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Original article

# A unique pattern of bisphenol A effects on nerve growth factor gene expression in embryonic mouse hypothalamic cell line N-44

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We investigated the toxicity of bisphenol A (BPA) by determining the gene expression of nerve growth factor (*Ngf*) in the embryonic mouse cell line mHypoE-N44 derived from the hypothalamus exposed to BPA dose range between 0.02 and 200 µmol $L^{-1}$ for 3 h. *Ngf* mRNA levels decreased in a dose-dependent manner, with significant reductions observed in the 2 to 50 µmol $L^{-1}$ BPA treatment groups compared to controls. However, at 100 to 200 µmol $L^{-1}$ the *Ngf* mRNA gradually increased and was significantly higher than control, while the expression of the apoptosis-related genes *Caspase 3* and transformation-related protein 73 decreased significantly. These results suggest that in an embryonic hypothalamic cell line the higher doses of BPA induce a unique pattern of *Ngf* gene expression and that BPA has the potential to suppress apoptosis essential for early-stage brain development.

**KEY WORDS:** Caspase 3*; developmental toxicity; foetal hypothalamus; nerve growth factor; transformation-related protein 73*

Bisphenol A (BPA) is one of the most abundantly produced chemicals worldwide that can disrupt endocrine function by mimicking the action of oestrogen. It has been detected in maternal serum, umbilical cord, foetal serum, and full-term amniotic fluid passing through the placenta (1, 2) and can also penetrate the blood-brain barrier (3). Because foetuses are extremely sensitive to chemicals with hormone-like activity, and even small changes induced by oestrogen-mimicking chemicals can lead to changes in brain function and behaviour (4), there is a growing concern that BPA exposure can disrupt foetal brain development/function or neuronal differentiation. Recent studies have demonstrated that BPA exposure can affect neurogenesis during gestation (5, 6) or in young adult mice (7).

Neurotrophins are crucial to the survival, development/differentiation, and function of neurons. The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 (8). NGF and BDNF initiate signalling by binding to specific high-affinity receptors, namely neurotrophic tyrosine kinase receptor type 1 (NTRK1) and type 2 (NTRK2), respectively (9). We previously showed that BPA decreased the *Bdnf* gene expression in embryonic hypothalamic cells and affected the BDNF-NTRK2 neurotrophin system (10). Seki et al. (11) have found that BPA causes morphological changes in NGF-induced differentiation and inhibits neurite extension in a PC12 cell line. It has also been reported to induce changes in both dendritic and synaptic development

in foetal hypothalamic cells (12). Because NGF regulates dendritic morphology and synaptic connectivity during development (13), these findings suggest that BPA would affect NGF-related development in embryonic hypothalamic cells and that the NGF-NTRK1 neurotrophin system could be a target of BPA. However, little is known about the relationship between BPA and NGF in developing hypothalamic cells.

Another mechanism potentially affected by BPA and regulated by neurotrophins is apoptosis (programmed cell death). In neural development, apoptosis plays an essential role in optimising synaptic connections, removing unnecessary neurons, and forming neuronal patterns (14). In embryonic neurons such apoptosis is regulated by neurotrophins (15, 16). Therefore, we also wanted to see how the effects of BPA on the NGF-NTRK1 neurotrophin system affect apoptosis in developing hypothalamic cells.

## MATERIALS AND METHODS

We focused our study on the previously unknown effects of BPA on the gene expression of *Ngf*, *Ntrk1*, *Caspase 3* (*Casp3*), and the transformation-related protein 73 (*Trp73*), which is also involved in apoptosis (17), in developing hypothalamic cells. We used the embryonic mouse hypothalamic cell line N-44 (mHypoE-N44) as the foetal hypothalamic cell model and assessed the effects of BPA on gene expression using real-time reverse transcription polymerase chain reaction (real-time RT-PCR).

### *Cell culture*

The mHypoE-N44 cell line was purchased from CELLutions Biosystems, Inc. (Toronto, ON, Canada). This cell line is immortalised from mouse embryonic hypothalamic primary cultures (days 15, 17, and 18) by retroviral transfer of SV40 T-Ag. We used this cell line as a suitable model for foetal hypothalamus. The cells were plated at a density of $2x10^4$ cells in 35-mm tissue culture dishes pre-coated with collagen type I (Corning, Inc., Corning, NY, USA) and were cultured in minimum essential medium alpha (MEMα; Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 10 % foetal bovine serum (FBS; Biowest, Nuaille, France). The cells were incubated at a permissive temperature (37 °C) in a humid atmosphere with 5 % $CO_2$.

### *Incubation of the mHypoE-N44 cells with BPA*

BPA (Junsei Chemical Co., Tokyo, Japan) in the concentration range of 0.02 to 200 μmol $L^{-1}$ was dissolved in 0.1 % (final concentration) dimethyl sulphoxide (DMSO; Wako, Osaka, Japan). The choice of the upper range limit was based on an *in vitro* study by Kim et al. (18) showing no notable cytotoxicity of BPA in concentrations ≤200 μmol $L^{-1}$. We therefore assumed that treatment with ≤200 μmol $L^{-1}$ BPA for 3 h would have no effect on cell viability. In addition, the 0.1 % DMSO vehicle was not toxic to the mHypoE-N44 cells and had no effect on cell viability or cell division. The cells were first cultured in phenol-red-free MEMα supplemented with 0.5 % charcoal-stripped FBS at 37 °C for 24 h. Then the medium was supplemented with BPA, and incubation continued for another 3 h. The cells treated with 0.1 % DMSO served as controls. All experiments were performed in triplicate.

### *Separation of total RNA and real-time RT-PCR*

Total cellular RNA was extracted from the mHypoE-N44 cells using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase-treated RNA (1 μg) was reverse transcribed to cDNA using the Super Script III First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA, USA). Gene expression was examined using quantitative RT-PCR. The cDNA was amplified through PCR with primer sets specific to mouse *Ngf*, *Ntrk1*, *Casp3, Trp73*, and oestrogen-related receptor γ (*Errγ*), which is a putative BPA receptor. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, UK) with LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green I mix (Roche Diagnostics Ltd.). Primer sequences for *Ngf*, *Ntrk1*, *Trp73*, and glyceraldehyde-3'-phosphate dehydrogenase (*Gapdh*) have been described previously (10, 19). *Casp3* primer sequences were 5'-CTG CCG GAG TCT GAC TGG AA-3' and 5'-ATC AGT CCC ACT GTC TGT CTC AAT G-3'. *Errγ* primer sequences were 5'-TCA AAG CCC TCA CCA CAC TCT-3' and 5'-GCC AGG GAC AGT GTG GAG AA-3'. Amplification of *Gapdh* mRNA was used as an internal positive control. The level of *Gapdh* mRNA was stable and similar between each sample, and the amounts of each mRNA were normalised to the *Gapdh* mRNA level in each sample.

*Statistical analysis*

Statistical analyses were performed using one-way analysis of variance (ANOVA) and Bonferroni–Dunn *post-hoc* tests with the StatView software (version 5.0; SAS Institute Inc., Cary, NC, USA). The data for each BPA-treated group were compared with those for the controls. *P* values of less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

Figure 1 shows the effects of the 3 h of exposure to BPA in the range between 0.02 and 200 μmol $L^{-1}$ on the expression of *Ngf* in mHypoE-N44 cells. The *Ngf* mRNA level gradually decreased in a dose-dependent manner. A significant drop ($P<0.01$) was observed in the cells treated with 2, 20, and 50 μmol $L^{-1}$ BPA compared to control. An *in vitro* study by Yokosuka et al. (12) has shown that BPA induces changes in both dendritic and synaptic development in foetal rat hypothalamic cells via alteration of microtubule-associated protein 2 (MAP2) and synapsin I expression, which serve as protein markers of neuronal growth and synaptogenesis, respectively (20). In addition to MAP2 and synapsin I, we believe that hypothalamic cell dendritic and synaptic development is also affected by changes in *Ngf* expression, given the important role of NGF in both dendritic morphology and synaptic connectivity (13). Further studies such as Western blotting should be conducted to evaluate the changes at the protein level.

Unexpectedly however, the *Ngf* mRNA level significantly increased with exposure to ≥100 μmol $L^{-1}$ BPA ($P<0.01$) compared to control. Lee et al. (21) reported that the effect of BPA on neuronal cells derived from the E18 rat cortex differed between low (below 50 μmol $L^{-1}$) and high (higher than 100 μmol $L^{-1}$) doses of BPA. In addition, the authors suggested that a concentration of 50 μmol $L^{-1}$ BPA might be a cut-off dose for the induction of adverse effects in neuronal cells. In our study, the dramatic change in *Ngf* mRNA expression was observed between 50 and 100 μmol $L^{-1}$. At the moment, we can not pinpoint the exact mechanism of or the reason for this switching effect on *Ngf* mRNA expression, but our results suggest that embryonic hypothalamic cells may have a similar flexion point (also around 50 μmol $L^{-1}$ BPA) to that reported for cortical cells by Lee et al. (21).

Despite the surprising reversal in *Ngf* mRNA expression observed at 200 μmol $L^{-1}$ BPA ($P<0.01$ compared to control), the gene expression of the high-affinity NGF receptor *Ntrk1* did not change (Figure

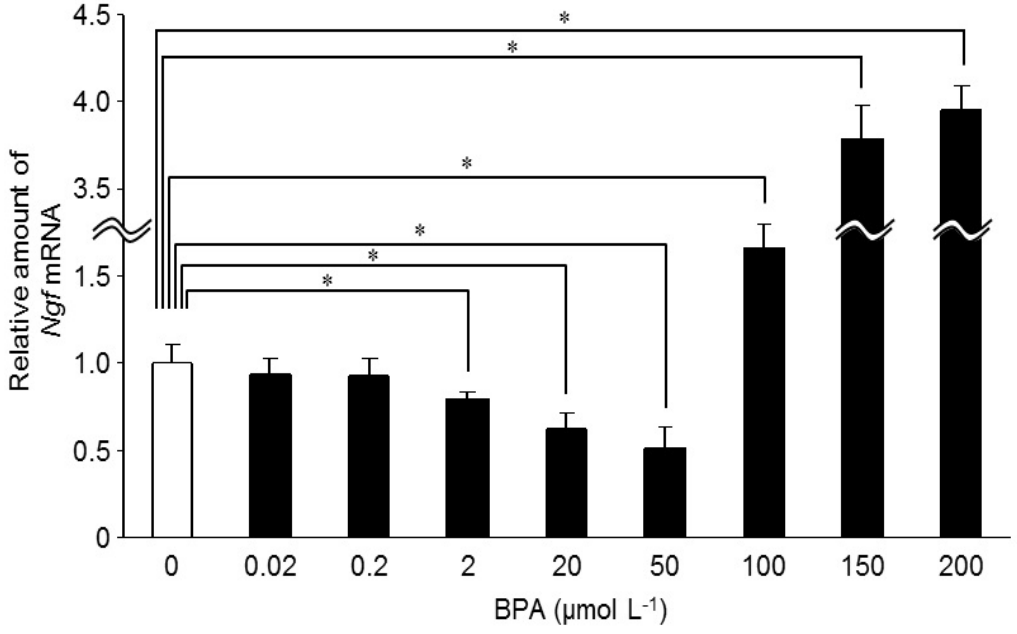


**Figure 1** *Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of* Ngf *mRNA levels in mHypoE-N44 cells treated with BPA for 3 h.*
*The data have been normalised to the* Gapdh *mRNA level in each sample and are expressed as a value relative to this internal control. Each column represents the mean ± SD (standard deviation) (n=3 for each group).* *P<*0.01 (significantly different from control)*

2). We have previously reported that treatment with 200 μmol $L^{-1}$ BPA decreases *Bdnf* gene expression without affecting the BDNF receptor gene *Ntrk2* (10). Both our studies suggest that the adverse effects of higher BPA doses on the NGF/NTRK1 and BDNF/NTRK2 systems occur through the alteration of gene expression of the ligands, not of their receptors.

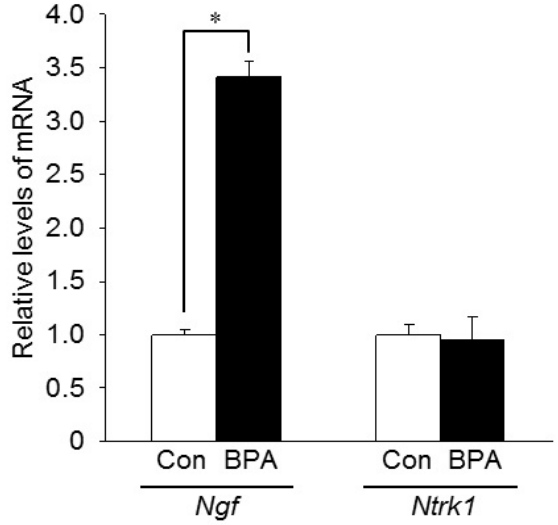


**Figure 2** *Quantitative RT-PCR analysis of* Ngf *and* Ntrk1 *mRNA levels in mHypoE-N44 cells treated with 200 μmol $L^{-1}$ of BPA for 3 h.*
*The data have been normalised to the* Gapdh *mRNA level in each sample and are expressed as a value relative to this internal control. Each column represents the mean ± SD (n=3 for each group).* *P*<0.01 (significantly different from control)*

On the other hand, *Casp3* and *Trp73* mRNA expression did not drop significantly in the cells treated with lower doses of BPA (data not shown), but only in those treated with 200 μmol $L^{-1}$ BPA ($P<0.01$ and $P<0.05$, respectively, compared to the controls) (Figure 3). In the early developmental stage, apoptosis is an essential physiological process for the formation of a normal central nervous system (CNS), and Casp3 plays an important role in apoptosis during brain development (14). TRP73 is also reported to induce apoptosis (22, 23). Therefore, our results suggest that BPA has the potential to suppress normal apoptosis in embryonic hypothalamic cells and are in line with the findings of Negishi et al. (24), who reported that BPA significantly inhibited Casp3 activity in foetal rat neurons treated with staurosporine, an inducer of apoptosis. Given that our highest dose of BPA decreased the expression of *Casp3* and *Trp73* and increased the expression of *Ngf,* which supports the survival and maintenance of neurons in the brain during embryonic development (25), we assume that high levels of BPA adversely affect normal development of the foetal hypothalamus by allowing the survival of cells that are destined for apoptotic thinning necessary for normal formation of the CNS. Furthermore, because the effect of BPA on the *Ngf* gene expression significantly differed with doses, adverse effects resulting from the change in *Ngf* levels may also be dose-dependent. It is possible that the effects shift from altering dendritic and synaptic development to affecting naturally occurring essential cell death.

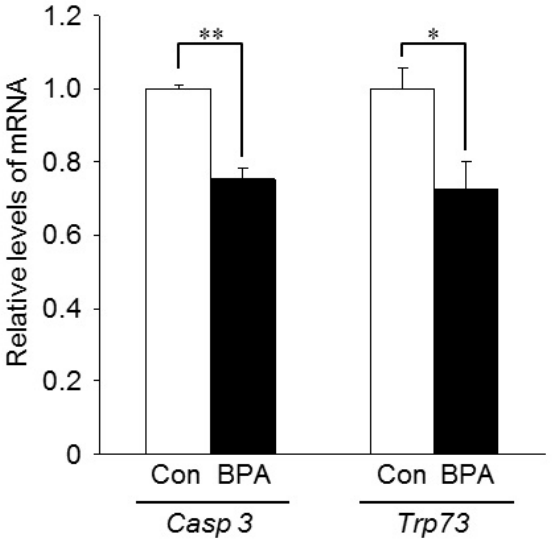


**Figure 3** *Quantitative RT-PCR analysis of* Casp3 *and* Trp73 *mRNA levels in mHypoE-N44 cells treated with 200 μmol $L^{-1}$ of BPA for 3 h.*
*The data have been normalised to the* Gapdh *mRNA level in each sample and are expressed as a value relative to this internal control. Each column represents the mean ± SD (n=3 for each group).* *P*<0.05,* **P*<0.01 (significantly different from control)*

Matsushima et al. (26) have reported that BPA binds strongly to ERRγ, a receptor highly expressed in the placenta (27), which suggests that BPA has a high potential for accumulating in the placental tissue. In our study, *Errγ* mRNA expression gradually started to decrease from 100 μmol $L^{-1}$ BPA only to reach significant reduction at 200 μmol $L^{-1}$ (Figure 4). Although it is unclear how much a dose of BPA can affect the embryonic hypothalamus through the placenta, higher doses may downregulate *Errγ* gene expression in foetal hypothalamic cells.

In summary, our findings suggest that in an embryonic hypothalamic cell line BPA has the potential to suppress apoptosis by lowering *Casp3* and *Trp73* mRNA levels and that higher doses of BPA may modulate NGF-mediated neuronal development by altering *Ngf* gene expression, but the effect is strongly

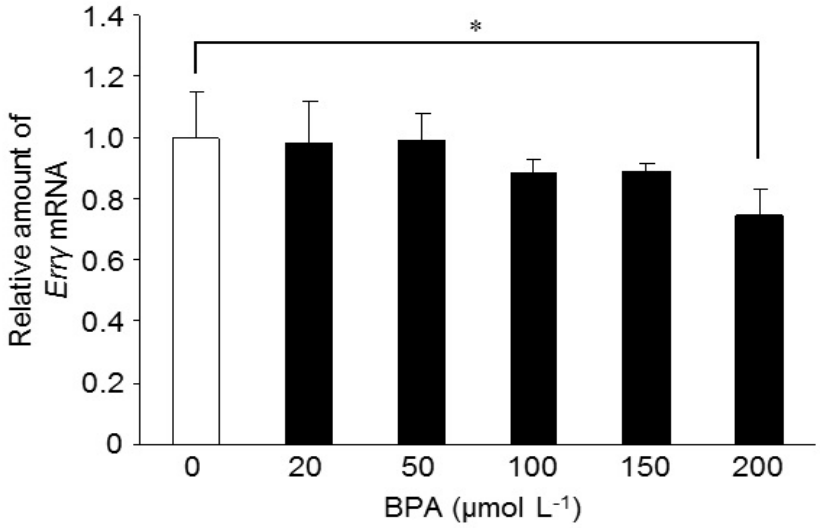


**Figure 4** *Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of* Errγ *mRNA levels in mHypoE-N44 cells treated with BPA for 3 h.*
*The data have been normalised to the* Gapdh *mRNA level in each sample and are expressed as a value relative to this internal control. Each column represents the mean ± SD (n=3 for each group).* *P*<0.05 (significantly different from the control)*

dose-dependent. Further investigation is needed to determine the significance of these results at the level of organism.

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*Conflicts of interest*

The authors declare no conflict of interest.

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***Sažetak***

**Jedinstveni obrazac djelovanja bisfenola A na ekspresiju gena čimbenika rasta živca embrionske mišje stanične linije N-44 dobivene iz hipotalamusa**

U istraživanju toksičnosti bisfenola A (BPA) utvrđena je ekspresija gena čimbenika rasta živca (eng. *nerve growth factor* - NGF) embrionske mišje stanične linije mHypoE-N44 dobivene iz hipotalamusa nakon trosatnog izlaganja BPA-u u rasponu doza od 0,02 do 200 μmol $L^{-1}$. Razine *Ngf* mRNA snizile su se ovisno o dozi, a značajne razlike od kontrolne skupine zamijećene su za raspon od 2 do 50 μmol $L^{-1}$. Međutim, počevši od doze od 100 do 200 μmol $L^{-1}$, razine *Ngf* mRNA značajno su se povećale u odnosu na kontrolu, a ekspresija gena kaspaze 3 i transformacijskog proteina 73 značajno snizila. Ti rezultati upućuju na to da visoke doze BPA u embrionskoj hipotalamičkoj staničnoj liniji stvaraju jedinstveni obrazac ekspresije gena *Ngf* te da BPA može suprimirati apoptozu koja je nužna za rani razvoj mozga.

**KLJUČNE RIJEČI**: *čimbenik rasta živca; fetalni hipotalamus; kaspaza 3; razvojna toksičnost; transformacijski protein 73*

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