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ORGAN TOXICITY AND MECHANISMS

New insights into mechanism of bisphenol analogue neurotoxicity: implications of inhibition of O-GlcNAcase activity in PC12 cells

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Abstract

Bisphenol analogues including bisphenol A and its derivatives are ubiquitous environmental contaminants and have been linked to adverse neurodevelopment effects on animals and humans. Most toxicological research focused on estrogen receptor mediated pathways and did not comprehensively clarify the observed toxicity. O-GlcNAcase (OGA), the highest level in brain, plays a critical role in controlling neuronal functions at multi-levels from molecule to animal behaviors. In this work, we intend to investigate the underlying molecular mechanisms for the neurotoxicity of bisphenol analogues by identifying their cellular targets and the resultant effects. The inhibitory actions of seven bisphenol analogues on the OGA activity at molecular level were investigated by our developed electrochemical biosensor. We found that their potency varied with substituent groups, in which tetrabromo bisphenol A (TBBPA) was the strongest. The seven bisphenol analogues (0–100 μM exposure) significantly inhibited OGA activity and up-regulated protein O-GlcNAcylation level in PC12 cells. Inhibition of OGA by bisphenol analogues further induced intracellular calcium, ROS, inflammation, repressed proliferation, interfered with cell cycle, induced apoptosis. And especially, 10 μM tetrabromo bisphenol A (TBBPA) exposure could impair the growth and development of neurite in human neural stem cells (hNSCs). Molecular docking for OGA/bisphenol analogue complexes revealed the hydrophobicity-dominated inhibition potency. OGA, as a new cellular target of bisphenol analogues, would illuminate the molecular mechanism of bisphenol analogues neurotoxicity.

Keywords Bisphenol analogues · O-GlcNAcase · Inhibition · Neurotoxicity

Introduction

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is widely used to produce polycarbonate plastics and epoxy resins, as well as various consumer products including food containers, thermal receipts paper, water pipes, toys, medical equipment and electronics (Rochester 2013). A great deal of research on experimental animals has showed toxic effects of BPA for reproduction and development, metabolism, nervous, cardiovascular and immune systems (Richter et al. 2007). The ubiquitous BPA in the environment may threaten human health and safety via various routes, e.g. dermal absorption, dust intake, inhalation and dietary ingestion (Geens et al. 2012). Actually, BPA has been detected in human serum, urine, placental tissue, umbilical cord blood and breast milk (Vandenberg et al. 2007). Also, in human epidemiological studies, BPA exposure has been linked to a variety of health symptoms including reduced sperm quality (Li et al. 2011) and fertilization success (Fujimoto et al. 2011), polycystic ovarian syndrome (Takeuchi and Tsutsumi 2002), altered neural development (Kajta and Wojtowicz 2013; Negri-Cesi 2015), obesity (Shankar et al. 2012), cardiovascular disease (Melzer et al. 2012a, b) and type 2 diabetes (Shankar and Teppala 2011). Due to the toxicities of BPA, some countries including North America (Government of Canada 2010) and the European Union (The European

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Commission 2011) prohibited the production and usage of BPA. The public attention and governmental regulations for BPA motivated the development, production and usage of alternative substances. A number of chemicals that are similar in structure to BPA have already been developed and synthesized as the major substitutes for BPA added into products (Liao et al. 2012). These chemicals possess a same structure with two hydroxyphenyl functionalities known collectively as bisphenol analogues. Several bisphenol analogues have been found in indoor dust, sediment, water, soil, and sewage sludge, sometimes in levels similar to or higher than that of BPA (Chen et al. 2016). Available limited studies have revealed diverse adverse effects similar to those observed for BPA, including endocrine disruption (Rochester and Bolden 2015), neurotoxicity (Ji et al. 2013), and reproductive toxicity (Kinch et al. 2015). To date, toxicity studies remain remarkably limited in the determination of modes of action and quantitative toxic end points or benchmarks, both in vitro and in vivo, for bisphenol analogues. Actually, the endocrine disrupting toxicity of BPA has been widely reported. Recently, it is also becoming evident that exposure of animal models (rodent, zebrafish, caenorhabditis elegans) to BPA or its analogues, particularly during development, can cause several neurobehavioral disorders, including cognitive and socio-sexual deficiencies, impaired parental care ability, multiplied anxiety and hyperactive behaviors and retardant habitual responses (Wolstenholme et al. 2011). Some of these neurological effects are likely due to BPA acting as a weak estrogen and binding to estrogen receptors (ER, e.g. ERα, ERβ, membrane ER) or estrogen-related receptors (ERR, e.g. ERR-γ, GPR30) within various brain regions. Others are presumably through engagement of other steroid/non-steroid receptor pathways (Rosenfeld 2017). In order to more fully understand the neurotoxic effects of BPA and its analogues and their health risks to humans, their potential molecular targets need to be further identified.

The modification of serine or threonine residues with a single *N*-acetylglucosamine (GlcNAc) moiety via a β-O-glycosidic linkage, termed as O-GlcNAcylation, is an abundant, dynami and reversible type of protein post-translational modification in mammals. It involves in multiple signaling pathways connected with innate immune, stress and growth factor response, transcription, translation and protein degradation (Hart et al. 2007). Dysregulation of O-GlcNAc modification coordinately catalyzed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) is implicated in the etiology of some chronic diseases of aging, cancer, type 2 diabetes, cardiovascular and Alzheimer's disease (AD) (Vaidyanathan et al. 2014). OGT and OGA abundantly expressed in the brain suggest that O-GlcNAc modification plays a crucial role in controlling neuronal functions at multi-levels from molecule to animal behaviors (Yuzwa and Vocadlo 2014). The deficit of OGA in mice results in death throughout embryo-genesis or perinatal lethality, and hence keeping proper levels of O-GlcNAcylation are necessitated for embryonic development (Keembiyehetty et al. 2015). Genetic disruption of OGA caused not only constitutively increased O-GlcNAcylation in embryos, but also fetal lethality with delayed development. Also, increased O-GlcNAcylation from OGA disruption weakened cell proliferation and led to mitotic defects with down-regulated mitosis, such as cytokinesis failure and binucleation, multiplied lagging chromosomes and micronuclei formation (Yang et al. 2012). Notably, brain-specific deletion of OGA in mice induced a tardive brain differentiation and neurogenesis and aberrant proliferation with a delayed development. And the ability of mouse embryonic stem cells (mESCs) derived from the Oga knockout mice to differentiate into neuronal lineage was dramatically suppressed (Olivier-Van Stichelen et al. 2017). $Oga^{+}/^{-}$ mice showed damaged spatial learning and memory, a defect in hippocampal synaptic plasticity, and the impairment of both long-term potentiation and long-term depression (Yang et al. 2017b). Although the biological roles of OGT have been extensively studied, how OGA is targeted, how OGA activity is governed and what the toxicological effects are when OGA is disturbed in vivo remain unclear.

In the present study, we intend to explore the neurotoxicity mechanism of bisphenol analogues by identifying their biological targets within cells. First, at molecular level, we employed an electrochemical biosensor-based assay to investigate the inhibitory actions of bisphenol analogues on OGA activity. Then, at cellular level, the inhibition reactions and subsequent toxic effects, especially potential impacts on human health, were further investigated. The chosen seven bisphenol analogues (supplementary material Fig. S1), existing ubiquitously in the environment, are structural diversity, with different substituent groups, which would allow us to explore some structural characteristics of the inhibition potency.

Materials and methods

OGA activity and global O-GlcNAcylation level within cells

For OGA activity assay, PC12 cells were lysed in RIPA lysis buffer and the OGA activity was measured using spectrophotometry. OGA hydrolyzes the β-linked glycosidic bond of PNP-GlcNAc to generate GlcNAc and p-nitrophenol. Generally, OGA enzyme reactions are monitored after quenching with a basic solution. The chromophoric product, p-nitrophenol (protonated form), released from the enzymatic reaction, has a relatively weaker absorbance than its

deprotonated form, the p-nitrophenolate ion. Therefore, the released p-nitrophenol can be spectrophotometrically measured in a deprotonated form in alkaline solution at 400 nm (Gao et al. 2001). Briefly, OGA was immunoprecipitated from cell lysates by using anti-OGA antibody conjugated dynabeads for 6 h at 4 °C. After cleaning up unbound lysate, reaction mixtures containing 50 mM sodium cacodylate, pH 6.5, 0.3% bovine serum albumin and 2 mM PNP-GlcNAc were added and then incubated for 40 min at 37 °C. After being quenched by adding 0.5 M sodium carbonate, the reaction mixtures were centrifuged at 12,000*g* for 30 min. The absorbance of the supernatant at 400 nm was measured. Meanwhile, global O-GlcNAcylation level in cells was determined. PC12 cells were cultured in 60 × 60 mm dish for 12 h and next stimulated with nerve growth factor (NGF) (50 ng/mL, 5 days) and then exposed to bisphenol analogues (0–100 μM) or control medium (VC) for 24 h. The exposed cells were treated with RIPA buffer. The protein concentration of each specimen was determined by BCA Protein Assay Kit. The specimens were isolated by SDS-PAGE gel electrophoresis and then transferred to a PVDF membrane (0.45 μm). The membrane was blocked in 5% nonfat milk and rinsed in TBST (containing 0.1% Tween 20), followed by incubating with anti-O-GlcNAc primary antibody (1:1000 dilution) at 4 °C overnight and the HRP-conjugated secondary antibody (1:10000 dilution) at 25 °C for 1 h, rinsed by TBST and then imaged on X-ray film.

Immunostaining and high-content screening assay

Human neural stem cells (hNSCs) were seeded in poly-L-lysine and laminin coated 96-well plates (100 μL/well) at a density of 3000 cells/well, in maintenance medium supplemented with 5 μM Y-27632, 100 U/mL penicillin and 100 μg/mL streptomycin. After 24 h, cells were incubated with complete maintenance medium deprived of Y-27632. Starting from the next day, cells were exposed to 0.1% DMSO, PUGNAc (100 μM), TBBPA (1, 5 and 10 μM), or coexposed to PUGNAc (100 μM) and TBBPA (1, or 5, or 10 μM), respectively. The exposure media were refreshed daily. After three-day treatments, hNSCs were fixed with 4% formaldehyde for 15 min at room temperature and blocked with 5% goat serum (Solarbio, Beijing, China) and 0.3% Triton X-100 (Solarbio, Beijing, China), in Dulbecco's phosphate-buffered saline (DPBS) (Gibco, MA, USA). After incubation with a primary antibody against MAP2 (Santa Cruz Biotechnology, CA, USA), at 4 °C overnight and three DPBS washes, cells were incubated with the secondary antibody anti-rabbit IgG Alexa Fluor (R) 488 molecular probe (Cell Signaling Technology, MA, USA), at 4 °C for 1–2 h. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Solarbio, Beijing, China) for 5 min at room temperature. Fluorescent images were captured with an EVOS FL Auto II cell imaging system (Thermo Fisher Scientific, MA, USA). A Cellomics Cellinsight CX7 instrument (Thermo Fisher Scientific, MA, USA) was used to quantify neurite number and length.

Statistical analysis

Results are expressed as mean ± SD ($n = 4$, SD represents the standard deviation of four determinations). The statistical analysis was operated using SPSS statistics software (Chicago, IL). The normality and homogeneity of variance of all data were analyzed by one-way analysis of variance (ANOVA) and then the multiple comparisons for differences in means were executed by Bonferroni's post hoc test. A p value < 0.05 was considered statistically significant.

Results

Inhibitory actions of bisphenol analogues on OGA activity at molecular level

We first developed an electrochemical biosensor for the determination of OGA enzyme activity. The biosensor works on the sensing mechanism we reported previously in which protease displays a large distinction in the hydrolysis of glycosylated and unglycosylated versions of peptide (Yang et al. 2017a). In the biosensor (supplementary material Fig. S2a), in the presence of OGA, glycosylated peptides were converted to unglycosylated peptides, which could be cut facilely with proteinase K and produced a low current. Upon adding an inhibitor, the signal increased due to less conversion of glycosylated peptides. The signal enhanced gradually with the concentration of PUGNAc (a known OGA inhibitor) until the maximum reached (supplementary material Fig. S2b). The IC_{50} was estimated to be 0.21 ± 0.05 μM from dose–response curve. K_i values were calculated to be 0.08 ± 0.01 μM, which was in consistent with that reported in the literature ($K_i = 0.05$ μM) (Macauley et al. 2005).

After the electrochemical biosensor assay was established and validated, seven bisphenol analogues were investigated for their inhibitory effects on OGA. As shown in supplementary material Fig. S2c, bisphenol analogues inhibited OGA activity in a concentration-dependent manner. However, their potency varied significantly, as manifested by IC_{50} values summarized in supplementary material Table S1. It follows the order of TBBPA > TCBPA > BPAF > BPB > BPA > BPF > BPS. Among them, halogen atom-substituted bisphenol analogues showed stronger potency than any other ones. TBBPA, a BPA analogue containing four bromine atoms, exhibited the strongest inhibitory effect on OGA activity with an IC_{50} of 8.30 μM.

Inhibition of OGA activity by bisphenol analogues in PC12 cells

PC12 cells were chosen to investigate the effects of bisphenol analogues on OGA activity in biological systems. Cells were exposed to one of bisphenol analogues at non-cytotoxic concentrations for 24 h (supplementary material Fig. S3). Then OGA was immunoprecipitated from cell lysates, and its activity was measured by spectrophotometry. All bisphenol analogues were found to inhibit OGA activity in a dose-dependent manner (Fig. 1a). At 50 μM exposure, TBBPA, TCBPA, BPAF, BPB, BPA, BPF and BPS inhibited the OGA activity by 49%, 38%, 45%, 27%, 25%, 22% and 31%, respectively.

Actually, the enzymatic activity of a protein lies with both total content and specific activity of the protein; then we examine gene expression and protein levels of OGA within cells after bisphenol analogues' exposure. The results of RT-PCR indicated that at 25 μM exposure, BPB, BPS and BPAF induced the gene expression of OGA, whereas no apparent effect was detected after exposure to one of the others (supplementary material Fig. S4a). After treatment, the OGA protein level in PC12 cells was quantified utilizing Western-blotting (supplementary material Fig. S4b and 4c). At 25 μM exposure, BPB increased the protein level by 1.38 folds. At 100 μM exposure, BPA induced the increase of OGA protein level by 1.46 folds. No marked difference was observed after exposure to the others individually. Either gene or protein level of OGA was not repressed by bisphenol analogues. This demonstrated that the decrease of OGA activity was actually derived from the inhibition of bisphenol analogues, rather than from the changes of OGA expression level.

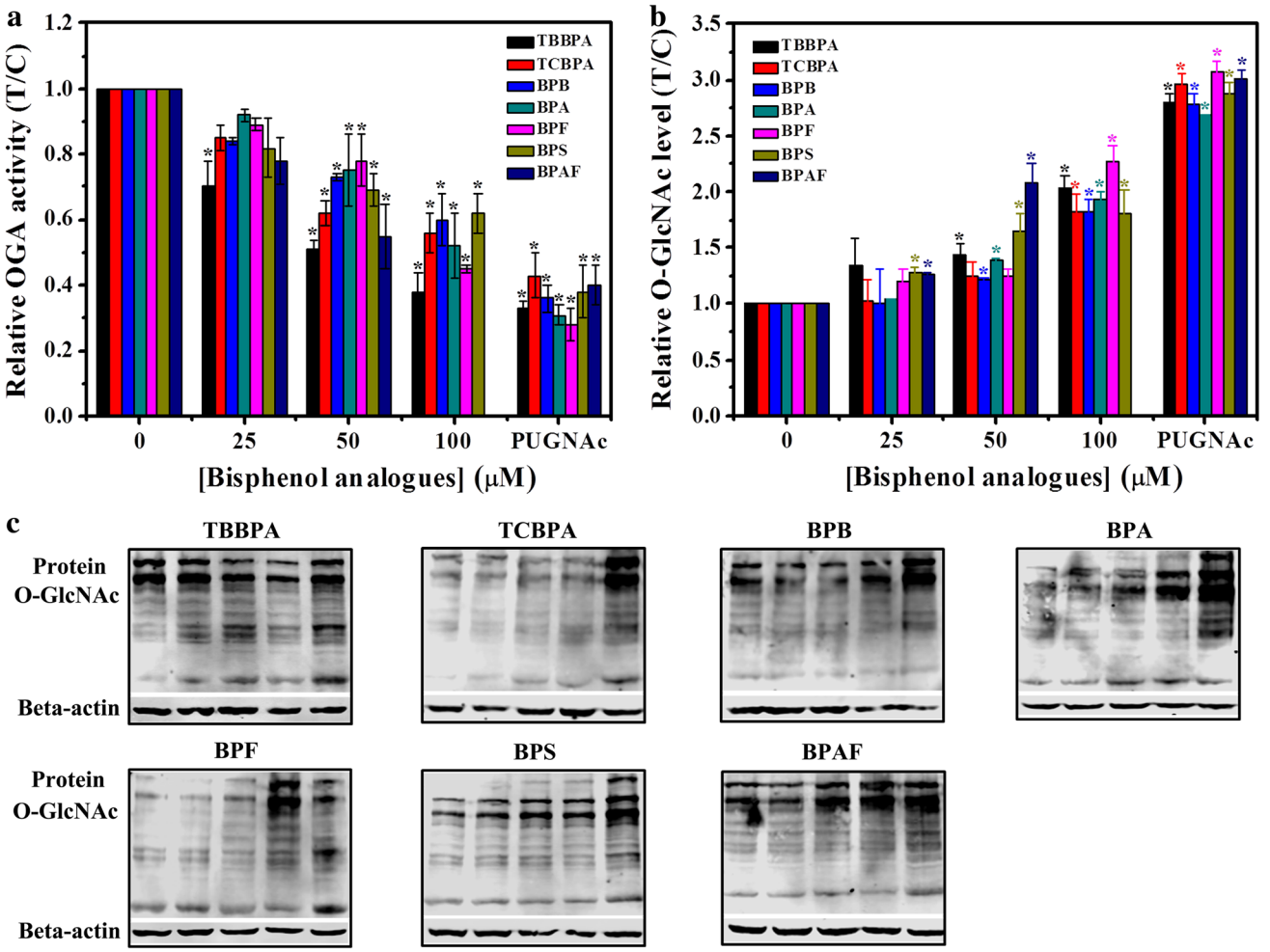

Fig. 1 OGA activity (**a**) and global O-GlcNAc levels (**b**), representative images of Western-blot analysis on OGA level in PC12 cells (**c**) exposed to bisphenol analogues for 24 h. PC12 cells treated with PUGNAc (100 μM, 24 h) were used as positive control. T/C means treated/control. The bands of global protein O-GlcNAcylation are in the range of 48-180KD. Data are normalized against *β*-actin

Change of protein glycosylation level after bisphenol analogues exposure

The level of protein O-GlcNAcylation in cell is manipulated by cooperative effects of OGT and OGA. Since OGA is responsible for hydrolysing the O-GlcNAc glycosidic bond and thus returning proteins modified by OGT to their unmodified state, its inhibition by bisphenol analogues should result in a cumulation of protein O-GlcNAcylation. The global O-GlcNAcylation level was detected. Contrasted with control group, bisphenol analogue-treated cells had a notable dose-related increment for the global O-GlcNAcylation level (Fig. 1b), illustrated as the higher signal intensity of treated groups (Fig. 1c). Then, we chose AKT and CREB, two typical physiologic substrates of OGA, to explore the effects of bisphenol analogues' inhibitions on intracellular protein O-GlcNAcylation. After exposure to one of the bisphenol analogues, PC12 cells were lysed and the levels of both protein and its O-GlcNAcylated content were determined using immunoblotting. Compared with control group, bisphenol analogue-treated groups induced an evident dose-dependent enhancement in the relative level of O-GlcNAcylated protein. For AKT (supplementary material Fig. S5a and 5c), at 50 μM exposure, TBBPA, TCBPA, BPB, BPA, BPF, BPS and BPAF promoted the O-GlcNAcylation level of AKT by 1.65, 1.41, 1.90, 1.55, 2.59, 1.56 and 2.08 folds, respectively, while, for CREB (supplementary material Fig. S5b and 5d), at 50 μM exposure, TBBPA, TCBPA, BPB, BPA, BPF, BPS and BPAF increased the O-GlcNAcylation level by 1.46, 1.95, 1.98, 1.36, 1.71, 2.11 and 2.10 folds, respectively.

Ca^{2+} influx ($[Ca^{2+}]_i$), ROS and secretion of inflammatory cytokines in cells

Furthermore, TBBPA was chosen as a typical representative and some biological effects involving $[Ca^{2+}]_i$, ROS, proinflammatory cytokines were investigated. Calcium is a central signal that regulates diverse cellular processes from neuron growth to cell communication and adhesion. Precise control for calcium homeostasis not only promotes brain function but also assists neuronal physiology and survival. Emerging knowledge shows that calcium homeostasis is crucial for normal cell function and health, but when deregulated, can result in neurodegeneration by complicated and multiple mechanisms implicated in selective neuronal damages and death (Nanou and Catterall 2018). In order to determine whether inhibition of OGA activity contributes to $[Ca^{2+}]_i$ or not, PUGNAc, a well-known OGA inhibitor, was employed. As shown in Fig. 2a, PUGNAc exposure evoked robust increase of F340/F380 ratios, suggesting that OGA inhibition could induce calcium ion influx and resulted in elevated $[Ca^{2+}]_i$. Meanwhile, contrasted with that in untreated, PUGNAc- or TBBPA-treated cells, accumulation of $[Ca^{2+}]_i$ in PUGNAc and TBBPA coexposed cells was further amplified with the increase of TBBPA concentration and then arrived a maximum at 25 μM, demonstrating the crucial role of TBBPA in eliciting Ca^{2+} influx.

Accumulating evidence suggests that ROS should be considered as second messengers involved in numerous signaling pathways in health and disease. ROS maintain the oxidative status of the eukaryotic cell, promoting normal physiology from the innate immunity to neuron growth. Especially, ROS play an important function in the differentiation, development and regeneration of neurons of both the central and peripheral nervous systems (Dasuri et al. 2013). Analogously, in order to clarify whether inhibition of OGA activity devoted to ROS generation or not, OGA inhibitor-PUGNAc was available to expose cells. As given in Fig. 2b, in contrast to control group, TBBPA-treated cells produced a marked increase of ROS amounts as PUGNAc did. Additionally, in PUGNAc- and TBBPA-coexposed cells, the generation of ROS amounts were also enlarged with the increase of TBBPA concentration and then approached the saturation value at 25 μM.

Neuroinflammation has important impacts on the pathogenesis of some neurological disorders, such as Alzheimer's disease (AD), stroke and depression. Some proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, are described as biomarkers and are correlated with inflammatory response and adverse neurologic outcomes. Most studies revealed that high levels of inflammatory cytokines were in connection with neurodevelopment impairments (Rodney et al. 2018). As shown in Fig. 2c–e, PUGNAc exposure induced active release of inflammatory cytokines IL-1β, IL-6 and TNF-α as TBBPA did, which suggested that OGA inhibition could induce the inflammatory response. Also, in PUGNAc and TBBPA coexposed cells, secretion of these inflammatory cytokines were significantly increased, which indicated that the introduction of TBBPA further aggravated the occurrence of inflammation.

Cell proliferation, cycle and apoptosis in exposed cells

Thereafter, the effects of OGA inhibition on cell functions, including cell proliferation, cycle and apoptosis were investigated. Cell proliferation is essential to appropriate growth, development and maintenance of cells or tissues (Yang and Herrup 2007). As given in Fig. 2f, compared with control group, cell proliferation was reduced by 11% and 18%, respectively, after 50 or 100 μM TBBPA exposure. It suggested that TBBPA exposure could suppress the proliferation. Moreover, compared with control, PUGNAc or TBBPA-treated group, 25, 50 or 100 μM TBBPA and

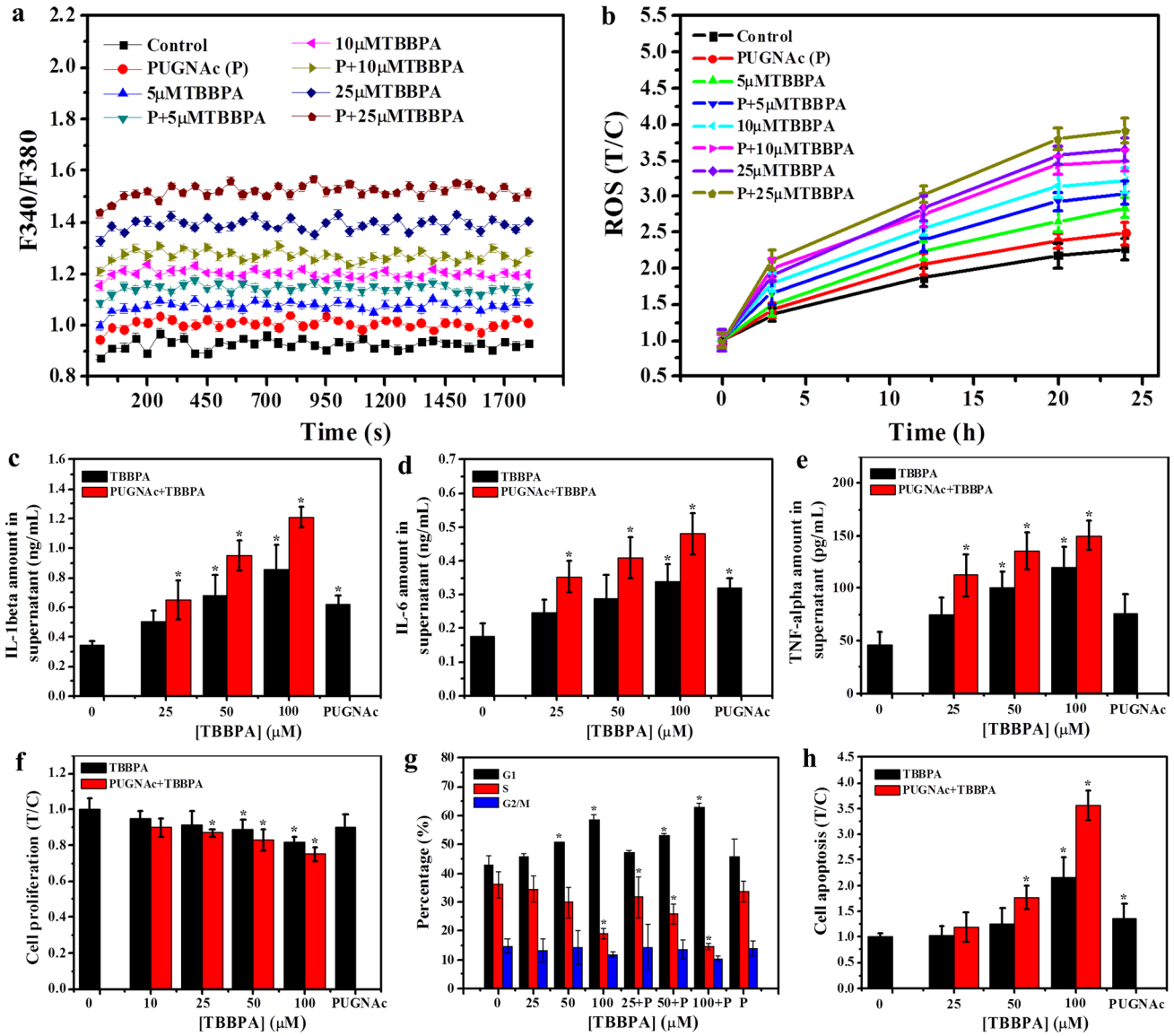

Fig. 2 The change of calcium ion level (**a**), ROS amounts (**b**), inflammatory cytokines (**c**–**e**), cell proliferation (**f**), cell cycle (**g**) and cell apoptosis (**h**) in exposed cells. P is the abbreviation for PUGNAc in **g**

PUGNAc coexposure substantially reduced the cell proliferation by 13%, 17% and, 25% respectively.

Cell cycle is a delicately controlled process involving cell growth and division that creates two new daughter cells. It comprises three stages: G1 phase (The period of growth preceding any commitment to division), S phase (The period of DNA synthesis) and G2/M phase (Anaphase of DNA synthesis and mitosis phase) (Wang et al. 2009). Due to the difference of DNA content in different phases of cell cycle, the G1 phase of normal cells has the DNA content of diploid cells (2 N), While the G2/M phase has the DNA content of tetraploid cells (4N). The content of DNA in S phase was between diploid and tetraploid. As shown in Fig. 2g and supplementary material Fig. S6a–h, after exposure to 50 or 100 μM TBBPA, the cell percentages of G1 phase were significantly increased up to 50.8% and 58.4%, respectively, compared with 43.0% in the control group. It indicated that TBBPA exposure could arrest cell cycle in G1 phase. Also, compared with control, PUGNAc or TBBPA-treated group, 50 or 100 μM TBBPA and PUGNAc coexposure could induce a higher percentage of G1 phase, with 53.1% and 62.9%, respectively. Meantime, the cell percentage of S phase decreased gradually from 36.1% to 31.7%, 25.9% and 14.6%, respectively, after 25, 50 or 100 μM TBBPA and PUGNAc coexposure. It suggested that a remarkable increase of cell population in G1 phase was accompanied by a reduction in S phase. TBBPA

and PUGNAc coexposure further augmented the arrest of G1 phase and impeded the DNA synthesis of S phase.

Apoptosis is characterized by programmed cell death that occurs in mammalian cells. It is critical for the maintenance of normal development and tissues homeostasis (Yuan and Yankner 2000). As shown in Fig. 2h and supplementary material Fig. S7a–h, PUGNAc or TBBPA exposure significantly induced cell apoptosis. Moreover, compared with control, PUGNAc or TBBPA-treated group, 50 or 100 μM TBBPA and PUGNAc coexposure drastically induced apoptosis in a dose-dependent manner, by 1.77 and 3.56 folds, respectively.

hNSCs generation and neurite growth

Pluripotent stem cells (PSCs) are capable of indefinite proliferation and directional differentiation into virtually all the cell types of the body. Thus, PSCs can be employed for multi-directional toxicological evaluations (Liang et al. 2019). Here, in order to dissect the potential inhibition of TBBPA on neurite development and growth during the differentiation of neural precursor cells into immature neurons, we employed human PSCs and performed neural induction in vitro. First, we examined the effects of TBBPA on human neural stem cells (hNSCs) viability to determine non-cytotoxic concentrations. We found that hNSC viability was reduced in a dose-dependent manner after TBBPA exposure, with a significant decrease starting at 25 μM (supplementary material Fig. S8). We then investigated the effects of TBBPA non-cytotoxic concentrations on neurite growth (Fig. 3a–h), including neurite total count and length. As shown in Fig. 3i, neurite total count was decreased by 43.1% after PUGNAc exposure. It was reduced by 32.2% and 89.7%, respectively, after 1.0 or 10.0 μM TBBPA exposure, while 1.0, 5.0 or 10.0 μM TBBPA and PUGNAc coexposure lowered the neurite total count by 61.9%, 58.0% and 86.1%, respectively.

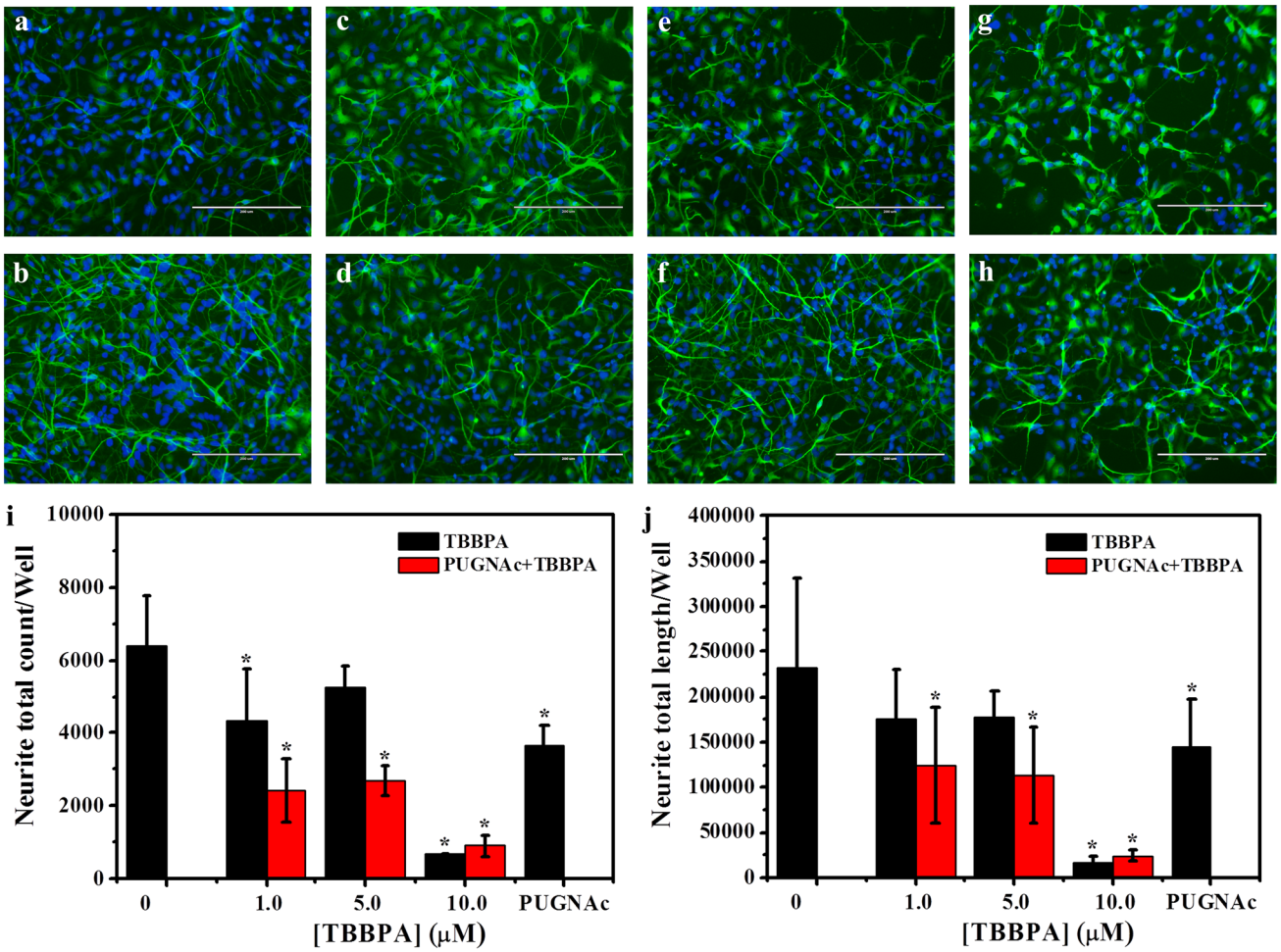

Fig. 3 Fluorescence images in hNSCs exposed to control medium (**a**), 100 μM PUGNAc (**b**), 1 μM TBBPA (**c**), 100 μM PUGNAc and 1 μM TBBPA (**d**), 5 μM TBBPA (**e**), 100 μM PUGNAc and 5 μM TBBPA (**f**), 10 μM TBBPA (**g**), 100 μM PUGNAc and 10 μM TBBPA (**h**), using high-content screening assay. The change of neurite total count (**i**) and total length (**j**)

Similarly, PUGNAc or 10.0 μM TBBPA exposure reduced neurite total length by 37.8%.and 93.4%, respectively. Also, 1.0, 5.0 or 10.0 μM TBBPA and PUGNAc coexposure decreased the neurite total length by 46.5%, 51.5% and 89.5%, respectively (Fig. 3j).

Molecular docking

In order to illuminate the inhibition mechanism of bisphenol analogues on OGA, we studied their interactions with OGA by molecular docking. OGA is a nucleocytoplasmic protein that consists of three distinct regions: an N-terminal catalytic domain that exhibits sequence similarity with glycoside hydrolases of the GH84 family and is responsible for O-GlcNAc removal, an intervening stalk domain and a C-terminal pseudo-histone acetyltransferase (HAT) domain. The residues in the active site of OGA catalytic domain are Gly187, Tyr189, Lys218, Asp297, Asp298, Tyr335, Thr366, Val370, Val371, Trp394, Asn396, Asp401, Tyr402, Asn429 (Rao et al. 2006).

Using the software of Discovery Studio, seven bisphenol analogues were docked into the binding pocket of OGA, respectively. The binding energies and some non-covalent bond interactions for the docked complexes are summarized in supplementary material Table S1. All the bisphenol analogues bound to the active site of OGA, which would impede the combination between substrate and OGA. The optimal steric conformations of the complexes are shown in supplementary material Fig. S9.

Discussion

One of the major toxicities of bisphenol analogues on developing animals is neurotoxicity. However, the molecular mechanism responsible for the neurotoxicity of bisphenol analogues is not yet understood. We speculate that some key biomolecules might be participating in mediating these effects. OGA is abundantly expressed in brain and confined to the chromosome 10 arm, a location closely related to late-onset Alzheimer's disease (AD) (Bertram et al. 2000). Many studies indicate that OGA plays a vital role in both normal brain functions and the etiology of neurodegeneration. The inhibition of OGA activity could in principle lead to neurological disorder or diseases. Therefore, in our work, we conducted a series of tests to explore OGA as a cellular target of bisphenol analogue neurotoxicity.

First, at molecular level, we found that all the seven bisphenol analogues inhibited OGA activity for the deglycosylation of glycosylated peptide. The inhibition potency relied upon their structures and it has a positive relationship with their hydrophobicity that was related to molecular volume and polarity. By comparing bisphenol analogues with different groups, it was obvious that halogen atom-substituted bisphenol analogues were more potent than the other ones. Also, For the three bisphenol analogues substituted with halogen group, the inhibition potency follows the order of TBBPA > TCBPA > BPAF, with TBBPA being the strongest (supplementary material Table S1). Molecular docking for the interactions between bisphenol analogues and OGA provided theory foundation for the diversity in their hydrophobicity-dominated inhibition potency. For the seven bisphenol analogues, the calculated binding energy of the docked complexes decreased with their hydrophobicity and it follows the order of TBBPA > TCBPA > BPAF > BPB > BPA > BPF > BPS, with TBBPA being the lowest and BPS being the highest (supplementary material Table S1). This tendency was in agreement with the electrochemical measured inhibition potency (IC_{50}) of bisphenol analogues, and thus supports the validity of molecular docking approach.

For the docked complex, the bisphenol analogues all bound in a hydrophobic pocket constructed by the residues of Trp490, Asn429, Tyr402, Asp401, Val399, Asn396, Trp394, Val371, Val370, Tyr335, Asp298, Lys218 and Tyr189 of OGA in an N-terminal catalytic domain (supplementary material Fig. S9a–n). A common hydrogen bond existed between the oxygen atom of aromatic ring of bisphenol analogue and the hydrogen atom of Asn396 residue of OGA. Also, a common π-alkyl interaction was formed between the aromatic ring of bisphenol analogue and the oxygen (or nitrogen) atom of Val370 residue of OGA. Although their binding modes shared some similarities, they are stylistically very different. For TBBPA, TCBPA, BPB and BPA, there were two same hydrogen bonds between the oxygen atom of bisphenol analogue and the hydrogen atoms of Asn396 and Asn429 residues of OGA (supplementary material Fig. S9a, 9c, 9g and 9i). Also, a similar π-anion interaction existed between the electron-deficient aromatic ring of bisphenol analogue and the nearest oxygen atom of Asp401 residue of OGA (supplementary material Fig. S9b, 9d, 9h and 9j). For BPAF, one hydrogen bond existed between the oxygen atom of BPAF and the hydrogen atom of Asn396 residue of OGA (supplementary material Fig. S9e). For BPF, three hydrogen bonds were formed between the oxygen atom of BPF and the hydrogen atoms of Asn396, Asn429 and Trp394 residues of OGA (supplementary material Fig. S9k). For BPS, three hydrogen bonds existed between the oxygen atom of aromatic ring of BPS and the hydrogen atoms of Asn396, Asn429, Asp401 residues of OGA. And one hydrogen bond was formed between the oxygen atom of sulfuryl group of BPS and the hydrogen atom of Tyr335 residue of OGA (supplementary material Fig. S9m). Moreover, other non-covalent interactions, including van der Waals, π–π stacked, etc., coordinately contributed to the organization of enzyme-inhibitor complexes (supplementary material Fig. S9b, 9d, 9f, 9h, 9j, 9l and 9n).

For the OGA activity assays within cells, the inhibitory effects of the seven bisphenol analogues were further verified. The inhibition potency follows the order of TBBPA > BPAF > TCBPA > BPS > BPB > BPA > BPF. Remarkably, bisphenol analogues containing halogen atoms were superior to others. The inhibition potency of TBBPA containing four bromine atoms was the strongest, which coincided with the results from the biosensor. While, for the order of inhibition potency of other bisphenol analogues, there was a discrepancy between in vitro and cell-based OGA activity. The cause lays in the fact that cell microenvironment is more sophisticated as various elements, like cell permeation, properties of bisphenol analogues, as well as the disturbance of coexisting protein, exert influences on the inhibition reaction.

Then, intracellular O-GlcNAc level stemmed from the OGA inhibition were investigated. Since OGA participates in the regulation of intracellular protein O-GlcNAcylation level by catalyzing the hydrolytic cleavage of O-GlcNAc from modified proteins, inhibition of OGA activity should induce a increase in O-GlcNAc level in cells. We found the increased global O-GlcNAc level in whole cells. Intracellular O-GlcNAc modification has a significant influence on neuron functions at multiaspect from intrinsic neuron characteristics to synaptic plasticity and animal behaviors. Dysregulation of O-GlcNAcylation is highly related to some human diseases, for example neurodegeneration, diabetes and cancer (Bertram et al. 2000). Then, AKT and CREB, two representative substrates of OGA, were chosen to assess the effect of OGA inhibition in PC12 cells. AKT, which is regulated by O-GlcNAcylation, is a central module in signal transduction pathway and regulates multiple functions including cell growth, metabolism, survival and angiogenesis (Risso et al. 2015). CREB, whose activity is regulated by O-GlcNAcylation, is a key transcription factor. O-GlcNAcylation of CREB plays a major role on the gene regulation of neuron, the development of axons and dendrites and the formation of memory (Lonze and Ginty 2002). As anticipated, the seven bisphenol analogues provoked evident increase of protein O-GlcNAc level in PC12 cells. Abnormal O-GlcNAc level of protein might have deleterious effects on the biological functions of protein.

In addition, other subsequent effects involving $[Ca^{2+}]_i$, ROS, proinflammatory cytokines were also investigated. In the cells exposed to PUGNAc (an OGA inhibitor), we found the distinct increase of above biological effects, indicating that OGA inhibition was capable of triggering these changes. TBBPA and PUGNAc coexposed cells could enhance the degrees of these changes, demonstrating the crucial role of TBBPA in inducing these effects. Virtually, anomalies in $[Ca^{2+}]_i$, ROS and proinflammatory cytokines may threaten the health of the nervous system in the organism. Many researches have revealed that neurons need extraordinarily accurate control of Ca^{2+} concentration in specific compartments for such vital functions as synaptic plasticity. Dysregulation in cellular $[Ca^{2+}]_i$ homeostasis is implicated in some neuropsychiatric diseases, such as Parkinson's disease (PD), AD, Huntington's disease, amyotrophic lateral sclerosis (ALS), spinocerebellar ataxias, schizophrenia, bipolar disorder and depression (Nanou and Catterall 2018). Meanwhile, abnormal ROS productions contribute to the dysfunction of neuronal development, including neurogenesis, polarization and maturation of neurons. It is also involved in the occurrence of some neurodegenerative diseases, including AD, PD, ALS and stroke (Dasuri et al. 2013). Moreover, neuroinflammation is considered to be not only as a consequence of cell death but also as a feedback mechanism promoting further abnormal neuron clearance. The inflammatory process affects proliferation and differentiation of neuron and glial cell and enhances cell death rate. The increase in proinflammatory cytokines represents a risk factor for neural development and function (Rodney et al. 2018).

Furthermore, the effects of OGA inhibition on cell functions, including cell proliferation, cycle and apoptosis were investigated. Compared with control group, TBBPA or PUGNAc exposure remarkably suppressed cell proliferation, arrested the G1 phase of cell cycle and induced cell apoptosis. While, in PUGNAc and TBBPA coexposed group, these effects were further enhanced. Actually, abnormal cell proliferation can lead to malignant transition and cancer pathology (Yang and Herrup 2007). Moreover, failure of cell cycle regulation might be a common pathway of several neurodegenerative disorders and for other CNS diseases. Many researches have demonstrated correlations between cell cycle dysregulation and neuron death, both in acute CNS damage (e.g. trauma and stroke) and in neurodegenerative diseases (e.g. AD, PD, ALS and Niemann–Pick disease type C) (Wang et al. 2009). Also, inappropriate apoptosis (e.g. too much or too little) can either aggravate ischaemic conditions and induce neurodegenerative diseases or trigger cancer and autoimmune disorders (Yuan and Yankner 2000). Moreover, in order to extrapolate the biological effects to humans, hNSCs were selected to further investigate the effect of OGA inhibition on the neurite development. PUGNAc exposure led to the decrease of neurite total count and length, which indicated that OGA inhibition could significantly impair the growth and development of neurites. TBBPA and PUGNAc coexposure caused a more serious damage, indicating the critical role of TBBPA in restraining the growth of neurite.

Based on all above results that TBBPA exposure could bring some biological effects as PUGNAc (a well-known OGA inhibitor) did, and TBBPA also inhibited OGA activity in cells, we deduced that the changes caused by TBBPA were at least partly mediated by inhibition of OGA activity. Certainly, there may be other unknown pathways induced by TBBPA, leading to the generation of these effects.

Conclusions

In sum, by different experimental means involving in vitro inhibitory action measurements, cell-based tests and molecular simulation, we have demonstrated OGA as a new biological target of bisphenol analogues. Within cells, seven bisphenol analogues incline to target OGA, inhibit the OGA activity, further induce the increase of protein O-GlcNAcylation level, and consequently, result in some detrimental biological effects on cell functions and neurite development. The new target OGA might give a hint about in vivo toxic and pathogenic mechanism of bisphenol analogues. Further researches will be needed to explore the impacts of OGA inhibition on synaptic function, as well as its deep connection with the neurological disorders observed on experimental animals.

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Compliance with ethical standard

Conflict of interest None of the authors have any conflict of interest to declare.

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