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Role of glutamate receptors in tetrabrominated diphenyl ether (BDE-47) neurotoxicity in mouse cerebellar granule neurons

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Abstract

The polybrominated diphenyl ether (PBDE) flame retardants are developmental neurotoxicants, as evidenced by numerous *in vitro*, animal and human studies. PBDEs can alter the homeostasis of thyroid hormone and directly interact with brain cells. Induction of oxidative stress, leading to DNA damage and apoptotic cell death is a prominent mechanism of PBDE neurotoxicity, though other mechanisms have also been suggested. In the present study we investigated the potential role played by glutamate receptors in the *in vitro* neurotoxicity of the tetrabromodiphenyl ether BDE-47, one of the most abundant PBDE congeners. Toxicity of BDE-47 in mouse cerebellar neurons was diminished by antagonists of glutamate ionotropic receptors, but not by antagonists of glutamate metabotropic receptors. Antagonists of NMDA and AMPA/Kainate receptors also inhibited BDE-47-induced oxidative stress and increases in intracellular calcium. The calcium chelator BAPTA-AM also inhibited BDE-47 cytotoxicity and oxidative stress. BDE-47 caused a rapid increase of extracellular glutamate levels, which was not antagonized by any of the compounds tested. The results suggest that BDE-47, by still unknown mechanisms, increases extracellular glutamate which in turn activates ionotropic glutamate receptors leading to increased calcium levels, oxidative stress, and ultimately cell death.

Keywords

Flame retardants; BDE-47; Developmental neurotoxicity; Glutamate receptors; NMDA; AMPA/Kainate

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been extensively used in recent years as flame retardants in a variety of consumer products. Since they are not chemically bound to the polymer product, they easily leach out into the environment. Levels of PBDEs in biota and in human tissues have significantly increased in the past three decades, and levels in North America are particularly high (USEPA, 2010; EFSA, 2011). There are 209 possible congeners of PBDEs; the most utilized penta- and octa-BDE mixtures have been banned in

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the E.U. and in several states in the U.S.A. Five congeners (BDE-47, -99, -100, -153, -154) predominate in human tissues, usually accounting for 90% of the total body burden, with BDE-47 (2, 2', 4, 4'-tetrabrominated diphenyl ether) alone accounting for about 50% (USEPA, 2010).

Body burden of PBDEs is much higher (by 3–9-fold) in infants (because of exposure through breast milk), and in toddlers (because of exposure through house dust and the diet), raising concerns for their potential developmental toxicity and neurotoxicity (Costa and Giordano, 2007; Williams and DeSesso, 2010; Kodavanti et al., 2011; Dingemans et al., 2011). A plethora of animal studies indicate that prenatal and/or postnatal exposure to different PBDEs causes long-lasting behavioral abnormalities, particularly in the domains of motor activity and cognition (Branchi et al., 2002; Dufault et al., 2005; Viberg et al., 2003; Cheng et al., 2009; Kodavanti et al., 2010; Ta et al., 2011). Animal evidence is supported by human studies which are suggestive of neurodevelopmental effects of PBDEs, including poorer attention, decreased motor coordination and cognition, altered adaptive behavior, and increased activity/impulsivity behaviors (Herbstman et al., 2010; Chao et al., 2011; Shy et al., 2011; Eskenazi et al., 2013).

The mechanisms of PBDEs' developmental neurotoxicity have not been fully defined. However, two general modes of action have emerged, one indirect, mediated by interference of PBDEs with thyroid hormones, and the other involving direct effects of PBDEs on the developing brain (Costa and Giordano, 2007; Alm and Scholz, 2010; Costa et al., 2014). There is evidence that PBDEs, possibly through their hydroxylated metabolites, may alter thyroid hormone homeostasis, and this would undoubtedly contribute to adverse neurodevelopmental effects; however, there is also evidence of developmental neurotoxicity of PBDEs in the absence of alterations in thyroid hormones (Gee et al., 2008; Kim et al., 2012; Costa et al., 2015). Evidence abounds indicating that PBDEs directly affect brain cells. PBDEs induce oxidative stress in various brain cells system *in vitro* (He et al., 2008; Costa et al., 2010a; Tagliaferri et al., 2010; Giordano et al., 2008), as well as *in vivo* (Cheng et al., 2009; Costa et al., 2015). Oxidative stress may result from the ability of PBDEs to disrupt mitochondria, though the exact molecular mechanisms need further scrutiny. The downstream effects of PBDE-induced oxidative stress are DNA damage (He et al., 2008; Gao et al., 2009; Pellacani et al., 2012) and apoptotic cell death (Giordano et al., 2008; He et al., 2008; Tagliaferri et al., 2010; Costa et al., 2015). In addition, a variety of other potential mechanisms have also been investigated in *in vitro* and *in vivo* studies across different PBDE congeners. Some relate to effects on signal transduction, others to receptor and neurotransmission modulation, mostly related to effects on the cholinergic, dopaminergic, GABAergic, and glutamatergic systems (Costa et al., 2014).

The present study was prompted by the findings of Reistad et al. (2006, 2007) who showed that *in vitro* cytotoxicity of DE-71 (a pentaBDE mixture) and that of tetrabromobisphenol-A (TBBPA; a brominated flame retardant) in rat cerebellar neurons was reduced by MK-801, an antagonist of the *N*-methyl-D-aspartate (NMDA) receptor. Findings with DE-71 were later confirmed by Yu et al. (2008) in human neuroblastoma cells. The aim of the present study was to further explore the role of glutamate receptors in modulating the neurotoxicity of BDE-47 *in vitro* and to explore potential underlying mechanisms.

2. Materials and methods

2.1. Materials

BDE-47 (99.9%) was purchased from AccuStandard Inc. (New Haven, CT). The glutamate receptor antagonists 2,3-dihydroxy-6-nitro-sulfamoylbenzo [f] quinoxaline (NBQX), (RS)-1-aminoin-dan-1,5-dicarboxylic acid (AIDA), (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), and (RS)- α -methylserine-*O*-phosphate (MSOP) were from Tocris Cookson (Ellisville, MO), while (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-iminemaleate (MK-801) was from Sigma–Aldrich (St. Louis, MO). Neurobasal-A medium, gentamycin, fetal bovine serum and 5-(and-6)-carboxy-2'-7'-dichloro-fluorescein diacetate were from Invitrogen (Carlsbad, CA). The protein bicinchoninic acid assay was from Thermo Fischer Scientific (Rockford, IL). Anhydrous dimethylsulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and all other chemicals were from Sigma–Aldrich, while the Thiobarbituric Acid Reactive Substances (TBARS) assay kit was purchased from Cayman Chemical Company, Ann Arbor, MI.

2.2. Cell culture

Cultures of cerebellar granule neurons (CGNs) were prepared from 7-day-old C57Bl/6J mice killed by decapitation after CO₂ narcosis, as described in detail by Giordano et al. (2006, 2008). After removal of meninges from the whole brain, cerebella were rapidly dissected and cut into small cubes. The matrix was digested at 37 °C for 30 min in 1.5 mg/ml papain solution. After addition of HBBS buffer, tissue was sedimented by centrifugation, the supernatant carefully removed, and the cerebellar tissue was re-suspended in HBBS buffer. The tissue was mechanically dissociated using a long-stem Pasteur pipette and the cell suspension was centrifuged at $900 \times g$ for 5 min. The cell pellet was re-suspended in complete growth medium consisting of Neurobasal A medium containing 1.5 mM GlutaMAX, 250 mg/ml fungizone, gentamycin, KCl 26 mM, and B27, a medium supplement with a newly improved formulation that substitutes serum. Neurons were seeded in poly- D-lysine-coated 48-well plates at the density of $5 \times 10^4/\text{cm}^2$, and cultured for 10 days before treatments. Four days after seeding, neurons were treated with cytosine arabinofuranoside (AraC; 3 μM) in complete Neurobasal A medium, containing B27 minus AO (antioxidants) to prevent glial proliferation and obtain neuron-enriched cultures. Four days after AraC treatment, 50% of medium was replaced with fresh complete medium.

2.3. Cell treatments

BDE-47 was dissolved in DMSO to obtain stock solution of 12.5 mM, which was diluted appropriately at the time of use in medium-B27 minus AO. The ionotropic or metabotropic glutamate receptor antagonists and BAPTA-AM were added 30 min before BDE-47.

2.4. Cytotoxicity assay

Cell viability was quantified using the metabolic dye MTT, as previously described (Giordano et al., 2006, 2008). Briefly, cells were treated with BDE-47 for 24 h at 37 °C. At

the end of exposure, the medium was removed and cells were incubated with 500 µl/well of Locke's buffer solution containing 2 mg/ml MTT for 30 minutes. MTT was then removed and the reaction product was dissolved in 0.25 ml DMSO/well. Absorbance was read at 570 nm, and the results expressed as the percentage of viable cells relative to the DMSO exposed controls. Untreated controls and blanks were incubated in the same plates and under the same conditions.

2.5. Measurement of glutamate release

Extracellular glutamate was measured essentially as described by Giordano et al. (2006). CGN were exposed to BDE-47 for various times (5–60 min), after which supernatants were collected, and cells were scraped to determine protein content. Determination of L-glutamate was carried out using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay kit (Molecular Probes, Invitrogen). The kit is designed for detecting glutamic acid by virtue of its oxidation by glutamate oxidase to produce α-ketoglutarate, NH₃ and H₂O₂. The latter reacts with Amplex Red reagent in a 1:1 stoichiometric reaction catalyzed by horseradish peroxidase to generate the fluorescent product, resorufin. Fluorescence was measured at 560 and 590 nm for excitation and emission, respectively. A standard curve with glutamate indicated linearity between 20 nM and 10 µM.

2.6. Measurement of reactive oxygen species (ROS)

ROS formation was determined by fluorescence using 2',7'-dichlorofluorescein diacetate (DCFH₂-DA), as previously described (Giordano et al., 2006, 2008). DCFH₂-DA is readily taken up by cells and is subsequently de-esterified to DCFH₂, which can be oxidized to dichlorofluorescein (DCF) by hydrogen peroxide, peroxyxynitrite, and other ROS/reactive nitrogen species. Cells were washed with Locke's buffer and treated with BDE-47 in Locke's buffer for 1 h at 37 °C. After 40 min, DCFH₂-DA diluted from a stock solution in DMSO in Locke's solution was added to measure the level of ROS formation. After 20 min incubation in DCFH₂-DA, cells were then washed twice with Locke's buffer to remove extracellular DCFH₂-DA, and fluorescence was immediately read at excitation 488 nm, emission 525 nm, using a fluorescent microplate reader.

2.7. Measurement of lipid peroxidation

Lipid peroxidation, a major indicator of oxidative stress, was measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay, with a commercial kit used according to manufacturer's instructions, as described by Giordano et al. (2006, 2008). The TBARS assay measures the changes in levels of malondialdehyde (MDA), a natural byproduct of lipid peroxidation, which, upon binding to thiobarbituric acid forms a fluorescent red adduct. After exposure to BDE-47 and wash out, cells were scraped with 1% SDS lysis buffer (pH 7.4) and aliquots were removed to determine protein content. Butylhydroxytoluene (1 µM), an antioxidant, was added to the lysis buffer to prevent sample oxidation, and cells were centrifuged at 3000 × g for 10 min to remove large particles, before proceeding according to the TBARS assay protocol.

2.8. Measurement of intracellular calcium levels

Changes in intracellular calcium levels were measured as previously described (Giordano et al., 2006). 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, before addition of BDE-47. The plates were placed on the stage of a fluorescence microscope. The dye in the cytoplasmic portion of the cells was excited, and fluorescence images were captured at 20-s intervals by using a MicroMax cooled CCD camera (Princeton Instruments, Trenton, NJ) and images were analyzed using Metamorph software. Fifty cells in each treatment group were analyzed in each experiment. Fluorescence measurements were normalized as F/F_0 ($F - F_0/F$, where F is the intensity value obtained during the experiment and F_0 was the baseline intensity). Time-course experiments (not shown) indicated that peak calcium concentrations occurred between 10 and 15 min and the latter time was chosen to compare results of antagonist treatments, as shown in Fig. 3. Antagonists were added together with BDE-47.

2.9. Statistical analysis

Results are expressed as the mean ($\pm$ SD) of at least three independent experiments. Statistical analyses were carried out by one way ANOVA followed by Bonferroni's multiple comparison tests.

3. Results and discussion

The potential involvement of glutamate receptors in PBDE neurotoxicity was tested by investigating the cytotoxicity of BDE-47 (5 μM , a concentration previously shown to cause approximately 40–50% cell death) in mouse CGNs in the presence of several glutamate receptor antagonists: these included a NMDA receptor antagonist (MK-801, 5–10 μM), an AMPA/Kainate receptor antagonist (NBQX, 5–10 μM), a Group I metabotropic receptor antagonist (AIDA, 100, 250, 500 μM), a Group II metabotropic receptor antagonist (LY341495, 10, 50, 100 μM), and a Group III metabotropic receptor antagonist (MSOP, 100, 250, 500 μM). As shown in Fig. 1, MK-801, and to a lesser extent NBQX, protected against BDE-47-induced cytotoxicity. In contrast, antagonists of all three subtypes of glutamate metabotropic receptors provided no protection. Together, these results indicate the involvement of ionotropic glutamate receptors in BDE-47-induced cytotoxicity. In order to investigate whether ionotropic receptors were also involved in the induction of oxidative stress by BDE-47, we measured ROS production and lipid peroxidation in mouse CGNs upon BDE-47 treatment, in the absence or presence of ionotropic glutamate receptor antagonists. As shown in Fig. 2, both MK-801 and NBQX (at 10 μM) inhibited BDE-47-induced oxidative stress suggesting that induction of ROS production and of lipid peroxidation was secondary to activation of ionotropic glutamate receptors.

In further experiments we also measured the ability of BDE-47 to increase intracellular calcium levels, as reported by various investigators (Coburn et al., 2008; Dingemans et al., 2010). BDE-47 increased calcium levels, with a peak at 10–15 min, followed by a slow decrease (not shown). The increase of calcium levels was inhibited by both glutamate

ionotropic receptor antagonists (Fig. 3), suggesting that it involved prior activation of glutamate receptors. In order to assess the involvement of calcium in BDE-47 cytotoxicity, CGNs were exposed to the permeable calcium chelator BAPTA-AM. Fig. 4 shows that BAPTA-AM was able to protect CGNs against BDE-47 cytotoxicity. Furthermore, BAPTA-AM also antagonized BDE-47-induced oxidative stress (Fig. 4B and C). These findings suggest that BDE-47 induced alterations in calcium homeostasis are involved in its neurotoxicity, and that oxidative stress is down-stream of calcium increases.

In a final series of experiments we sought to determine whether BDE-47 may increase the concentration of extracellular glutamate. Time-course experiments indicated that BDE-47 caused a rapid increase in extracellular glutamate levels, with a doubling by 5 min (Fig. 5A). Extracellular glutamate levels peaked at 30 min, then slightly decreased, but remained significantly higher than controls even at 60 min. These results are in agreement with those of Reistad et al. (2007) who reported that TBBPA, another brominated flame retardant, increased extracellular glutamate levels in rat CGNs for up to 3 h. Blocking either NMDA or AMPA/kainate receptors did not antagonize the effect of BDE-47 on extracellular glutamate levels (Fig. 5B). Similarly, BAPTA-AM did not affect BDE-47-induced increase in extracellular glutamate levels (Fig. 5B). These findings suggest that the increase in extracellular glutamate is not secondary to activation of glutamate ionotropic receptors and by increases in intracellular calcium, as had been shown instead for the marine neurotoxin, and AMPA/kainate receptor agonist, domoic acid (Giordano et al., 2006).

Altogether, these findings indicate that BDE-47 increases glutamate levels outside the cells, by a still unknown mechanism. Elevated levels of extracellular glutamate activate NMDA and AMPA/kainate receptors and this in turn causes an increase in intracellular calcium, followed by increased production of ROS and of lipid peroxidation, ultimately resulting in cell death. The latter has been shown by several studies to be apoptotic in nature (Giordano et al., 2008; He et al., 2008; Tagliaferri et al., 2010; Costa et al., 2015). This line of events is suggested by the time course of BDE-47-induced alterations in extracellular glutamate and intra-cellular calcium levels, and by the findings with the glutamate receptor antagonists and the calcium chelator.

All experiments were carried out in CGNs, glutamatergic neurons that require high potassium levels in the medium to remain viable in culture. CGNs are an established model of primary neuronal cultures and have been extensively utilized in neurobiology and neurotoxicology (Giordano and Costa, 2011), including the study of PBDE neurotoxicity (Reistad et al., 2006, 2007; Giordano et al., 2008). Though other brain regions (*e.g.*, hippocampus) are also known to be targeted by PBDEs, cerebellar neurotoxicity, as evidenced by increased oxidative stress and apoptotic cell death, have been reported upon *in vivo* exposure to BDE-47 (Costa et al., 2015). The concentration of BDE-47 used in the present study (5 μ M) is in the same range as in most of published *in vitro* studies (see Costa et al., 2014 for references), slightly higher than those found *in vivo*. In various animal studies, brain levels of PBDEs up to $\sim$ 1.0 μ M have been reported (Cheng et al., 2009; Ta et al., 2011; Koenig et al., 2012; Costa et al., 2015). In humans, BDE-47 levels of 41 nM and 10–20 nM have been reported in brain and blood, respectively (Toms et al., 2009; Woods et al., 2012).

Our findings confirm and expand the earlier observations by Reistad et al. (2006, 2007) and by Yu et al. (2008) on an involvement of NMDA receptors in PBDE cytotoxicity. They also show that the ionotropic AMPA/kainate receptors may be involved in the effect of BDE-47, while the metabotropic glutamate receptors are not. Based on the time sequence of the biochemical events measured in the present study, we propose that BDE-47 first causes an increase in extracellular glutamate levels. The mechanism of such effect is not known and may involve an increased release of glutamate by still unknown mechanisms. Another possibility, suggested by Reistad et al. (2007), is that BDE-47 may inhibit glutamate re-uptake. Indeed, Mariussen and Fonnum (2003) reported that TBBPA non-selectively inhibited glutamate uptake in nerve terminals, though the penta-BDE mixture DE-71 was much less effective in this regard, and BDE-47 was not tested. Preliminary experiments in our laboratory with two glutamate uptake inhibitors (*L-trans*-pyrrolidine-2,4 dicarboxylic acid and *DL-threo*- β -benzyloxyaspartic acid) failed to modify the effects of BDE-47 on extracellular glutamate (Costa, Tagliaferri et al. unpublished observation). Additional experiments with release inhibitors and sodium channel blockers should be carried out to elucidate the molecular mechanism(s) by which BDE-47 increases extracellular glutamate levels. Independent of the underlying mechanism leading to its extracellular accumulation, the additional glutamate will activate NMDA and AMPA/kainate receptors on CGNs. This in turn would cause an elevation of intracellular calcium, followed by oxidative stress and ultimately by cell death, previously shown to be apoptotic (Giordano et al., 2008; He et al., 2008; Tagliaferri et al., 2010; Costa et al., 2015). This progression of events is supported by the findings that ionotropic glutamate receptor antagonists did not block BDE-47-induced glutamate release (Fig. 5B), but antagonized BDE-47-induced increases of intracellular calcium, oxidative stress, and cytotoxicity. In addition, BAPTA-AM antagonized BDE-47-induced oxidative stress and cytotoxicity, but had no effect on glutamate release.

A possible involvement of the glutamatergic system in PBDE developmental neurotoxicity has been previously suggested by a few *in vivo* and *in vitro* studies. A single exposure of mice to BDE-47 on postnatal day 10 was found to decrease post-synaptic protein involved in glutamate receptor signaling (*e.g.*, glutamate receptor subunits NR2B and GluR1), and to reduce hippocampal long-term potentiation and post-tetanic potentiation (Dingemans et al., 2007). Similar findings were obtained by Yan et al. (2012) in rats exposed for 30 days to BDE-47. They reported decreased levels of the NMDA receptor subunits NR1 and NR2B and of their mRNAs in the hippocampus, which was accompanied by decreased performance in the Morris water maze. In contrast, in young adult mice, a 30 day treatment with DE-71 did not alter NR2B levels, but increased expression of a vesicular glutamate transporter (Bradner et al., 2013). In another study in rats, prenatal exposure to BDE-99 was shown to increase the activity of the glutamate-nitric oxide-cGMP pathway, as assessed by microdialysis (Llansola et al., 2007), possibly as a result of an effect on calmodulin.

The involvement of glutamate ionotropic receptors in PBDE neurotoxicity may also be of relevance with respect to possible co-exposures. For example, PBDEs, and BDE-47 in particular, are contaminating mussels and other marine shellfish all over the globe (Dodder et al., 2014; Munschy et al., 2015). These same shellfish may also contain the marine toxin domoic acid, a known neurotoxin and developmental neurotoxin (Costa et al., 2010b), which

acts by activating AMPA/Kainate receptors and at higher concentrations, NMDA receptors, through domoic acid-induced glutamate release (Giordano et al., 2006, 2007). Co-exposure to domoic acid and BDE-47 may thus lead to additive neurotoxicity. Furthermore, as low concentrations of BDE-47 have been shown to synergize with other PBDEs (*e.g.*, BDE-99) (Tagliaferri et al., 2010), the possibility of glutamate receptor involvement in such interactions should be investigated.

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HIGHLIGHTS

- Toxicity of BDE-47 in cerebellar granule neurons is decreased by antagonists of ionotropic glutamate receptors.
- BDE-47 increases intracellular calcium and causes oxidative stress.
- These effects of BDE-47 appear to be secondary to an increase of extracellular glutamate.

Fig. 1.

(A) Viability of mouse CGNs, as measured by the MTT assay, after 24 h exposure to BDE-47 (5 μ M) in the absence or presence of the NMDA receptor antagonist MK-801 (5 and 10 μ M) or the AMPA/Kainate receptor antagonist NBQX (5 and 10 μ M). (B) Viability of mouse CGNs cell after 24 h exposure to BDE-47 (5 μ M) in the absence or presence of group I metabotropic receptor antagonist AIDA (100–500 μ M), group II metabotropic receptor antagonist LY341495 (10–100 μ M), or group III metabotropic receptor antagonist MSOP (100–500 μ M). Results represent the mean (SD) of three separate experiments. Significantly different from BDE-47, * p < 0.05, ** p < 0.01.

Fig. 2. Reactive oxygen species (ROS) levels (top) and lipid peroxidation (MDA levels; bottom) upon exposure to BDE-47 (5 μ M) in the absence or presence of MK-801 or NBQX (both at 10 μ M). ROS were measured 1 h after treatment, as described in Section 2, while lipid peroxidation was assessed after 12 h. Results represent the mean ($\pm$ SD) of three separate experiments. Significantly different from control, ** $p < 0.01$; significantly different from BDE-47, # $p < 0.05$, ## $p < 0.01$.

Fig. 3.

Change in intracellular calcium levels in mouse CGNs after a 15 min treatment with BDE-47 (5 μ M) alone or in the presence of MK-801 and NBQX (both at 10 μ M). Results represent the mean ($\pm$ SD) of three separate experiments. Significantly different from control, ** $p < 0.01$; significantly different from BDE-47, ## $p < 0.01$.

Fig. 4.

Effect of BAPTA-AM (5 μ M) on changes in cell viability (A), ROS levels (B) and lipid peroxidation (C) induced by BDE-47 (5 μ M) in mouse CGNs. Results are the mean ($\pm$ SD) of three separate experiments. Significantly different from control, ** $p < 0.01$; significantly different from BDE-47, # $p < 0.05$, ## $p < 0.01$.

Fig. 5. Effect of BDE-47 on extracellular glutamate levels. (A) Mouse CGNs were treated with BDE-47 (5 μ M) and the levels of extracellular glutamate were measured over time. Results are the mean ($\pm$ SD) of three separate experiments. Significantly different from control, * p < 0.05. (B) Extracellular glutamate levels in mouse CGNs after a 10 min incubation with BDE-47 (5 μ M) in the absence or presence of MK-801 (10 μ M), NBQX (10 μ M) or BAPTA-AM (5 μ M). Results represent the mean ($\pm$ SD) of three separate experiments. All

treatment groups are significantly different from control ($p < 0.05$), and do not differ among themselves.