GLUTATHIONE LEVELS MODULATE THE NEUROTOXICITY OF POLYBROMINATED DIPHENYL ETHER (PBDE) FLAME RETARDANTS IN MOUSE NEURONS AND ASTROCYTES

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Abstract
Polybrominated diphenyl ether (PBDE) flame retardants have become widespread environmental contaminants. Body burden in the U.S. population has been shown to be higher than in other countries. Exposure is believed to occur primarily through diet and dust. Infants and toddler have highest exposure through maternal breast milk and household dust. The primary concern for adverse health effects of PBDEs relates to their potential developmental neurotoxicity, which has been found in a number of animal studies. Information on the possible mechanisms of PBDE neurotoxicity is limited, though some studies have suggested that PBDEs may elicit oxidative stress. The present study examined the in vitro neurotoxicity of DE-71, a pentaBDE mixture, in primary neurons and astrocytes obtained from wild-type and *Gclm* knockout mice, which lack the modifier subunit of glutamate-cysteine ligase and, as a consequence, have very low levels of glutathione (GSH). These experiments show that neurotoxicity of DE-71 is modulated by cellular GSH levels. Cerebellar granule neurons and hippocampal neurons, as well as hippocampal astrocytes, from *Gclm* (-/-) mice displayed high sensitivity to DE-71 toxicity, which was antagonized by antioxidants. These findings suggest that the developmental neurotoxicity of PBDE may involve oxidative stress, and that individual with genetic polymorphism leading to lower GSH levels may be more susceptible to their adverse effects.

Introduction
Polybrominated diphenyl ethers (PBDEs), a group of flame retardants widely used in a variety of consumer products, have become ubiquitous environmental contaminants. They have been detected in soil, air, sediments, birds, marine species, fish, house dust and human tissues, blood and breast milk. Diet and house dust appear to be the major sources of PBDE exposure in the general population[1]. Levels of PBDEs in human tissues, particularly in breast milk, are particularly high in North America[2], resulting in high exposure of infants to PBDEs[3]. In addition, for toddlers, dust has been estimated to account for a large percentage of exposure[4]. PBDEs can also cross the placenta, and concentrations as high as 40 ng/g lipid and 98.5 ng/g lipid have been found in fetal blood and liver, respectively[5,6]. The current greatest concern for potential adverse effects of PBDEs relates to their developmental neurotoxicity[7,8]. Exposure of neonatal mice or rats to various PBDEs (e.g BDE-47, -99, -153, -209) has been shown to cause long-lasting changes in spontaneous activity, mostly characterized as hyperactivity (decreased habituation) and to disrupt performance in learning and memory tests[9-12]. Such behavioral alterations have also been seen in other studies with BDE-99[13-15], and with the pentaBDE mixture DE-71[16]. While a reduction of T4 by PBDEs[17,18] may contribute to their developmental neurotoxicity, direct effects on the developing brain have also been reported. Among these, PBDEs have been shown to affect signal transduction pathways (protein kinase C, calcium homeostasis, arachidonic acid release;[19-21]), and to cause oxidative stress[22-24]. The latter observations prompted us to investigate whether PBDEs would induce oxidative stress, and whether glutathione (GSH) would modulate their neurotoxicity.

Materials and Methods
Experiments were carried out in cerebellar granule neurons (CGNs) isolated from wild-type and *Gclm* knockout mice. *Gclm* (-/-) mice lack the modifier subunit of glutamate-cysteine ligase, the first and limiting step in the synthesis of GSH. As a result, CGNs from *Gclm* (-/-) mice have very low GSH levels (2.4 vs. 12.4 nmol/mg protein), and are more sensitive to the toxicity of agents causing oxidative stress, such as domoic acid[25]. Hippocampal neurons and hippocampal astrocytes, prepared from the same animals, were also utilized. DE-71, dissolved in DMSO, was utilized in these studies. The mixture is reported to contain primarily BDE-99 (44%), BDE-47 (32%), BDE-100 (9%) and BDE-153 (4%). These are the PBDE congeners most commonly found in

human tissues and milk. Cytotoxicity was assessed by the MTT assay, while apoptosis was assessed by Hoechst staining. Reactive oxygen species were measured by DCF fluorescence. GSH levels were measured by HPLC. All methods have been previously described[25].

Results and Discussion

The cytotoxicity of DE-71 was initially assessed by the MTT assay in CGNs from wild-type [*Gclm* (+/+)] and *Gclm* (-/-) mice. DE-71 was more toxic (by 8.5-fold) in CGNs from *Gclm* (-/-) mice (IC50 = 0.9 +/- 0.2 uM) than in CGNs from wild-type mice (IC50 = 7.7 +/- 2.3 uM). When CGNs from *Gclm* (+/+) mice were exposed to the GSH synthase inhibitor buthionine sulfoximine (BSO; 25 uM for 24 h), levels of GSH decreased from 12.5 to 3.7 nmol/mg protein, similar to the levels present in CGNs from *Gclm* (-/-) mice (2.4 nmol/mg protein). Under this condition, the toxicity of DE-71 was significantly increased (IC50 = 0.74 +/- 0.10 uM), and *Gclm* (+/+) CGNs were as sensitive as CGNs from *Gclm* (-/-) mice, not treated with BSO. Treatment of CGNs with the membrane permeable GSH delivery agent GSH ethylester (2.5 mM for 30 min), significantly increased intracellular GSH levels, and prevented the toxicity of DE-71 in CGNs of both genotypes. The antioxidant melatonin (200 uM) also significantly reduced DE-71 toxicity. DE-71 (5 uM) also caused a time-dependent increase in the levels of reactive oxygen species, which was more pronounced in CGNs from *Gclm* (-/-) mice. The nature of DE-71-induced cell death was also investigated. DE-71 (0.1-50 uM) caused a concentration-dependent apoptotic cell death, which was more pronounced in CGNs from *Gclm* (-/-) mice, particularly at low concentrations (e.g 1.0 uM). A comparison of the MTT and Hoechst staining experiments suggests that DE-71-induced cell death is mostly apoptotic in nature.

Hippocampal neurons from wild-type mice display lower GSH levels than CGNs (5.5 nmol/mg protein vs. 12.4 nmol/mg protein), and levels of GSH in hippocampal neurons from *Gclm* (-/-) mice were only 1.4 nmol/mg protein. Values of IC50 in the MTT assay for DE-71 in hippocampal neurons were 2.2 +/- 0.7 uM [*Gclm* (+/+) mice], and 0.31 +/- 0.15 uM [*Gclm* (-/-) mice]. Hippocampal neurons thus appear to be more sensitive than CGNs to the toxicity of DE-71, possibly because of their lower GSH content. The ratio between the IC50s in the two genotypes is 7.1, comparable to what observed in CGNs.

Toxicity of DE-71 was also assessed in hippocampal astrocytes, whose levels of GSH were 21.4 nmol/mg protein and 8.3 nmol/mg protein for *Gclm* (+/+) and *Gclm* (-/-) mice, respectively. Values of IC50 (MTT assay) for DE-71 in hippocampal astrocytes were 52.3 +/- 3.4 uM [*Gclm* (+/+)] and 28.3 +/- 3.4 uM [*Gclm* (-/-)]. This finding indicates that hippocampal astrocytes are approximately 20-fold less sensitive to DE-71 than hippocampal neurons. This, and the relatively small difference in sensitivity between the two genotype (ratio = 1.8), are in agreement with the respective GSH levels. As in CGNs, cell death in hippocampal astrocytes was primarily apoptotic in nature.

Altogether, these results provide evidence that GSH levels play a prominent role in modulating the neurotoxicity of DE-71 in vitro, and suggest that induction of oxidative stress may be involved in the toxicity of PBDEs. The *Gclm* (-/-) mouse represents a useful model to study the role of GSH in modulating toxicity, amenable to in vitro as well as in vivo studies. Of note is that a relatively common C588T polymorphism has been discovered in the 5'-flanking region of the human *GCLM* gene[26], and that individuals with the T allele have significantly lower plasma GSH levels. These individuals, as well as others with different mutations leading to decreased GSH levels[27], would be expected to display enhanced susceptibility to the adverse effects of environmental chemicals that elicit oxidative stress.

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