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Changes in cholesterol biosynthetic and transport pathways after excitotoxicity

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Abstract
The present study was carried out to elucidate changes in the gene expression and activity of cholesterol biosynthetic enzymes and transporters in the rat hippocampus after kainate excitotoxicity. Significantly increased cholesterol level was detected in the degenerating hippocampus, reaching double normal levels at 1 week after kainate injury. RT-PCR analyses of hippocampal homogenates showed significantly decreased mRNA expression of the transcription factor controlling cholesterol biosynthesis SREBP-2, and the rate-controlling enzyme HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase at all time points after kainate injection; and decreased lanosterol synthase and CYP51 at 1 and 2 weeks post-kainate injection respectively. GC-MS analyses showed a significant increase in cholesterol biosynthetic precursors lanosterol, desmosterol and 7-dehydrocholesterol at 1 day after kainate injection presumably reflecting biosynthesis in injured neurons, and significant decreases in precursors at 1 and 2 weeks post-kainate injection, at time of gliosis in the degenerating hippocampus. Levels of cholesterol autooxidation including 7 ketocholesterol and cholesterol epoxides were elevated in the kainate lesioned hippocampus. Furthermore, loss of expression of the cholesterol transporter, ABCA1 was detected in neurons, but increased expression in astrocytes was detected after kainate lesions. The results suggest that increased cholesterol biosynthesis and loss of ABCA1 expression in injured neurons might result in increase in cholesterol in the degenerating hippocampus. The increased cholesterol might predispose to increased formation of cholesterol oxidation products which have been shown to be toxic to neurons. © 2009 International Society for Neurochemistry.

Keywords
cholesterol, biosynthetic, changes, transport, excitotoxicity, pathways, after

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Changes in cholesterol biosynthetic and transport pathways after excitotoxicity

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The brain has higher concentration of cholesterol compared with other body parts (25% of the body’s free cholesterol) (Dietschy and Turley 2001). It is an integral component of neural membranes, where it has been implicated in the assembly and maintenance of lipid rafts. Cholesterol not only affects the physicochemical properties of neural membranes, and facilitates exocytosis, but also modulates activities of the membrane-bound enzymes, receptors, and ion channels (Dietschy and Turley 2001). The blood brain barrier effectively blocks uptake of cholesterol from the circulation, and thus brain cholesterol is derived mostly from de novo synthesis (Björkhem et al. 1998).

Cholesterol biosynthesis in brain involves several intermediates and mediating enzymes. The regulation of biosynthesis of cholesterol is believed to involve insulin-induced genes (INSIGs) and Sterol Regulatory Element-Binding Proteins, in particular SREBP-2 (DeBose-Boyd 2008) and controlled through feedback regulation by sterols, including cholesterol itself (Brown and Goldstein 1997). HMG-CoA reductase, the rate-controlling enzyme for cholesterol biosynthesis is the main target of cholesterol regulation (Wang et al. 2008), but other enzymes further downstream in the cholesterol synthetic pathways include squalene synthase, lanosterol synthase and cytochrome P450 (CYP51) which produce squalene, lanosterol, and cholesterol respectively.
Excitotoxicity changes cholesterol pathways

Kojima et al. (2004). There are two pathways of cholesterol synthesis. The Bloch pathway occurs via zymosterol and desmosterol, and the Kandutsch–Russell pathway is through lathosterol and 7-dehydrocholesterol (Lütjohann et al. 2002). The Bloch pathway contributes more to cholesterol biosynthesis in young animals, whereas the alternative pathway is more involved in aged animals (Thelen et al. 2006).

Cholesterol is excreted from the brain into the circulation predominantly through conversion to 24-hydroxycholesterol and to a lesser extent 27-hydroxycholesterol, which are formed by hydroxylation of cholesterol side chain. The enzyme mediating the conversion of cholesterol into 24-hydroxycholesterol has been characterized at the molecular level (CYP46) and is mainly located in neurons (Russell 2000; He et al. 2006). 24-hydroxycholesterol plays a pivotal role in promoting altered inflammatory signaling, apoptotic responses and Alzheimer’s disease-type changes in brain (the A and G subfamilies play important roles in cholesterol efflux (Borst et al. 2000). ABCA1 is known to play a critical role in peripheral lipid transport by regulating cholesterol efflux from the plasma membrane to the lipid acceptor protein-AI (apoA-I). High expression of ABCA1 occurs in neurons of hypothalamus, thalamus, amygdala, cholinergic basal forebrain, and hippocampus in the adult rat brain (Koldamova et al. 2003). ABCA1 expression is known to be regulated by Liver X receptors (LXRs) (Venkateswaran et al. 2000).

Alterations in cholesterol metabolism have been reported in Alzheimer’s disease (Björkhem and Diecfałusy 2002; Björkhem and Meaney 2004; Michikawa 2004; Lukiw 2006), vascular dementia (Kolsch et al. 2000), multiple sclerosis and its animal model (Leoni et al. 2003; Teunissen et al. 2007) and traumatic brain injury (Cartagena et al. 2008). Our previous study in the rat hippocampus after excitotoxic injury induced by intracerebroventricular injection of the glutamate analog, kainate has shown increases in the level of cholesterol, 24-hydroxycholesterol and various other oxysterols, including cholesterol oxidation products (COPs) formed by direct attack of reactive oxygen species such as 7-ketocholesterol and 5,6-alpha and beta cholesterol epoxides (Ong et al. 2003; He et al. 2006). These COPs act as biomarkers of lipid peroxidation, and indicate that kainate excitotoxicity induces oxidative stress and lipid damage. Elevated COPs in human neurodegenerative diseases including Alzheimer’s disease and multiple sclerosis (Leoni et al. 2005; Hascalovici et al. 2009) have provided further evidence for oxidative damage during neurodegeneration and illustrated their potential as important biomarkers of oxidative damage in brain.

Increased cholesterol and oxysterols may be due to increased biosynthetic activity of cholesterol, altered metabolism or disruption of the cholesterol transport system. The present study was carried out to elucidate changes in the gene expression and activity of cholesterol biosynthetic enzymes and transporters in the rat hippocampus after kainate excitotoxicity.

Materials and methods

GC-MS analyses

Twenty four adult male Wistar rats weighing approximately 200 g each were used for this portion of the study. The rats were divided into four groups of six rats each. The first group consisted of untreated controls. The second to fourth groups were injected with kainate. Rats were anaesthetized by ketamine and xylazine cocktail (prepared with 7.5 mL ketamine (75 mg/kg), 5 mL xylazine (10 mg/kg), and 7.5 mL sterile water) and kainate 1 μL of 1 mg/mL was injected through a small craniotomy as previously described (Kim and Ong 2009). The kainate injected rats were killed at 1 day, 1 week and 2 weeks after injection. The animals were deeply anesthetized and decapitated. The lesioned right hippocampi were quickly removed and snap frozen in liquid nitrogen, and kept at −80°C till analyses. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

All reagents for gas chromatography-mass spectrometric (GC-MS) analysis were of analytical grade. Standards for 7-ketocholesterol, 7-alpha-hydroxycholesterol, 7-beta-hydroxycholesterol, cholesterol 5 beta-6-epoxide, cholesterol 5 alpha-6-epoxide, and 5 alpha-cholestanol were purchased from Sigma (St. Louis, MO, USA) and of at least 95% purity. 26 (27)-Hydroxycholesterol was obtained from Steraloids (Newport, RI, USA). 5 alpha-cholestanol and other compounds were used as internal standards. 7-alpha-hydroxycholesterol-d5, 7 beta-hydroxycholesterol-d7, 26 (27)-OH cholesterol-d3, 7-ketocholesterol-d5, lathosterol, and lathosterol-d4 were purchased from CDN Isotopes (Quebec, Canada). All standards were of high purity (≥95%). Standard solutions of oxysterols, 5-alpha cholestane, and other cholesterol biosynthetic precursors were diluted in ethanol. Formic acid (Lancaster, England), potassium hydroxide, butylated hydroxytoluene, ethanol, acetic acid (Merck, Darmstadt, Germany), and hexane (Tedia, OH, USA) were used as analytical grade. Methanol (EM Science, Darmstadt, Germany) and ethyl acetate (Fisher Scientific, Loughborough, UK) were of HPLC grade. N,O-bis(trimethylsilyl)triﬂuoroacetamide +1% trimethylchlorosilane (BSTFA + TMCS) silylating agent was obtained from Pierce Chemicals (Rockford, IL, USA). Oasis mixed anion-exchange cartridges were from Waters Corp. (Milford, MA, USA).

Extraction of lipids was carried out using the Folch method with slight modification (Folch et al. 1957). Hippocampal specimens of 1 day, 1 week, and 2 weeks post-kainate injected and control hippocampi were homogenized at 4°C with 0.75 mL phosphate-buffered saline (PBS) and 3.25 mL Folch organic solvent mixture (chloroform/methanol 2:1, containing 0.05% butylated hydroxytoluene). The homogenates were centrifuged at 1000 g for 10 min at 4°C. The upper phase was discarded and the lower organic phase carefully transferred to a glass vial and evaporated under a stream of N2. 1 mL of 0.5 M KOH (in 100% methanol) and 1 mL of water was added with a mixture of heavy isotopes, 40 ng of 7 alpha-hydroxycholesterol-d5, 40 ng of 7 alpha-hydroxycholesterol-d5, 40 ng of 7 alpha-hydroxycholesterol-d5.
beta-hydroxycholesterol-d$_{5}$, 40 ng of 26 (27)-hydroxycholesterol-d$_{5}$, 80 ng of 7-ketcholesterol-d$_{5}$, 0.2 µg 5-alpha cholestanate, 0.2 µg of lathosterol-d$_{4}$, 0.2 µg of campesterol-d$_{4}$, and 0.2 µg of beta-sitosterol-d$_{4}$ in 25 µL of ethanol were added to the sample and mixed. The tube was purged with argon gas and closed with a Teflon cap. After gentle mixing, samples were incubated at for 15 h at 23°C in order to measure the total (free + esterified) forms of cholesterol, COPs, and oxysterols.

Mixed anion-exchange SPE columns were preconditioned with 2 mL methanol; followed by 2 mL of 20 mM formic acid pH 4.5. 0.5 mL of 0.4 M acetic acid was added to the hydrolysed samples and then neutralized with 0.85 mL of 1 M HCl. The hydrolyzed samples (pH 4.5) were loaded onto the columns, and the columns were washed with 2 mL of 40% methanol/formic acid pH 4.5. After the wash, 2 mL of hexane and then 2 mL of ethyl acetate/hexane (30 : 70) were added to elute cholesterol and oxysterols. The eluted samples were evaporated under a stream of nitrogen. The aliquots were derivatized with 25 µL acetonitrile and 25 µL BSTFA + TMCS for an hour at 22°C. For oxysterols measurement, the derivatized samples were analyzed using Agilent 5975 inert XL mass selective detector. Helium was used as the carrier gas at a flow rate of 0.8 ml/min, derivatized samples (1 µL) were injected splitless into the GC injection port (280°C). Column temperature was increased from 160°C to 300°C at 40°C/min after 1 min at 160°C, then held at 300°C for 6 min. Selective ion monitoring was performed using electron ionization mode at 70 eV (with ion source maintained at 230°C and the quadrupole at 150°C) to monitor one target ion and 2 qualifier ions selected from each compound's mass spectrum to optimize sensitivity and specificity. Quantification of cholesterol and oxysterols was achieved by relating its peak area of target ion to its corresponding internal standard peak. Statistical analysis of results was done using one-way ANOVA with Bonferroni’s multiple comparison post-hoc test. $p < 0.05$ was considered significant in all cases.

**RT–PCR analyses**

Another 20 adult male Wistar rats weighing approximately 200 g each were used for this portion of the study. The first group consisted of untreated controls. The second to fourth groups were injected with kainate and killed at 1 day, 1 week and 2 weeks after injection (5 rats per group). The animals were deeply anesthetized and decapitated. The lesioned right hippocampi were quickly removed and immersed in RNAlater/C210 and decapitated. The lesioned right hippocampi were quickly injected (5 rats per group). The animals were deeply anesthetized in a 1 : 200 dilution in Tris-buffered saline (Abcam, Cambridge, UK) at 4°C. After washing with 0.1% Tween-20 in Tris-buffered saline, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin IgG (Amersham) for 1 h at 22°C. The protein was visualized with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

**Immunohistochemistry**

Three untreated control rats and three 1 week post-kainate injected rats were used for this portion of the study. The hippocampus was harvested and homogenized in 10 volumes of ice-cold buffer containing 0.32 M sucrose, 4 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 0.25 mM dithiothreitol. After centrifugation at 1000 g for 10 min, the supernatant was collected and protein concentrations in the preparation were measured using the BioRad protein assay kit (Bio-Rad Laboratories, CA, USA). Total proteins (60 µg) were resolved in 10% SDS polyacrylamide gels under reducing conditions and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Nonspecific binding sites on the PVDF membrane were blocked by incubation with 5% non-fat milk for 1 h. The PVDF membrane was then incubated overnight with rabbit polyclonal antibody to ABCA1 [1 : 500 dilution in Tris-buffered saline] (Abcam, Cambridge, UK) at 4°C. After washing with 0.1% Tween-20 in Tris-buffered saline, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin IgG (Amersham) for 1 h at 22°C. The color reaction was stopped with several washes of Tris-buffer. Some sections were mounted on glass slides and lightly counterstained with methyl green before coverslipping. Control sections were incubated with antigen-absorbed antibody instead of primary antibodies. They showed absence of staining.

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Results

GC-MS analyses

Cholesterol and oxysterols
Significant increase in cholesterol was detected in the kainate lesioned hippocampus at 1 week after kainate injection (Table 1). The same result was observed using different internal standards (data not shown). Significant increase in 5,6-beta cholesterol epoxide \((p = 0.028)\) was observed in the hippocampus at 1 day after kainate injection (Fig. 1a), and significant increases in levels of 5,6-beta cholesterol epoxide \((p = 0.002)\), 5,6-alpha cholesterol epoxide \((p = 0.014)\) and 7-ketocholesterol \((p = 0.001)\) were detected at 1 week after kainate injection, compared with controls. The levels of 7-beta hydroxycholesterol \((p = 0.003)\) 5,6-beta cholesterol epoxide \((p = 0.036)\) and 7-ketocholesterol \((p = 0.004)\) were significantly elevated at 2 weeks after kainate injection (Fig. 1a). Small increases in 7-alpha hydroxycholesterol at 1 and 2 weeks post-kainate injection were not statistically significant. In approximately 50% of samples, 27-hydroxycholesterol levels were below the limit of detection and the data for this oxysterol is not reported.

Cholesterol biosynthetic precursors
A significant increase in cholesterol biosynthetic precursors lanosterol \((p < 0.001)\), desmosterol \((p = 0.001)\), and 7-dehydrocholesterol \((p < 0.001)\) was detected in the 1 day post-kainate injected hippocampus, compared to controls. In contrast, all biosynthetic precursors, such as lanosterol \((p = 0.015)\) and 7-dehydrocholesterol \((p = 0.002)\) were significantly decreased in the 1 week post-kainate injected hippocampus, compared with controls (Fig. 1b and c). At 2 weeks post-kainate injection, lathosterol \((p = 0.004)\), 7-dehydrocholesterol \((p = 0.012)\), and lanosterol \((p = 0.002)\) were significantly decreased while other compounds showed a non-significant trend to a decrease, compared with controls (Fig. 1b and c).

RT-PCR analyses

Cholesterol biosynthetic enzymes
Significant decrease in mRNA levels of SREBP-2 \((p = 0.026)\), and HMG-CoA reductase \((p = 0.031)\) were detected in the degenerating hippocampus at 1 day post-kainate injection (Fig. 2a and b). Significant decrease in mRNA expression of the biosynthetic regulator SREBP-2 was detected in the degenerating hippocampus at 1 week post-kainate injection \((p = 0.042)\). The mRNA expression of HMG-CoA reductase \((p = 0.031)\) and CYP51 \((p = 0.047)\) was also decreased at this time. The mRNA expression of INSIG1 \((p = 0.005)\), SREBP-2 \((p = 0.016)\), HMG-CoA reductase \((p = 0.004)\) and lanosterol synthase \((p = 0.042)\) were significantly decreased compared with controls at 2 weeks post-kainate injection (Fig. 2a and b).

Table 1

<table>
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<tr>
<th>Concentration of total cholesterol per mg rat hippocampus after kainate-induced injury using gas chromatography-mass spectrometry</th>
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<tr>
<td>Control</td>
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<td>μg/mg tissue ± SD</td>
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*Significant difference, by one-way ANOVA with Bonferroni’s multiple comparison post-hoc test. \(p < 0.05\).

Cholesterol transporters
Significant increase in the level of mRNA expression of the cholesterol efflux protein, ABCA1 was detected in the
degenerating hippocampus at 1 and 2 weeks post-kainate injection (\(p = 0.042\) and \(0.03\) respectively) (Fig. 2).

**Western blot analyses**

ABCA1 antibody labeled a single band at 210 kDa consistent with the expected molecular weight of the protein. Significantly greater ABCA1 expression was observed after kainate lesions compared with controls (Fig 3).

**Immunohistochemistry**

Baseline immunoreactivity to ABCA1 was observed in the cell bodies and dendrites of neurons in the hippocampus (Fig. 4a). Decreased ABCA1 expression in neurons, but increased expression of in astrocytes in the lesioned areas was observed at 1 week after kainate injection, compared to controls (Fig. 4b). Sections incubated with antigen-absorbed antibody showed absence of staining (Fig. 4c).

**Discussion**

The present study was carried out to elucidate changes in the gene expression and activity of cholesterol biosynthetic enzymes and transporters in the rat hippocampus after kainate excitotoxicity. Increased cholesterol levels were found in the degenerating hippocampus after kainate injury, reaching a maximum at 1 week after kainate injury. At this time, cholesterol concentration was double that of the normal hippocampus and accompanied by an increase in oxysterols in the degenerating hippocampus. RT-PCR analyses of hippocampal homogenates showed significant decrease in mRNA expression of the transcription factor controlling cholesterol biosynthesis SREBP-2, and the rate-controlling enzyme HMG-CoA reductase at all time points after kainate injection; and decrease in lanosterol synthase and CYP51 at 1 and 2 weeks post-kainate injection respectively. GC-MS analysis showed a significant increase in cholesterol biosynthetic precursors lanosterol, desmosterol and 7-dehydro-cholesterol at 1 day after kainate injection and significant decreases in precursors at 1 and 2 weeks post-kainate injection.

The above observations suggest that the brain mounts an early acute response to excitotoxic injury by inducing an increase in cholesterol synthesis even though there is no increase in mRNA expression of these enzymes. Since increases in cholesterol precursors occur at 1 day after kainate injection (Fig. 1b and c) at a time before substantial glial reaction, this suggests increased cholesterol biosynthesis in injured neurons through both the Bloch and Kandutsch-Russell pathways. On the other hand, no net increase in cholesterol precursors was observed at 1 and 2 weeks after kainate injection at a time of significant gliosis in the degenerating hippocampus (Sandhya et al. 1998), suggesting that a primarily glial source of the increased cholesterol is unlikely. It is possible that ongoing neuronal injury due to...
Excitotoxicity could continue to contribute to cholesterol biosynthesis for some time after the kainate injection, leading to significantly elevated cholesterol in the hippocampus, at 1 week after kainate injection (Table 1). This notion is supported by our previous immunohistochemical results of increased SREBP-2 immunoreactivity in pyramidal neurons at the edge of the glial scar, but absence of SREBP-2 in the glial scar itself (Kim and Ong 2009). Another factor which could result in cholesterol accumulation in the hippocampus is the changing pattern of expression of the cholesterol transporter ABCA1. RT-PCR analyses revealed a net increase in mRNA of this transporter at 1 and 2 weeks after kainate lesion. Immunohistochemical analysis, however, revealed that this transporter is normally expressed in neurons, but there is loss of immunoreactivity in neurons, and increased immunoreactivity in astrocytes after kainate injection. It is possible that decreased expression of ABCA1 in neurons could contribute to increased cholesterol accumulation in these cells. A third possibility which could account for the increase in cholesterol in the brain is that some of the cholesterol may be in the form of cholesterol esters, which may not be good substrates for cholesterol transporters (Chang et al. 2006). Preliminary NMR analysis has suggested the presence of cholesterol esters in kainate injected hippocampus (data not shown). Further studies examining kainate-induced cholesterol accumulation are necessary to elucidate the exact mechanisms responsible and its importance in the progression of the hippocampus lesion.

Our analysis of oxysterols formed by autooxidation (COPs) revealed increases in 7-ketocholesterol at 1 week and 2 weeks after kainate injection, and increases in cholesterol 5,6, alpha and beta epoxide at 1 week, and 1 day, 1 week and 2 weeks after kainate injection respectively. These findings confirm and add to our earlier results showing increases in cholesterol and COPs in the hippocampus after kainate lesions (Ong et al. 2003; He et al. 2006). In addition, increased level of 24-hydroxycholesterol was found in the degenerating hippocampus after kainate lesions (He et al. 2006). Oxysterols have also been demonstrated to reduce the level of cholesterol biosynthesis and down-regulate mRNA expression of many cholesterol synthesizing enzymes (Wang et al. 2008), which may explain the net decrease in expression of the cholesterol biosynthetic enzymes after kainate lesions. Increase in 24-hydroxycholesterol might also explain the overall increase in ABCA1 expression in the kainate lesioned hippocampus. ABCA1 expression is known to be regulated by LXR (Liver X receptor) (Venkateswaran et al. 2000). Since their major ligand is oxysterols (Repa et al. 2007; Wojcicka et al. 2007), the increase of ABCA1 after kainate neurodegeneration might be due to increased oxysterols which in turn activate LXR that modulate ABCA1 expression (Cao et al. 2007). Anticipated increase in another LXR target gene, ABCG1 was not statistically significant, but differential regulation of target genes by different synthetic and endogenous LXR ligands has recently been reported (Trasino et al. 2009). In

![Fig. 4 Immunohistochemical analyses of the hippocampus. ABCA1](image-url)
particular, ABCG1 mRNA expression was regulated predominantly by activation of the LXR beta subtype rather than the alternative LXR alpha isofrom.

There is now increasing evidence that alterations in formation and metabolism of oxysterols, including COPs, may contribute to the pathophysiology of neurodegeneration including Alzheimer’s disease (Lütjohann et al. 2002; Hascalovici et al. 2009). Oxysterols such as 25-hydroxycholesterol, and 24-hydroxycholesterol stimulate secretory phospholipase A2 (sPLA2) IIa promoter and sPLA2 activity in an oligodendrocyte cell line (Trousson et al. 2009). Both sPLA2 and cytosolic phospholipase A2 (cPLA2) enzymatic activity are also increased after kainate injury (Sandhya et al. 1998; Thwin et al. 2000), and, along with cyclooxygenases, are associated with the release of arachidonic acid, generation of eicosanoids, and production of reactive oxygen species (Farooqui et al. 2001; Farooqui and Horrocks 2007). Deposition of accumulated cholesterol may promote further neurodegeneration by generation of neurotoxic COPs. Treatment of PC12 cells with the COP 7-ketocholesterol results in nuclear damage, decrease in the mitochondrial transmembrane potential, facilitation of release of cytochrome c, activation of caspase 3, increase in the formation of reactive oxygen species, and these effects were modulated by N-acetylcysteine, trolox, carboxy-PTIO and Mn-TBAP (Kim et al. 2006; Han et al. 2007). Finally, treatment of rat hippocampal slice cultures with 7-ketocholesterol and cholesterol alpha or beta epoxides results in decrease in neuronal markers and cell death, and these effects were modulated by treatment with the antioxidant, reduced glutathione (Ong et al. 2003). Collectively, these findings indicate that dysregulation of cholesterol metabolism in kainate-mediated neurotoxicity may promote neural cell death, possibly through the induction of cholesterol oxidation products.

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References


