after glutamate exposure. At DIV14, glial cell growth was still largely suppressed by a treatment of AraC (Fig. 4), and 200 μM glutamate was sufficient to trigger chronic and acute cell death. In acute glutamate toxicity, after 10 min exposure, neuronal cell death was very rapid and the cells showed about 60% cytotoxicity after 3 h incubation. In chronic toxicity, cell death was slow, ~10% after 3 h and only ~30% after 6 h (Fig. 5). The amount of LDH released from the neurons after 24 h was almost identical for both exposures, but the LDH release in acute exposure was more rapid than in chronic exposure. These results suggest that glutamate triggers different cell death pathways depending on the duration of exposure. Furthermore, chronic exposure may provide neurons with some resistance to glutamate toxicity in early cell death; that is slow release of LDH. The presence of AIDA had no significant effect on the temporal requirements of the two glutamate toxicities. This shows that inactivation of group I mGluRs, even increasing neuronal sensitivity to glutamate, does not result in the same temporal requirement for glutamate toxicity between chronic and acute exposure.

### 3. Discussion

In conclusion, receptor-mediated glutamate toxicity could be triggered by chronic exposure at DIV7 and 14 in neuronal cultures. Neuronal sensitivity to glutamate during chronic exposure increased as the neurons were cultured in vitro. Cell density-dependency, activation of mGluRs and temporal requirement for toxicity were significantly different in chronic and acute glutamate toxicity.

The low level expression of iGluRs at early in vitro culture maybe an explanation for why relatively high concentrations of glutamate are required in oxidative glutamate toxicity, while very little amount of glutamate induce excitotoxicity in older cultures. It has been proposed that developmental changes in NMDA-induced excitotoxicity reflect changes in the subunit composition of NMDA receptors (Zhou and Baudry, 2006). In primary hippocampal neuron culture, excitotoxic vulnerability increased with age in culture and was associated with increased NR1 and NR2A subunit expression (Brewer et al., 2007). In our cortical neuron culture, using serum-free NB medium, the sensitivity to glutamate in acute exposure was slightly increased from DIV7 to DIV14. However, in chronic exposure the sensitivity increased significantly and the EC\textsubscript{50} at DIV14 was reduced approximately 20-fold compared to the younger culture (Table 1). Interestingly, the sensitivity to glutamate in acute exposure at DIV7 increased with cell density. This result is consistent with dense cultures being more sensitive to oxygen and glucose deprivation than thin cultures (Yavin and Billia, 1997). The most likely
Chronic glutamate toxicity is distinguished from oxidative toxicity by the involvement of iGluRs. Oxidative glutamate toxicity occurs in primary neuron cultures that do not express glutamate receptors (DIV2) and in iGluR-deficient HT22 cells (Schubert and Piasecki, 2001). However, activation of mGluRs protects nerve cells from oxidative stress (Sagara and Schubert, 1998), which resembles the chronic toxicity mediated by iGluRs in primary neuron culture. It is unclear if the rapid decline in intracellular glutathione, one of the initial events in oxidative glutamate toxicity (Tan et al., 1998), is involved in chronic glutamate toxicity. 12-lipoxygenase is the predominant brain lipoxygenase and several studies have demonstrated its role in linking glutathione depletion to neuronal cell death during oxidative glutamate toxicity (Li et al., 1997; Murphy et al., 1989). Interestingly, the presence of glial cells increases the sensitivity to glutathione depletion and inhibition of lipoxygenase prevents glial-dependent toxicity. However, reducing the glutathione level has no effect on survival in enriched neuronal cultures (Mytilineou et al., 1999). This suggests that receptor-mediated chronic glutamate toxicity in our neuron-enriched culture does not result from the processes observed in oxidative glutamate toxicity, because the high resistance to glutamate at DIV7 is presumably derived from the high-level expression of mGluRs compared to other culture stages (Schubert and Piasecki, 2001), and early in vitro culture (DIV2) without using cytosine arabinoside possibly contains glial cells (Fig. 4A). The composition of the glutamate receptors, including iGluRs and mGluRs, thus appears differentially involved in glutamate toxicity during chronic exposure.

Chronic glutamate toxicity is observed at higher glutamate concentrations than acute glutamate toxicity, at any stage of primary neuron culture. Therefore, if glutamate is removed shortly (within 10 min) after exposure to levels sufficient for chronic toxicity, the neurons may be damaged by acute toxicity. If not, the protective functions of the mGluRs are activated and chronic toxicity may contribute to glutamate toxicity. Activation of the mGluRs is potentially associated with sensitization and desensitization of receptors (Hermans and Challiss, 2001), and iGluR interactions (Alagarsamy et al., 1999). We speculate that more complicated modulations of the glutamate receptors play a role in the different forms of glutamate toxicity.

4. Experimental procedures

4.1. Materials

Glutamate, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate), 3,5-dihydroxypheglycine (DHPG), and (RS)-1-Aminooindan-1,5-dicarboxylic acid (AIDA) were purchased from Sigma (St. Louis, MO). Anti-microtubule-associated protein 2 (MAP2) rabbit polyclonal and anti-glial fibrillary acidic protein (GFAP) monoclonal antibodies were purchased from Millipore (Billerica, MA) and Abcam (Cambridge, MA), respectively. Anti-rabbit fluorescein isothiocyanate and anti-mouse Cy3-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Neurobasal medium, B-27 Supplement Minus AO, and laminin were purchased from Invitrogen (Carlsbad, CA).

4.2. Primary neuron culture

Mouse neuronal cultures were prepared from embryonic day 14 embryos as previously described (Schubert and Piasecki, 2001). Cortical neurons were plated at 1×105 cells per well, unless otherwise indicated, in poly-D-lysine and laminin-coated 96-well microtiter plates (Nunc) in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum, or B27-neurobasal (NB) medium with or without 10% fetal bovine serum. After two days, non-neuronal cell division was inhibited by incubation with 10 μM cytosine arabinoside (AraC) for 24 h followed by incubation in serum-free NB medium for all three culture conditions. Subsequently, the medium was replaced every 3 days.

4.3. Measurement of neurotoxicity

To measure the chronic glutamate toxicity, the cortical neurons were exposed to glutamate in serum-free NB medium for 24 h and cytotoxicity measured via lactate dehydrogenase (LDH) release using the Cytotox 96 assay kit (Promega) as previously described (Williamson et al., 2002). To measure the acute glutamate toxicity, neurons initially cultured in NB medium were exposed to increasing concentrations of glutamate for 10 min in a HEPES-buffered salt solution (HCSS) (Hartley et al., 1993) containing 20 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 15 mM glucose, and 20 mM HEPES, pH 7.4. After 10 min at room temperature, the HCSS solution was replaced with the original culture medium and cytotoxicity monitored 24 h later by measuring released LDH (Schubert and Piasecki, 2001). Data represent the percentage relative to control treated with lysis buffer.

4.4. Immunocytochemistry

Neuronal cells (1×105 cells/cm2) cultured in B27-NB medium for 14 days were washed twice with phosphate-buffered saline (PBS) and fixed with formaldehyde solution (1% formaldehyde, 5% fetal bovine serum, and 4% sucrose in PBS) for 20 min at 4 °C. After permeabilization with 0.2% Triton X-100 in PBS for 30 min at 4 °C, cells were placed in blocking buffer (PBS with 0.1% bovine serum albumin and 3% fetal bovine serum) for 1 h. The primary antibodies to MAP2 (1:100) and GFAP (1:200) were applied overnight at 4 °C, followed by incubation with fluorescent dye-conjugated secondary antibodies (1:200) for 1 h. Fluorescence images were obtained via a confocal laser microscope (LSM 510 META, Carl Zeiss).

4.5. Data analysis

The values were expressed as means±SD. ANOVA with repeated measures and followed by t-test were used to
evaluate significance within group differences and individual points between groups. Probability values of <0.05 were considered of statistical significance.

Acknowledgments

This work was supported by grants from the MOST of Korea (M10642040003, M10503010002, and M10601000164 to K.-S. K.).

REFERENCES


Murphy, T.H., Miyamoto, M., Sastre, A., Schnaar, R.L., Coyle, J.T., 1989. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2, 1547–1558.


学霸图书馆
www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。
图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：
图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具