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Optimizing GluR1 and PSD 95 for slot blot analysis and the neonatal effects on mouse after exposure to ALPi, ZS or TBBPA

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Contents

Abstract

Flame retardants are used as an additive in many different products such as plastics, textiles and building material to reduce the ability to catch flame or to inhibit combustion. These chemical compounds are known to be persistent in nature and has an ability to bioaccumulate in animals and humans. Luckily several of these chemical compounds have been or are going to be phased out from the market. To meet the demands of the market and the safety regulations of the authorities, new types of "environmental friendly" flame retardants have entered the market such as Aluminium diethylphosphinate (ALPi), Zinc stannate (ZS) and Tetrabromobisphenol A (TBBPA). In this study we investigated the possible effects of ALPi, ZS and TBBPA on the two neuroproteins Glutamate receptor 1 (GluR1) and Post-synaptic

density protein 95 (PSD 95) after a single oral dose at postnatal day 10 (PND 10) in mouse cerebral cortex. The results showed now statistically significant change in protein levels for either GluR1 or PSD 95 compared to controls.

Introduction

Flame retardants

Flame retardants are commonly used as a chemical additive in several petroleum based products such as plastics, textiles, and coatings to inhibit combustion and make the material less prone to catch flame (WHO, 1994a). These chemicals are widely used in electronic equipment such as TVs and computers, in cabling, foam products for furniture and in building material. Since the 1950's, following an increased global use of plastics, to meet the fire safety regulations, several types of flame retardants and chemicals with flame retarding abilities like PCBs (polychlorinated biphenyls) and PBDEs (polybrominated diphenyl ethers) have entered the market. Like other persistent organic pollutants (POPs), these flame retardants are often highly persistent, lipophilic molecules which are hard to break down in nature, leading to bioaccumulation in the brain and adipose tissue of animals and humans (de Wit, 2002). Several studies have indicated that neonatal and postnatal exposure to flame retardants can effect neuroproteins in the brain and cause adverse effects on reproduction and change in behavior for several species, including humans (Darnerud, 2003; Kuriyama *et al*., 2005; Meeker and Stapleton, 2009).

The most common route of exposure for humans to flame retardants has shown to be lactational exposure for toddlers, ingestion of animal fat and inhalation and ingestion of household dust for both adults and toddlers (Schecter *et al*., 2004, Wang *et al*., 2010; Stapleton *et al*., 2012).

Even though, several types of flame retardants have been banned in most countries (PCBs in 1997, PBDEs in 2008) the use of flame retardants around the world is still increasing and new types of flame retardants is continuously manufactured to meet the demands of the market (Council directive 85/467/EEC, 1985; Council directive 2003/11/EC, 2003). The potentially toxic effects of these new chemical compounds needs to be examined.

Aluminium diethylphosphinate (ALPi)

ALPi ($C_{12}H_{30}AlO_6P_3$) belongs to the phosphor-based halogen-free type of flame retardants and is commonly used in plastics as an additive together with other halogen free flame retardants to create synergistic effects, which increases its flame retardant properties (Braun and Schartel, 2000; Gallo *et al.,* 2009, 2010).

Zinc stannate (ZS)

ZS (Zn_2SnO_4) belongs to the metallic oxide type of synergetic "environmental-friendly" flame retardants and is commonly used together with brominated flame retardants in several types of polymers as a smoke suppressant (Horrocks *et al*., 2009). ZS is also known to reduce the CO emission during combustion when combined with other types of flame retardants (Horrocks *et al*., 2012).

Tetrabromobisphenol A (TBBPA)

TBBPA ($C_{15}H_{12}Br_4O_2$) belongs to the group of brominated flame retardants (BFRs) and is currently the most used flame retardant in electronic equipment and many types of polymers (Ogunbayo and Michelangeli, 2007). Several studies have indicated that TBBPA can induce reproductive harm and endocrine disruption in fish and mouse (Kuipera *et al*., 2007; Ven *et al*., 2008). Bioaccumulation of the compound has been seen occurring in both animals and humans alike (Darnerud, 2003).

Vulnerable periods and brain development

The mammalian brain development includes a number of stages of which the brain growth spurt (BGS) is the most eventful. During the BGS a series of profound changes occur including rapid growth of axons and dendrites, myelinization, synaptogenesis and formation of neural connections (Davison and Dobbing, 1968). This phase varies amongst different species but occurs postnatal in rodents, spanning from the first 3-4 weeks after birth and peaking around postnatal day 10 (PND 10). In humans on the other hand, the BGS occurs from the third trimester of pregnancy and peaks around birth (Dobbing and Sands, 1979). A number of studies have shown that the brain is particularly sensitive to adverse effects caused by xenobiotics during this phase (Eriksson *et al.,* 1992; Eriksson, 1997; Viberg *et al.,* 2008). Because the BGS occurs postnatally in mouse, this animal is well suited for studying adverse effects following neonatal exposure to xenobiotics.

Neuroproteins; Glutamate receptor 1 (GluR1) and Post-synaptic density protein 95 (PSD 95)

Glutamate receptors are integral membrane protein belonging to the group of ligand-gated ion channels. These receptors are divided into three different categories, based on their respective agonist neurotransmitter; *N*-Methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainite (Hollmann and Heinemann, 1994). The AMPA receptors are mainly expressed in the central nervous system (CNS) of the mammalian brain, where they act as major excitatory receptors mediating fast signal transmission between neurons. The AMPA receptors consists of four subunits, GluR1, GluR2, GluR3 and GluR4 which agonist neurotransmitters such as glutamate can bind to (Dingledine *et al*., 1999). Recent studies have shown that alterations in the proliferation of glutamate receptors in the mammalian brain is linked to changes in cognitive development, affecting learning and

memory to induce development of schizophrenia and bipolar disorders (Wakabayashi *et al*., 1999*)*.

PSD 95 is a scaffold protein, belonging to the membrane-associated guanylate kinase family known as the MAGUK family. The protein is mainly located in the post-synaptic density of the neuron, where it interacts with several neuroproteins and play a big role in the organization and stabilization of neuroproteins in the post-synaptic density (Kim *et al*., 1998; Sturgill *et al.,* 2009). Studies have shown that alterations in the proliferation of PSD 95 in the post-synaptic density can affect the function of other neuroproteins like AMPA receptors (Béïque *et al*., 2006). Other studies have shown that alterations of PSD95 protein levels in the brain also can be linked to cognitive dysfunction and neurological diseases like Alzheimers disease (Proctor *et al.*, 2010).

Aims

The aims of this study were to; 1) Optimize GluR1 and PSD 95 antibodies for use in Slot blot analysis, as a complement to already used protein markers for studying neonatal toxicity, 2) Study the effects on GluR1 and PSD 95 in mouse cerebral cortex, following neonatal exposure to ALPi, ZS or TBBPA.

Materials and methods

Animal treatment

Pregnant C57bl/6 mice were obtained from Harland (Horst, the Netherlands) and housed individually in plastic cages in a room with a temperature of 22ºC and 12/12 hour cycle of light and dark. The animals had free access to standardized food pellets and tap water *ad libitum*. The day of birth was assigned as PND 0 and the litters contained pups of both sexes. The litter sizes were culled to 10-14 pups, within the first 48h after birth. Only male mice were used in this study. Experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals.

Exposure and sample treatment

At PND 10, eight control mice were given a 1:10 (wt/wt) egg lecithin and peanut oil vehicle, sonicated with water to obtain a 20% (wt/wt) fat: water emulsion. Seven mice were given 48.9 mg (211 µmol)/kg bw of ALPi, eight mice were given 48.9 mg (211 µmol)/kg bw of ZS and eight mice were given 115 mg (211 µmol)/kg bw of TBBPA as a single oral dose administered via a metal gastric tube. The mice were sacrificed on PND 17 and the cerebral

cortices were dissected out, frozen in liquid nitrogen and stored in -80ºC until homogenization.

The cerebral cortex samples was weighed and homogenized in RIPA cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% sodium deoxycholate and 0.1% SDS) with the addition of 5 µL protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem) per mL of RIPA cell lysis buffer, using a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 14000 x g for 10 min at 4ºC. The supernatant was collected and stored in -80ºC until use. The protein content of the supernatant was measured using the BCA protein assay method (Pierce).

Optimizing GluR1 and PSD 95 for Slot blot analysis

The optimization of GluR1 and PSD 95 was carried out using Slot blot analysis and a previously aliquoted NMRI mouse control sample. The total protein content used for the first titration of GluR1 and PSD 95 was 1, 2, 3, 4, 5, 6, 8 and 10 µg, diluted in sample buffer to a final volume of 200 µL for each sample. The diluted supernatant was then applied in duplicates to a nitrocellulose membrane (0.45 mm, Bio-Rad) soaked in TBS buffer [NaCl (0.9%), Tris–HCl (42.1 mM) and Tris–Base (7.5 mM)], using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membranes were dried at 60ºC for 5 min, fixed in a 25% isopropanol and 10% acetic acid solution, washed in 1% TBS, and blocked for 1h at room temperature in 5% non-fat dry milk containing 0.03% Tween-20. The membranes were then cut in two and placed in separate trays incubated overnight at 4°C with either a rabbit monoclonal GluR1 primary antibody (Millipore AB1504, 1:1000 and 1:2000) or a mouse monoclonal PSD 95 primary antibody (Millipore, MABN68, 0,1 µg/ml and 0,05 µg/ml). Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody against mouse (KPL 074-1806, 1:20 000) or rabbit (KPL 074-1506, 1:20 000). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of the bands was quantified using IR-LAS 1000 Pro (Fuji Film).

A second titration was made according to the method stated above except that the total protein content used for GluR1 was changed to 2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5 µg and that the dilution of the primary GluR1 antibody used was changed to 1:1000 and 1:500. The total protein content and the primary antibody concentration used for PSD 95 were left unchanged.

Slot blot analysis for GluR1 and PSD 95

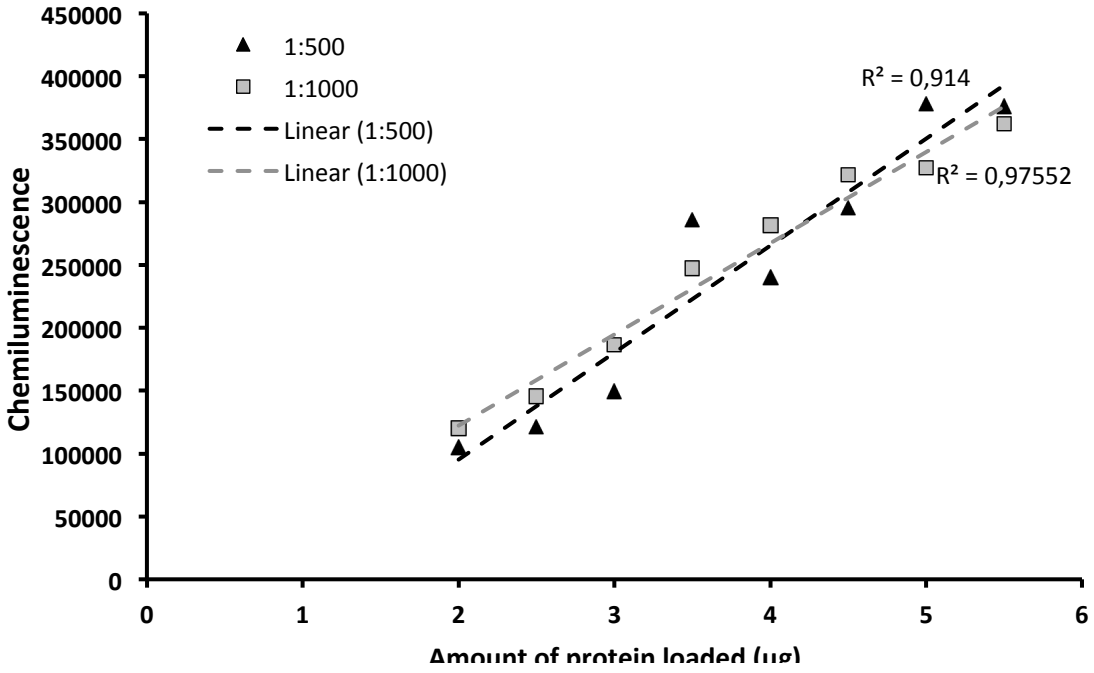
For determining the possible effects of ALPi, ZS and TBBPA on GluR1 and PSD 95 in neonatal mouse brain cortices, a Slot blot analysis was made using a sample protein amount of 3 µg and a primary antibody dilution of 1:1000 for GluR1 and a sample protein amount of 5 µg and a primary antibody concentration of 0,1 µg/ml for PSD 95.

Results

Optimization of GluR1 and PSD 95 antibodies

A titration was made to optimize the GluR1 and PSD 95 protein amounts and concentrations for Slot blot analysis and to verify that an increase in protein amount gave a linear increase in chemilumenescence.

GluR1 showed a high correlation with the linear equation of the first order (R^2=0.976). A protein amount of 3 µg and a primary antibody dilution of 1:1000 were found to be most



suitable (Fig. 1).

Figure 1. Slot blot titration of GluR1. Band chemiluminescence versus the amount of protein loaded (2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5 µg) with two different concentrations of primary antibody (1:500 and 1:100).

PSD 95 showed a high correlation with the linear equation of the first order (R^2=0.980). A protein amount of 5 µg and a primary antibody concentration of 0.1 µg/ml were found to be most suitable (Fig. 2).

Both GluR1 and PSD 95 showed a high linear correlation between the amount of protein loaded and chemiluminescence. Therefore the chemilumenescence will correspond to an increase of protein amount in the sample.

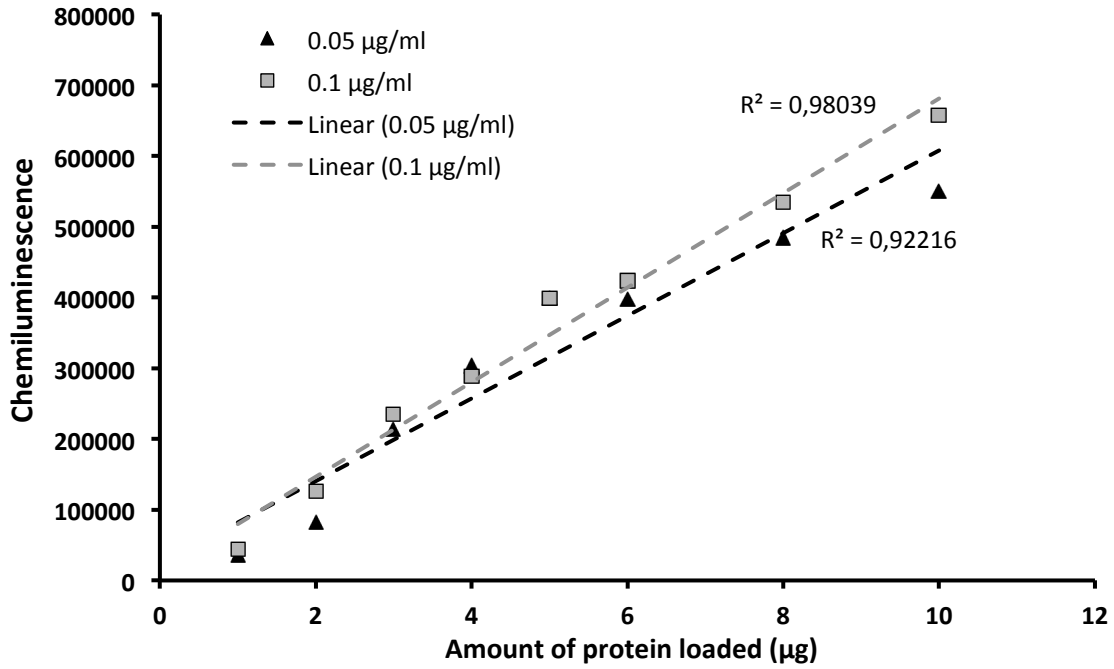


Figure 2. Slot blot titration of PSD 95. Band chemiluminescence versus the amount of protein loaded (1, 2, 3, 4, 5, 6, 8 and 10 µg) with two different concentrations of primary antibody (0.05 µg/ml and 0.1 µg/ml).

The effects of ALPi, ZS and TBBPA on GluR1 and PSD 95 in cerebral cortex

The levels of GluR1 and PSD 95 in cerebral cortex were quantified on PND 17, 7 days after exposure to a single oral dose of fat emulsion vehicle or 48.9 mg/kg bw of ALPi, 48.9 mg/kg bw of ZS or 115 mg/kg bw of TBBPA on PND 10. The results presented in Fig. 3a show that there were no statistically significant changes in the levels of GluR1 in cerebral cortex compared to controls. The levels of PSD 95 did not show any statistically significant changes in protein levels between exposed animals and controls.

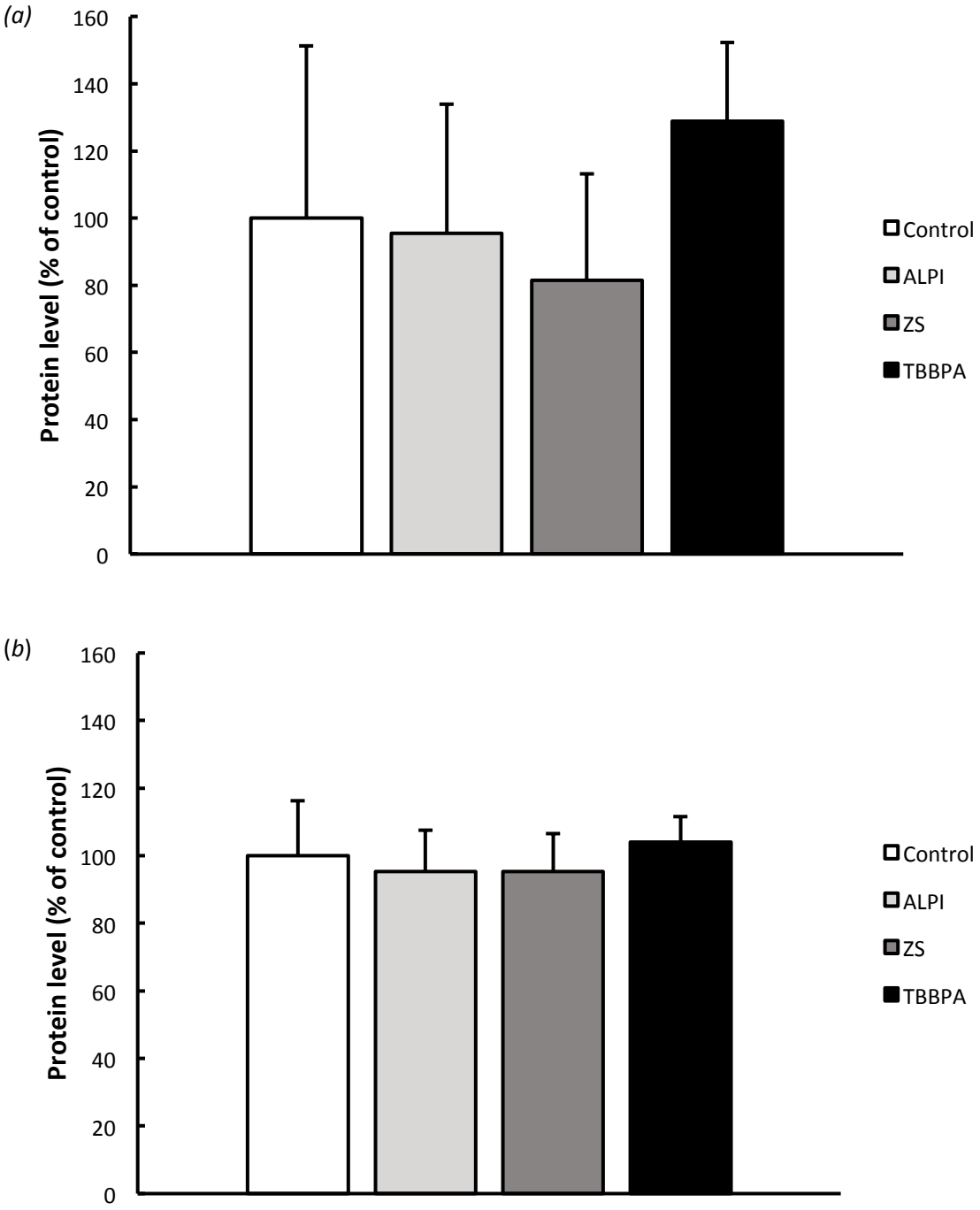


Figure 3. Levels of (a) GluR1 and (b) PSD 95 in cerebral cortex of animals exposed to a single oral dose of fat emulsion vehicle or 48.9 mg/kg bw of ALPi, 48.9 mg/kg bw of ZS or 115 mg/kg bw of TBBPA on PND 10 and sacrificed 7 days later on PND 17. The data was statistically analyzed with a one-way ANOVA test which displayed no statistical significance. The height of the bars represents the mean value ± SD. The number of observations for each group (*n*) is 6.

Discussion

A titration of the GluR1 and PSD 95 antibodies was made to optimize the two antibodies for further use in Slotblot analysis, as a complement to the already used protein markers used to study the effects of neonatal neurotoxicity; CaMKII, GAP-43, synaptophysin and Tau. As multiple studies have indicated, neonatal exposure to flame retardants can alter the expression of neuroproteins such as CaMKII (Viberg *et al*., 2008), GAP-43 (Viberg and Eriksson, 2011) and synaptophysin (Viberg, 2009). Therefore it was interesting to see if there were any effects on GluR1 and PSD 95 following neonatal exposure to the supposedly ”environmental friendly” flame retardants ALPi, ZS and TBBPA, as these two neuroproteins are known to play an important role in cognitive behaviour (Wakabayashi *et al*., 1999; Proctora *et al.*, 2010).

For the Slot blot titration, a suitable sample protein amount of 3 µg and an antibody concentration of 1:1000 for GluR1 was chosen (Fig. 1). For PSD 95 (Fig. 2), a suitable sample protein amount of 5 µg and a concentration of 0.1 µg/ml was chosen to be used in further Slot blot analysis. The high correlation of chemiluminescence vs. protein amount for both antibodies (R^2=0.976 for GluR1 and R^2=0.980 for PSD 95) indicates a good linear correlation between the amount of protein loaded and chemiluminescence, which make them well suited for use in further Slot blot analysis.

In this study the results showed no significant changes in protein levels of either GluR1 or PSD 95 compared to controls in cerebral mouse cortex, following neonatal exposure to ALPi, ZS or TBBPA. As no previous studies on GluR1 or PSD 95 have been conducted with the Slot blot analysis method, it is hard to determine whether this two neuroproteins is commonly affected by flame retardants or not. As both ALPi and ZS is regarded as “environmental friendly” flame retardants, as they are free of halogens (Horrocks *et al*., 2009), one would expect little or no effect on the protein expression of GluR1 and PSD 95 after exposure (Waaijers *et al*., 2013). TBBPA on the other hand is a well-known environmental toxicant known to bioaccumulate in both animals and humans (Darnerud, 2003), so one could expect possible effects on GluR1 and PSD 95 following exposure and by visually studying the bars of Fig. 3a we can see a tendency towards increased protein expression for GluR1 following exposure to TBBPA, although the data is not statistically significant compared to the controls. Therefore it would be of interest to repeat this study to get a lower spreading within the groups and thus get a higher significance value. An increase in the number of samples (*n*) in future studies would be preferable as it would decrease the total spread within the groups. As other studies have shown, protein changes can occur in either cerebral cortex or hippocampus tissue (Johansson et al., 2009) and because only cerebral cortex was used in this study, future studies should also include cerebral hippocampus tissue and also other kinds of neuroproteins such as CaMKII, GAP-43, synaptophysin, and Tau.

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