Glutathione levels modulate domoic acid-induced apoptosis in mouse cerebellar granule cells

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Running title: Domoic acid causes oxidative stress-mediated apoptosis
Abstract

Exposure of mouse cerebellar granule neurons (CGNs) to domoic acid induced cell death, either by apoptosis or by necrosis, depending on its concentration. Necrotic damage predominated in response to domoic acid above 0.1 µM. In contrast, cell injury with apoptotic features (assessed by Hoechst staining and DNA laddering assay) was evident after exposure to lower concentrations of domoic acid (≤ 0.1 µM). The AMPA/kainate receptor antagonist NBQX, but not the NMDA receptor antagonist MK-801, prevented domoic acid-induced apoptosis. To evaluate the role of oxidative stress in domoic acid-induced apoptosis, experiments were carried out in CGNs isolated from wild-type mice [Gclm (+/+)], and mice lacking the modifier subunit of glutamate-cysteine ligase, the first and rate limiting step of glutathione (GSH) biosynthesis [Gclm (-/-)]. CGNs from Gclm (-/-) mice have very low levels of GSH, and were more sensitive to domoic acid-induced apoptosis and necrosis than Gclm (+/+). The antioxidant melatonin (200 µM), and the membrane permeant GSH delivery agent GSH ethylester (2.5 mM) prevented domoic acid-induced apoptosis. Domoic acid increased formation of reactive oxygen species, but did not affect intracellular GSH levels. Domoic acid also increased cytosolic and mitochondrial calcium levels, increased oxidative stress in mitochondria, and altered mitochondrial membrane potential, which ultimately caused cytochrome c release, activation of caspase-3 and degradation of poly (ADP-ribose) polymerase (PARP). These results indicate that low concentrations of domoic acid cause apoptotic neuronal cell death mediated by oxidative stress.
Key words: Apoptosis, Domoic acid, Glutamate-cysteine ligase, Glutamate receptors, Glutathione, Oxidative stress.
Introduction

Following a 1987 outbreak of toxicity in Eastern Canada, due to consumption of cultured mussels contaminated with the potent neuroexcitatory toxin domoic acid, increasing attention has been devoted to the features and mechanisms of domoic acid neurotoxicity (Jeffery et al., 2004). Domoic acid is a structural analog of kainic acid (KA), an excitatory amino acid that exerts its toxicity by activating the AMPA/KA subtype of glutamate receptors (Hampson and Manalo, 1998). Activation of AMPA/KA receptors by domoic acid (10 μM) causes an increase in [Ca^{2+}]_i, resulting in the release of L-glutamate, which in turn activates N-methyl-D-aspartate (NMDA) receptors and promotes neurotoxicity (Berman et al., 2002; Giordano et al., 2006). Activation of NMDA receptors causes apoptotic and necrotic neuronal cell death, depending on the intensity of the insult (Ankarcrona et al., 1995; Bonfoco et al., 1995).

In contrast to NMDA, less is known on the features of neuronal cell death mediated by AMPA/KA receptors. There is evidence indicating that KA induces apoptosis, both in cells in culture and after in vivo administration (Cheung et al., 1998; Giardina et al., 1998; Dje baili et al., 2001; Liu et al., 2001; Verdaguer et al., 2002). However, contrasting findings have been reported with regard to domoic acid. Ananth et al. (2001) concluded that neuronal degeneration observed during domoic acid-induced excitotoxic damage was mostly necrotic. Similarly, domoic acid neurotoxicity in mixed hippocampal cell cultures was found to be necrotic, and protection afforded by two virally-derived caspase inhibitors (p35 and crmA), was ascribed to their ability to sequester free radicals, upstream of the execution phase of oxidative stress-induced apoptosis, and to stabilize the mitochondrial membrane potential, respectively (Sah et al., 1999; Shimizu et al., 1996; Roy et al., 2003). However, Erin and Billingsley (2004)
reported activation of caspase-3 and apoptotic cell death in rat brain slices following exposure to domoic acid. Given the paucity of available information, the present study was designed to investigate features of domoic acid-induced cell death in cerebellar granule neurons (CGNs).

In a previous study (Giordano et al., 2006), we had shown that domoic acid neurotoxicity was mediated by oxidative stress. To investigate whether domoic acid may elicit oxidative stress-dependent apoptotic cell death, we carried out the present studies in CGNs derived from Gclm(-/-) mice. These mice lack the modifier subunit of glutamate cysteine ligase, which carries out the first and rate-limiting reaction in the synthesis of glutathione (GSH) (Yang et al., 2002). In the absence of GCLM, the ability of glutamate cysteine ligase catalytic subunit (GCLC) to synthesize GSH is drastically reduced (Yang et al., 2002). Indeed, GCNs from Gclm(-/-) mice have very low GSH levels, and are more sensitive than Gclm(+/+) neurons to the neurotoxic effects of domoic acid (Giordano et al., 2006).
Materials and Methods

Materials. DL-2-amino-5-phosphonovaleric acid, domoic acid, poly-D-lysine, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine-maleate (MK-801), staurosporine, carbonyl cyanide m-chlorophenylhydrazone (CCCP); melatonin, glutathione ethyl ester (GSHEE), horseradish peroxidase-conjugated anti-mouse, horseradish peroxidase-conjugated anti-rabbit IgG, mouse anti-poly (ADP-ribose) polymerase (PARP), dimethylsulfoxide (DMSO) and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse anti-ß-actin antibody was from ABCAM (Cambridge, MA, USA). Rabbit anti-cytochrome c antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA,USA). 2,3-Dihydroxy-6-nitro-sulfamoylbenzo [ f ] quinoxaline (NBQX) and ruthenium red were from Tocris Cookson (Ellisville, MO, USA). The reagents for enhanced chemiluminescence were from Amersham (Arlington Heights, IL, USA). The dNTPs, apoptotic DNA ladder kit, protease inhibitors and DNase I were from Roche (Indianapolis, IN, USA), while the Taq polymerase was from Qiagen, Inc. (Valencia, CA, USA). Neurobasal, Hibernate A and B27 MinusAO media, Hank’s balanced salt solution, GlutaMax, dispase, fetal bovine serum and gentamycin were from Invitrogen (Carlsbad, CA, USA). Naphthalene dicarboxaldheyde (NDA), 2,7’-dichlorofluorescin diacetate (DCF-DA), dihydrorhodamine (DHR), Rhod-2, tetramethylrhodamine ethyl ester (TMRE) and Hoechst DNA binding dye were from Molecular Probes (Eugene, OR, USA). The caspase -3 detection kit was from Oncogene (San Diego, CA, USA), while the caspase inhibitor I zVAD-fmk and monobromobimane (MBB) were from EMD.
Biosciences, Inc. (San Diego, CA, USA). Bicinchoninic acid assay was from Pierce (Rockford, IL, USA). Tissue culture plasticware was from Corning Costar (Cambridge, MA, USA).

**Generation of Gclm-null mice and genotyping.** All procedures for animal use were in accordance with the National Institute of Health Guide for the Use and Care of Laboratory Animals, and were approved by the University of Washington Animal Care and Use Committee. *Gclm*-null [Gclm (-/-)] mice were derived by homologous recombination techniques in mouse embryonic stem cells, as previously described in detail by Giordano et al. (2006). Pups were genotyped as described by Giordano et al. (2006).

**Cultures of cerebellar granule neurons and cell treatments.** Cultures of cerebellar granule neurons (CGN) were prepared from 7 day-old mice, as described by Giordano et al. (2006). Neurons were grown for 10-12 days before treatments. All compounds were dissolved in Locke’s solution, with the exception of zVAD-fmk and staurosporine, which were dissolved in DMSO. Unless indicated, in all experiments CGNs were incubated with domoic acid for 1h in Locke’s buffer, followed by washout and an additional 23 h incubation (Giardina et al., 1998; Berman et al., 2002). Antagonists were added 1 h before domoic acid.
**Cytotoxicity Assays.** Cell viability was quantified by a colorimetric method utilizing the metabolic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Giordano et al., 2006), and by the Trypan blue exclusion assay.

**Measurements of apoptosis.** To visualize nuclear morphology following domoic acid treatment, cells were fixed with methanol and stained in 10 μg/mL Hoechst DNA binding dye for 15 min. All cells exhibiting either classical apoptotic bodies, or shrunken, rounded nuclei were scored as being apoptotic. Five fields placed across the diameter of each well were assessed using a 20× objective on an inverted fluorescence microscope.

DNA fragmentation was detected with a commercial kit. Briefly, cells were washed twice with Locke’s buffer, and incubated for 10 minutes with an equal volume of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 6 M guanidine-HCl, 10 mM urea plus EDTA, and 0.2% Triton X-100. The samples were passed through glass fiber fleece by centrifugation and the nucleic acid bound to the glass fibers was eluted. The DNA was applied to a 1.5% agarose gel, and the bands were visualized by ethidium bromide staining.

**Assay of caspase-3 activation.** The activation of caspase-3 was examined using a caspase-3 inhibitor (DEVD-FMK) conjugated to FITC as the fluorescent *in situ* marker in living cells (Oncogene, San Diego, CA). FITC-DEVD-FMK is cell permeable, nontoxic, and irreversibly binds to activated caspase-3 in apoptotic cells. After exposure of neurons to domoic acid, cultures were washed twice with HBSS containing 0.1% BSA and then lysed with a buffer containing: 25 mM HEPES (pH 7.5), 5 mM EDTA, 1 mM EGTA, 5 mM MgCl₂, 0.5 mM DTT, and a mixture of protease inhibitors (Roche, Indianapolis, IN).
The cell lysates were placed on ice for 20 min, sonicated, and centrifugated for 60 min at 16,000 x g. The protein concentrations in the supernatant were determined using a protein assay kit. The FITC label in apoptotic cells was examined immediately using a fluorescence microplate reader (Molecular Devices Corp. Sunnyvale, CA) with excitation and emission set at 488 and 538 nm respectively.

*Measurements of GSH levels and GSH efflux.* Total intracellular GSH levels were measured spectrophotometrically, and GSH efflux from neurons was measured by HPLC, as described by Giordano et al. (2006).

*Measurement of L-glutamate release.* Exposure conditions in L-glutamate release studies were identical to those used in the cytotoxicity assay. Buffers from treated CGN were analyzed spectrophotometrically using the GLN-kit (Sigma), as described by Giordano et al. (2006).

*Assay of Reactive Oxygen Species formation.* ROS formation was determined by fluorescence using 2,7′-dichlorofluorescin diacetate (DCF-DA). Upon entering cells DCF-DA is de-esterified to DCFH, which is then oxidized by ROS to form the fluorescent 2,7′-dichlorofluorescein (DCF). In a typical experiment, cells were first washed with Locke’s solution, and then preincubated for 30 min (37°C) with DCF-DA (50 nmol/mg cell protein) in Locke’s solution. DCF-DA was added from a stock solution in DMSO; the quantity of DMSO never exceeded 0.1% and was also added to the blank. Cells were then washed with Locke’ solution to remove extracellular DCF-DA and
fluorescence was immediately read using a fluorescence microplate reader (excitation 488 nm, emission 538 nm).

**Fluorescence imaging of cytoplasmic free Ca$^{2+}$ in single cells.** CGNs were loaded with the Ca$^{2+}$-sensitive fluorescent dye fluo-3/AM (3 µM) at 37°C for 60 min in culture medium. Cells were then washed and incubated for an additional 30 min in a fluo-3/AM-free Locke’s buffer to remove extracellular traces of the dye and to complete intracellular de-esterification. The plates were placed on the stage of a fluorescence microscope. In some cases, a Ca$^{2+}$-free condition was achieved in Ca$^{2+}$-free Locke’s buffer containing 0.1 mM EGTA. The dye in the cytoplasmic portion of the cells was excited, and fluorescence images were captured at 20-s intervals by a MicroMax cooled CCD camera (Princeton Instruments, Trenton, NJ) using Metamorph software.

**Immunoblotting analysis.** Neurons were scraped in lysis buffer (Tris 50 mM pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 µg/ml leupeptin, and 2 µg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM NaF, 0.25% SDS). Whole homogenates were subjected to SDS-PAGE and immunoblotting using rabbit antibodies against cytochrome c protein (diluted at 1:1000), mouse anti-poly (ADP-ribose) polymerase (PARP) (1:500), rabbit anti COX-4 (1:2500), or mouse anti-β-actin antibody (1:2000). After electrophoresis, proteins were transferred to PDVF membranes and incubated with the above antibodies. Membranes were rinsed in TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG for
cytochrome c (1:1000) or with horseradish peroxidase-conjugated anti-mouse IgG for actin and PARP at the appropriate dilutions (1:5000 and 1:2000, respectively).

**Isolation of Nuclei, Mitochondria and Cytosol.** Mitochondria were isolated from mouse CGNs by centrifugation. The cells, washed with Locke’s buffer, were removed from the incubation Petri dishes in the mitochondria isotonic buffer containing 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin, 10 µg/mL aprotinin, 200 mM mannitol and 75 mM sucrose, and gently homogenized with a glass homogenizer. The homogenate was centrifuged at 750 x g for 10 minutes at 4°C to remove nuclei and unbroken cells, and the supernatant was centrifuged at 10,000 x g for 15 minutes. The supernatant was separated from the mitochondrial pellet and centrifuged again to obtain the cytosolic fraction. The mitochondrial pellet was lysed in 50 µL of 20 mM Tris (pH 7.4), 100 mM NaCl, 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin. Protein was determined in both mitochondrial and cytosolic fractions by the bicinchoninic acid assay. To detect poly (ADP-ribose) polymerase, nuclei were isolated from CGNs using the protocol described by Giordano et al., (2005).

**Measurement of mitochondrial Ca²⁺.** CGNs were loaded with 1µM of rhod-2 AM for 1 h at room temperature, followed by overnight incubation in normal culture medium (Limke et al., 2003). Since rhod-2 can fluoresce in both the cytosol and mitochondria, this protocol was used to ensure that mitochondrial loading occurred, and to provide time for the cytosolic dye to leak out of the cell. Multiple cells were imaged simultaneously. The
efficiency of mitochondrial loading was confirmed by using an 8-min exposure to 5µM CCCP, which uncouples oxidative phosphorylation, and 10 µM oligomycin, which dissipates $\psi_m$ (Kruman and Mattson, 1999). Thus, mitochondrial uptake of Ca$^{2+}$ during Ca$^{2+}$ influx was prevented using CCCP and oligomycin. Mitochondrial localization of rhod-2 was examined using granule cells loaded with rhod-2, and then loaded with MitoTracker Green. Images of cells loaded with MitoTracker Green and/or rhod-2 were obtained using excitation wavelengths of 488 and 535 nm, respectively, on a Leica MP laser scanning confocal microscope using a 40× objective.

**Assay of mitochondrial membrane potential.** The fluorescent dye JC-1 (Molecular Probes) was used as a measure of mitochondrial membrane potential (MMP), as described by Almeida et al. (1999). JC-1 is a membrane potential-sensitive probe that accumulates in energized mitochondria and subsequently forms J-aggregates from monomers. In brief, cells were incubated for 30 min in the presence of 10 µM of the dye, and then washed in Locke's solution. Fluorescence images were captured using a MicroMax cooled CCD camera (Princeton Instruments, Trenton, NJ) using Metamorph software and 535 and 595 emission wavelength (with excitation at 490 nm), corresponding to the fluorescence peak of the monomer and that of the aggregate, respectively. Some reports (Scanlon et al., 1998; Chinopoulos et al., 1999) indicate that the fluorescence response at 595 nm produced by oxidative stress-causing agent (such as H$_2$O$_2$) is largely unrelated to MMP and cannot be considered as a reflection of mitochondrial depolarization. However, changes in the fluorescence of the monomer (535 nm) in the presence of H$_2$O$_2$ do appear to be related to MMP (Chinopoulos et al., 1999).
Therefore, to study the effect of oxidative stress-mediated apoptosis caused by domoic acid on MMP, we were limited to considering only fluorescence emission monitored at 535 nm corresponding to J-monomer (Chinopoulos et al., 1999); thus, in the experiments presented only fluorescence at this wavelength was taken into consideration and analyzed. Determination of the MMP was also carried out using tetramethylrhodamine ethyl ester (TMRE) with similar results (data not shown). CGNs were loaded with 150 nM of TMRE for 30 min in Locke’s buffer and treated. After dye loading, the cells were rinsed and examined by microscopy as was done with JC-1, except that the TMRE fluorescence was measured at 590 nm.

Measurement of intramitochondrial ROS. Dihydrorhodamine 123 is the uncharged and nonfluorescent reduction product of the mitochondrion-selective dye rhodamine 123. This leuco dye passively diffuses across most cell membranes where it is oxidized to cationic rhodamine 123. Rhodamine 123 is fluorescent dye that is rapidly sequestered by active mitochondria showing to be a specific probe for mitochondria in living cells (Johnson et al., 1980).

Following experimental treatment, cells were loaded with 10 μM DHR for 30 min at 37°C in a 5% CO₂ incubator, and then washed with Locke's solution. Cellular fluorescence was quantified as described for cells loaded with 2,7'-dichlorofluorescin diacetate (DCF-DA), using excitation at 488 nm and emission at 538 nm.

Measurement of mitochondrial GSSG. Mitochondrial GSSG was measured by HPLC, using monobromobimane (MBB) as previously reported (Thompson et al., 2000) with
modifications as follows: briefly, mitochondria were isolated and centrifuged for 5 min at 300 x g. The supernatant was discarded and the cell pellet resuspended in 150 µl of Locke’s buffer. An aliquot of 50 µl was taken to measure the protein level while a second aliquot was diluted (1:1) with 10 % SSA to avoid oxidation of GSH and to induce mitochondria lysis. Two aliquots containing the same amount of protein were taken from each sample; one aliquot was reduced via addition of TCEP (10 µl, 10 mM) to determine total glutathione, while to the second aliquot a volume of 10 µl of water was added for 15 min at 4ºC to determine reduced glutathione. A volume of 20 µl of MBB solution (12.5 mM) was added for 30 min. GSSG was calculated by subtracting reduced glutathione (GSH) from total glutathione. To ensure that TCEP effectively reduced all of the GSSG in the sample to GSH, known amounts of GSSG were added to the extract and incubated in presence of TCEP. HPLC analysis indicated that other compounds present in the extract did not consume TCEP. The values for GSH and GSSG were calculated from the mean of triplicate runs for each sample. The coefficient of variation (CV) for measurements of GSH and GSSG were 4.5% and 15.1 %, respectively.

Statistical Analysis. Data are expressed as the mean ± SD of three independent experiments. Statistical analysis was performed by one way ANOVA followed by Bonferroni's Multiple Comparison Test.
Results

Domoic acid causes neurotoxicity in mouse CGNs

We had previously shown that domoic acid causes a concentration-dependent toxicity in mouse CGNs, as assessed by the MTT assay, and that neurons from Gclm (-/-) mice were more sensitive than neurons from Gclm(+/+) animals (Giordano et al., 2006). This finding was replicated in the present study (Fig. 1A), and confirmed by assessment of cell viability with the Trypan blue exclusion assay (Fig. 1B). To determine whether cell death could be ascribed to apoptosis, CGNs were treated with domoic acid (1h incubation, followed by 23 h washout) and apoptotic cell death was assessed by the chromatin dye Hoechst 33342 (Fig. 1C) and by measurement of DNA laddering (Fig. 1D). Results indicate that a low concentration of domoic acid (0.1 µM) was maximally effective in inducing apoptosis, while a concentration causing high toxicity (10 µM) induced very limited apoptosis. Based on these findings, a concentration of domoic acid of 0.1 µM was used to characterize apoptosis, while 10 µM domoic acid were utilized in some experiments to compare apoptotic and necrotic processes. Results of these experiments also show that apoptosis is more pronounced in CGNs from Gclm (-/-) mice (Fig. 1C,D), suggesting that oxidative stress is involved in both apoptosis and necrosis.

Pharmacological characterization of domoic acid-induced apoptosis

Domoic acid (10 µM) toxicity in CGNs had been shown to be antagonized by NBQX, an AMPA/KA receptor antagonist, as well as by MK-801, an antagonist of NMDA receptors (Giordano et al., 2006). This was attributed to the ability of domoic acid to
induce release of glutamate, which, in turn, activates NMDA receptors (Giordano et al., 2006). In contrast, domoic acid (0.1 µM)-induced apoptosis was antagonized by NBQX, but not by the NMDA antagonists MK-801 (5 µM Fig. 2A,C) or 2-amino-5-phosphonovalerate [100 µM, a concentration previously shown to block Ca²⁺ influx in primary neuronal cultures (Lee et al. 1998) (not shown)].

Role of oxidative stress in domoic acid-induced apoptosis

Domoic acid-induced apoptosis is more pronounced in CGNs from Gclm(-/-) mice, suggesting a role for oxidative stress. This was further confirmed by the following findings. First, treatment of cells with GSH ethyl ester, which significantly increases intracellular GSH levels (Giordano et al., 2006), prevented domoic acid-induced apoptosis (Fig. 2B,C). Second, the antioxidant melatonin (200 µM) rescued CGNs of both genotypes from domoic acid-induced apoptotic death (Fig. 2B,C). Third, domoic acid (0.1 µM) caused an increase of ROS production. The value of DCF fluorescence (expressed as % of control) after domoic acid (0.1µM) treatment in Gclm (+/+) neurons was 170.1±10.4, while in Gclm (-/-) neurons it was 252.3 ± 27.6 (n=3; for both p<0.05 vs control; p<0.05 Gclm (-/-) vs Gclm (+/+)).

We had previously shown that 10 µM domoic acid caused a time-dependent decrease of intracellular GSH, which was due to a net efflux of GSH from CGNs (Giordano et al., 2006). In contrast, at 0.1 µM, domoic acid did not alter intracellular GSH levels, nor did it cause any efflux of GSH (not shown).
Effect of domoic acid on cytosolic and mitochondrial calcium levels

Increases in intracellular calcium ([Ca^{2+}]_i) have been implicated in both apoptotic and necrotic cell death (Nicotera et al., 1992). Fig. 3A shows that 0.1 μM domoic acid caused a small and delayed increase in [Ca^{2+}]_i, with a full recovery by 20 minutes. This increase was abolished by removing extracellular calcium (not shown), indicating that it was due to calcium entry from outside the cell, and was antagonized by NBQX, but not by MK-801 (Fig. 3B). In contrast, the higher concentration of domoic acid (10 μM) caused a rapid and robust increase in [Ca^{2+}]_i, which was still elevated after 25 min (Fig. 3A). This effect was antagonized by both NBQX and MK-801 (Fig. 3B). Results shown in Fig. 3A and 3B are for CGNs from Gclm (+/+) mice, but findings were almost identical in neurons from Gclm (-/-) mice. This confirms that the differential susceptibility of the two genotypes to domoic acid toxicity is not due to difference in receptor expression or early events limited to receptor activation (Giordano et al., 2006).

We also measured changes in calcium levels of mitochondria ([Ca^{2+}]_M), by using the fluorescent probe rhod-2 (Kruman and Mattson, 1999). Fig. 3C shows that 0.1 μM domoic acid increases [Ca^{2+}]_M by about 3-fold, with a delay of about 15 minutes. This increase was identical in CGNs from Gclm (+/+) and Gclm (-/-) mice, and was antagonized by NBQX, but not by MK-801 (not shown). In contrast, no changes in [Ca^{2+}]_M were observed following a high concentration (10 μM) of domoic acid (Fig. 3C). CCCP, which uncouples oxidative phosphorylation, abolished rhod-2 fluorescence changes in response to the domoic acid treatments, confirming that rhod-2 was measuring changes in mitochondrial, not cytosolic fluorescence. When CGNs were co-
incubated with CCCP and domoic acid, the maximum increase, seen after 23 minutes, was 1.32 ± 0.18-fold over basal.

To assess whether the increase in \([\text{Ca}^{2+}]_M\) would be involved in apoptotic cell death, CGNs were treated with ruthenium red (RR), an inhibitor of mitochondrial calcium uptake (Kruman and Mattson, 1999). RR (10 µM) prevented the increase in \([\text{Ca}^{2+}]_M\); levels of rhod-2 fluorescence 15 min after domoic acid (0.1 µM) treatment were (arbitrary intensity units): control, 3.1 ± 0.3; domoic acid, 7.4 ± 0.6; RR + domoic acid, 3.9 ± 0.4 (n = 3).

As shown in Fig. 3D, RR blocked domoic acid-induced apoptosis in GCNs of both genotypes, while it had no effect on cytotoxicity induced by 10 µM of domoic acid (not shown).

It is important to note that RR, whilst being a potent inhibitor of the calcium uniporter of mitochondria (Carafoli, 1987), can also inhibit the \(\text{Ca}^{2+}\) efflux from ryanodine-sensitive \(\text{Ca}^{2+}\) stores (Berridge, 1993). This possibility, however, was ruled out by the observation that ryanodine, at the concentration of 20 µM which inhibits the efflux of \(\text{Ca}^{2+}\) from ryanodine receptors (Guidarelli et al., 1997), did not modify the extent of the apoptosis induced by domoic acid (data not shown).

**Domoic acid induces changes in mitochondrial membrane potential**

A rise in \([\text{Ca}^{2+}]_M\) may be expected to depolarize the mitochondrial membrane potential (MMP) through promotion of \(\text{Ca}^{2+}\) exchange, increased ATP consumption, or activation of \(\text{Ca}^{2+}\)-dependent mitochondrial dehydrogenases (Almeida et al., 1999). We therefore
measured MMP using the fluorescent probe JC-1 (White and Reynolds, 1996). CGNs showed very high mitochondrial activity along the neurite (Fig. 4A, left panel); treatment with domoic acid (0.1 μM for 1 hr) resulted in a markedly decreased MMP at 6hr, causing a shift in JC-1 fluorescence from orange to green (Fig. 4A, right panel). A quantitative analysis showed that 0.1 μM domoic acid caused a significant reduction of MMP (indicated by an increase in 535 nm intensity), and that this decrease was antagonized by NBQX, but not by MK-801 (Fig. 4B). Furthermore, RR and BAPTA-AM antagonized the decrease in MMP induced by domoic acid, indicating that increases in [Ca^{2+}]_{i} and [Ca^{2+}]_{M} are a requirement for the changes in MMP (Fig. 4B).

One consequence of Ca^{2+} accumulation in mitochondria is the activation of the mitochondrial membrane permeability transition pore (PTP) (Kruman and Mattson, 1999). The PTP is thought to be a large channel in the inner mitochondrial membrane, which is normally closed and can be opened by Ca^{2+} overload, and by other factors including oxidative stress (Naderi et al., 2006). In addition, studies in non-neuronal cells have indicated a linkage between sustained changes in MMP and formation of PTP (Takeyama et al., 2002). We found that cyclosporin A, an inhibitor of PTP (Ruan et al., 2004) prevented domoic acid-induced apoptosis (Fig. 3D), and inhibited the decrease in MMP caused by 0.1 μM domoic acid (Fig. 4B). This effect was not due to inhibition of calcineurin, another target of cyclosporin A (Kung et al., 2001), since FK506, a calcineurin inhibitor that does not block mitochondrial PTP (He et al., 2000), did not antagonize the effect on MMP induced by domoic acid (not shown).

Domoic acid induces oxidative stress in mitochondria
Opening of PTP uncouples mitochondria, thereby causing a drop in MMP and a net oxidation of the pyridine nucleotide redox state (Zago et al., 2000), and this correlates with increases in ROS production by isolated mitochondria (Naderi et al., 2006). We measured oxidative stress in mitochondria using the fluorescent probe dihydrorhodamine 123 (DHR), which accumulates in mitochondria and fluoresces when oxidized by ROS or RNS (Wei et al., 2000). Domoic acid (0.1 µM) caused a 3-fold increase in DHR fluorescence. This occurred between 1 and 2 hours, and was higher in CGNs from *Gclm* (-/-) mice (Fig. 5A). This increase in ROS was antagonized by RR, indicating that it is a consequence of an increase in [Ca²⁺]M, and by cyclosporin A, indicating a role for the mitochondrial PTP (Fig. 5B).

Changes in levels of oxidized glutathione (GSSG) have been shown to be a marker of oxidative stress in primary neurons (Ehrhart and Zeevalk, 2001). Fig. 6 shows that domoic acid (0.1 µM) caused an increase in mitochondrial GSSG levels; this was antagonized by NBQX (but not by MK-801), by the antioxidant GSHEE, and by RR.

**Domoic acid induces cytochrome c release, caspase-3 activation, and PARP degradation**

PTP opening uncouples mitochondria, thereby causing a drop in ΔΨ, a net oxidation in the pyridine nucleotide redox state and a loss of cytochrome c (Kowaltowski et al., 2000). Fig. 7 shows that domoic acid stimulates the release of cytochrome c, and that this effect is more pronounced in neurons from *Gclm* (-/-) mice. Released cytochrome c elicits a cascade of events ultimately leading to activation of caspase-3. Caspase-3 activation was measured using a caspase 3 inhibitor (DEVD-FMK) conjugated to FITC, which
irreversibly binds to activated caspase 3 in apoptotic cells. Fig. 8A,B show activation of caspase 3 in CGNs by 0.1 µM domoic acid. The effect was more pronounced in neurons from Gclm (-/-) mice, and was abrogated by an antagonist of AMPA/KA receptors, and by antioxidants (not shown). The broad-spectrum caspase inhibitor zVAD-fmk (100 µM) antagonized domoic acid (0.1 µM)-induced apoptotic cell death (Fig. 8C), while having no effect on cytotoxicity induced by 10 µM domoic acid (Fig. 8C), a concentration that caused no activation of caspase-3 (not shown). Poly ADP ribose polymerase (PARP) is a major substrate for activated caspase-3. During the induction of apoptosis, activated caspase-3 cleaves active PARP (116 kDa) into an inactive fragment of 89 kDa, and the formation of the inactive fragment is one of the hallmarks of caspase-mediated apoptosis. Fig. 9 shows that 0.1 µM domoic acid induces a time-dependent degradation of PARP, which is more pronounced in CGNs from Gclm (-/-) mice.
Discussion

The results of this study show that a low concentration of domoic acid (0.1 µM) caused apoptotic cell death of mouse CGNs. Domoic acid is an analog of KA, and is believed to exert its neurotoxicity by activating both AMPA/KA and NMDA receptors (Berman et al., 2002; Giordano et al., 2006). However, while both receptors are involved in necrotic cell death caused by higher concentrations (10 µM) of domoic acid (Giordano et al., 2006), domoic acid-induced apoptosis was solely due to activation of AMPA/KA receptors (Fig. 2). Indeed, at 10 µM, domoic acid caused the release of L-glutamate, which in turn activates NMDA receptors (Giordano et al., 2006), while low, apoptotic concentrations of domoic acid do not affect L-glutamate release. KA has been shown to induce neuronal apoptosis, both in cells in culture, and after in vivo administration (Cheung et al., 1998; Giardina et al., 1998; Djebaili et al., 2001; Liu et al., 2001; Verdaguer et al., 2002). In contrast, whether domoic acid may induce apoptosis is still controversial. While some in vitro and in vivo studies have found domoic acid neurotoxicity to be primarily necrotic (Roy et al., 2003; Ananth et al., 2001), others have reported apoptotic cell death following exposure to domoic acid (Erin and Billingsley, 2004).

In the present study we show that domoic acid causes both apoptotic or necrotic cell death of mouse CGNs, depending on its concentration. Necrotic damage predominates in response to higher concentrations (10 µM), while cell death with apoptotic features is more pronounced following exposure to a low concentration (0.1 µM) of domoic acid. Such findings are similar to what has been previously observed for the neurotransmitter glutamate (Ankarcrona et al., 1995), for NMDA (Bonfoco et al.,...
1995), or for the neurotoxicant trimethyltin (Gunasekar et al., 2001). Our findings also show that, as previously shown for necrosis (Giordano et al., 2006), domoic acid-induced apoptosis is mediated by oxidative stress. Indeed, apoptosis was inhibited by antioxidants, and was more pronounced in CGNs from Gclm (-/-) mice, which display very low levels of GSH (Giordano et al., 2006). Furthermore, 0.1 µM domoic acid caused an increase in the levels of ROS (Fig. 5).

Activation of AMPA/KA receptors by a low concentration of domoic acid caused a small increase in [Ca\textsuperscript{2+}]\textsubscript{i}, which returned to control values within 10-15 min. This increase in [Ca\textsuperscript{2+}]\textsubscript{i} was prevented by NBQX, but not by MK-801, and indicates that mouse CGNs possess AMPA/KA channels that are directly permeable to calcium, as observed in other neurons (Lu et al., 1996; Yin et al., 1999). The decrease in [Ca\textsuperscript{2+}]\textsubscript{i} was paralleled by an increase in mitochondrial calcium levels, indicating that sequestration of calcium in mitochondria is a primary initial event in domoic acid-induced apoptosis. In agreement with this finding, RR, an inhibitor of mitochondrial calcium uptake, blocked domoic acid-induced apoptosis. In contrast, a high concentration of domoic acid (10 µM) caused a robust and prolonged increase in [Ca\textsuperscript{2+}]\textsubscript{i}, but did not alter [Ca\textsuperscript{2+}]\textsubscript{M}, and domoic acid-induced necrosis was not antagonized by RR.

The increase in [Ca\textsuperscript{2+}]\textsubscript{M} caused a loss of membrane mitochondrial potential, an increase in mitochondrial oxidative stress (evidenced by increased mitochondrial ROS and GSSG levels), and the opening of the PTP. The latter can be inferred by the finding that cyclosporin A, a PTP inhibitor (Ruan et al., 2004), blocked domoic acid-induced apoptosis, as also shown for other compounds (Pastorino et al., 1996; Waring and Beaver, 1996). The release of cytochrome c from mitochondria induced by 0.1 µM
domoic acid, and the subsequent activation of caspase-3, are the probable consequence of PTP. The pore opening has indeed been shown to be an irreversible step in the cell death cascade that leads directly to marked mitochondrial depolarization and release of "apoptotic factors" that produce nuclear chromatin condensation and DNA fragmentation (Kluck et al., 1997; Kroemer et al., 1997).

In agreement with our findings, apoptosis caused by domoic acid in rat brain slices, had been found to involve activation of caspase-3 (Erin and Billingsley, 2004). Similarly, in hippocampal slices cultures, KA had been shown to induce a caspase-3-dependent apoptosis (Liu et al., 2001). In contrast, KA-induced apoptosis in rat CGNs has been reported not to be antagonized by caspase inhibitors (Verdaguer et al., 2002), and these authors suggested that KA may induce its neurotoxicity via a caspase-independent pathway. However, KA neurotoxicity including apoptosis, was examined at relatively high concentrations (500 µM), where necrotic cell death would predominate. Indeed, we show that apoptosis induced by a low concentration of domoic acid is antagonized by a caspase inhibitor, while neurotoxicity (mostly due to necrosis) induced by higher concentration of domoic acid, was not affected by caspase inhibition (Fig. 8). Furthermore, domoic acid (0.1 µM) induced PARP degradation, a hallmark of caspase-mediated apoptosis (Fig. 9).

In summary, our findings indicate that a relatively low concentration of domoic acid (0.1 µM) causes apoptotic cell death of mouse CGNs, while higher concentrations cause primarily necrosis, thus extending to domoic acid previous observations obtained with glutamate, NMDA and trimethyltin (Ankarcrona et al., 1995; Bonfoco et al., 1995; Gunasekar et al., 2001). An additional relevant finding of this study is that domoic acid -
induced apoptosis was more pronounced in CGNs from Gclm (-/-) mice, suggesting a primary role of GSH and oxidative stress in this process. Low GSH levels are found in individuals with polymorphisms in the GCLM gene (Nakamura et al., 2002), as well as in other individuals carrying other polymorphisms in glutamate cysteine ligase (Dalton et al., 2004). Such individuals would be expected to be particularly susceptible to neurotoxicity induced by low doses of domoic acid.
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References


Legend to Figures

**Figure 1.** Neurotoxicity of domoic acid (DomA) in mouse CGNs. A,B CGNs from Gclm (+/+) or Gclm (-/-) mice were exposed to different concentrations of domoic acid at 37 °C for 60 min. Cell survival was quantified at 24 h by assaying MTT reduction (A) or trypan blue exclusion (B). C. CGNs from Gclm (+/+) or Gclm (-/-) mice were exposed to different concentrations of domoic acid at 37 °C. At 24 hr neurons were stained with Hoechs 33342, and nuclei of apoptotic cells were counted and expressed as a percentage of the total number of nuclei. Results from A,B and C represent the mean (± SD) of at least three experiments. *Significantly different from untreated control (p < 0.001). 

a Significantly different from Gclm (+/+) (p < 0.001). D. Domoic acid (DomA)-induced apoptosis as assessed by DNA laddering. Treatment conditions were the same as in C, and figure is representative of three separate experiments.

**Figure 2.** Pharmacological characterization of domoic acid-induced apoptosis. A. An AMPA/kainate receptor antagonist (NBQX; 10 µM), but not an NMDA receptor antagonist (MK-801; 5µM) prevents domoic acid (0.1 µM)-induced apoptosis. *Significantly different from domoic acid-treated CGN (p < 0.001). aSignificantly different from Gclm (+/+) (p < 0.05) B. The antioxidants GSH ethyl ester (GSHEE; 2.5 mM) and melatonin (mel; 200 µM) prevent domoic acid (0.1 µM)-induced apoptosis. *Significantly different from domoic acid-treated CGN (p < 0.001). aSignificantly different from Gclm (+/+) (p < 0.05). Each bar represents mean (± SD) of three experiments done in triplicate. C. Effects of receptor antagonists and of antioxidants on
domoic acid (0.1 μM)-induced apoptosis, as assessed by DNA laddering. Staurosporine (0.5 μM) was used as a positive control. Figure is representative of three separate experiments.

**Figure 3.** Effect of domoic acid on calcium levels in cytosol and mitochondria of mouse CGNs. **A.** Effect of domoic acid (0.1 and 10 μM) on intracellular [Ca²⁺] levels, as measured by fluo-3 fluorescence. **B.** Effect of NBQX (10 μM) and MK-801 (5 μM) on intracellular [Ca²⁺] increase induced by 0.1 and 10 μM of domoic acid. **C.** Effect of domoic acid (0.1 and 10 μM) on intramitochondrial calcium levels, as measured by rhod-2 fluorescence. **D.** Effect of ruthenium red (RR; 10 μM) and cyclosporin A (CspA, 10 μM) on domoic acid (0.1 μM)-induced apoptosis. Results shown are for CGNs from Gclm (+/+ ) mice, unless indicated. Data in A, B and C are expressed as arbitrary units of fluorescence and represent the mean ± SD of three experiments. *Significantly different from control CGN (p < 0.01). **Significantly different from control CGN (p < 0.001). aSignificantly different from Gclm (+/+) (p < 0.05).

**Figure 4.** Effect of domoic acid on mitochondrial membrane potential as assessed by Fluorescence change of JC-1 at 535 nm. **A.** Microphotographs of control (left panel) and domoic acid (0.1 μM)-treated (right panel) CGNs from Gclm (+/+) mice. Neurons were stained using the fluorescent probe JC-1 to measure changes in mitochondrial membrane potential. **B.** NBQX (10 μM), but not MK-801 (5 μM), antagonizes the effect of domoic acid. Ruthenium red (RR, 10 μM), BAPTA-AM (5 μM) and cyclosporin A (CspA, 10 μM), antagonize the decrease of mitochondrial membrane potential induced by domoic
acid. Values are the means ± SD of determinations made in three cultures. *Significantly different from control CGN (p < 0.001).

**Figure 5.** Effect of domoic acid on mitochondrial ROS/RNS levels. A. CGNs from Gclm (+/+) and Gclm (-/-) mice were exposed to 0.1 µM domoic acid, and DHR fluorescence (a measure of mitochondrial ROS/RNS levels) was quantified at the indicated time points. Values are the means ± SD of three separate determinations. B. Ruthenium red (RR; 10 µM) and cyclosporin A (CspA; 10 µM) inhibit the increase in ROS induced by domoic acid (shown are results in Gclm (+/+) neurons). *p < 0.001 compared with domoic acid -treated CGNs.

**Figure 6.** Effect of domoic acid on intramitochondrial GSSG levels in CGNs from Gclm (+/+) and Gclm (-/-) mice. Domoic acid (0.1 µM) causes an increase in GSSG levels in mitochondria after 3 h of treatment, as assessed by HPLC. This increase is prevented by the AMPA/kainate receptor antagonist NBQX (10 µM), by GSH ethyl ester (GSHEE; 2.5 mM) and by Ruthenium red (RR, 10 µM), but not by the NMDA receptor antagonist MK-801 (5 µM). Each values represents the mean (± SD) of three experiments done in triplicate. *Significantly different from domoic acid -treated CGNs (p < 0.001).

**Figure 7.** Domoic acid (0.1 µM) causes cytochrome c release from mitochondria of Gclm (+/+) and Gclm (-/-) CGNs. Six hours after the beginning of the exposure, mitochondrial and cytosolic fractions were isolated and subjected to Western blot
analysis using COX-4 as a loading control. Blot shows a typical experiment that was repeated three times with similar results.

**Figure 8.** Effect of domoic acid on caspase 3 activity as assessed using a caspase-3 inhibitor (DEVD-FMK) conjugated to FITC. A. Time-course of caspase-3 activation by 0.1 µM domoic acid in CGNs from Gclm (+/+) and (-/-) mice (n=3; *p<0.05 vs Gclm (+/+). B. Fluorescence micrographs of domoic acid (0.1 µM; 12h)-induced caspase-3 activation in CGNs from Gclm (+/+) and Gclm (-/-) mice. C. The caspase inhibitor zVAD-fmk (100 µM) inhibits domoic acid -induced apoptosis (left panel), but not domoic acid -induced necrosis (right panel). Values are the means ± SD of three separate determinations. *p< 0.001 compared with domoic acid -treated CGNs. *Significantly different from Gclm (+/+ (p < 0.05).

**Figure 9**

Time-course of PARP fragmentation induced by domoic acid (0.1 µM) in CGNs from Gclm (+/+) and Gclm (-/-) mice. At the indicated time-points (9 and 12 h) nuclear fractions were isolated and subjected to Western blot analysis using β-actin as loading control. Arrows indicate intact (116 kDa) and cleaved (89-kDa) PARP. Blot shows a typical experiment that was repeated three times with similar results.
**Figure 1**

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Figure 2

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Figure 3

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Fig. 9

**Figure 9**
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