DISEASE IN WILDLIFE OR EXOTIC SPECIES

Oxidative Stress and Redistribution of Glutamine Synthetase in California Sea Lions (Zalophus californianus) with Domoic Acid Toxicosis

J. E. Madl*, C. G. Duncan†, J. E. Stanhill†, P.-Y. Tai*, T. R. Spraker† and F. M. Gulland‡

*Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, †Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO and ‡The Marine Mammal Center, 2000 Bunker Road, Sausalito, CA 94965, USA

Summary

The aim of this study was to test the hypothesis that oxidative stress and glutamine synthetase (GS) redistribution occur in domoic acid (DA) toxicosis in California sea lions (CSLs, Zalophus californianus). Sections of archived hippocampi from seven control and 13 CSLs diagnosed with DA toxicosis were labelled immunohistochemically for GS and for two markers of oxidative stress, malondialdehyde (MDA) and 3-nitrotyrosine (NT). The distribution and intensity of labelling were compared with the pathological changes seen in haematoxylin and eosin-stained sections. Increased expression of MDA and NT occurred in neurons of the hippocampal formation of CSLs with lesions consistent with DA toxicosis. The degree of oxidative stress was not affected significantly by the chronicity or severity of hippocampal damage. In six out of seven CSLs with chronic effects of DA toxicosis, in addition to the normal glial distribution of GS, GS expression was very strong in some neurons of the subiculum. However, neuronal GS labelling was also seen in one control CSL, an effect that may have been due to previous exposure to DA. GS expression in neurons was associated with decreases in GS labelling in neighbouring glial cell processes. DA toxicosis therefore induces increased expression of markers of oxidative stress in neurons consistent with oxidative stress contributing to the initial DA insult and also the epilepsy that often develops in chronic DA toxicosis. GS redistribution occurred primarily in chronic DA toxicosis, perhaps leading to alterations of the glutamine—glutamate—GABA (gamma-aminobutyric acid) cycle and contributing to the excitotoxicity and seizures often seen in DA toxicosis.

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Introduction

Domoic acid (DA) is a glutamate agonist that causes hippocampal damage in California sea lions (CSLs, Zalophus californianus) following ingestion of this phytoxin in their diet (Silvagni et al., 2005; Goldstein et al., 2008). Two clinical syndromes are recognized in sea lions exposed to DA. Initial, acute, damage occurs in specific regions of the hippocampal formation (Silvagni et al., 2005). Animals that survive the acute disease often develop a second syndrome resembling temporal lobe epilepsy in man (Goldstein et al., 2008). In these chronic cases the recurrent seizures can become progressive and cause additional excitotoxicity of their own. Therefore, in more chronic cases of DA toxicosis it may be difficult to separate the damage caused by the initial DA insult(s) from the damage due to the seizures.

DA toxicosis results from overstimulation of kainate and AMPA glutamate receptors (Giordano et al., 2007; Hogberg and Bal-Price, 2011). This leads to excessive excitotoxicity in vulnerable types of neurons with large numbers of these glutamate receptors. The influxes of Na⁺ and Ca²⁺ ions due

Correspondence to: J. E. Madl (e-mail: James.Madl@ColoState.edu).

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to the excessive stimulation of glutamate receptors have long been considered to be the main contributors to neuronal death (Olney, 1990). However, recent studies in laboratory animals and cell culture suggest that an additional process, oxidative stress, may also contribute substantially to excitotoxic neuronal death (Gluck et al., 2000; Giordano et al., 2007, 2008). Excitotoxicity caused by ischaemia, seizures and glutamate agonists may involve the formation of reactive oxygen species (ROS) and reactive nitrogen species, presumably due to oxidation of inappropriate substrates by mitochondria during excessive excitatory input (Waldbaum and Patel, 2010). Such excitotoxicity may be reduced or prevented by antioxidants (Golechha et al., 2011). Exposure of cultured cerebellar granule cells to DA has been shown to increase the formation of ROS and much of the DA-induced neuronal death can be prevented by antioxidants (Gluck et al., 2000; Giordano et al., 2007). Measures of oxidative stress include malondialdehyde (MDA), a product of lipid peroxidation that has been used frequently in the past several decades as a measure of oxidative stress in various diseases (Nielsen et al., 1997), and 3-nitrotyrosine (NT), a product of nitration of the tyrosines of proteins by reactive nitrogen species formed from nitric oxide (Darwish et al., 2007).

Decreased levels of glutamine synthetase (GS) may also play a role in the development of hippocampal damage and seizures induced by DA. Inhibition of GS causes seizures (Rowe and Meister, 1970; Wang et al., 2009) and GS levels are reduced in human temporal lobe epilepsy (Eid et al., 2004; van der Hel et al., 2005), a disease associated with hippocampal sclerosis and astroglisis similar to DA toxicity (Silvagni et al., 2005). GS has been reported to be one of the proteins in the central nervous system (CNS) most vulnerable to oxidation/nitration, which results in inhibition of GS activity (Gorg et al., 2006; Swamy et al., 2009) and increases in its degradation (Gorg et al., 2007). The two clinical syndromes identified in CSLs with DA toxicosis resemble the two stages of toxicosis induced by kainic acid in rats, in which the acute insult leads to initial increases of GS in glial cells, followed by a drop in GS after spontaneous seizures increase in frequency (Hammer et al., 2008).

The aim of this study was to determine whether oxidative stress and changes in glial GS levels occur in CSLs with DA toxicosis. The hypothesis was that acute DA toxicosis may induce damage through oxidative stress and ion fluxes, leading to progressive seizures and epilepsy with chronic hippocampal damage. Furthermore, we hypothesize that CSLs with DA toxicosis would have decreased GS levels that may contribute to seizure formation.

Materials and Methods

Case Selection

CSLs included in this study stranded along the central California coast, were brought to the Marine Mammal Center in Sausalito, California for treatment and then died or were humanely destroyed due to poor prognosis. Following post-mortem examination, formalin-fixed tissues were examined by two pathologists at Colorado State University’s Veterinary Diagnostic Laboratory. CSL cases from 2000 to 2010 were reviewed and designated as controls or DA toxicosis cases based on clinical history, microscopic lesions and DA levels in urine or faeces (Goldstein et al., 2008). CSLs with DA toxicosis were classified as having chronic disease if they were destroyed after more than 14 days in captivity or had severe hippocampal atrophy; otherwise they were considered to be acute cases (Goldstein et al., 2008). CSLs designated as controls died of unrelated causes and lacked clinical signs of neurological damage and histological lesions typical of DA toxicosis.

Immunohistochemistry

Serial sections (5 μm) from the hippocampal formation of each case were placed on positively charged slides for immunohistochemistry (IHC). Sections were labelled for MDA, NT and GS using an avidin–biotin method (Alyahya et al., 2007; Chen et al., 2008). Briefly, sections were dewaxed in xylene and graded ethanol before antigen retrieval by autoclaving for 15 min in a 0.1 M citrate buffer, pH 6.0. Sections were incubated in primary antibody (diluted 1 in 1,000 in phosphate buffered saline, pH 7.4) overnight. A Vectastain Elite™ kit for either rabbit or mouse antibodies (Vector Laboratories, Burlingame, California, USA) was then used to ‘visualize’ the binding of primary antibody with peroxidase, with 3,3'-diaminobenzidnate as chromogen. Slides were dehydrated through graded ethanol, cleared in xylene and coverslipped. Controls for specificity included omission of the primary antibody and in separate experiments substitution of normal rabbit serum or mouse isotype control IgG2a (Sigma–Aldrich, St Louis, Missouri, USA) for the primary antibodies. In all cases only very minimal background was observed in the control sections (data not shown). The mouse monoclonal antibody specific for GS was obtained from Chemicon International (Temecula, California, USA). The
rabbit anti-MDA was obtained from Alpha Diagnostic International (San Antonio, Texas, USA), while the rabbit anti-NT was obtained from EMD Millipore Corporation (Billerica, Massachusetts, USA).

For comparisons of immunohistochemical labelling density between animals, sections from the CSLs were labelled at the same time using the same batch of reagents and sections were handled in an identical manner. Digital images were captured using a Zeiss Axiosplan 2 microscope with Axiosvision 3.1 software (Carl Zeiss MicroImaging, Inc., Thornwood, New York, USA) using an oil immersion ×40 objective. For measurements of labelling density, images were analysed using ImageJ 1.34 (National Institutes of Health, Bethesda, Maryland, USA). After inversion, background densities of labelling of blank areas of the slide were subtracted from each image as previously described (Alyahya et al., 2007; Chen et al., 2008). The average densities in three of the most intensely labelled neuronal cell bodies in each region were quantified for MDA and NT expression. For GS labelling of neuropil, the density of bands of neuropil approximately 10 μm wide adjacent to three neuronal cell bodies was measured for each image. Measures of labelling intensity (grey scale) were compared within animals using repeated measures ANOVA with post-hoc testing, and between animals with t-tests and ANOVA (SPSS, IBM Inc, Chicago, Illinois, USA).

Results

Histopathological Findings

Microscopical lesions in the CSLs with DA toxicosis were consistent with previous reports describing acute (Silvagni et al., 2005) and chronic (Goldstein et al., 2008) lesions when present. Of the six cases classified as acute, histological lesions were only identified in three animals; however, only one hippocampal formation was examined from each animal. In acute cases changes were characterized by spongiosis, acute neuronal necrosis of the granule cells in the dentate gyrus and/or pyramidal cells in the cornu ammonis (CA) and variable gliosis. Consistent with previous reports (Silvagni et al., 2005) CA2 was less affected, with mild gliosis devoid of significant neuronal degeneration. Prominent cell swelling was observed in a single case and one animal had mild, lymphoplasmacytic meningitis. Changes extended into the subiculum, but tended to be less severe than those seen within the dentate gyrus or hippocampus.

Lesions in the CSLs with chronic DA toxicosis were more variable. Overall, there was significantly more parenchymal loss and collapse relative to animals with acute disease. Both the dentate gyrus and CA were affected, predominantly by neuronal loss, but in many cases active neuronal necrosis was also identified. Gliosis was prominent in most cases and resulted in an overall hypercellular appearance to the affected area. Lymphoplasmacytic meningitis was observed in four cases of chronic disease and one animal also had perivascular aggregates of leucocytes within the brain. No histological lesions were seen in the brains of the seven control CSLs.

Oxidative Stress

Labelling of NT appeared stronger in hippocampal neurons of animals diagnosed with DA toxicosis than in control CSLs (Fig. 1). Strong immunoreactivity appeared in some pyramidal cells of CA 1, 3 and 4, the subiculum and in dentate granule cells. Semic quantitative measures of neuronal labelling density were performed on the dentate gyrus, the subiculum and CA1. In animals with DA toxicosis, NT labelling densities were not significantly different between the three regions (P >0.05 by repeated measures ANOVA; data not shown), so the mean value of all measurements was used for subsequent analysis. Overall, NT labelling density was significantly different between cases of DA toxicosis and controls; more labelling was present in the neurons of CSLs with DA toxicosis than in control animals (Fig. 2A; P <0.05, ANOVA). When cases of DA toxicosis were subclassified as acute or chronic, there was no significant difference between the NT labelling densities of the animals with acute or chronic disease (Table 1; P =0.63, Tukey–Kramer LSD) or between the control CSLs and the animals with acute DA toxicosis (Table 1; P >0.05, Tukey–Kramer LSD), but there was a significant difference between controls and cases of chronic disease (Table 1; P >0.05, Tukey–Kramer LSD). To compare the degree of oxidative stress with the severity of damage, NT expression in granule cells of the dentate gyrus, reported to have the most severe damage (Silvagni et al., 2005), was compared with the subiculum with the least severe damage. No significant difference was seen between NT labelling density in neurons of the regions using a paired t test (P =0.28), suggesting that NT levels were not increased in areas with increased damage.

Subjectively and semiquantitatively, the pattern of MDA expression was similar to that of NT labelling. No differences were seen between labelling densities in the subiculum, CA1 and the dentate gyrus (P >0.05, repeated measures ANOVA). Overall, there was significantly more MDA labelling in the CSLs with acute and chronic DA toxicosis relative to controls.
(Figs. 2B and 3; \(P = 0.005\) by ANOVA, Tukey–Kramer LSD, \(P < 0.05\)). In contrast to NT, however, there was strong expression of MDA in the white matter of the brain, presumably due to the prevalence of lipids in the white matter (data not shown). No significant differences in MDA labelling density were found between the granule cells of the dentate gyrus and neurons of the subiculum (\(P = 0.67\); paired \(t\) test; data not shown), suggesting that the degree of MDA expression was not affected by the severity of damage.

### Glutamine Synthetase

In control CSLs and those with DA toxicosis, GS labelling was seen predominantly in cells with glial morphology in the hippocampal formation or other examined regions (Fig. 4A). However, in nine CSLs strong GS immunoreactivity was also seen in some cells with neuronal morphology (Figs. 4B and 5D). In the hippocampal formation, neurons with strong GS expression were most common in the subiculum, with no apparent labelling of granule cells of the dentate gyrus.

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**Fig. 1.** NT expression in the hippocampal formation. (A) Labelling for NT in the dentate gyrus of a case of chronic DA toxicosis. Arrows indicate labelled granule cells. (B) Labelling for NT in CA1 of the same case of chronic DA toxicosis. Arrows indicate labelled pyramidal cells. (C) Labelling for NT in the subiculum of the same case of chronic DA toxicosis. Arrows indicate labelled neurons. (D) Labelling for NT in the dentate gyrus of a control animal. (E) Labelling for NT in CA1 of the same control animal. (F) Labelling for NT in the subiculum of the same control animal. IHC. Bar, 25 μm.

**Fig. 2.** NT and MDA labelling densities in control CSLs and CSLs with DA toxicosis. (A) NT expression was increased in neurons of the hippocampal formation in DA toxicosis. Columns represent the mean labelling densities (grey scale) of three neurons from each of three hippocampal areas from each animal. (B) MDA labelling was also increased in neurons of the hippocampal formation in DA toxicosis. Columns represent the mean labelling densities (grey scale) of three neurons from each of three hippocampal areas from each animal. *\(P < 0.05\) compared with control by Tukey–Kramer LSD. **\(P < 0.01\) compared with control using Tukey–Kramer LSD. Bars indicate SEM.
dentate gyrus (Fig. 4C). In one CSL with chronic DA toxicosis, many neurons outside the hippocampal formation had strong GS immunoreactivity, including Purkinje cells of the cerebellum (Fig. 4D).

GS labelling of neurons was often seen in CSLs exposed to DA, especially in cases of chronic DA toxicosis (Figs. 5 and 6); five out of seven cases of chronic DA toxicosis had high levels of GS in neurons of the subiculum. One out of six cases of acute DA toxicosis also had high levels of GS in neurons of the subiculum, while one out of seven control CSLs had high levels of GS in neurons of the subiculum. GS labelling appeared to be decreased in glial cells in those areas where neurons were intensely labelled for GS. Expression of GS appeared reduced in the neuropil immediately surrounding labelled neurons of the subiculum (Figs. 5A, D). This visual impression was confirmed in measurements of the density of GS labelling in the neuropil adjacent to heavily-labelled neurons compared with unlabelled neurons in the same section (Fig. 6B; \( P < 0.01 \) for paired \( t \) test). In areas of the subiculum where no GS expression was apparent in neurons, there were no significant differences in GS labelling in the neuropil among control CSLs and those with acute or chronic DA toxicosis (\( P = 0.38 \), ANOVA).

### Discussion

For this study, classification of animals as controls or cases affected by DA toxicosis was based on clinical signs rather than known exposure. DA is a toxin that may be found commonly in the diet of CSLs, especially during periods of *Pseudo-nitzschia* spp. blooms. Testing for DA was performed on some clinical specimens obtained from these CSLs. When measurements of DA in clinical specimens (made by enzyme linked immunosorbent assay) were compared with the classification system described above, one of the two control CSLs that had DA testing performed on faeces had detectable levels of DA. All six of the

<table>
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<th>Animals</th>
<th>Number</th>
<th>NT expression</th>
<th>MDA expression</th>
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<tr>
<td>Control</td>
<td>6</td>
<td>20.7 ± 2.49</td>
<td>11.2 ± 2.59</td>
</tr>
<tr>
<td>CSLs with acute DA toxicosis</td>
<td>6</td>
<td>32.1 ± 2.93</td>
<td>31.5 ± 4.76*</td>
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<tr>
<td>CSLs with chronic DA toxicosis</td>
<td>7</td>
<td>37.1 ± 4.96*</td>
<td>35.0 ± 6.55*</td>
</tr>
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Values indicate the mean grey scale densities of three neurons from the three regions of each hippocampus ± SEM.

*Significantly different from control (\( P < 0.05 \) by Tukey–Kramer LSD).

**Table 1** Effects of acute and chronic DA toxicosis on NT and MDA expression in the hippocampus
cases of acute DA toxicosis had detectable levels of DA in their faeces or at least one body fluid (i.e. blood, milk, bile or aqueous humour), while none of the five cases of chronic toxicosis tested for DA had detectable levels of DA in any test performed. The apparently frequent exposure of CSLs to at least low levels of DA makes it difficult to identify control animals with no exposure or toxicosis cases with only acute exposure to DA.

Recent studies suggest that oxidative stress may occur during excitotoxicity induced by DA (Giordano et al., 2007) or similar glutamate agonists, including kainic acid (Gluck et al., 2000; Milatovic et al., 2002), and during epilepsy (Waldbaum and Patel, 2010), and that antioxidants decrease excitotoxic neuronal loss in vitro (Giordano et al., 2007) and in vivo (Golechha et al., 2011; Hsieh et al., 2011). In CSLs with DA toxicosis, immunohistochemical labelling for NT and MDA was increased in neurons of the hippocampal formation, suggesting that oxidative stress occurs in DA toxicosis. Overall, the results of the present study suggest that oxidative stress occurs in the initial damage of acute DA toxicosis and in the progressive epileptic syndrome that follows the initial insult.

The increases in NT expression may be especially important to affected CSLs. GS is inactivated and has increased rates of degradation due to NT formation (Gorg et al., 2007; Saitoh and Araki, 2010). The decrease in GS activity may then lead to increases in seizures, possibly due to the accumulation of extracellular glutamate from low levels of GS (Perez et al., 2012). Increases in nitric oxide may be due to increases in nitric oxide synthase (NOS), which is present in endothelial cells, glial cells and neurons. Reactive glial cells are one likely source of increased NOS. DA toxicity in CSLs is reported to cause gliosis in the hippocampus, which may lead to increased expression of the inducible glial form of NOS (iNOS or NOS2). Significant reductions in

Fig. 4. GS Expression. (A) CA1 of a control animal with DA levels in faeces below detectable limits. GS immunoreactivity was seen in the neuropil surrounding neurons and in small cell bodies with morphology consistent with glial cells. GS immunoreactivity was not apparent in the numerous cell bodies of pyramidal cells (arrows). (B) CA1 of a CSL with chronic DA toxicosis. Note the many pyramidal cells with large amounts of GS immunoreactivity (arrows). (C) Dentate gyrus of a case of chronic DA toxicosis. In the dentate gyrus, GS immunoreactivity was not apparent in granule cells (arrows) in either controls or cases of DA toxicosis. (D) Cerebellum of a CSL with chronic DA toxicosis. Note large amounts of GS immunoreactivity in three Purkinje cells (arrows) and in some Purkinje cell dendrites (arrowheads). IHC. Bar, 25 μm.

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GS, which may be related to increases in NT, were detected in the neuropil of CSLs with DA toxicosis.

GS is an essential enzyme in the glutamine–glutamate–GABA cycle of the CNS. GS is normally found in astrocytes and oligodendrocytes, where it catalyzes the formation of glutamine from glutamate. The glutamine is then released into the extracellular fluid where it is taken up by neurons and converted to glutamate by glutaminase. The glutamate in neurons may serve many purposes, including acting directly as an excitatory transmitter, serving as the precursor for the inhibitory transmitter GABA and serving as a precursor for the antioxidant glutathione. If GS is inhibited, synaptic transmission is quickly interrupted, especially in the retina (Bui et al., 2009). Loss of GS is associated with several diseases of animals in which

Fig. 5. GS labelling may be decreased in glial processes near neurons with high GS expression. (A) and (D) Neurons of the subiculum of cases of chronic DA toxicosis often show high levels of GS labelling (arrows). However, the neuropil surrounding the labelled neurons appears to have less GS immunoreactivity than in neighbouring regions of the subiculum of the same animals (B) and (E) or the subiculum of controls (C) and (F). (B) and (E) Regions of the subiculum near (A) and (D) where neurons (arrows) lack obvious GS immunoreactivity. Note the neuropil near the unlabelled neurons appears to contain more GS immunoreactivity than in (A) and (D). (C) and (F) Subiculum of control CSLs. Note the lack of immunoreactivity in neurons (arrows), but large amounts of immunoreactivity in putative glial processes of the surrounding neuropil. IHC. Bar, 25 μm.

Fig. 6. GS expression in the subiculum. (A) GS labelling was increased in some neurons of the subiculum. GS labelling was significantly increased (**P < 0.01 by paired t test) in some GS-positive neurons (GS+ neurons) compared with neighbouring neurons with normal expression (GS− neurons). Columns represent the mean labelling density of three neurons per section from eight CSLs. (B) GS expression was decreased in the neuropil near neurons that contained GS immunoreactivity. Columns represent the mean GS labelling densities of neuropil surrounding three GS+ neurons and three GS− neurons from each section. ***P < 0.001 by paired t test.
there is severe neuronal loss, such as in canine glaucoma (Chen et al., 2008) and equine recurrent uveitis (Hauck et al., 2007).

Inhibition of glial GS or decreased GS levels in glia may lead to increased seizure activity (Wang et al., 2009; Vohora et al., 2010). The mechanism by which this occurs is not entirely understood. In addition to increasing glutamate in glial cells, inhibition of GS is reported to cause a buildup of glutamate in the extracellular fluid (Perez et al., 2012), which would have an excitatory effect on nearby neurons and contribute to seizure formation. Increases in extracellular glutamate are presumed to be due, indirectly, to the increased levels of glutamate in glial cells (Chen et al., 2008).

One of the novel findings of this study is that DA toxicity in CSLs is associated with an altered distribution of GS within the hippocampal formation. GS accumulation was observed in pyramidal cells, but other neurons, such as granule cells, did not appear to accumulate GS. Improper expression of GS in neurons may lead to altered synaptic transmission and may contribute to damage. Expression of GS in adult neurons in vivo is very unusual. It has been reported in cultured neurons (Fernandes et al., 2010) and some neurons in damaged regions in Alzheimer’s disease (Robinson, 2000, 2001). The expression of GS in neurons in chronic DA toxicity may contribute to abnormal synaptic transmission in at least two ways. First, if GS is expressed in neurons, GS would catalyze a reduction in the amount of glutamate available for loading into vesicles and lead to decreased release of this major excitatory transmitter. Second, if less glutamate is available for GABA synthesis via glutamic acid decarboxylase, less GABA may be released at many inhibitory synapses. This may be important for seizure formation, since the loss of GABAergic input is one mechanism by which epilepsy is believed to occur (Avoli and de Curtis, 2011).

In the present study, GS expression was decreased in the neuropil in the subiculum of CSLs with DA toxicity near neurons that had high levels of GS. The GS labelling in the neuropil appeared to be contained primarily in glial processes. By reducing the amount of GS in glial cells, it is likely that seizure activity was potentiated (see above). The larger fraction of cases with chronic DA toxicity (six out of seven) with GS redistribution compared with cases of acute DA toxicity (one out of six) suggests that the loss of GS as the disease progresses may contribute to the progressive spontaneous seizures that characterize the chronic epileptic stage of the disease. However, it should be noted that two control CSLs also had GS redistribution. These control CSLs did not exhibit lesions in the single hippocampus examined for each animal, but one of the two had detectable levels of DA in its faeces, while the other did not have DA assays performed. The GS redistribution in these two CSLs without evidence of lesions suggests that GS may redistribute in very acute cases before clinical signs develop or with very low doses of DA that do not produce clinical signs.

Why do the neurons express GS in DA toxicosis? Recent studies of GS expression in cell culture suggest that GS expression may be induced in neurons by a lack of glutamine or GS in nearby glial cells (Fernandes et al., 2010). Our results are consistent with a similar mechanism occurring in DA toxicosis.

The results of this relatively small study suggest that oxidative stress and GS redistribution may occur in DA toxicosis in CSLs. This suggests that several mechanisms of pathogenesis may be occurring in DA toxicosis and that these were not investigated in previous studies. If oxidative stress and GS redistribution contribute substantially to the toxicity, it is likely that therapies based on inhibiting these mechanisms may prove to be effective in treating affected animals. If oxidative stress, especially from the increased formation of nitric oxide by NOS, is an important contributor to the pathogenesis, then antioxidants and NOS inhibitors that penetrate the blood–brain barrier might prove to be useful in combined therapy for the disease. Another promising type of therapy might be the use of non-steroidal anti-inflammatory drugs (NSAIDs) that may indirectly decrease oxidative stress by preventing NOS induction or activation of microglia. A recent study suggests that NSAIDs may prove effective in decreasing neuronal damage due to DA in mice (Ryan et al., 2011).

Another approach for therapy suggested by the redistribution of GS in chronic DA toxicity may be the prevention of improper GS expression in DA toxicosis. GS expression is known to be affected by many factors, including the amount of glutamine present in the extracellular fluid, glucocorticoids, tumour necrosis factor-α and other cytokines. If the distribution of GS is returned to a more normal pattern, CNS function may become more normal and fewer seizures might occur.

It should be noted that the sample sizes of the groups studied were relatively small and that the work should be repeated in a larger study. Perhaps more importantly, it was not possible to quantify the dose of DA or the times of exposure of the CSLs to DA. It is possible that the controls and acute cases of DA toxicosis had been exposed to DA at different times in their lives. Indeed, of the two control cases that had high levels of GS in neurons, one had detectable levels of DA in its faeces and the other did not.
had samples taken for DA analysis. We hope that the results of the current study will encourage future studies to address these issues.

Conflict of Interest Statement

The authors declare no conflict of interest.

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