COMPARATIVE EFFECTS OF BROMINATED FLAME RETARDANTS AND POLYCHLORINATED BIPHENYLS ON RAT CEREBELLAR GRANULE CELLS

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Introduction

Organohalogen compounds, such as brominated flame retardants (BFR) and polychlorinated biphenyls (PCBs) have attracted considerable concern since they are persistent, widely distributed in the environment and potential harmful for human and wildlife. It is now established that several of the chlorinated environmental contaminants, such as the PCBs and pesticides, are neurotoxic, especially in occupationally exposed individuals. Even exposure to relatively low concentrations through food may adversely affect neurological development in children, such as cognitive and motor functions [1] In contrast to the extensive research on the neurotoxic effects of polychlorinated biphenyls (PCB), there is little knowledge about the toxicity of BFRs, especially as neurotoxicity is concerned. However, recent investigations have indicated that BFRs and PCBs share some similar neurotoxic effects, both in vivo and in vitro.[1]

Cerebellar granule cells are primary nerve cells, which is a useful model for the investigation of neurotoxic mechanisms.[2,3] It has been shown that BFRs and PCBs at relatively low concentrations induce cell death, and that some of the observed effects have been attributed to oxidative stress.[4,5,6] Excess production of reactive oxygen species (ROS) is involved in tissue injury in the brain, and has been implicated in degenerative processes like aging, amyotrophic lateral sclerosis (ALS), and Alzheimer's-, Parkinson's-, and Huntington's disease.[7,8,9]

The link between oxidative stress and cell damage is not obvious and the aim of this presentation is to compare the effects of the main BFRs and selected PCBs on cell death and formation of oxidative stress based on previous findings from our laboratory. Further, cerebellar granule cells are primarily glutamatergic and extensive release of glutamate may induce both oxidative stress and excitotoxic cell death. We therefore aim to elucidate the effects of these environmental contaminants on glutamate release in CGCs

Materials and Methods

Preparation of cerebellar granule cells

Primary cultured neurons from rat cerebellum were isolated as previously described.[4] The cerebella from 6- to 8-day-old pups were dissected under sterile conditions and the cells were grown for 6-8 days in basal medium Eagle's containing foetal bovine serum, penicillin/streptomycin, KCl and glutamine before exposure.

Assay for measuring reactive oxygen species and cell death

Cell death was measured by using the trypan blue exclusion assay, which is a marker for necrotic cell death. Formation of ROS was elucidated by the use of the fluorescent probe DCFH-DA.[4,10] DFCH-DA is permeable across cell membranes and inside the cell the acetate moieties are cleaved by cellular esterases. DCFH readily reacts with ROS such as peroxynitrite (ONOO-) and lipid peroxides to the fluorescent DCF. The cells, preincubated with DCFH-DA, where incubated with BFRs in Hepes buffered HBSS with glucose. The incubated cells were then transferred to 96 wells microtiter plate reader.

Assay for glutamate analysis

The cells were exposed to BFRs and PCB for 90 minutes and an aliquot of the growth media attributed to amino acid analysis. The level of amino acids was analyzed by HPLC and fluorescence detection after pre-column derivatization with o-phtaldialdehyd using a-aminoadipate as internal standard as previously described.[11] An equimolar mixture of the amino acids of interest was run as external standard.

Measurement of intracellular free calcium [Ca2+]i in cerebellar granule cells
[Ca2+]i was measured in a plate reader using the fluorescent Ca2+-sensitive probe fura-2/AM. The cells were cultured in chambers on coverslips (Reistad et al., 2005).[6] The cells, preincubated with fura-2/AM, where incubated with BFRs in standard saline solution. Excitations were obtained from filters at 340 nm and 380 nm and emission was at 510 nm. [Ca2+]i was estimated using the equation previously described.[12]

Results and Discussion
Several reports have used cerebellar granule cells as a model to investigate mechanisms of toxicity of environmental contaminants such as BFRs and PCBs. The cerebellum accounts for some 10% of the total weight of the human brain, but it may contain more than 50% of the total number of neurons. More than 90% of its neurons are granule cells, which primarily are glutamatergic and constitute the largest homogeneous neuronal population in the mammalian brain. Due to their postnatal generation and the feasibility of well-characterized primary in vitro cultures, CGCs is a model well suited for the study of toxic effects and cellular and molecular mechanisms.[2,3]

This work attempt to compare the effect of selected BFRs, the hexabromocyclododecan (HBCD), tetrabromobisphenol A (TBBPA) and the commercial pentaBDE mixture DE-71, the commercial PCB mixture A1254 on CGCs. All the chemicals induce cell death at relatively low concentrations, of which HBCD appears most toxic (table 1). CGCs are primarily glutamatergic and activation of glutamate receptors may induce excitotoxic events, such as oxidative stress and extensive calcium influx, which may be followed by cell damage. Vitamin E, as a scavenger of lipid peroxyl radicals, is probably the most important inhibitor of the free radical chain reaction of lipid peroxidation in animals.[9] Vitamin E has also membrane stabilizing properties.[13, 14] The addition of 50µM Vitamin E was protective against both BFR and PCB induced cell death indicating involvement of oxidative stress. The protective effect with TBBPA was somewhat less pronounced than with the more lipid-soluble toxic compounds.

Table 1. Effect of BFRs and A1254 on viability of cerebellar granule cells and the relative (+ indicate small, but significant effect whereas +++ indicate large effect) effects of the potential protective chemicals, the antioxidant vitamin E and the glutamate receptor antagonist MK801.

	TBBPA	HBCD	DE-71	A1254
Cell death (EC50)	7µM	3µM	7µM	10µM
+ Vitamin E	++	+++	+++	+++
+ MK801	+++	+	++	+++

The involvement of ROS formation in the PCB and BFR induced toxicity, were further assayed using the fluorescence probe DCFH-DA. This is a sensitive and rapid method, which is well suited for detecting overall oxidative stress.[10] Only TBBPA and A1254 induced oxidative stress as measured with DCF, and TBBPA induced ROS much more potently than A1254 (table 2). DCF primarily measure ROS formation in the cytosolic compartment of the cell. Vitamin E is expected to be protective in the membrane compartment and did not significantly inhibit TBBPA induced ROS formation, but did inhibit A1254 induced ROS formation. These findings indicate that both TBBPA and A1254 induce oxidative stress, but in different cellular compartments.

Oxidative stress in CGCs may be induced as a consequence of glutamate receptor activation, primarily via NMDA receptors. MK801, a NDMA receptor antagonist, was protective against both BFR and A1254 induced cell death, albeit with different sensitivity (Table 1). It appears that MK801 is especially effective against A1254 and TBBPA induced cell death, whereas the least protection was towards HBCD. MK801 also inhibited A1254 induced ROS formation indicating that this PCB mixture induce cell death and ROS formation through activation of glutamate receptors (Table 2). Since the protective effects of MK801 could not be linked to TBBPA induced ROS formation, these findings indicate that the oxidative stress induced by TBBPA is not the prime mechanism of cellular damage, but probably a secondary effect.

Table 2. Effect of BFRs and the PCB mixture A1254 on ROS formation in cerebellar granule cells and the relative (- indicates no apparent effect, (+) indicates an effect, but non-significantly) effects of the potential protective compounds, the anti-oxidant vitamin E and the glutamate receptor MK801

	TBBPA	HBCD	DE-71	A1254
ROS	+++	-	-	+
+ Vitamin E	(+)	-	-	++
+ MK801	(+)	-	-	++

To further elucidate the importance of glutamate in the effects on CGCs, we investigated if the selected chemicals could induce elevated extracellular concentrations of glutamate, implying an increase in glutamate receptor activation. Previously all the test compounds are shown to inhibit glutamate uptake in synaptosomes, of which TBBPA is shown as the most powerful.[15, 16] All the chemicals induced an increase in extracelluar glutamate, and TBBPA was the most efficient. Interestingly HBCD also induced a considerable elevation in glutamate concentration. This effect is not obvious since HBCD do not induce ROS formation, MK801 was less protective against cell death and we have not been able to show that HBCD disrupt intracellular calcium homeostasis.[6] HBCD is shown, however, to affect glutamate transport in synaptosomal preparations at low concentrations.[16] Both PCB and TBBPA induce influx of calcium in CGCs, and there is some evidence in literature for similar effects by pentaBDEs.[17, 18]

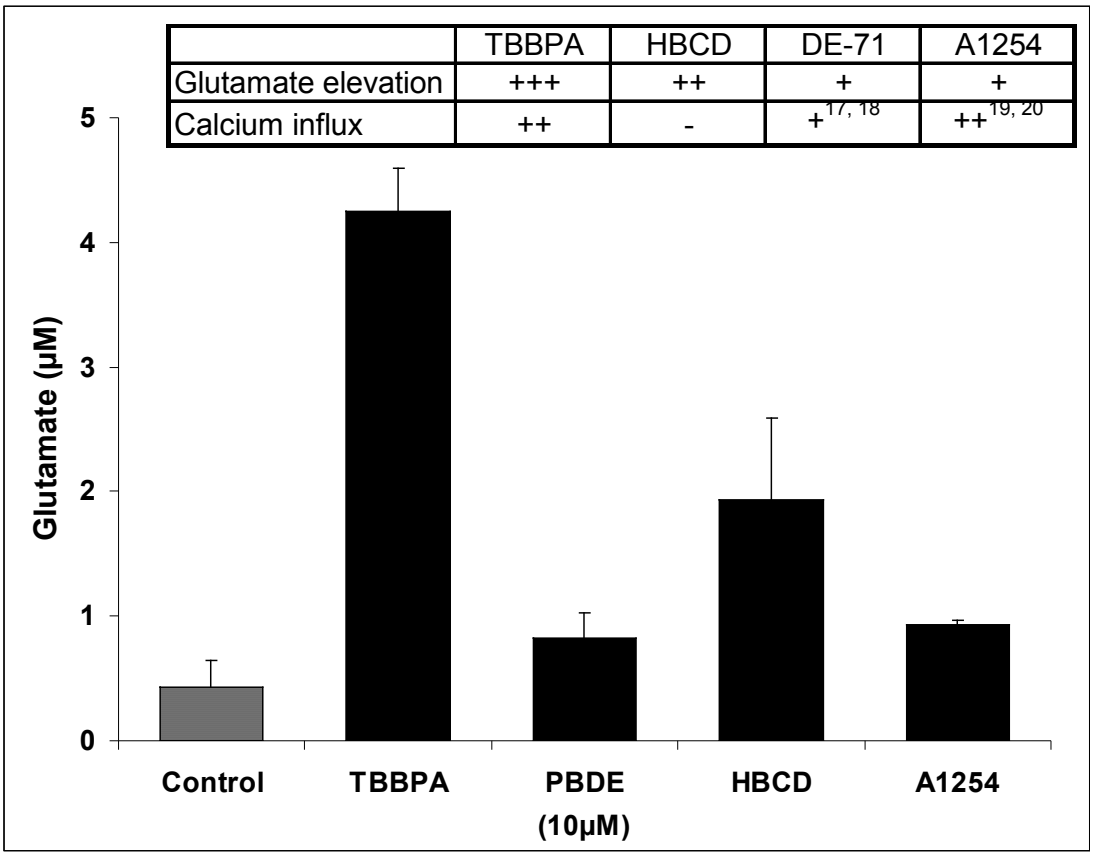

Fig 1. The figure shows elevation of glutamate concentration in the extracellular environment of CGCs exposed to 10µM BFRs and A1254 in 90 minutes. The table indicates the relative effects between the chemicals on glutamate elevation and their suggested relative potential for disrupting cellular calcium homeostasis.

It appears that both TBBPA and PCB have an effect on glutamatergic events and calcium homeostatic processes. The effect of DE-71 and especially HBCD are less defined. This has been confirmed in recent works where the effects of HBCD and DE-71 on CGCs were evaluated.[6] Both compounds induce necrotic cell death, but the mechanisms of effects are unknown. It is however important to continue to elucidate more closely the effects of HBCD on brain function since it evidently reach the brain following exposure.[6] Recent investigations have also revealed that it may affect neurobehavior in vivo.[21] PCBs on the other side induce several effects, which may be linked to cellular signalling pathways such as, PKC, PLA2 and the mitochondrial transition pore.[4, 20] All are pathways that can be connected to glutamate receptor activation and disruption of the calcium homeostasis. Recent investigation has also revealed a possible influence on glutamatergic events in connection to the effect of PCBs on long-term potentiation (LTP).[22] LTP is a model for the development of learning and memory.

As shown all the components investigated induce cell death of CGCs at relatively low and similar concentrations, but clearly with different mechanisms of action. Previous studies on PCBs have shown a connection between cell death and formation of reactive oxygen species. This is, in our studies, apparently not evident for TBBPA, HBCD and DE-71 even though TBBPA potently induces oxidative stress. Precautions have to be taken about the sensitivity of the method used to evaluate oxidative stress, but bearing in mind that vitamin E in general is protective it is reason to believe that the effects are localized to membrane compartments and that the cellular localisation and types of ROS formation is of high importance when anticipating damage made by oxidative stress.

References

1. Mariussen E, Fonnum F. Crit. Rev. Toxicol. 2006;36:253
2. Drejer J, Larsson OM, Schousboe A. Neurochem. Res. 1983;8:231
3. Kingsbury AE, Gallo V, Woodhams PL, Balazs R. Brain Res. 1985;349:17-25
4. Mariussen E, Myhre O, Reistad T, Fonnum F. Toxicol. Appl. Pharmacol. 2002;79:137
5. Reistad T, Fonnum F, Ring A, Mariussen E. *Organohal. Comp.2003* **65**:5
6. Reistad T, Fonnum F, Mariussen E. Arch. Toxicol. 2006 In press
7. Hastings TG, Lewis DA, Zigmond MJ. *Adv. Exp. Med. Biol. 1996;***387**:97.
8. Fonnum F. Excitotoxicity in the brain. *Arch. Toxicol. 1998; Suppl* **20**:387.
9. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford university press Inc, New York, 1999
10. Myhre O, Andersen JM, Aarnes H, Fonnum F. Biochem Pharmacol. 2003;65:1575.
11. Hassel B, Bachelard H, Jones P, Fonnum F, Sonnewald U. J Cereb Blood Flow Metab.1997;17:1230
12. Grynkiewicz G, Poenie M, Tsien RY. J. Biol. Chem.1985;260, 3440
13. Erin AN, Gorbunov NV, Brusovanik VI, Tyurin VA, Prilipko LL. Brain Res. 1986;398:85.
14. Urano S, Inomori Y, Sugawara T, Kato Y, Kitahara M, Hasegawa, Y, Matsuo M, Mukai K J Biol. Chem . 1992;267:18365
15. Mariussen E, Fonnum F. Toxicol. 2001;159:11.
16. Mariussen E, Fonnum F. Neurochem. Int. 2003;43:533
17. Kodavanti PR, Ward TR. Toxicol. Sci. 2005;85:952.
18. Reistad T, Mariussen E. Toxicol. Sci. 2005;87: 57
19. Kodavanti PRS, Shin DS, Tilson HA, Harry GJ. Toxicol. Appl. Pharmacol.1993;123:97
20. Mundy WR, Shafer TJ, Tilson HA., Kodavanti PRS . Toxicology 1999;136:27
21. Eriksson P, Fischer C, Wallin M, Jakobsson E, Fredriksson A. Environ. Toxicol. Pharmacol. 2006;21:317-322
22. Kodavanti PRS, Derr-Yellin EC, Mundy WR, Shafer TJ, Herr DW, Barone S, Choksi NY, MacPhail RC, Tilson HA. Toxicol. Appl. Pharmacol. 1998;153:186
23. Gilbert ME, Lasley SM. Environ. Toxicol. Pharm. 2002;12:105