MODULATION OF NICOTINIC ACETYLCHOLINE RECEPTORS BY BROMINATED AND ALTERNATIVE HALOGEN-FREE FLAME RETARDANTS

Hendriks H, van Kleef R, Westerink R*

Neurotoxicology Research Group, Institute for Risk Assessment Sciences (IRAS), Utrecht University, NL-3508 TD Utrecht, The Netherlands.

Introduction

The large scale use of brominated flame retardants (BFRs) is associated with ecological and toxicological concerns[1-2], with the (developing) nervous system being among the most vulnerable targets for the toxic actions of BFRs[3-5]. PCBs and PBDEs as well as organophosphorous compounds can interfere with the cholinergic system and contribute to neurodevelopmental abnormalities[6-9]. The $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptor is an abundant excitatory neurotransmitter receptor in the human central and peripheral nervous system. Previous *in vitro* research demonstrated that nACh receptors are a direct target for e.g., organophosphates[10], PCBs, PBDEs[11] and TBBPA[12]. These (neuro)toxic effects of BFRs argue for replacement by safe(r) and less persistent alternatives.

Suggested halogen-free substitutions include phosphorous flame retardant compounds such as triphenylphosphate (TPP), resorcinol bis (biphenyl)phosphate (RDP), bisphenol A bis (biphenol) phosphate (BDP), 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) and aluminium dietyhlphosphinate (Alpi); inorganic halogen-free flame retardants and synergists such as aluminium trihydroxide (ATH), ammonium polyphosphate (APP), antimony trioxide (ATO), magnesium hydroxide (MHO), zinc hydroxystannate (ZHS) and zinc stannate (ZS); the nanoclay cloisite 30B (montmorillonite, MMT) and the nitrogen-based organic flame retardant melamine polyphosphate (MPP). However, the (neuro)toxic potential of proposed halogen-free flame retardants (HFFRs) is largely unknown[13].

It is essential to assess the (neuro)toxic potential of these proposed HFFRs before they are used on large scale and in high volume. We therefore measured the effects of three frequently used BFRs and 13 possible halogen-free substitutions on the function of human $\alpha_4\beta_2$ nACh receptors, expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp technique. This assessment can be an important step in prioritizing viable halogen-free alternatives for the replacement of BFRs prior to large scale and global use of these HFFRs.

Materials and methods

Xenopus laevis (provided by Dr Wim Scheenen, Radboud University, Nijmegen, The Netherlands) oocytes were isolated and injected with cDNA coding for the human α_4 and β_2 subunits of the human neuronal nACh receptors (provided by Janssen Pharmaceutica N.V., Beerse, Belgium) as described previously[11,14,15]. Following expression of functional neuronal nACh receptors, effects of BFRs and HFFRs on $\alpha_4\beta_2$ nACh receptor function were measured with the two-electrode voltage-clamp technique using a Gene Clamp 500B amplifier (Axon Instruments) with high-voltage output stage as described previously[11,14,15].

Voltage-clamped (-60 mV) oocytes were continuously superfused with saline. Oocytes were exposed for 20-40 s by switching the perfusate from saline to flame retardant- and/or ACh-containing saline using a servomotor-operated valve. Aliquots (10-100 mM) of BFRs and phosphorous flame retardants in DMSO were diluted in the saline solution immediately before the experiments to obtain final concentrations of 0.3 to 100 µM. The other HFFRs (Alpi, ATH, APP, ATO, MMT, MHO, MPP, ZHS and ZS) are poorly soluble in DMSO (or other solvents) and were directly dissolved in saline solution at the maximal water solubility and dilutions thereof.

Peak amplitudes of ACh-evoked ion currents were measured and normalized to the maximal amplitude (at 1 mM) of agonist-evoked control responses to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitudes over time[11,14,15]. The percentage of flame retardant-induced inhibition of the ACh-evoked ion current was calculated from the quotient of the maximum amplitude of the ACh-congener co-application response and the maximum amplitude of the ACh response. Flame retardants were initially tested at two concentrations and complete concentration-response curves were measured where appropriate.

Data represent mean ± standard error of the mean (SEM) of *n* oocytes. Statistical differences ($p < 0.05$) were calculated using unpaired two-tailed Student's *t*-test. The concentration-dependence of the inhibiting effects was determined by one-way ANOVA ($p < 0.05$) and posthoc Bonferroni testing. Observed changes in ion current < 5 % were considered irrelevant.

Results and discussion

Voltage-clamped (-60 mV) oocytes expressing human $\alpha_4\beta_2$ nACh receptors display clear concentration-dependent receptor activation upon superfusion with ACh-containing saline. Superfusion of ACh-responsive oocytes with saline containing BPS, BDE-209 or TBBPA (1 and 10 µM) did not result in a detectable ion current, clearly indicating that none of the tested BFRs can act as full agonist of the $\alpha_4\beta_2$ nACh receptor (not shown). However, co-application of ACh-containing saline with BDE-209 (10 µM) resulted in a small inhibition (8 ± 1 %; $p < 0.01$) of the ACh-evoked response. As described previously[12], co-application of ACh with TBBPA induced a concentration-dependent inhibition of the ACh-evoked ion current, with a lowest observed effect concentration (LOEC) of 3 µM ($p < 0.01$) and a calculated effective concentration producing 50 % inhibition of the maximal response (IC_{50}) of 7 ± 1 µM. At ≥ 30 µM, TBBPA almost completely abolished the ACh-evoked ion current, indicating TBBPA is a strong antagonist of the $\alpha_4\beta_2$ nACh receptor (Figure 1A). On the other hand, co-application of ACh with BPS (up to 10 µM) did not affect the ACh-evoked ion current.

Similarly, superfusion of ACh-responsive oocytes with saline containing any of the phosphorous flame retardants (up to 100 µM) did not result in a detectable ion current, clearly indicating that none of these compounds act as full agonist of the $\alpha_4\beta_2$ nACh receptor (not shown). Also, co-application of ACh with DOPO (100 µM) or BDP (100 µM) did not affect the ACh-evoked ion current (Figure 1D). Co-application of ACh with RDP (100 µM) resulted in a small inhibition of the ACh-evoked ion current (10 ± 2 %; $p < 0.001$), whereas co-application of ACh with TPP resulted in a concentration-dependent inhibition of the ACh-evoked ion current, with a LOEC of 1 µM ($p < 0.001$; calculated IC_{50} 7.9 ± 1.4 µM) and a maximum inhibition at ≥ 30 µM (76 ± 6 %; Figure 1B). The metal-phosphinate Alpi also induced a concentration-dependent inhibition of the ACh-evoked ion current with a LOEC of 27.9 µM, i.e., 10 % of the maximum water solubility ($p < 0.05$). At the highest concentration tested (279 µM, i.e., maximum water solubility), Alpi inhibited the ACh-evoked ion current with 60 ± 6 % (Figure 1C), thus precluding reliable calculation of the IC_{50}.

Superfusion of ACh-responsive oocytes with saline containing any of the inorganic halogen-free flame retardants, nitrogen-based organic flame retardant or nanoclay (up to S_{max}) did not result in a detectable ion current, demonstrating that none of these compounds can act as full agonist of the $\alpha_4\beta_2$ nACh receptor (not shown). Co-application of ACh with ZS at S_{max} (0.8 µM) did not affect the ACh-evoked ion current, whereas ATO, ATH, MHO and ZHS all induced a small inhibition at their S_{max} (not shown).

The inorganic flame retardant APP and the nitrogen-based organic flame retardant MPP both induced a more robust inhibition of the ACh-evoked ion current, amounting to 55 ± 2 % (at $S_{max;}$ 1.3 µM, $p < 0.001$; LOEC 0.13 µM, $p < 0.001$) and 23 ± 3 % (at $S_{max;}$ 70 nM, $p < 0.001$), respectively. The poorly soluble nanoclay MMT also induced an inhibition of the $\alpha_4\beta_2$ nACh receptor in the nanomolar range, with a LOEC of 40 nM ($p < 0.001$) and a maximum inhibition amounting to 79 ± 4 % at its S_{max} (400 nM; calculated IC_{50} 140 ± 1.2 nM; not shown).

Based on the determined no observed effect concentration (NOEC, 'potency') and maximal effect size, an initial rank order potency can be established. Based on these criteria, TBBPA, TPP, Alpi, APP and MMT can be classified as 'highly potent', whereas BDE-209, ATH, ATO, MHO, ZHS and MPP can be classified as 'moderately potent'. On the other hand, the brominated flame retardant BPS and the alternative flame retardants BDP, RDP, DOPO and ZS can be classified as 'not potent' with respect to modulation of $\alpha_4\beta_2$ nACh receptors function *in vitro*.

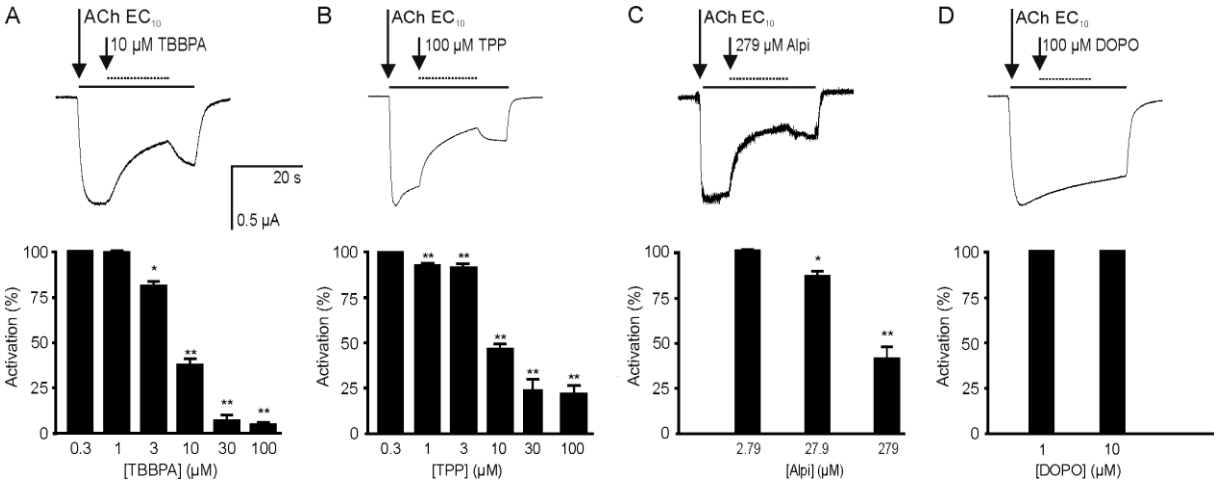

Figure 1. Example recordings (top) and bar graphs of concentration-response curve of the inhibitory effects of TBBPA (A), TPP (B) and Alpi (C) on the $\alpha_4\beta_2$ nACh receptor during co-application with ACh (EC_{10}). Note that DOPO (D) does not exert antagonistic effects on the ACh-evoked response. Scale bar applies to all traces. Data are presented as mean activation ± SEM (100% activation demonstrates the absence of antagonistic effects of the tested flame retardant). n = 3-7 oocytes. * $p < 0.05$ versus control; ** $p < 0.001$ versus control.

Ideally, HFFRs replacing existing BFRs should pose lower risks to the environment and human health. However, there is a general lack of toxicological information regarding the suggested HFFRs, which makes it hard to assess their toxic potential. Consequently, there is an urgent need for more research on the (eco)toxicological effects of these compounds before they are globally used on large scale. Our initial rank-order potency based on the *in vitro* inhibition of nACh receptors clearly indicates the neurotoxic potential of TBBPA, TPP, Alpi, APP and MMT, though additional studies, also focusing on expected concentrations in humans and the environment, are required before these compounds can be excluded as viable alternatives. Importantly, five out of the sixteen tested compounds (BPS, BDP, RDP, DOPO and ZS) are classified as not potent. Based on this specific neurotoxic endpoint, these five compounds could therefore be selected for additional testing to further assess the viability of these HFFRs as alternatives to replace current BFRs.

Acknowledgements
We thank Ing. Aart de Groot (Neurotoxicology Research Group, Institute for Risk Assessment Sciences) for excellent technical assistance, Drs Pim Leonards and Sicco Brandsma (Institute for Environmental Studies, VU University, Amsterdam, The Netherlands) for helpful discussions and ICP-MS measurements, Janssen Pharmaceutica N.V. (Beerse, Belgium) for providing the cDNA encoding the human $\alpha_4\beta_2$ nACh receptor subunits, and Dr Wim Scheenen (Radboud University, Nijmegen, The Netherlands) for providing the *Xenopus leavis* frogs. This research was funded by the European Union Union (ENFIRO; grant agreement FP7-ENV-2008-1-226563) and the faculty of veterinary sciences of Utrecht University.

References:

1. Covaci A, Harrad S, Abdallah MA, Ali N, Law RJ, Herzke D, de Wit CA. (2011) *Environ Int.* 37: 532-56
2. Shaw SD, Blum A, Weber R, Kannan K, Rich D, Lucas D, Koshland CP, Dobraca D, Hanson S, Birnbaum LS. (2010) *Rev Environ Health* 25: 261-305
3. Costa LG, Giordano G. (2007) *Neurotoxicology* 28: 1047-67
4. Dingemans MM, van den Berg M, Westerink RH. (2011) *Environ Health Perspect.* 119: 900-7
5. Fonnum F, Mariussen E. (2009) *J Neurochem.* 111: 1327-47
6. Coccini T, Roda E, Castoldi AF, Goldoni M, Poli D, Bernocchi G, Manzo L. (2007) *Toxicology* 238: 34-48
7. Eriksson P, Ankarberg E, Viberg H, Fredriksson A. (2001) *Neurotox Res.* 3: 37-51
8. Johansson N, Viberg H, Fredriksson A, Eriksson P. (2008) *Neurotoxicology* 29: 911-9
9. Slotkin TA. (2004) *Toxicol Appl Pharmacol.* 198: 132-51
10. Smulders CJ, Bueters TJ, Vailati S, van Kleef RG, Vijverberg HP. (2004) *Toxicol Sci.* 82: 545-54
11. Hendriks HS, Antunes Fernandes EC, Bergman A, van den Berg M, Westerink RH. (2010) *Toxicol Sci.* 118: 635-42
12. Hendriks HS, van Kleef RG, van den Berg M, Westerink RH. (2012) *Toxicol Sci.* in press
13. Waaijers SL, Kong D, Hendriks HS, de Wit CA, Cousins IT, Westerink RH, Leonards PE, Kraak MH, Admiraal W, de Voogt P, Parsons JR. (2012) Rev Environ Contam Toxicol. 221: in press
14. Antunes Fernandes EC, Hendriks HS, van Kleef RG, van den Berg M, Westerink RH. (2010) *Environ Sci Technol.* 44: 2864-9
15. Hondebrink L, Meulenbelt J, van Kleef RG, van den Berg M, Westerink RH. (2011) *Neurotoxicology* 32: 823-7.